

Decline of chaperone-mediated autophagy in aging impairs macrophage clearance of senescent cells

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Supplementary Information

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- **Source Data 1 (separate files):** Raw data and statistical analysis that contribute to the data shown in Main and Extended Data Figures prepared as individual files for Fig.1, 3-7, ED Fig. 1, 3, 4, 7-10 and Fig. S1.
- **Source Data 2 (separate files):** Uncropped membranes with molecular weight markers of the blots shown in Main and Extended Data Figures prepared as individual pdf files for Fig. 3-5, 7 and ED Fig. 1, 5-8, 10.

Supplementary Note 1: Justification of the experimental model of CMA blockage

Modulation of L2A remains the most selective approach to reduce CMA activity, as, unlike other CMA components (e.g., Hsc70), no additional functions have been described for this *Lamp2* spliced variant, which differs from the other two L2 isoforms in only 20 of 410 amino acids^{1, 2}. Furthermore, because L2B and L2C are more abundant than L2A in lysosomes, and all share identical luminal region (comprising more than 90% of the protein), it is well established that L2A deletion does not affect lysosomal stability, enzyme targeting or composition, nor does it reduce the activity of other autophagic pathways such as macroautophagy or eMI³⁻⁷.

Supplementary Note 2: Senescence-induced changes in individual metabolites.

Analysis of individual metabolites revealed that induction of senescence in young control cells decreased the ratio of carbon-nitrogen shuttle amino acids such as alanine (Ala) and glutamate (Glu) relative to metabolites involved in anabolic (e.g., glutamine, Gln) or immunometabolic (e.g., kynurenine, Kyn) pathways. In contrast, old and CMA-deficient cells displayed the opposite trend, with increased ratios upon senescence induction (**Extended Data Fig. 4e**). Old and CMA-deficient cells also had lower ATP/choline ratios, indicative of lower energy availability and of aberrant phospholipid breakdown (**Extended Data Fig. 4f**) and displayed opposite changes in NAM/NAD and in carbohydrate metabolism to those observed in young cells (**Extended Data Fig. 4g**).

Supplementary Note 3: Impact of CMA loss on the extracellular metabolome.

We used mass spectrometry to compare the extracellular metabolomes of CMA-competent and CMA-deficient cells. Under basal conditions, L2A^{KO} cells displayed a strikingly distinct metabolic secretory profile with 44 metabolites increased and 3 (glutathione, kynurenine, and salicylate glucuronide) decreased (**Extended Data Fig. 6c-f**). Senescence induction caused only minor changes in control cells (19 changing metabolites, but only β -alanine reached >1.5-fold increase) (**Extended Data Fig. 6e**). In contrast, senescent L2A^{KO} cells displayed significant alterations in 95 metabolites, with 40 showing >1.5-fold increases and 15 showing >0.5-fold reductions (**Extended Data Fig. 6e**; top 10 changing metabolites are shown in **Extended Data Fig. 6f**).

Comparison of the changes in extracellular metabolites in fibroblasts from old mice relative to young mice revealed that 69 metabolites changed in the same direction as in L2A^{KO} fibroblasts (**Extended Data Fig. 6g**). However, old senescent cells lacked the pronounced metabolite upregulation seen in senescent L2A^{KO} cells (**Extended Data Fig. 6g**), suggesting that CMA decline accounts for only part of age-related metabolic changes.

Notably, over half of the metabolites altered in both L2A^{KO} and aged cells regulate immune responses, particularly macrophage function (**Extended Data Fig. 6h**). As summarized in Supplementary Table 1, L2A^{KO} media was enriched in pro-inflammatory metabolites that promote cytokine and inflammatory signal amplification and skew macrophages toward an M1-like phenotype (e.g., eicosanoid chemoattractants such as 12-HETE and 5-HETE, polyamines, arachidonic and quinolinic acid, branch chain amino acids (BCAAs), and disease-associated microglia (DAM)-like molecules such as xanthine), alongside reduced levels of anti-inflammatory metabolites such as pyridoxine and NF- κ B signaling inhibitors (**Extended Data Fig. 6h**). Some anti-inflammatory molecules (e.g., bile acids, omega-3 PUFAs) were paradoxically increased.

Senescence further enhanced pro-inflammatory metabolites, including glycolytic intermediates that reinforce inflammatory metabolism, while some anti-inflammatory molecules that were reduced under basal conditions were now elevated (**Extended Data Fig. 6h**). Overall, CMA-deficient cells release metabolites that sustain pro-inflammatory signaling and contribute aberrant inflammatory cues to the immune system upon senescence induction.

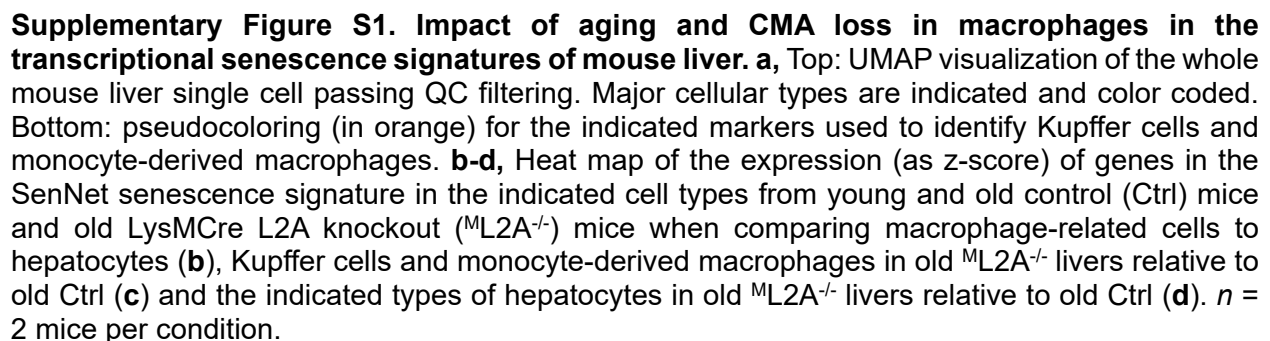
Supplementary Note 4: Characteristics of the selective CMA activator CA77.1.

The first-in-class small molecule CMA activators, including CA77.1, were designed to selectively activate the CMA transcriptional program controlled by retinoic acid receptor (RAR α)⁸ without affecting other cellular transcripts under RAR α regulation. This selectivity, attained by targeting the interaction of RAR α with its co-repressor NCoR1⁹, allows for their long-term administration without noticeable toxic effects³. CA77.1 (CA) This small molecule has been extensively used in mice *in vivo*, where it has proven to activate CMA in peripheral tissues and the brain, as it can cross the blood-brain barrier^{3, 9}. CA has not shown toxicity upon 6 months of daily administration at the concentration used in this work^{3, 9}, at which it has proven effective in restoring functionality of old hematopoietic stem cells¹⁰, decreasing pathology in Alzheimer's mice³, reducing age-related seizure propensity¹¹, improving brown-adipose tissue thermogenesis¹² and boosting T cell function in aged mice¹³.

References (for supplementary notes)

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Supplementary Table S1. Extracellular metabolome of CMA-defective cells

	WT	WT-P	L2AKO	L2AKO-P	O-WT	OW-P	pro-inflamm anti-inflamm	
Xanthine								Oxidant/urate-axis DAMP; tends to promote inflammation.
2-hydroxyglutaric acid								Effects vary by concentration/cell state.
12-HETE;5-HETE								Eicosanoid chemoattractants; boost cytokines/ROS.
3-hydroxyglutaric acid								TCA-reinforce inflammatory metabolism/signaling.
Arachidonic acid								Substrate for prostaglandins/leukotrienes.
Galact;Gluc;Mann;Fruct								Glycolytic reprogramming
Quinolinic acid								Inflammatory Trp catabolite; amplifies cytokines.
Ketoisocaproic acid								BCAAs: nutrient-rich M1 bias.
Isoleucine Leucine								BCAAs: nutrient-rich M1 bias.
Betaine;Valine;Norvaline								BCAAs: nutrient-rich M1 bias.
Histidine								promotes inflammatory signaling
Valine								BCAAs: nutrient-rich M1 bias.
Putrescine								Polyamines can enhance inflammatory programs (dose/context sensitive).
2-oxoglutaric acid								TCA-reinforce inflammatory metabolism/signaling.
Citric acid								TCA-reinforce inflammatory metabolism/signaling.
N1-acetylspermidine								Polyamines can enhance inflammatory programs (dose/context sensitive).
Isocitric acid								TCA-reinforce inflammatory metabolism/signaling.
cis-Aconitic acid								TCA-reinforce inflammatory metabolism/signaling.
Glyceraldehyde-3-phosphate								TCA-reinforce inflammatory metabolism/signaling.
N-acetylputrescine								Polyamines can enhance inflammatory programs (dose/context sensitive).
S-adenosylhomocysteine								Methylation brake; pro-inflammatory epigenetic tone.
Malic acid								TCA-reinforce inflammatory metabolism/signaling.
Glutathione								Can act as a DAMP (pro-inflammatory) but redox buffering may be protective at low concentrations.
PCae(16:0)								Intact PCs vs oxidized lysoPCs have opposite effects (anti- vs pro-inflammatory).
PCae(18:0)								Intact PCs vs oxidized lysoPCs have opposite effects (anti- vs pro-inflammatory).
PCee(16:0)								Intact PCs vs oxidized lysoPCs have opposite effects (anti- vs pro-inflammatory).
Malonic acid								Chronic exposure tends to pro-inflammatory
Ascorbic acid								Antioxidant/anti-inflammatory effects on macrophages
Hyochoic acid								Bile acids: suppress cytokines
Inosine								Adenosine-receptor signaling → dampens TNF/IL-6.
Docosahexaenoic acid								Omega-3/PUFAs: inhibit NF-κB, foster resolution
Docosapentaenoic acid								Omega-3/PUFAs: inhibit NF-κB, foster resolution
Eicosatrienoic acid								Omega-3/PUFAs: inhibit NF-κB, foster resolution
5-hydroxytryptophan								AhR/serotonergic axes; immunoregulatory
Taurolithocholic acid								Bile acids: suppress cytokines
Docosatetraenoic acid								Omega-3/PUFAs: inhibit NF-κB, foster resolution
3-hydroxybutyric acid								inhibits NLRP3 inflammasome

Glycochenodeoxycholic acid						
Taurochenodeoxycholic acid						
Riboflavin						
Indole-3-carboxyaldehyde						
Tryptophan						
Cholic acid						
Indole-3-acrylic acid						
Ornithine						
Methionine						
Niacinamide						
Phenylethanolamine						
Pyridoxine						
1-methylnicotinamide						
Phenyllactic acid						
Phenylacetyl glycine						
Proline						
4-hydroxyphenylacetic acid						
Beta-alanine						

Bile acids: suppress cytokines

Bile acids: suppress cytokines

antioxidant/anti-inflammatory effects on macrophages

AhR activation → tolerizing programs

AhR/serotonergic axes; immunoregulatory

Bile acids: suppress cytokines

AhR activation → tolerizing programs

Pro-repair/epigenetic support of M2 programs (Arg1/SAM/collagen remodeling).

Pro-repair/epigenetic support of M2 programs (Arg1/SAM/collagen remodeling).

Raise NAD⁺/pro-resolving, lower inflammasome activity

Suppresses pro-inflammatory cytokines.

Antioxidant/anti-inflammatory effects on macrophages

Raise NAD⁺/pro-resolving, lower inflammasome activity

Dampen NF-κB.

Dampen NF-κB.

Pro-repair/epigenetic support of M2 programs (Arg1/SAM/collagen remodeling).

Dampen NF-κB.

Reduces oxidative/inflammatory outputs

Supplementary Table S2. Sources of antibodies and dilutions used in this study.

Primary Antibodies		
Goat anti-CD47	1:1000 IB	ThermoFisher PA5-47215
Rat anti-CD68	1:1000 IF	Invitrogen MA5-16674
Rabbit anti-Collagen3 α 1	1:500 IF	Novus NB600-594SS
Rabbit anti-Dendra-2 (for fibroblasts and macrophages)	1:1000 IF	abin361314
Mouse anti-Dendra-2 (for lung)	1:500 IF	Origene TA180094
Rat anti-F4/80	1:000 IF	Abcam ab6640
Rabbit anti-CD4	1:500 IHC	Abcam ab183685
Rabbit anti- CD8 α	1:100 IHC	Cell Signaling 98941T
Rabbit anti- Foxp3	1:100 IHC	Invitrogen PA146126
Rabbit anti-Glucocerebrosidase	1:100 IB	Sigma G4171
Rabbit anti-Histone H2A.X	1:1000 IB	Cell Signaling 2595s
Rabbit anti-HMGB1	1:1000 IF, 1:5000 IB	Abcam ab18256
Rabbit anti-iNOS	1:500 IF, 1:1000 IB	Abcam ab15323
Rabbit anti-Ki67	1:1000 IF	Abcam ab16667
Rat anti-LAMP1	1:1000 IF, 1:1000 IB	Hybridoma Bank 1D4B
Rabbit anti-LAMP-2A	1:1000 IB	Invitrogen 512200
Rabbit anti-LC3	1:1000 IB	Cell Signaling 2775
Rabbit anti-LC3B (for macrophages)	1:1000 IB	Sigma-Aldrich L7543
Rabbit anti-p16 ^{INKA}	1:500 IB	Cell Signaling D7C1M
Mouse anti-p16 ^{INKA}	1:1000 IB	ThermoFisher MA5-17142
Rabbit anti-p21	1:1000 IF, 1:1000 IB	Abcam ab109199
Rabbit anti-p53-rodent specific	1:000 IB	Cell Signaling D2H90
Rabbit anti-p62/SQSTM1	1:1000 IB	Abcam ab155686
Rabbit anti-PAI1/Serpine1	1:1000 IB	Novus Biologicals nbp1-19773
Rabbit anti-Phalloidin (conjugated AF647)	1:500 IF	ThermoFisher A22287
Rabbit anti-Sirp α	1:500 IF, 1:1000 IB	Abcam ab259357
Rabbit anti-SHP-1	1:500 IF, 1:1000 IB	ThermoFisher Ma5-31989
Chicken anti-Vimentin	1:1000 IF	ThermoFisher PA1-16759
Secondary Antibodies		
Anti-rabbit Alexa Fluor 488	1:500 IF	ThermoFisher A11008
Anti-mouse Alexa Fluor 555	1:500 IF	ThermoFisher A32727
Anti-Mouse IgM Alexa Fluor 488	1:500 IF	ThermoFisher A21042
Anti-rat Alexa Fluor 555	1:500 IF	ThermoFisher A21434
Anti-rat Alexa Fluor 488	1:500 IF	ThermoFisher A21470
Anti-rat Alexa Fluor 647	1:500 IF	ThermoFisher A21247
Anti-mouse Alexa Fluor 647	1:500 IF	ThermoFisher A21242
Anti-rat HRP	1:5000 IB	ThermoFisher 31470
Anti-rabbit HRP	1:5000 IB	ThermoFisher 31460
Anti-mouse HRP	1:5000 IB	ThermoFisher 31430

Flow-cytometry Antibodies		
Mouse anti- CD11b Alexa Fluor 700	1:500	Invitrogen
Mouse anti- CD11c Brilliant Violet 650	1:500	Biolegend
Mouse anti- CD19 PE-Cyanine5.5	1:700	Invitrogen
Mouse anti- CD24 Pacific Blue	1:500	Biolegend
Mouse anti- CD3 PerCP/Cyanine 5.5	1:500	Biolegend
Mouse anti- CD45 Brilliant Violet 605	1:700	Biolegend
Mouse anti- CD64 PE	1:500	Biolegend
Mouse anti- CD170 Brilliant Violet 711	1:500	Biolegend
Mouse anti- F4/80 FITC	1:500	Biolegend
Mouse anti- F4/80 Spark UV 387 (for efferocytosis assay)	1:500	Biolegend
Mouse anti- I-A/I-E APC/Fire/750	1:500	Biolegend
Mouse anti- Ly-6c Alexa Fluor 647	1:800	Biolegend
Mouse anti- Ly-6g Spark UV 387	1:800	Biolegend
Mouse anti- NKp46 Brilliant Violet 421	1:500	Biolegend

Supplementary Table S3. Source and sequences of primers used in this study.

Oligonucleotides	
Forward primer 18s GTAACCCGTTGAACCCCAT	Integrated DNA Technologies
Reverse primer 18s CATCCAATCGGTAGTAGCG	Integrated DNA Technologies
Forward primer Akt1 ATCCCCTCCAACAACCTTCTCAGT	Integrated DNA Technologies
Reverse primer Akt1 CCTTCCCGTCCACTCTTCTCTTTC	Integrated DNA Technologies
Forward primer Akt2 ACGTGGTGAATACATCAAGACC	Integrated DNA Technologies
Reverse primer Akt2 GGGCCTCTCCTTATACCCAAT	Integrated DNA Technologies
Forward primer β -Actin AAGGACTCCTATAGTGGGTGACGA	Integrated DNA Technologies
Reverse primer β -Actin ATCTTCTCCATGTGCTCCAGTTG	Integrated DNA Technologies
Forward primer B2M CACTGAATTCACCCCCACTGA	Integrated DNA Technologies
Reverse primer B2M GGGCCTCTCCTTATACCCAAT	Integrated DNA Technologies
Forward primer Cathepsin A CAGCCCTCTTTCCGGCAATA	Integrated DNA Technologies
Reverse primer Cathepsin A TTTGGGTCGTTCTGCGACTC	Integrated DNA Technologies
Forward primer Cdkn1a CGGTGTCAGAGTCTAGGGGA	Integrated DNA Technologies
Reverse primer Cdkn1a GCCTGTGGCTCTGAATGTCT	Integrated DNA Technologies
Forward primer Col1 α 1 GCTCCTCTTAGGGGCCACT	Integrated DNA Technologies
Reverse primer Col1 α 1 ATTGGGGACCCCTTAGGCCAT	Integrated DNA Technologies
Forward primer Col3 α 1 CTGTAAACATGGAACTGGGGAA	Integrated DNA Technologies
Reverse primer Col3 α 1 CCATAGCTGAACTGAAAACCACC	Integrated DNA Technologies
Forward primer Dendra AAGGGCATCTGCACCATCCG	Integrated DNA Technologies
Reverse primer Dendra CGTGCTCGTACAGCTTCACCTTG	Integrated DNA Technologies
Forward primer Dnajb1 TTCGACCGCTATGGAGAGGAA	Integrated DNA Technologies
Reverse primer Dnajb1 CACCGAAGAACTCAGCAAACA	Integrated DNA Technologies
Forward primer Eef1a1 GAAGATCGGCTACAACCCAG	Integrated DNA Technologies
Forward primer Gfap CGGAGACGCATCACCTCTG	Integrated DNA Technologies
Reverse primer Gfap TGGAGGAGTCATTCGAGACAA	Integrated DNA Technologies
Forward primer Hspa8 TCTCGGCACCACCTACTCC	Integrated DNA Technologies
Reverse primer Hsp8a CCCGCATCAGACGTTTGGCA	Integrated DNA Technologies
Forward primer Hsp90aa1 GACGCTCTGGATAAAATCCGTT	Integrated DNA Technologies
Reverse primer Hsp90aa1 TGGAATGAGATTGATGTGCAG	Integrated DNA Technologies
Forward primer Hsp90ab1 AAACAAGGAGATTTTCTCCGC	Integrated DNA Technologies
Forward primer II-1 α AGGGAGTCAACTCATTGGCG	Integrated DNA Technologies
Reverse primer II-1 α TGGCAGAACTGTAGTCTTCGT	Integrated DNA Technologies
Forward primer II-1 β TGCCACCTTTTGACAGTGATG	Integrated DNA Technologies
Reverse primer II-1 β CGAGATTTGATGTGCTGCTG	Integrated DNA Technologies
Forward primer II-6 CACGGCCTTCCCTACTTCAC	Integrated DNA Technologies
Reverse primer II-6 CTGCAAGTGCATCATCGTTGT	Integrated DNA Technologies
Forward primer Lamp1 TAGTGCCACATTACGCATCTCCA	Integrated DNA Technologies
Reverse primer Lamp1 TTCCACAGACCCAAACCTGTCACT	Integrated DNA Technologies
Forward primer Lamp2a GCAGTGCAGATGAAGACAAC	Integrated DNA Technologies
Reverse primer Lamp2a AGTATGATGGCGCTTGAGAC	Integrated DNA Technologies
Forward primer Mmp10 TGTTGCTCTTCAGTATGTGTGT	Integrated DNA Technologies
Reverse primer Mmp10 CCAGGAATTGAGCCACAAGT	Integrated DNA Technologies

Forward primer Mmp12 GCTCCTGCCTCACATCATAC	Integrated DNA Technologies
Reverse primer Mmp12 GGCTTCTCTGCATCTGTGAA	Integrated DNA Technologies
Forward primer Ncor1 CCCATTTCCACCGTGTTAGTG	Integrated DNA Technologies
Reverse primer Ncor1 AAGGTGAAGCATTTTGGTCATCT	Integrated DNA Technologies
Forward primer Nfatc1 GGAGAGTCCGAGAATCGAGAT	Integrated DNA Technologies
Reverse primer Nfatc1 TTGCAGCTAGGAAGTACGTCT	Integrated DNA Technologies
Forward primer Nfe2i2 CTTTAGTCAGCGACAGAAGGAC	Integrated DNA Technologies
Reverse primer Nfe2i2 AGGCATCTTGTTTGGGAATGTG	Integrated DNA Technologies
Forward primer p16 ^{INKA} CTCTGCTCTTGGGATTGGC	Integrated DNA Technologies
Reverse primer p16 ^{INKA} GTGCGATATTTGCGTTCCG	Integrated DNA Technologies
Forward primer p53 ATGGCCATCTACAAGAAGTCACAG	Integrated DNA Technologies
Reverse primer p53 ATCGGAGCAGCGCTCATG	Integrated DNA Technologies
Forward primer Phlpp1 AGCTTCCCGGCAAGTA AAAT	Integrated DNA Technologies
Reverse primer Phlpp1 GGTGGCAGGTTTTCTGGTAA	Integrated DNA Technologies
Forward primer Rab11a AGGAGCGGTACAGGGCTATAA AAAT	Integrated DNA Technologies
Reverse primer Rab11a ATGTGAGATGCTTAGCAATGTCA	Integrated DNA Technologies
Forward primer Rara CTAGCCGATTTGCACGGATG	Integrated DNA Technologies
Reverse primer Rara GGGTCTCGATGGAGTGGTTT	Integrated DNA Technologies
Forward primer Rac1 ACGGAGCTGTTGGTAAACCT	Integrated DNA Technologies
Reverse primer Rac1 AGACGGTGGGGATGTACTCTC	Integrated DNA Technologies
Forward primer Rictor GCTGCGCTATCTCATCCAAGA	Integrated DNA Technologies
Reverse primer Rictor GGGTTCTGAAGTGCTAGTTCAC	Integrated DNA Technologies
Forward primer Serpine1 CGTGTCAGCTCGTCTACAG	Integrated DNA Technologies
Reverse primer Serpine1 CTATGGTGAAACAGGTGGACT	Integrated DNA Technologies
Forward primer Tbp GAAGCTGCGGTACAATTCCAG	Integrated DNA Technologies
Reverse primer Tbp CCCCTTGTAACCTTCAACCAAT	Integrated DNA Technologies
Forward primer Tgfβ CCACCTGCAAGACCATCGAC	Integrated DNA Technologies
Reverse primer Tgfβ CTGGCGAGCCTTAGTTTGGAC	Integrated DNA Technologies
Forward primer Tnfa AGCCGATGGGTTGTACCTTG	Integrated DNA Technologies
Reverse primer Tnfa GCAGCCTTGTCCCTTGAAGA	Integrated DNA Technologies