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(54) Title: USE OF CHAPERONE-MEDIATED AUTOPHAGY ACTIVATORS FOR TREATING OR PREVENTING BLOOD CANCERS AND MYELODYSPLASTIC SYNDROMES

(57) Abstract: The disclosure provides a method of increasing the activity of a population of hematopoietic (blood-forming) stem cells (HSCs) in vivo or in vitro, the method comprising contacting the population of HSCs with a sufficient concentration of a Chaperone-mediated Autophagy (CMA) activating compound to increase the number of blood cells produced by the HSCs in a time period relative to the number of blood cells produced by the HSCs in the same time period prior to being contacted with the CMA.



**USE OF CHAPERONE-MEDIATED AUTOPHAGY ACTIVATORS FOR
TREATING OR PREVENTING BLOOD CANCERS AND MYELODYSPLASTIC
SYNDROMES**

STATEMENT OF GOVERNMENT SUPPORT

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BACKGROUND

[0001] Hematopoietic (blood-forming) stem cells (HSCs) residing in bone marrow produce all mammalian blood cells, including key immune cells that provide protection from bacteria and viruses. As mammals age, HSCs become less efficient and less able to make healthy new blood cells. Applicants have found that this reduction in HSC efficiency is caused in part by the deterioration of chaperone-mediated autophagy (CMA), the housekeeping process that removes damaged proteins and other waste materials that interfere with cells' ability to function.

SUMMARY

[0002] The disclosure provides a method of increasing the activity of a population of hematopoietic (blood-forming) stem cells (HSCs) in vivo or in vitro, the method comprising contacting the population of HSCs with a sufficient concentration of a Chaperone-mediated Autophagy (CMA) activating compound to increase the number of blood cells produced by the HSCs in a time period relative to the number of blood cells produced by the HSCs in the same time period prior to being contacted with the CMA. The disclosure also includes a method of improving the proteostasis of a population of hematopoietic (blood-forming) stem cells (HSCs) in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a Chaperone-mediated Autophagy (CMA) activating compound to sufficient to improve a marker of proteostasis in the population of HSCs relative to the marker of proteostasis in the population of HSCs prior to being contacted with the CMA.

BRIEF DESCRIPTION OF THE DRAWINGS

[0003] FIGURE 1. CMA activity is required for HSC self-renewal. FIG. 1A, B, Dendra fluorescence in sorted HSC from 4m, 12m and 30m old KFERQ-Dendra mice (FIG. 1A) and HSC and myeloid progenitors (Lin[−] cKit⁺Sca-1[−]) from KFERQ-Dendra mice before and at the indicated days after single injection of 5-fluorouracil (5FU) (FIG. 1B). *Right*: Quantification of Dendra⁺ puncta per cell in n=9 fields from 4 (FIG. 1A) or 5 (FIG. 1B) individual mice. FIG. 1C, HSC frequency in BM from Ctrl and L2AKO mice untreated (Basal) or 8 days after a single 5FU injection. n=6 mice. FIG. 1D, Survival curve of Ctrl and L2AKO mice after serial injections of 5FU 7 days apart. n=5 (Ctrl) and 4 (L2AKO) mice. FIG. 1E, White blood cell counts 7 days after first and second 5FU injection. n=8,10,7,6 mice, respectively. FIG. 1F-H, Serial transplantation and competitive BM repopulation with Ctrl or L2AKO BM cells: experimental strategy (FIG. 1F); frequency of donor-derived HSC 16 weeks after the first competitive transplantation n=5 mice (FIG. 1G) and donor cell (from 3-4m CD45.2 mice) contribution in recipients' peripheral blood after competitive secondary transplantation. n=7 mice (FIG. 1H). FIG. 1I, Number of colonies at day 10 after the indicated plating of serial colony formation assay with HSC from Ctrl or L2AKO mice. n=6 (1st plating) and 4 (2nd and 3rd plating) mice. FIG. 1J, Fold change relative to Ctrl of LTC-IC frequency of LSK cells from Ctrl and L2AKO mice after 4 weeks of culture. n=3 mice. Data shows individual values and mean $\pm$ SEM. One-way ANOVA with Tukey's multiple comparison post-hoc test (FIG. 1A,B), two-way ANOVA with Sidak's multiple comparison post-hoc test (FIG. 1E,H,I), Log-rank (Mantel-Cox) test (FIG. 1D) and unpaired two tailed t-test (FIG. 1C,G) were used. P values are shown and statistical source data are provided. ns: no statistical significance.

[0004] FIGURE 2. Consequences of CMA blockage on HSC function. FIG. 2A-C, Cycling cells (a), ATP levels (b) and median fluorescence intensity (MFI) of ROS (FIG. 2C) in HSC from control (Ctrl) and L2AKO mice untreated (basal) or at day 8 after 5FU injection (5FU day8), n= 7,5,15 (basal) and 6,4,5 (5FU) mice in FIG. 2A-C, respectively. FIG. 2D, ROS levels in BM-derived HSC from transplanted recipients. n=5 mice. FIG. 2E,F, HSC gene expression (FIG. 2E) and enrichment pathway analysis (FIG. 2F). FIG. 2G,H, STRING analysis (FIG. 2G) and Venn diagram (FIG. 2H) of proteins decreased in Ctrl or accumulated in L2AKO LSK post-5FU, n=3 independent experiments (ie) (3 mice per experiment). FIG. 2I-K, LSK metabolic phenotypes: unsupervised principal component analysis (i), hierarchical clustering of top 25 significant metabolites (FIG. 2J) and Omicsnet analysis of top affected pathways in L2AKO cells (FIG. 2K), n=3 ie (3 mice pool per experiment). FIG. 2L,

Extracellular acidification rates (ECAR) of LSK cells upon glucose (Glu), oligomycin (Oligo) and 2-Deoxy-D-glucose (2DG) addition, n=3 ie. FIG. 2M, LSK cells basal glycolysis, glycolytic capacity and glycolytic reserve, n=3 ie. FIG. 2N, GAPDH and pyruvate kinase (PK) activity in LSK cells, n=3 ie. FIG. 2O, Percentage of oxidized GAPDH and PK in LSK cells. n=3 ie. FIG. 2P, Percentage of total cellular proteins oxidized and carbonylated peptides number in LSK cells. n=3 ie. FIG. 2Q,R Oxidized proteins (FIG. 2Q) and protein inclusions (FIG. 2R) in resting HSC. n=15 fields from 5 mice. Data shows individual values and mean $\pm$ SEM. Two-way ANOVA with Sidak's (FIG. 2A-C) or Tukey's (FIG. 2P) multiple comparison post-hoc test, Chi-square test (FIG. 2K), multiple time point paired t-test (FIG. 2L) and unpaired two tailed t-test (FIG. 2D, M-O, Q, R) were used. P values are shown and statistical source data are provided.

[0005] FIGURE 3. CMA increases linoleic acid metabolism upon HSC activation. FIG. 3A, Top lipid metabolism pathways different between control (Ctrl) and L2AKO LSK cells 8 days post-5FU. FIG. 3B-D, Abundance (FIG. 3B) and relative changes in levels (FIG. 3D) of substrate and metabolite in the linoleic acid and α -linolenic pathways (shown in FIG. 3C) in LSK cells, n=3 independent experiments (ie). FIG. 3E,F, Effect of FADS2 inhibitor SC-26196 (FIG. 3E) or γ -linolenic acid (GLA) (FIG. 3F) in total (left) and GEMM (right) colony formation from HSC, n=3 (FIG. 3E) and 4 (FIG. 3F) ie. FIG. 3G, Predicted FADS2 acetylation-generated CMA-targeting motif (green) and its acetylation levels in LSK cells, n=9 mice in 3 ie. FIG. 3H,I, Representative immunoblot (3 repeats) of Ctrl (FIG. 3H) and L2AKO (FIG. 3I) HSC expressing wild-type (VIDRK) or mutant (VIDRQ or VIDRA) Flag-tagged FADS2. N/L: NH₄Cl/Leupeptin. FIG. 3J, Oxygen consumption rate (OCR) of LSK cells post-5FU, n=5 ie. FIG. 3K, Mitochondrial fatty acid β -oxidation in LSK cells, n=5 ie. FIG. 3L, Effect of methyl-pyruvate on colony formation from HSC, n=7 (None) and 4 (Methyl-pyruvate) mice. FIG. 3M, Effect of NAC on ROS levels (left) and colony formation from HSC 388 (right), n=7 (None) and 4 (NAC) mice. FIG. 3N,O, Ratio γ -linolenic acid (GLA) to linoleic acid (LA) (n) and acetylated to total K42FADS2 peptide (o) in young and old mice LSK cells, n=3 ie. FIG. 3P, Effect of GLA on total (left) and GEMM (right) colony formation from old mice HSC. Top: Representative images, n=5 mice. FIG. 3Q, Effect of GLA on absolute (left) and relative (right) frequency of GEMM colonies from human CD34+ cells, n=3 patients. Data shows individual values and mean $\pm$ SEM. Two-way ANOVA with Tukey's (FIG. 3D,F) or Sidak (FIG. 3E,L,M) multiple comparison test, unpaired two-tailed t-tests (FIG. 3G,K,N,O,P), time point-paired two-tailed t-test (FIG. 3J), and Chi-square test (FIG. 3Q) were used. P values are shown and statistical source data are provided.

[0006] FIGURE 4. Modulation of CMA restores old HSC function. FIG. 4A-G, Comparison of young and old Ctrl mice and hL2AOE mice: (FIG. 4A) Percentage of BM HSC, n= 5,18,13 mice per group, (FIG. 4B,C) HSC ROS levels (representative FACS in FIG. 4B), n=5 mice, (FIG. 4D) PK activity, (FIG. 4E) GAPDH activity and (FIG. 4F) extracellular acidification rates (ECAR) in LSK cells, n=3 mice (Oligo: oligomycin, 2DG: 2-deoxy-D-glucose) and (FIG. 4G) FADS2-generated fatty acids, n= 9,18,9 mice. FIG. 4H. Donor derived cells in mice transplanted with old Ctrl and hL2AOE mice BM, n=5 mice. FIG. 4I-L, Effect of *in vivo* administration of CMA activator (CA) to old Ctrl mice in HSC oxidized proteins, n=12 fields from 4 mice (i), LSK cells GAPDH activity, n=3 mice (FIG. 4J), ECAR, n=4 mice (FIG. 4K) and LTC-IC frequency, n=4 mice (FIG. 4L). (Veh.: vehicle). FIG. 4M-O, Effect of CA treatment of old mice LSK cells on (FIG. 4M) absolute (left) and relative (right) LTC-IC frequency, (FIG. 4N) cell viability and (FIG. 4O) Giemsa staining of cells from formed colonies, n=3 mice. FIG. 4P,Q. Effect of CA on GEMM (FIG. 4P) and total (FIG. 4Q) colonies from human CD34+ cells. n=3 patients (59, 65 and 71 years old). Data shows individual values and mean $\pm$ SEM. One-way ANOVA with Tukey's (FIG. 4A, C-E, G), or Dunnett's (FIG. 4J) post-hoc test, time points paired two tailed t-test (FIG. 4F,K), two-way ANOVA with Sidak's post-hoc test (FIG. 4H), unpaired two tailed t-test (FIG. 4I,M right, FIG. 4N, P, Q) and Chi-square test (FIG. 4L,M left) were used. P values are shown and statistical source data are provided. ns: no statistical significance.

DETAILED DESCRIPTION

TERMINOLOGY

[0007] Prior to setting forth the invention in detail, it may be helpful to provide definitions of certain terms to be used in this disclosure. Compounds are described using standard nomenclature. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. Unless clearly contraindicated by the context each compound name includes the free acid or free base form of the compound as well as all pharmaceutically acceptable salts of the compound.

[0008] The term "compounds of Formula I" encompasses all compounds that satisfy Formula I, including any enantiomers, racemates and stereoisomers, as well as all pharmaceutically acceptable salts of such compounds. A dash ("-") that is not between two letters or symbols is used to indicate a point of attachment for a substituent.

[0009] The terms “a” and “an” do not denote a limitation of quantity, but rather denote the presence of at least one of the referenced item. The term “or” means “and/or”. The open-ended transitional phrase “comprising” encompasses the intermediate transitional phrase “consisting essentially of” and the close-ended phrase “consisting of.” Claims reciting one of these three transitional phrases, or with an alternate transitional phrase such as “containing” or “including” can be written with any other transitional phrase unless clearly precluded by the context or art. Recitation of ranges of values are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. The endpoints of all ranges are included within the range and independently combinable. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”), is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention as used herein. Unless defined otherwise, technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs.

[0010] A dash (“-”) that is not between two letters or symbols is used to indicate a point of attachment for a substituent. For example, $-(C=O)OH$ is attached through carbon of the keto ($C=O$) group.

[0011] A bond represented by a combination of a solid and dashed line, i.e., ----- , may be either a single or double bond.

[0012] “Alkyl” is a branched or straight chain or cyclic saturated aliphatic hydrocarbon group, having the specified number of carbon atoms, generally from 1 to about 8 carbon atoms. The term C_1 - C_6 alkyl as used herein indicates an alkyl group having from 1, 2, 3, 4, 5, or 6 carbon atoms. Other embodiments include alkyl groups having from 1 to 8 carbon atoms, 1 to 4 carbon atoms or 1 or 2 carbon atoms, e.g. C_1 - C_4 alkyl and C_1 - C_2 alkyl. When C_0 - C_n alkyl is used herein in conjunction with another group, for example, $-C_0$ - C_2 alkyl(phenyl), the indicated group, in this case phenyl, is either directly bound by a single covalent bond (C_0 alkyl), or attached by an alkyl chain having the specified number of carbon atoms, in this case 1, 2, 3, or 4 carbon atoms. Alkyls can also be attached via other groups such as heteroatoms as in $-O-C_0$ - C_4 alkyl(C_3 - C_7 cycloalkyl). Examples of alkyl include, but are not limited to, methyl, ethyl, n-

propyl, isopropyl, cyclopropyl, cyclopropylmethyl, cyclopropylethyl, n-butyl, cyclobutyl, 3-methylbutyl, t-butyl, cyclobutyl methyl, n-pentyl, and sec-pentyl.

[0013] “Alkoxy” is an alkyl group as defined above with the indicated number of carbon atoms covalently bound to the group it substitutes by an oxygen bridge (-O-). Examples of alkoxy include, but are not limited to, methoxy, ethoxy, n-propoxy, i-propoxy, cyclopropyloxy, cyclopropylmethoxy, n-butoxy, 2-butoxy, t-butoxy, n-pentoxo, 2-pentoxo, 3-pentoxo, isopentoxo, neopentoxo, n-hexoxy, 2-hexoxy, 3-hexoxy, and 3- methylpentoxo.

[0014] “Aryl” indicates aromatic groups containing only carbon in the aromatic ring or rings. Typical aryl groups contain 1 to 3 separate, fused, or pendant rings and from 6 to about 18 ring atoms, without heteroatoms as ring members. When indicated, such aryl groups may be further substituted with carbon or non-carbon atoms or groups. Aryl groups include, for example, phenyl, naphthyl, including 1- naphthyl, 2-naphthyl, and bi-phenyl.

[0015] “Cycloalkyl” is a saturated hydrocarbon ring group, having the specified number of carbon atoms. Monocyclic cycloalkyl groups typically have from 3 to about 7 (3, 4, 5, 6, or 7) carbon ring atoms. Cycloalkyl substituents may be pendant from a substituted nitrogen, sulfur, oxygen or carbon atom, or a substituted carbon atom that may have two substituents may have a cycloalkyl group, which is attached as a spiro group. Examples of cycloalkyl groups include cyclopropyl, cyclobutyl, cyclopentyl, or cyclohexyl as well as bridged or caged saturated ring groups such as norbornane or adamantane.

[0016] “Haloalkyl” includes both branched and straight-chain alkyl groups having the specified number of carbon atoms, substituted with 1 or more halogen atoms, up to the maximum allowable number of halogen atoms. Examples of haloalkyl include, but are not limited to, trifluoromethyl, difluoromethyl, 2-fluoroethyl, and pentafluoroethyl.

[0017] “Haloalkoxy” is a haloalkyl group as defined herein attached through an oxygen bridge (oxygen of an alcohol radical).

[0018] “Halo” or “halogen” indicates any of fluoro, chloro, bromo, and iodo.

[0019] “Heteroaryl” is a stable monocyclic aromatic ring having the indicated number of ring atoms which contains from 1 to 4, or in some embodiments from 1 to 2, heteroatoms chosen from N, O, and S, with remaining ring atoms being carbon, or a stable bicyclic system containing at least one 5- to 7-membered aromatic ring which contains from 1 to 4, or in some embodiments from 1 to 2, heteroatoms chosen from N, O, and S, with remaining ring atoms being carbon. Monocyclic heteroaryl groups typically have from 5 to 7 ring atoms. In certain embodiments the heteroaryl group is a 5- or 6-membered heteroaryl group having 1, 2, 3, or 4 heteroatoms chosen from N, O, and S, with no more than 2 O atoms and 1 S atom.

[0020] The term “substituted,” as used herein, means that any one or more hydrogens on the designated atom or group is replaced with a selection from the indicated group, provided that the designated atom's normal valence is not exceeded. When the substituent is oxo (i.e., =O) then 2 hydrogens on the atom are replaced. When an oxo group substitutes aromatic moieties, the corresponding partially unsaturated ring replaces the aromatic ring. For example a pyridyl group substituted by oxo is a pyridone. Combinations of substituents and/or variables are permissible only if such combinations result in stable compounds or useful synthetic intermediates. A stable compound or stable structure is meant to imply a compound that is sufficiently robust to survive isolation from a reaction mixture, and subsequent formulation into an effective therapeutic agent. Unless otherwise specified substituents are named into the core structure. For example, it is to be understood that when aminoalkyl is listed as a possible substituent the point of attachment of this substituent to the core structure is in the alkyl portion.

[0021] In certain embodiments, groups that may be “substituted” or “optionally substituted” include, but are not limited to: monocyclic aryl, e.g., phenyl; monocyclic heteroaryl, e.g., pyrrolyl, pyrazolyl, thienyl, furanyl, imidazolyl, thiazolyl, triazolyl, pyridyl, pyrimidinyl; bicyclic heteroaryl, e.g., benzimidazolyl, imidazopyridizynyl, indolyl, indazolyl, quinoliny, isoquinoliny; and C₁-C₆alkyl in which any carbon-carbon single bond is optionally replaced by a carbon-carbon double or triple bond, any methylene group is optionally replaced by O, S, or NR¹².

[0022] Suitable groups that may be present on a “substituted” or “optionally substituted” position include, but are not limited to: halogen; cyano; CHO; COOH; hydroxyl; oxo; amino; alkyl groups from 1 to about 6 carbon atoms; alkoxy groups having one or more oxygen linkages and from 1 to about 8, or from 1 to about 6 carbon atoms; haloalkyl groups having one or more halogens and from 1 to about 8, from 1 to about 6, or from 1 to about 2 carbon atoms; and haloalkoxy groups having one or more oxygen linkages and one or more halogens and from 1 to about 8, from 1 to about 6, or from 1 to about 2 carbon atoms.

[0023] “Pharmaceutical compositions” are compositions comprising at least one active agent, such as a compound or salt of Formula I, and at least one other substance, such as a carrier. Pharmaceutical compositions optionally contain one or more additional active agents. When specified, pharmaceutical compositions meet the U.S. FDA's GMP (good manufacturing practice) standards for human or non-human drugs.

[0024] “Pharmaceutically acceptable salts” includes derivatives of the disclosed compounds in which the parent compound is modified by making inorganic and organic, non-toxic, acid or base addition salts thereof. The salts of the present compounds can be synthesized

from a parent compound that contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting free acid forms of these compounds with a stoichiometric amount of the appropriate base (such as Na, Ca, Mg, or K hydroxide, carbonate, bicarbonate, or the like), or by reacting free base forms of these compounds with a stoichiometric amount of the appropriate acid. Such reactions are typically carried out in water or in an organic solvent, or in a mixture of the two. Generally, non-aqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred, where practicable. Salts of the present compounds further include solvates of the compounds and of the compound salts.

[0025] Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. The pharmaceutically acceptable salts include the conventional non-toxic salts and the quaternary ammonium salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. For example, conventional non-toxic acid salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, pantoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, mesylic, esylic, besylic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, $\text{HOOC}-(\text{CH}_2)_n-\text{COOH}$ where n is 0-4, and the like.

[0026] The term “carrier” applied to pharmaceutical compositions/ combinations of the present disclosure refers to a diluent, excipient, or vehicle with which an active compound is provided. To be pharmaceutically acceptable a carrier must be safe, non-toxic and neither biologically nor otherwise undesirable.

[0027] “Increasing the activity of a hematopoietic (blood-forming) stem cells (HSCs)” includes contacting a population of HSCs with a CMA activating compound sufficient to increase the number of red blood cells produced by the population of HSCs in a given time period relative to the number of red blood cells produced by the HSCs in the same same period of time prior to being contacted with the CMA compound.

APPLICANTS PROVIDE HEREIN A NEW STRATEGY FOR MAKING BLOOD STEM CELLS HEALTHIER

[0028] While the aging of HSCs in our bone marrow is inevitable, it may be reversible. Mouse studies presented here suggest that CMA activating compounds, such as those listed in this disclosure, can restore the vitality of HSCs in aging mammals, including humans.

[0029] Decline in CMA allows waste to build up in cells, contributing to Parkinson's, Alzheimer's, and aging in general. Activating CMA can reduce or prevent those processes. Studies provided in this disclosure indicate age-related CMA plays a role in the fall-off of HSC activity.

[0030] Applicants first established that CMA in the HSCs of mice become less efficient with age. Applicants then showed that HSCs depend on CMA for their vitality—both to maintain a healthy balance of proteins and to switch from their normally quiescent state to their active, blood-cell forming state.

[0031] Applicants show that blocking CMA in the HSCs of young mice duplicates many of the features observed in the HSCs of aged mice. Conversely, young mice that were genetically engineered to prevent CMA in their HSCs from declining with age were able to repopulate bone marrow with healthy blood cells even into old age. Most importantly, Applicants pharmacologically activated CMA in old mice, and thereby restored HSC activity in those mice.

[0032] Although the studies discussed above were conducted in mice they can be applied to other mammals, including humans. HSCs from elderly humans have the same defects Applicants observed in mice in which CMA was blocked. Applicants treated HSCs from people over 70 with CMA activating compounds they had previously developed. those HSCs treated with CMA activating compounds recovered the ability to make healthy blood cells.

[0033] Certain blood cancers such as acute myeloid leukemia and myelodysplastic syndromes overwhelmingly affect older people and evolve from mutations that accumulate in HSCs. Activating CMA in the HSCs of elderly people may prevent those blood cancers from occurring. Activating CMA in HSCs of cancer patients undergoing chemotherapy or radiation therapy, both of which destroy large quantities of blood cells and compromise the immune response, is also likely to benefit those patients.

[0034] In this disclosure, we demonstrate that CMA is required for HSC maintenance and function by contributing to protein quality control in quiescent steady-state HSC. Contrary to other cell types where proteostasis is preserved upon CMA blockage by upregulating macroautophagy, CMA-deficient HSC do not activate this compensatory mechanism, thus explaining their early loss of proteostasis and worsening of the L2AKO HSC phenotype with age

[0035] CMA is also essential for the adaptation to the metabolic requirements of dividing HSC through regulation of FADS2-dependent fatty acid metabolism. The facts that

(i) the same polyunsaturated fatty acids decrease with age in blood from healthy volunteers, (ii) the molecular defects of L2AKO HSC phenocopy those in old HSC volunteers and (iii) that pharmacological reactivation of CMA in HSC is possible and beneficial at advanced age, provide translational rationale to targeting of CMA to restore hematopoietic stem cell function in aging.

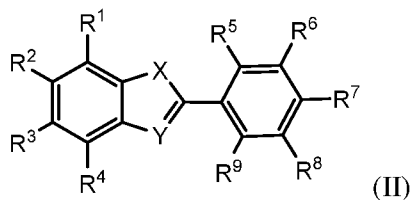
[0036] Other components of the proteostasis network have been reported to contribute to HSC homeostasis and function. Metabolic deregulation with massive accumulation of lipid droplets and increased glycolysis occurs upon macroautophagy blockage in neutrophil precursors. This metabolic phenotype is in clear contrast with the reduced glycolysis observed in CMA-deficient HSC. In further support that both autophagic pathways are non-redundant, loss of CMA in HSC cannot be compensated for by macroautophagy in these cells. Different from other cell types (i.e. fibroblasts, hepatocytes) that respond to CMA blockage with an upregulation of macroautophagy activity to preserve cellular proteostasis, HSC fall in the group of cells (also including retinal cells or activated T cells) in which macroautophagy activity remains unaltered upon CMA blockage. Differences in timing, signaling pathways and substrate recognition may be responsible for the inability of these proteolytic systems to compensate for each other in HSC.

[0037] As expected for a proteolytic system, the consequences of CMA failure in HSC may go beyond the changes in lipid metabolism reported in this work. Our comparative proteomic analysis demonstrate that CMA-deficient cells are unable to carry out the overall proteome remodeling required during the process of activation. Failure to timely modulate levels of proteins involved in processes such as cell cycle, cytoskeletal organization, or in the regulation of other proteostasis components, are likely to contribute to the functional impairment of HSC. The selectivity and timing of CMA degradation is likely behind the cell type-specific functions described for this type of autophagy. For example, CMA facilitates T cell activation by directly degrading proteins that actively repress T cell activation, whereas here we uncovered that CMA partakes in regulation of the metabolic state of HSC upon activation. Interestingly, the mechanisms and metabolic pathways regulated by CMA appear also to be cell type and context specific as, for example, CMA participates in maintenance of pluripotency of embryonic stem cells also through metabolic changes but in that case by limiting α -ketoglutarate production, which ultimately affects activity of DNA demethylases involved in pluripotency.

optionally substituted with one or more substituents independently chosen from halogen, hydroxyl, cyano, C₁-C₆alkyl, C₁-C₆alkoxy, C₁-C₂haloalkyl, and C₁-C₂haloalkoxy/

[0047] R¹² is hydrogen, C₁-C₆alkyl, or (C₃-C₇cycloalkyl)C₀-C₂alkyl.

[0048] The CMA Activating compound may be a compound of Formula II



or a pharmaceutically acceptable salt thereof. Within Formula II the variables carry the following definitions.

[0049] X is O, C(R¹⁰R¹¹), C=O, N(R¹²), S, or S=O and Y is CR¹⁰ or N.

[0050] R¹, R², R³, and R⁴ are independently chosen from hydrogen, halogen, hydroxyl, C₁-C₆alkyl, and C₁-C₆alkoxy.

[0051] R⁵, R⁶, R⁸, and R⁹ are independently chosen from hydrogen, halogen, hydroxyl, C₁-C₆alkyl, and C₁-C₆alkoxy.

[0052] R⁷ is -NR²⁰COR²¹ or -NR²⁰SO₂R²¹, or R⁷ is a phenyl, naphthyl, pyridyl, pyrimidinyl, pyrazinyl, thienyl, thiazolyl, imidazolyl, oxazolyl, triazolyl, quinolinyl, or isoquinolinyl group; each of which is optionally substituted with one or more substituents independently chosen from halogen, hydroxyl, cyano, -CHO, -COOH, amino, and C₁-C₆alkyl in which any carbon-carbon single bond is optionally replaced by a carbon-carbon double or triple bond, any methylene group is optionally replaced by O, S, or NR²², and optionally substituted with one or more substituents independently chosen from halogen, hydroxyl, cyano, amino, and oxo; and each of which is optionally substituted with one substituent chosen from -N(R²⁰)COR²¹ and -N(R²⁰)SO₂R²¹.

[0053] R¹⁰ and R¹¹ are independently chosen from hydrogen, halogen, hydroxyl, amino, cyano, C₁-C₆alkyl, C₁-C₆alkoxy, (C₃-C₆cycloalkyl)C₀-C₂alkyl, C₁-C₂haloalkyl, and C₁-C₂haloalkoxy.

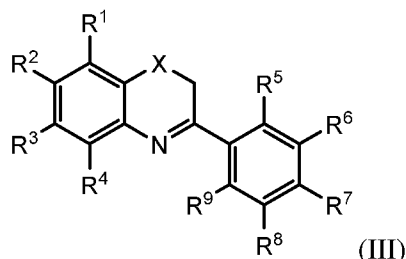
[0054] R¹² is hydrogen, C₁-C₆alkyl, or (C₃-C₆cycloalkyl)C₀-C₂alkyl.

[0055] R²⁰ is hydrogen or C₁-C₆alkyl.

[0056] R²¹ is independently chosen at each occurrence from hydrogen, C₁-C₆alkyl, C₁-C₂haloalkyl, monocyclic aryl and heteroaryl, each of which monocyclic aryl and heteroaryl is optionally substituted with one or more substituents independently chosen from halogen, hydroxyl, cyano, C₁-C₆alkyl, C₁-C₆alkoxy, C₁-C₂haloalkyl, and C₁-C₂haloalkoxy/

[0057] R^{22} is hydrogen, C_1 - C_6 alkyl, or $(C_3$ - C_7 cycloalkyl) C_0 - C_2 alkyl; with the proviso that the compound is not N-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)isobutyramide.

[0058] The CMA Activating compound may be a compound of Formula III



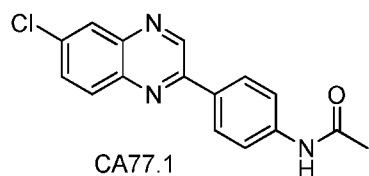
or a pharmaceutically acceptable salt thereof. The variables in Formula III carry the following definitions.

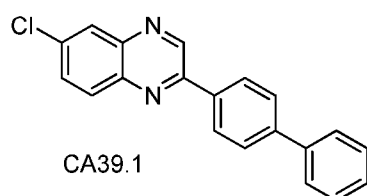
[0059] R^1 , R^2 , R^3 , R^4 , R^5 , R^6 , R^8 , and R^9 of formula (III) are independently H, hydroxyl, halogen, SH, NO_2 , CF_3 , $COOH$, $COOR^{10}$, CHO , CN , NH_2 , NHR^{10} , $NHCONH_2$, $NHCONHR^{10}$, $NHCOR^{10}$, $NHSO_2R^{10}$, OCR^{10} , COR^{10} , CH_2R^{10} , $CON(R^{10}R^{11})$, $CH=N-OR^{10}$, $CH=NR^{10}$, OR^{10} , SR^{10} , SOR^{10} , SO_2R^{10} , $COOR^{10}$, $CH_2N(R^{10}R^{11})$, $N(R^{10}R^{11})$, or optionally substituted lower alkyl, alkenyl, alkynyl, cycloalkyl, heterocycloalkyl, aryl, heteroaryl, aralkyl, or heteroaralkyl; wherein the optional substituent is one or more of F, Cl, Br, I, OH, SH, NO_2 , $COOH$, $COOR^{10}$, R^{10} , CHO , CN , NH_2 , NHR^{10} , $NHCONH_2$, $NHCONHR^{10}$, $NHCOR^{10}$, $NHSO_2R^{10}$, $HO CR^{10}$, COR^{10} , CH_2R^{10} , $CON(R^{10}R^{11})$, $CH=N-OR^{10}$, $CH=N R^{10}$, OR^{10} , SR^{10} , SOR^{10} , SO_2R^{10} , $COOR^{10}$, $CH_2N(R^{10}R^{11})$, $N(R^{10}R^{11})$.

[0060] R^7 of formula (II) is H, hydroxyl, halogen, CF_3 , CN , OCF_3 , $COOH$, $COOCH_3$, $COOR^{10}$, $COO(CH_2)_2Si(CH_3)_3$, $COOR^{10}Si(CH_3)_3$, $NHCOCH_3$, $C=C-CH_2OH$, $C=C-R^{10}-OH$ or optionally substituted alkyl, aryl, heteroaryl, aralkyl, heteroaralkyl, cyclic or heterocyclic; wherein the optional substituent is one or more of F, Cl, Br, I, OH, SH, NO_2 , CH_3 , R^{10} , $COOH$, $COOR^{10}$, CHO , CN , NH_2 , NHR^{10} , $NHCONH_2$, $NHCONHR^{10}$, $NHCOR^{10}$, $NHSO_2R^{10}$, $HO CR^{10}$, COR^{10} , CH_2R^{10} , $CON(R^{10}R^{11})$, $CH=N-OR^{10}$, $CH=N R^{10}$, OR^{10} , SR^{10} , SOR^{10} , SO_2R^{10} , $COOR^{10}$, $CH_2N(R^{10}R^{11})$, $N(R^{10}R^{11})$.

[0061] R^{10} and R^{11} are independently H or C^1 - C^6 alkyl; and X is C, $C=O$, N, O, S or $S=O$; and Y is N, NH or C.

[0062] CMA Activators of Formula I include:





[0063] The disclosure provides CMA Activators of Formula I, in which the activator is a compound or salt of Table 1.

TABLE 1. Benzoxazines and Quinoxalines		
Cpd. No.	Chemical structure	Name
1 (CA-3-55)		4'-(7-chloro-2H-benzo[b][1,4]oxazin-3-yl)-[1,1'-biphenyl]-3,4-diol
2 (CA-3-67)		4'-(7-chloro-2H-benzo[b][1,4]oxazin-3-yl)-[1,1'-biphenyl]-4-ol
3 (CA-3-69)		7-chloro-3-(2'-methoxy-[1,1'-biphenyl]-4-yl)-2H-benzo[b][1,4]oxazine
4 (CA-3-70)		7-chloro-3-(2'-hydroxy-[1,1'-biphenyl]-4-yl)-2H-benzo[b][1,4]oxazine
5 (CA39.1)		2-([1,1'-biphenyl]-4-yl)-6-chloroquinazoline
6 (CA77.1)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)acetamide

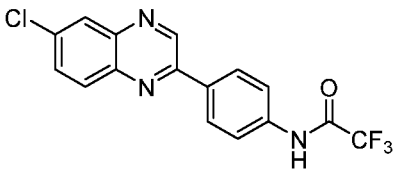
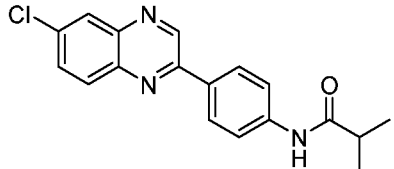
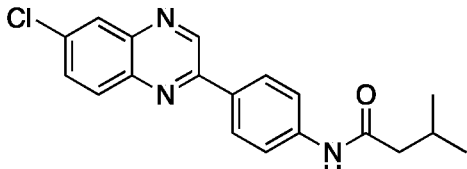
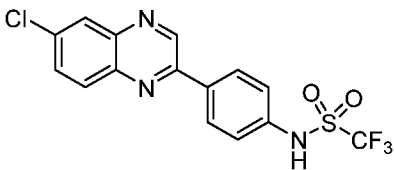
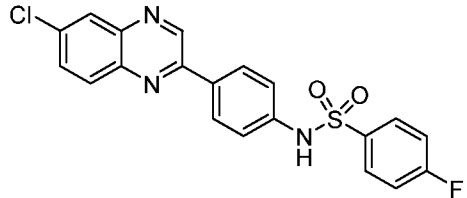
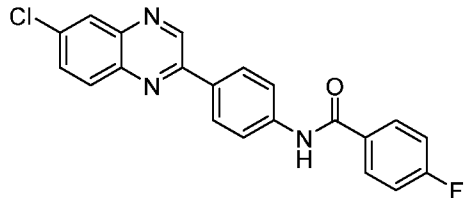
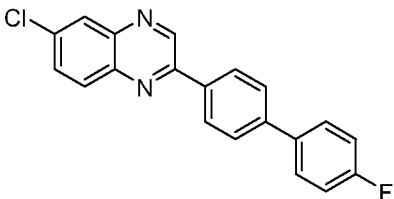
TABLE 1. Benzoxazines and Quinoxalines		
Cpd. No.	Chemical structure	Name
7 (CA77.11)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)-2,2,2-trifluoroacetamide
8 (CA77.12)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)isobutyramide
9 (CA77.13)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)butyramide
10 (CA77.14)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)-1,1,1-trifluoromethanesulfonamide
11 (CA77.15)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)-4-fluorobenzenesulfonamide
12 (CA77.16)		N-(4-(6-chloroquinoxalin-2-yl)phenyl)-4-fluorobenzamide
13 (CA39.12)		6-chloro-2-(4'-fluoro-[1,1'-biphenyl]-4-yl)quinoxaline

TABLE 1. Benzoxazines and Quinoxalines		
Cpd. No.	Chemical structure	Name
14 (CA39.13)		6-chloro-2-(2'-fluoro-[1,1'-biphenyl]-4-yl)quinoxaline
15 (CA39.14)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)-2,2,2-trifluoroacetamide
16 (CA39.15)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)isobutyramide
17 (CA39.16)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)butyramide
18 (CA39.17)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)-4-fluorobenzamide
19 (CA39.18)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)-4-fluorobenzenesulfonamide

TABLE 1. Benzoxazines and Quinazolines		
Cpd. No.	Chemical structure	Name
20 (CA39.19)		N-(4'-(6-chloroquinoxalin-2-yl)-[1,1'-biphenyl]-4-yl)-1,1,1-trifluoromethanesulfonamide

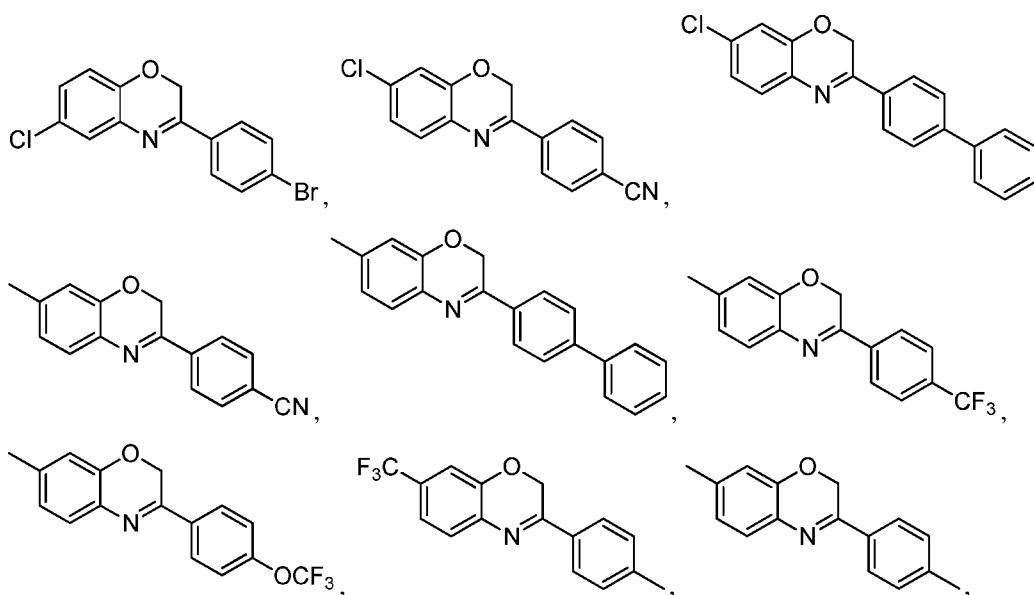
[0064] The CMA Activator can be a compound of Formula II as shown in Table 2 or a salt thereof.

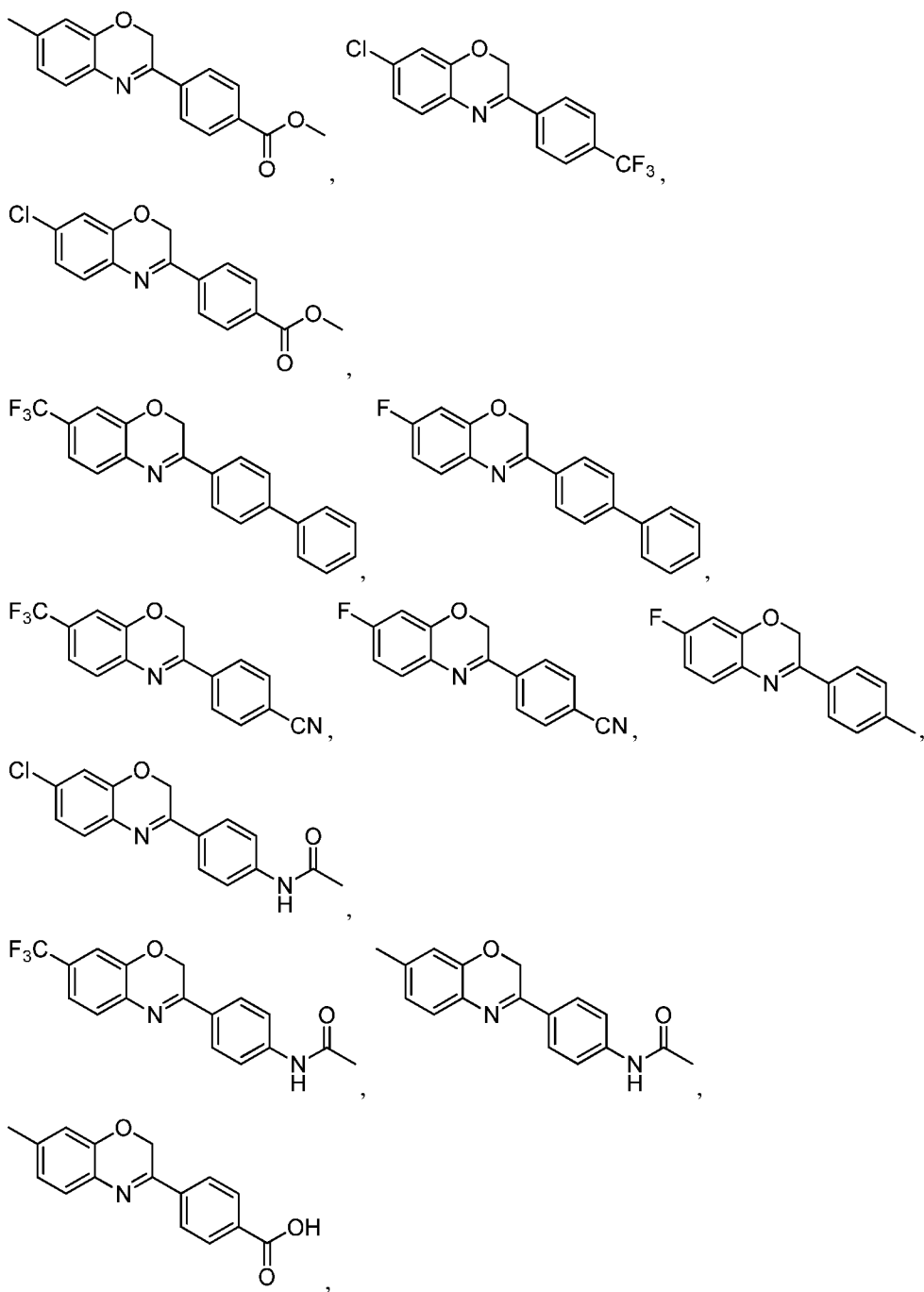
TABLE 2		
Cmp. #	Structure	Name
21		N-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)-2,4-difluorobenzamide
22		4'-(6-chlorobenzo[d]oxazol-2-yl)-[1,1'-biphenyl]-3,4-diol
23		N-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)pyrazine-2-carboxamide
24		methyl 4-(6-chlorobenzo[d]oxazol-2-yl)benzoate
25		6-chloro-2-(4-(5-methyl-4H-1,2,4-triazol-3-yl)phenyl)benzo[d]oxazole
26		cyclopropyl 4-(6-chlorobenzo[d]oxazol-2-yl)benzoate
27		5-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)pyrazine-2,3-diol
28		N-(5-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)pyrazin-2-yl)-2,2,2-trifluoroacetamide

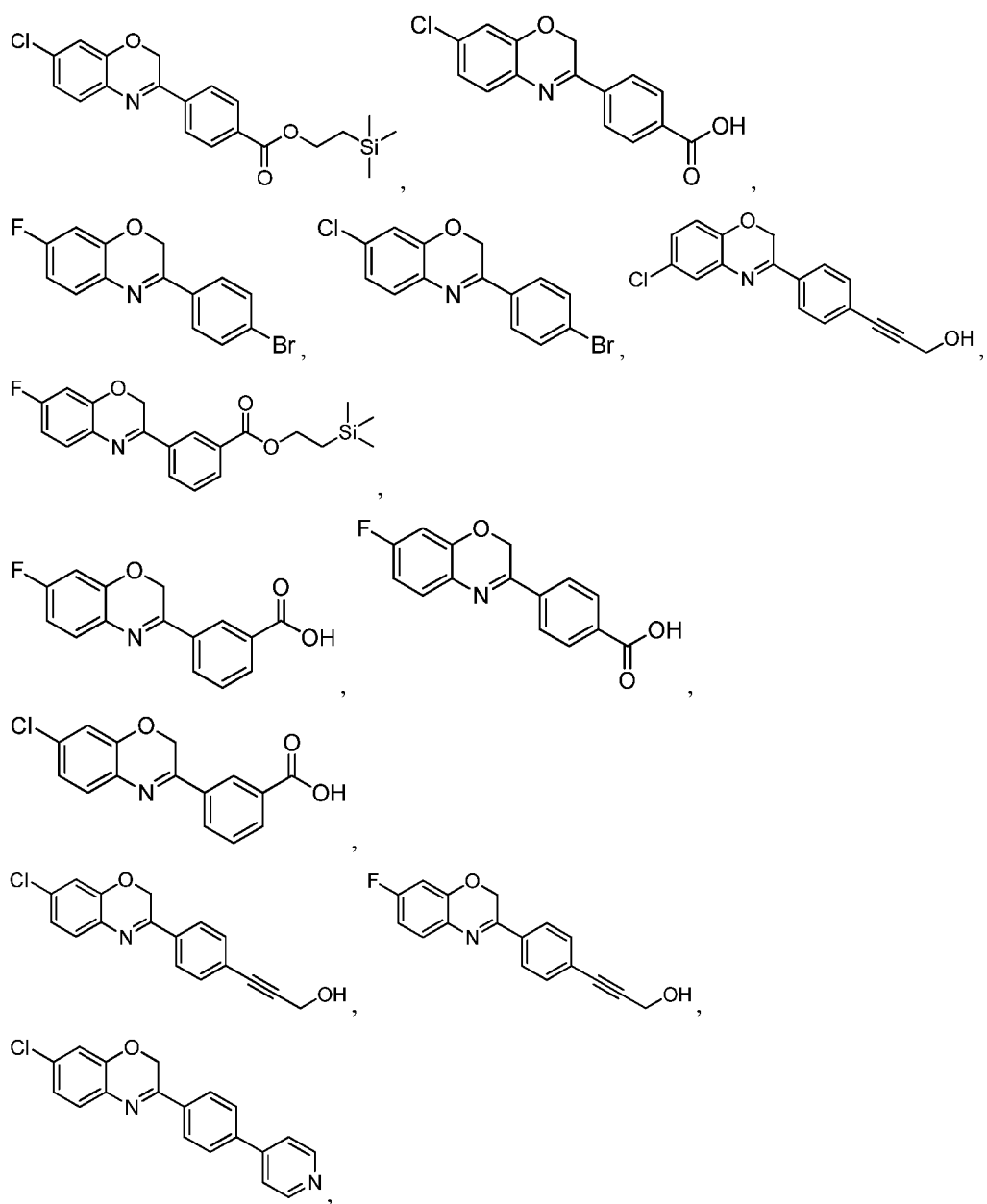
TABLE 2		
Cmp. #	Structure	Name
29		N-(5-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)pyrazin-2-yl)acetamide
30		4-(6-chlorobenzo[d]oxazol-2-yl)-N-(1,1,1-trifluoropropan-2-yl)aniline
31		N-(4-(6-chlorobenzo[d]oxazol-2-yl)phenyl)cyclopropanecarboxamide

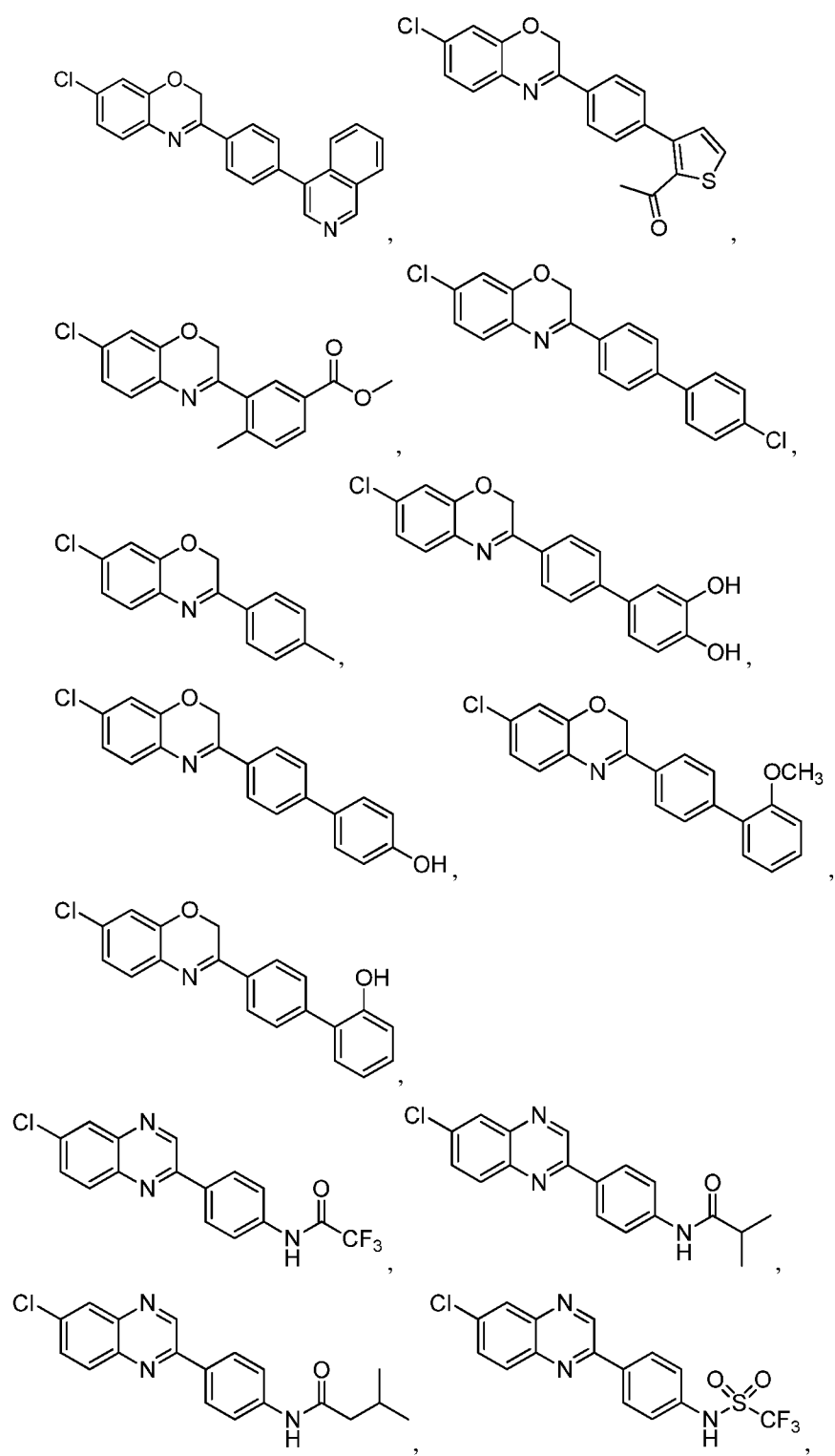
[0065] The CMA Activator can be a compound of Formula III as shown immediately below, or a salt thereof. Compounds of Formula III:

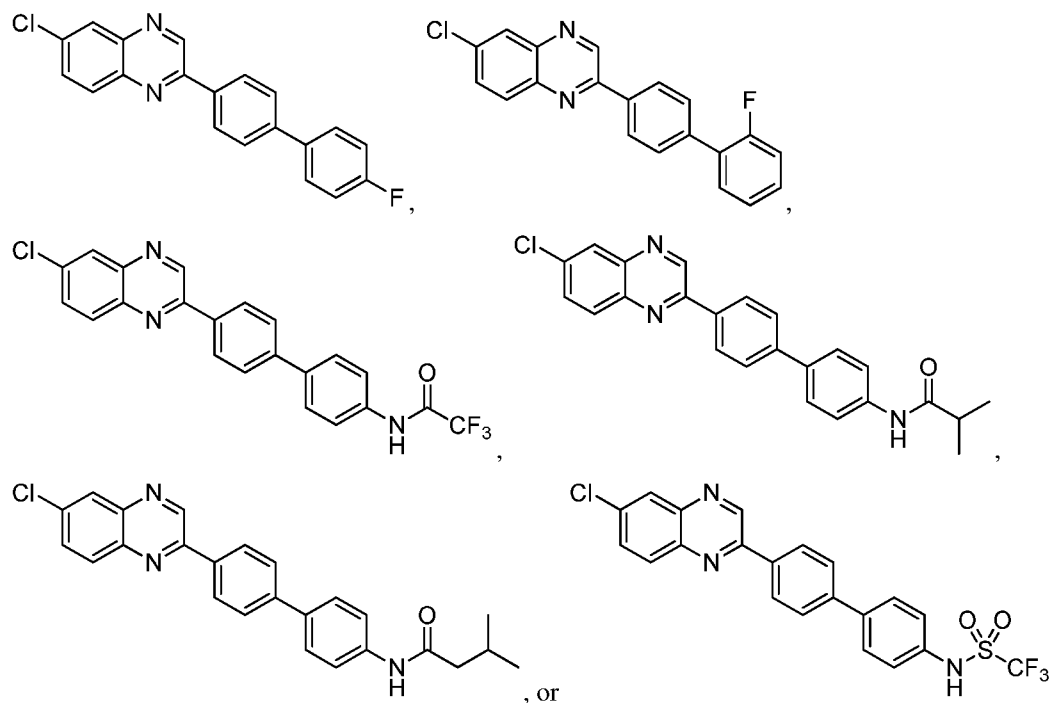
The compound of formula (III) can have the following structures:











PHARMACEUTICAL PREPARATIONS

[0066] CMA Activators can be administered as the neat chemical, but are preferably administered as a pharmaceutical composition. Accordingly, the disclosure provides pharmaceutical compositions comprising a compound or pharmaceutically acceptable salt of a CMA activator, such as a compound of Formula I, II, or III together with at least one pharmaceutically acceptable carrier. In certain embodiments the pharmaceutical composition is in a dosage form that contains from about 0.1 mg to about 2000 mg, from about 10 mg to about 1000 mg, from about 100 mg to about 800 mg, or from about 200 mg to about 600 mg of a compound of Formula I and optionally from about 0.1 mg to about 2000 mg, from about 10 mg to about 1000 mg, from about 100 mg to about 800 mg, or from about 200 mg to about 600 mg of an additional active agent in a unit dosage form.

[0067] The CMA activator may be administered orally, topically, parenterally, by inhalation or spray, sublingually, transdermally, via buccal administration, rectally, as an ophthalmic solution, through intravitreal injection or by other means, in dosage unit formulations containing conventional pharmaceutically acceptable carriers. The pharmaceutical composition may be formulated as any pharmaceutically useful form, e.g., as an aerosol, a cream, a gel, a pill, a capsule, a tablet, a syrup, a transdermal patch, or an ophthalmic solution for topical or intravitreal injection. Some dosage forms, such as tablets and

capsules, are subdivided into suitably sized unit doses containing appropriate quantities of the active components, e.g., an effective amount to achieve the desired purpose.

[0068] Carriers include excipients and diluents and must be of sufficiently high purity and sufficiently low toxicity to render them suitable for administration to the patient being treated. The carrier can be inert or it can possess pharmaceutical benefits of its own. The amount of carrier employed in conjunction with the compound is sufficient to provide a practical quantity of material for administration per unit dose of the compound.

[0069] Classes of carriers include, but are not limited to binders, buffering agents, coloring agents, diluents, disintegrants, emulsifiers, flavorants, glidants, lubricants, preservatives, stabilizers, surfactants, tableting agents, and wetting agents. Some carriers may be listed in more than one class, for example vegetable oil may be used as a lubricant in some formulations and a diluent in others. Exemplary pharmaceutically acceptable carriers include sugars, starches, celluloses, powdered tragacanth, malt, gelatin; talc, and vegetable oils. Optional active agents may be included in a pharmaceutical composition, which do not substantially interfere with the activity of the compound of the present disclosure.

[0070] The pharmaceutical compositions/ combinations can be formulated for oral administration. These compositions contain between 0.1 and 99 weight % (wt.%) of a compound of Formula I and usually at least about 5 wt.% of a compound of Formula I. Some embodiments contain from about 25 wt.% to about 50 wt.% or from about 5 wt.% to about 75 wt.% of the compound of Formula.

METHODS OF TREATMENT

[0071] The disclosure also provides methods of selectively activating chaperone-mediated autophagy (CMA) in a mammal in need thereof comprising administering to the mammal a compound of Formula I, II, or III in an amount effective to activate CMA in the mammal.

[0072] A therapeutically effective amount of a CMA Activator is an amount sufficient to inhibit the progression of a disease or disorder, cause a regression of a disease or disorder, reduce symptoms of a disease or disorder, or significantly alter a level of a marker of a disease or disorder, or reduce the probability of a treated mammal of developing the disease or disorder relative to an untreated mammal of the same species.

[0073] The disclosure includes methods in which the blood cancer is leukemia, lymphoma or myeloma. Leukemia is a blood cancer that originates in the blood and bone marrow. Leukemia can be of various types for example, Acute Lymphoblastic Leukemia, Acute Myeloid Leukemia, Chronic Lymphocytic Leukemia, Chronic Myeloid Leukemia, Hairy Cell Leukemia, Chronic Myelomonocytic Leukemia, Juvenile Myelomonocytic

Leukemia, Large Granular Lymphocytic Leukemia, Blastic Plasmacytoid Dendritic Cell Neoplasm, B-cell Prolymphocytic Leukemia, T-cell Prolymphocytic Leukemia, and others.

[0074] Non-Hodgkin lymphoma is a blood cancer that develops in the lymphatic system from cells called lymphocytes, a type of white blood cell that helps the body fight infections. Hodgkin lymphoma is a blood cancer that develops in the lymphatic system from cells called lymphocytes. Hodgkin lymphoma is characterized by the presence of an abnormal lymphocyte called the Reed-Sternberg cell.

[0075] Multiple myeloma is a blood cancer that begins in the blood's plasma cells, a type of white blood cell made in the bone marrow.

[0076] In addition to the methods of treatment and increasing proteostasis provided in the SUMMARY section this disclosure also provides such methods in which (i) the mammal treated is a human, aged 55 years or more, aged 60 years or more, aged 65 years or more, aged 70 years or more, aged 75 years or more, or aged 80 years or more, (ii) the mammal is a companion animal (such as a dog or cat) or a livestock animal (such as cow, pig, sheep, goat, or horse), (iii) the mammal is suffering from myeloid leukemia, (iv) the mammal has a blood cancer and the blood cancer is acute myeloid leukemia, (v) the mammal has a myelodysplastic syndrome or is at risk for myelodysplastic syndrome (vi) the mammal has myelodysplastic syndrome and the myelodysplastic syndrome is a syndrome of low red blood cell count, such as anemia, a syndrome of low white cell count, such as neutropenia, or a syndrome of low platelet count such as thrombocytopenia, (vii) wherein the blood cells are red blood cells, (viii) the blood cells are white blood cells, such as neutrophils, (ix) the blood cells are platelets, (x) the CMA activator is a compound of Formula I, Formula II, or Formula III as disclosed in the specification, and/ or (xi) the CMA activator is a compound of Table 1, Table 2, of a specific compound of Formula III as disclosed in the specification, or a salt of a compound of Table 1, Table 2, of a salt of a specific compound of Formula III as disclosed in the specification.

GENERAL METHODS

[0077] MICE. Hematopoietic system specific L2A KO mice were created by crossing C57BL/6 ^{Vav-i}Cre mice with C57BL/6 L2A^{ff} mice. Wild type, ^{Vav-i}Cre and L2A^{ff} male littermate mice were separately analyzed for each test and because no differences were detected among them, they were grouped in the results as “control” for the experimental group (^{Vav-i}Cre L2A^{ff}). For the aging studies, 3-4 months (labeled as 4m) and 25-30m (labeled as >25m) male mice were used in the young and old group, respectively. KFERQ-Dendra2 transgenic mice and KFERQ-Dendra mice defective in CMA (KFERQ-Dendra-L2AKO) were generated as described before (Dong, S., et al., Nature Communications, (2020) 11: 645). Transgenic mouse

models with targeted insertion of a human copy of L2A that can be induced by tamoxifen (hL2AOE) were generated by crossing h L2A^{ff} mice with ^{Tmx}ER-Cre. hLAMP2A expression was induced with 3 intraperitoneal (i.p.) injections of tamoxifen (2mg/20g body weight) every other day either at 4m and analyzed at 7m of age or at 12m and analyzed at 27m of age. Young and old controls were similarly injected with tamoxifen. Injected animals did not show differences in HSC frequency with non-injected controls, thus discarding any possible direct effect of tamoxifen at the time that the animals were analyzed. KFERQ-Dendra mice with extra copy of human L2A (KFERQ-Dendra-hL2AOE) were generated by breeding KFERQ-Dendra male mice with hL2AOE mice. Where indicated, mice were i.p. injected with a single dose of 5-fluorouracil (5FU, 150mg/kg body weight, Invitrogen, sud-5fu). Blood cell count was analyzed in tail blood drawn at day 0, 1, 4, 3, 6, 8 and 16 post 5FU injection using an Oxford Science Forcyte Blood Analysis Unit. BM was analyzed at the same times. Data is from the analysis 8 days post-injection, unless otherwise specified. For serial 5FU injections, mice were injected every week and 7 days post injection blood was taken and blood counts measured as above and dead mice and time of death were registered. For GLA supplementation, old mice (>25m) were injected with either saline or GLA (1mg/kg bw) for 40 days. The CMA activator (CA 20mg/Kg b.w. per mouse day for 2 months) was administered orally in the form of sucralose gelatin agar pills. The same delivery method was utilized in control mice but using drug-free pills. CA is a derivative from the previously generated first-in-class small molecules for selective activation of CMA in vitro³⁹, that has been modified to make it suitable for in vivo administration (Gomez et al., in preparation). Animals were maintained at 19-23°C at 40-60% relative humidity in 12h light/dark cycle. All genotyping, breeding, handling, and treatments in this study were done according to protocol and all animal studies were under an animal study protocols approved by the Institutional Animal Care and Use Committee of Albert Einstein College of Medicine.

[0078] HUMAN HEMATOPOIETIC STEM AND PROGENITOR CELLS. Mobilized peripheral blood from multiple myeloma patients (59-71 years old) was enriched for mononuclear cells by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Mono-nuclear cells were further enriched for CD34+ cells using immunomagnetic bead sorting (CD34 MicroBead Kit, human; Miltenyi Biotec) according to the manufacturer's protocol. The isolated CD34+ cells were then used for LTC-IC or colony formation assays. All human specimens used in this study were provided by Dr. Amit Verma and were fully deidentified left-over material from autologous transplants. Ethics approval for the use of human specimen

in this study was obtained from the Internal Review Board at the Albert Einstein College of Medicine (IRB protocol 2005).

[0079] COMPETITIVE BONE MARROW TRANSPLANTATION. Total nucleated BM cells (5×10^5) were injected retro-orbitally into lethally irradiated CD45.1 congenic mice in competition with the same amount of BM cells from CD45.1 mice. Transplanted mice were given antibiotic-containing water for 4 weeks post-irradiation. Reconstitution of donor derived (CD45.2) cells was monitored by staining blood cells with antibodies against CD45.2 (PE/Cy7-CD45.2, Biolegend 109830, 1:100) and CD45.1 (PE-CD45.1, Biolegend 110708, 1:500) at different times as indicated. For serial transplantation, donor-derived BM cells were collected from recipient mice 24 weeks after first BM transplantation. Cells were then transplanted into recipient mice with fresh competitor cells (CD45.1).

[0080] FLOW CYTOMETRY. For flow cytometric analyses of mouse cells, the following monoclonal antibodies were used: APC-CD117(c-kit) (Biolegend 105812, 1:100), PE/Cy7-Ly-6A/E (Sca-1) (Biolegend 108114, 1:100), Pacific blue-CD48 (Biolegend 103418, 1:100), PE-CD150 (Biolegend 115904, 1:100), APC-CD3 ϵ (Biolegend 100312, 1:100), Percp/Cy5.5-B220 (Biolegend 103236, 1:100), FITC-CD11b (Biolegend 101206, 1:100), PE-CD11b (Biolegend 101208, 1:500), FITC- Ly-6G/Ly-6C (Gr-1) (Biolegend 108406, 1:100), and FITC-CD34 (eBioscience, 11-0341-82, 1:100), PE-CD16/32 (Biolegend 101308, 1:100). Biotin-conjugated antibodies for lineage markers were as follows: CD3 (Biolegend 100244), CD4 (Biolegend 100404), CD8a (Biolegend 100704), IgM (Biolegend 406504), TER-119 (Biolegend 116204), B220 (Biolegend 103204), NK-1.1 (Biolegend 108704), CD19 (Biolegend, 115504). PerCP/Cy5.5 Streptavidin (Biolegend, 405214) was used against the biotin-conjugated antibodies, all lineage antibodies were diluted 1:100. Before analysis, BM from tibias and femurs were depleted from red blood cells with ACK lysis buffer, then stained with HSC markers (Lin-/cKit+/Sca1+/CD48-/CD150+) or myeloid progenitor markers (Lin-/cKit+/Sca1-) or GMP markers (Lin-/cKit+/Sca1-/CD34hi/CD16/32hi) followed by either ROS staining or cell cycle analysis. All antibodies used in this study were commercially available and the validation method followed by the vendor is described in the reporting summary associated with this manuscript. For ROS staining, cells were resuspended in staining buffer (2%FBS/2mM EDTA in PBS) with 5 μ M CellROX reagent (ThermoFisher C10444, 1:5000) and incubated at 37°C for 30 min. Cells were washed 3 times with staining buffer, resuspended in the same buffer with 1 μ g/ml propidium iodide for dead cell exclusion and then subjected to FACS analysis. For cell cycle analysis, after staining for cell surface markers, cells were fixed with Cytofix/Cytoperm buffer (BD Bioscience, 554722) for 20 min at 4°C, washed

twice with Perm/Wash buffer (BD Bioscience, 554723), re-suspended in Perm/Wash buffer containing Alexa Fluor 488 anti-mouse Ki-67 antibody (Biolegend, 652417, 1:500) and incubated overnight at 4°C. After 3 washes with Perm/Wash buffer, cells were resuspended in cell staining buffer with Hoechst 33342 (ThermoFisher H3570, 1:5000) for 5min at RT, and subjected to FACS analysis. For BrdU staining, mice were intraperitoneally injected with a single dose of 100mg/kg BrdU (Sigma-Aldrich, B5002) and provided with drinking water containing 0.8mg/ml BrdU and 5% glucose 24 hours before dissection. After staining with surface markers, cells were fixed, permeabilized, treated with DNase, and stained with anti-BrdU FITC antibody (Biolegend 364103, 1:200) following the BrdU labeling kit protocol (BD Pharmingen). Cells were then resuspended in staining buffer with Hoechst 33342. For sorting, BM cells from tibias, femurs and spine were centrifuged through Histopaque to isolate mononucleated cells. Cells were stained with HSC markers or LSK markers, resuspended in the staining buffer with 1µg/ml propidium iodide for dead cell exclusion and then subjected to sorting in a Beckman Moflo sorter with 70µm nozzle.

[0081] COLONY FORMATION AND SERIAL RE-PLATING ASSAYS. Sorted mouse HSC (180 cells Lin⁻/cKit⁺/Sca1⁺/CD48⁻/CD150⁺) or CD34-enriched human HSPC (5,000 cells) were plated into methylcellulose media (STEMCELL Technologies, MethoCult M 3434 for mouse or MethoCult H4434 for human) according to the manufacturer's protocols. Colonies were scored after 10 to 12 days for mouse and 14 to 16 days for human using an Inverted Infinity and Phase Contrast Microscope (Fisher Scientific). For serial re-plating assays, methylcellulose medium from primary platings were dissolved in PBS to dissociate the colonies into a single-cell suspension, washed three times and 20,000 cells (mouse) or 40,000 cells (human) were re-plated in 1ml of MethoCult M3434 or MethoCult H4434 medium for mouse and human cells, respectively. Where indicated, media was supplemented with 100 nM FADS2 inhibitor SC-26196 (Sigma, PZ0176), 100 µM γ-linolenic fatty acid (GLA) (Sigma, L2378), 100 µM NAC (Sigma, A7250) or 5 mM Methyl-pyruvate 514 (Sigma, 371173).

[0082] LTC-IC ASSAY. To assess LTC-IC frequencies, serial dilutions of FACS-sorted mouse Lin⁻Sca-1+c-Kit⁺ (LSK) cells or CD34⁺ enriched human HSPCs were plated in MyeloCult M5300 for mouse or MyeloCult H5100 for human (STEMCELL Technologies) mixed at 50:50 ratio with primary mouse stroma cell-conditioned medium or with HS5-conditioned medium for human. For each cell dose (1:10, 1:20, 1:40, 1:80, 1:160 for mouse LSK cells, 1:50, 1:100, 1:200, 1:400, 1:800 for human HSPC), 10 technical replicates were performed. After 4 weeks (mouse) or 5 weeks (human) in culture with half change of the medium every week, cells in each well were used for the colony formation assay in MethoCult

M3434 and MethoCult H4434 (STEMCELL technologies) for mouse and human, respectively. For CFU-C counting, in the case of mouse samples, wells 524 containing any type of colonies were scored as positive, while in the case of human samples, only wells with GEMM colonies were scored as positive. Where indicated, the CMA activator (10 μ M) or the same volume of DMSO were added to the mouse LSK cells every 24 hours during the 4 weeks in culture. Supplementation of the γ -linolenic fatty acid (GLA) was done by adding 100 μ M GLA (Sigma, L2378) or the same volume of IMDM medium to the human HSPC cells every week for 5 weeks.

[0083] LONG-TERM EX VIVO EXPANSION OF MOUSE HEMATOPOIETIC STEM CELLS AND ELECTROPORATION. The long-term *ex vivo* expansion of mouse HSC were conducted as previously described⁴³. Briefly, Ctrl or L2AKO HSC were sorted into a well of 96-well plates (3 plates for each genotype) containing F-12 medium supplemented with 10 mM HEPES, 1 $\times$ PSG, 1 $\times$ ITSX, 1 mg/ml PVA, 100 ng/ml TPO, and 10 ng/ml SCF. Cells were cultured for one month with half of the medium changed twice a week. Cells were then collected and subjected to gradient centrifugation using Ficoll (Sigma, Histopaque-1083) to remove any dead cells. Electroporation was performed using the Amaxa Human CD34+ cell Nucleofector kit (Lonza, VPA-1003) according to the manufacturer's instructions. Cells (2 $\times$ 10⁶) were electroporated with 3 μ g DNA coding for FADS2, FADS2-K42Q or FADS2-K42A together with 1.2 μ g of the plasmid pmaxGFP. G418 (1 mg/ml) was used for selection after 48 hours of electroporation and cells were cultured for another 3 weeks following the protocol for long-term *ex vivo* expansion. Twenty-four hours before the experiment, cells were sorted for HSC (CD48-CD150+LSK) and then treated with leupeptin (100 μ M) and NH₄Cl (20 mM) for 16 hours and collected for SDS-PAGE and immunoblot. The following antibodies were used for immunoblot: Flag (Sigma F1804, 1:5000), P62 (ENZO, bml-pw9860-0100, 1:2000), L2A (Invitrogen 512200, 1:7000).

[0084] SEAHORSE ASSAY. Oxygen consumption rates and extracellular acidification rates were measured using a 96-well Seahorse Bioanalyzer XF 96 according to the manufacturer's instructions (Agilent Technologies). In brief, LSK cells were sorted and plated into 96-well plates pre-coated with CELL-TAK (CORNING, 354240). For extracellular acidification rates to measure glycolysis, cells (100,000 cells per well) were plated into 180 μ l base media (100nM SCF, 100nM TPO, 2mM L-Glutamine, 1mM Pyruvate), spin down at 80 g for 1 min and incubated within a CO₂-free chamber at 37 °C for 1 hour. Once in the reader, plates were sequentially injected with 30mM glucose, 2 μ M oligomycin and 100mM 2-DG or just oligomycin and 2-DG where is indicated in the related figures. To determine the fraction

of oxygen consumption dependent on fatty acid β -oxidation, cells (200,000 cells/ well) were plated into 180 μ l KHB media (111mM NaCl, 4.7mM KCl, 1.25mM CaCl₂, 2mM MgSO₄, 1.2mM NaH₂PO₄) and in half of the samples etomoxir (40 μ M, Sigma, E1905) was added for 30 min before the analysis. Glycolysis was calculated as ECAR rate after adding saturating amount of glucose, maximal glycolytic capacity as maximum ECAR rate reached upon addition of oligomycin and glycolytic reserve as the difference between glycolytic capacity and glycolysis rate. Once in the reader, plates were sequentially injected with 1 μ M oligomycin, 2 μ M FCCP and 0.5 μ M Rotenone. Fatty acid β -oxidation rate was calculated as the difference in oxygen consumption in presence or absence of etomoxir. Data were normalized to cell number using CyQuant (ThermoFisher, C7026).

[0085] ENZYME ACTIVITY AND ATP MEASUREMENT. Activity of Pyruvate Kinase or GAPDH were measured using Biovision kit (K709 for Pyruvate Kinase, K608 for GAPDH) according to the manufacturer's instructions. Briefly, for Pyruvate Kinase, freshly sorted LSK cells (50,000) were collected by centrifugation, lysed with 50 μ l lysis buffer and after centrifugation, the supernatant was added to 96-well plate with clear bottom, followed by 50 μ l reaction mix. The plate was read in a Luminex Magpix 4.2 (Luminex-ThermoFisher) using fluorescence Ex/Em=535/587 nm every 10 minutes. The Pyruvate Kinase activity was calculated by the two readings within a linear range. For the GAPDH activity, LSK cells (50,000) were lysed with 25 μ l GAPDH Assay buffer by incubation on ice for 10 min followed by centrifugation. The supernatant (20 μ l per well) adjusted to a final volume of 50 μ l with GAPDH Assay Buffer was incubated with 50 μ l of reaction mix and the plate measured at 450 nm in kinetic mode for 10-60 min at 37°C. ATP was measured using ATPlite luminescence ATP detection assay kit (PerkinElmer). Briefly, HSC (10,000) were plated in a white bottom 96-well plate with 100 μ l 2% FBS in PBS and 50 μ l mammalian cell lysis solution for 5 min with shaking, 577 followed by additional 5 min incubation with 50 μ l substrate solution. Plates were measured in a 578 Tecan i.control 1.11 luminometer (Tecan).

[0086] IMMUNOFLUORESCENCE STAINING. HSC or myeloid progenitor cells were directly sorted into 16-well slides pre-coated with Cell-Tak Cell Tissue Adhesive (Corning, 354240) and then fixed with 4% PFA for 15 min at room temperature (RT). For LAMP2A, LAMP1, total human LAMP2 and FADS2 staining, slides were washed with PBS and incubated with blocking buffer (5% goat serum/0.3% TrionX-100 in PBS) for 1 hour at RT and incubated overnight at 4°C with the first antibody diluted in 1% BSA/0.3% TritonX-100 followed by 40 min incubation at RT with fluorescence-conjugated secondary antibodies. Cells were washed 3 times with PBS and mounted with mounting medium with DAPI (Southern

biotech). For LC3 staining, after fixation, cells were permeabilized with 0.015% (v/v) digitonin in PBS (Sigma) and then incubated with blocking buffer (10% FBS in PBS) for 45 minutes. Both the 1st and 2nd antibodies were diluted in blocking buffer and incubated for 30 minutes and 45 minutes at RT, respectively. Oxidized proteins were detected with OxyICC Oxidized Protein Detection Kit from Sigma (S7350) and protein inclusions were detected with PROTEOSTAT Aggresome detection kit from ENZO (ENZ-51035) following manufacturer's instructions. For LysoTracker staining, cells were incubated with 50nM LysoTracker green (Invitrogen, L7526) for 30 min at 37°C, washed and fixed for 10 minutes with 4% PFA and mounted. For analysis of CMA activity using direct fluorescence and the KFERQ-Dendra CMA reporter, cells isolated from KFERQ-Dendra2 transgenic mice were fixed with 2% PFA for 5 min at RT and mounted for direct puncta counting or subjected for immunofluorescence as described above. Quantification was performed in TIFF converted images upon thresholding using the 3D object counter tool of Image J software (1.52v, NIH). Average number of puncta per cell was determined for each of the cells in a field and at least 3 different fields per animal were counted. Where indicated, we also calculated the percentage of cells active for CMA, defined as those with at least two Dendra positive puncta. To determine CMA flux using the CMA reporter, cells were incubated or not in the presence of leupeptin (100 μ M, 16 hours), fixed with 4% PFA for 15 minutes followed by blocking with 5% goat serum/0.01% tritonX-100 for 1 hour at RT, and then incubated with the dendra2 antibody and the corresponding secondary antibody in 1% BSA/0.01% TritonX-100 in PBS sequentially. CMA flux was calculated as the increase in number of Dendra positive puncta upon leupeptin treatment. The following primary antibodies were used: anti-mouse LAMP2A (Invitrogen 512200, 1:2000), anti-mouse LAMP1 (Hybridoma Bank 1D4B, 1:5000), total human LAMP2 (Hybridoma Bank h4b4, 1:2000), FADS2 (Abclonal A10270, 1:1000), LC3 (MBL PM036, 1:1000), Dendra2 (antibodies abin361314, 1:5000). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Invitrogen, A-11008) and Alexa Fluor 488 goat anti-rat IgG (H+L) (Invitrogen, A-11006), Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A-21428), Alexa Fluor 555 goat anti-rat IgG (Invitrogen, A-21434), Alexa Fluor 635 goat anti-rabbit IgG (Invitrogen, A-31576). All the secondary antibodies were used at 1:1000 dilution. The LAMP2A antibody was validated by immunoblot using cells knock-out for LAMP2A (example in FIG. 3I). All the images were taken with a Confocal microscope (TCS SP8; Leica) using an HCX Plan Apo CS 63.0 $\times$ 1.40 NA oil objective and the Leica Application Suite X (LAS X).

[0087] TRANSMISSION ELECTRON MICROSCOPY. Cells (100,000 per condition) were pelleted and fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, followed by sequential fixation in 1% osmium tetroxide followed and 2% uranyl acetate. Samples were dehydrated through a graded series of ethanol dilutions and embedded in LX112 resin (LADD Research Industries). Ultrathin (80nm) sections were cut on a Leica EM Ultracut UC7, stained with uranyl acetate followed by lead citrate, and viewed on a JEOL 1200EX transmission electron microscope at 80kV.

[0088] RNA PURIFICATION, AMPLIFICATION, AND MICROARRAY ANALYSIS. Cells (10,000) were sorted, and total RNA was extracted using RNeasy Micro kit (Qiagen) according to the manufacturer's protocols. All total RNA samples were quantified with the RNA Quantification Kit (ThermoFisher Scientific, 902905). Using the GeneChip WT Pico Kit (ThermoFisher Scientific, 902622), samples were amplified to cRNA from *in vitro* transcription of one round of linear amplification reaction and then converted to sense-strand single stranded cDNA followed fragmentation of biotinylated cDNA. 5.5 micrograms of cDNA from each sample was hybridized to ThermoFisher Scientific (Affymetrix) GeneChip Mouse Gene 2.0 ST Array (902118). Hybridization cocktail was made using the hybridization kit (ThermoFisher Scientific, 900454) and array scans were performed according to the manufacturer's instructions using the high-resolution GeneArray Scanner 3000 7G (ThermoFisher Scientific, Affymetrix). The data were analyzed with the GeneChip Command Console Software version 4.0 from ThermoFisher Scientific (Affymetrix) default analysis settings.

[0089] QUANTITATIVE PROTEOMICS AND PROTEIN PATHWAY ANALYSIS. Freshly sorted LSK cells (500,000) were pelleted and flash-frozen in liquid nitrogen for shipment to the Biological Mass Spectrometry Core Facility at University of Colorado Denver. For analysis, cells were lysed with RIPA buffer (ThermoFisher) and subjected to GeLC-MS. Excised gel pieces were destained in ammonium bicarbonate in 50% acetonitrile and dehydrated in 100% acetonitrile, trypsin digested upon reduction and alkylation of unmodified cysteine residues, and analyzed by nano-UHPLC-MS/MS (Easy-nLC1000, QExactive HF-positive ion mode (ThermoFisher)). The peptide mixture was desalted and concentrated in a Thermo Scientific Pierce C18 Tip. Samples were analyzed on an Orbitrap Fusion mass spectrometer (ThermoFisher) coupled to an Easy-nLC 1200 system (ThermoFisher) through a nano-electrospray ion source according to manufacturer's instructions. MS/MS spectra were extracted from raw data files and converted into mgf files using a Proteome Discoverer Software (ver. 2.1.0.62, ThermoFisher). The mgf files were

then independently searched against the mouse database using an in-house Mascot server (Version 2.6, Matrix Science). Mass tolerances were ± 10 ppm for MS peaks, and ± 0.6 Da for MS/MS fragment ions. Trypsin specificity was used allowing for 1 missed cleavage. Met oxidation, protein N-terminal acetylation, and peptide N-terminal pyroglutamic acid formation were allowed as variable modifications while carbamidomethyl of Cys was set as a fixed modification. Scaffold (version 4.8, Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (<http://peptideprophet.sourceforge.net/>). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least two identified unique peptides. Allocation of proteins to functional groups was done using the IPA software (version 57662101, Ingenuity Systems) and STRING database (version 11.0, <https://string-db.org/>). Protein oxidation state was performed by analyzing oxidation of methionine, carbonylation of proline to pyroglutamate and various degrees of cysteine oxidation (disulfide, glutathionylation, sulfenic, sulfonic and beta-alanine). Total protein oxidation was determined by the total number and abundance of carbonylated residues via mass spectrometry. Oxidation of methionine and carbonylation were included in this work as they showed significant differences across genotypes and interventions. To determine the acetylation state of FADS2, integrated peak areas of the FADS2 acetylated intact peptides were obtained from their extracted ion chromatograms using Qual Browser of Xcalibur 2.2 (Thermo Fisher Scientific), while b and y series ions for the MS2 of those peptides were used for the assignment of the peptide sequence and position of the acetylation. This approach allows identification and quantitation of both N-terminus acetylation as well as acetylation at specific amino acid residues, such as lysines – K. For the FADS2 peptides KVYNVTK (SEQ ID NO.15) and its di-acetylated form, the predicted m/z 's were: 426.2529 $\pm$ 0.0043 and 468.2635 $\pm$ 0.0046; and for the WLVIDRK peptide (SEQ ID NO.16) and its acetylated form 465.2820 $\pm$ 0.0047 and 486.2873 $\pm$ 0.0049 m/z 's.

[0090] METABOLOMICS. Freshly sorted LSK cells (100,000) were centrifuged, and the pellet was flash-frozen in liquid nitrogen and shipped to the Biological Mass Spectrometry Core Facility at University of Colorado Denver. Cell pellets were lysed with lysis solution (methanol:acetonitrile: water 5:3:2 $v/v/v$), before ice cold extraction by vortexing for 30 minutes at 4°C. Insoluble proteins were pelleted by centrifugation (15,000 g for 10 min at 4°C) and supernatants were analyzed using a UHPLC system (Vanquish, ThermoFisher) coupled online to a mass spectrometer (Q Exactive, ThermoFisher). Samples were resolved over a

Kinetex C18 682 column (2.1 × 150 mm, 1.7 μm; Phenomenex) at 25°C using a 9-minute gradient at 400 μl/min from 5% to 95% B (A: water/0.1% formic acid; B: acetonitrile/0.1% formic acid). MS analysis and data analysis were performed as described before 46,47 . Metabolite assignments were performed using a metabolomics data analyzer (MAVEN). Metabolic pathway analysis was performed using the MetaboAnalyst software.

[0091] MEASUREMENT OF INTRACELLULAR PROTEIN DEGRADATION. Freshly isolated LSK cells (25,000 cells) were labeled with [³H]Leucine (2μCi/ml) in stem span medium containing 100nmol mSCF for 24 hours in a 48-well plate pre-coated with CELL-TAK. After extensive washing, media (with 2.8mM unlabeled leucine) was added and cells were incubated at 37°C. Where indicated, 20mM NH₄Cl and 100μM Leupeptin (Sigma) were added to the media. Aliquots of the medium taken at 12 hours were precipitated with trichloroacetic acid and proteolysis was calculated as the percentage of initial total acid precipitable radioactivity (protein) transformed to acid soluble (peptides and amino 694 acids) at each time point⁴⁸. Lysosomal proteolysis was determined as percentage of proteolysis 695 sensitive to the combination of lysosomal inhibitors.

[0092] OTHER PROCEDURES. For quantitative PCR, RNA was extracted using RNeasy Micro kit (Qiagen) according to the manufacturer's protocols. Transcripts were reverse transcribed according to manufacturer protocols (Invitrogen), and qPCR was performed using SYBR Green (Applied Biosystems) and data acquired using the StepOne Software 2.3 (ThermoFisher). The following primers were used: mouse FADS2, F-5'-GCTCTCAGATCACCGAGGAC-3' (SEQ ID NO. 1); R-5'-AGTGCCGAAGTACGAGAGGA-3' (SEQ ID NO. 2); mouse L2A, F-5'-GCAGTGCAGATGAAGACAAC-3' (SEQ ID NO. 3), R-5'-AGTATGATGGCGCTTGAGAC-3' (SEQ ID NO. 4); mouse L2B, F-5'-GGTGCTGGTCTTTCAGGCTTGATT-3' (SEQ ID NO. 5), R-5'-ACCACCCAATCTAAGAGCAGGACT-3' (SEQ ID NO. 6); mouse L2C, F-5'-ATGTGCTGCTGACTCTGA CCTCAA-3' (SEQ ID NO. 7), R-5'-TGGAAGCACGAGACTGGCTTGATT-3' (SEQ ID NO. 8); human L2A, F-5'-TGACGACA ACTTCCTTGTGC-3' (SEQ ID NO. 9), R-5'-AGCATGATGGTGCTTGAGAC-3' (SEQ ID NO. 10); mouse β-actin, F-5'-AAGGACTCCTATAGTGGGTGACGA-3' (SEQ ID NO. 11), R-5'-ATCTTCT CCATGTCGTCCCAGTTG (SEQ ID NO. 12); mouse TBP, F-5'-GTTGGGCTTCCCAGCTAAGT-3' (SEQ ID NO.13),

R-5'-CACAAGGCCTTCCAGCCTTA-3' (SEQ ID NO.14). Immunoblot for different tissues (100µg total protein) was performed on nitrocellulose membranes after tissue sonication in RIPA buffer. The pCMV6-Entry-FADS2-Myc-DDK plasmid was purchased from Origene (MR207091), single point mutation of FADS2 42K to Q and A was performed at GenScript.

[0093] STATISTICS, SAMPLE SIZE AND SOFTWARE. All data are presented both as individual values (symbols) and mean + standard error of the mean (sem). Unpaired t-test, two-way ANOVA test, one-way ANOVA tests, chi-square test, log-rank (Mantel-Cox) test were used for the statistics as indicated in each figure legend. The test statistic (F, t, R), effect size (calculated from Cohen's d), confidence interval and degrees of freedom are indicated in the source data file. In all instances, "n" refers to individual experiments or animals and is indicated in the figure legends. The number of animals used per experiment was calculated through power analysis based in previous results. Animals were randomly attributed to control or treatment groups after separating them according to genotype. No mouse was excluded from the analysis unless there were technical reasons or the mouse was determined to be in very poor health by the veterinarian. Outliers were determined by the ROUT method (Q=1%). Investigators were blinded to the treatment during data collection and analysis and unblinding was done when the analysis was completed for plotting. All parameters were analyzed using independent sample tests and were tested for normal distribution using Shapiro-Wilk normality test. For software, image analysis was performed using Image J (NIH), pathway analysis using the Ingenuity Pathway Analysis(Ingenuity Systems) and STRING database (<https://string-db.org/>),HSC frequency within the tested cell population was estimated using ELDA software (version 4.9, ELSA), all graphical plots were made using GraphPad Prism software 7.04 (GraphPad) and images were assembled with Adobe Photoshop 6.0CC software.

EXAMPLES

ABBREVIATIONS

[0097] The following abbreviations will be helpful in the examples set forth below.

BM – Bone Marrow

CMA – Chaperone Mediated Autophagy

GLA – γ -Linolenic Fatty Acid

HSC - Hematopoietic Stem Cells

ROS - Reactive Oxygen Species

EXAMPLE 1. MEASUREMENT OF CMA ACTIVITY IN VITRO

[0098] Suitability of compounds for use in the disclosed methods as a CMA Activator can be determined by measurement of In vitro CMA activity. The photoactivatable CMA reporter assay was constructed by inserting a sequence of 21 amino acid of Ribonuclease A bearing the CMA-targeting motif in the N-terminus multicloning site of the photoactivatable protein mCherry1 or the photoswitchable protein.

[0099] NIH 3T3 fibroblasts were stably transduced with a photoconvertible CMA reporter, KFERQ-Dendra and were photoswitched by exposure to a 3.5 MA (constant current) LED (Norlux, 405 nm) for 10 minutes and at the desired times fixed in 3% formaldehyde. Test cells are exposed to the indicated concentrations of the compounds, e.g. for 12 hours or 24 hours. Cells are imaged, e.g., by using high content microscopy (Operetta, Perkin Elmer) or by capturing images with an Axiovert 200 fluorescence microscope (Zeiss) with apotome and equipped with a 63× 1.4 NA oil objective lens and red (ex. 570/30 nm, em. 615/30 nm), cyan (ex. 365/50 nm and em. 530/45 nm) and green (ex. 475/40 nm and em. 535/45 nm) filter sets (Chroma). Images were acquired with a high-resolution CCD camera after optical sectioning through the apotome. CMA activity is measured as the average number of fluorescent puncta (CMA active lysosomes) per cell. Values are expressed relative to values in untreated cells that were assigned an arbitrary value of 1 and are mean of >2,500 cells counted per condition. The S.D. in all instances was <0.01% mean value.

EXAMPLE 2. CMA IS UPREGULATED IN HSC

[0100] Using mice expressing a CMA reporter (KFERQ-Dendra2 that highlights lysosomes as fluorescent puncta upon CMA activation, we found reduced CMA activity per cell and a lower fraction of HSC active for CMA in old mice, but no changes in CMA in granulocyte-monocyte progenitor (GMP) cells (FIG. 1A). Levels of LAMP2A, essential for CMA, were reduced in HSC from old mice, although their lysosomal abundance (LAMP1 positive vesicles) remained unchanged. CMA may also decline with age in humans, as we found higher abundance of proteins bearing CMA-targeting motifs among proteins reported to accumulate with age in human HSC.

[0101] Young mice quiescent HSC showed higher basal CMA activity than myeloid progenitors, and further upregulated CMA upon *in vivo* HSC activation with the myeloablative agent 5- fluorouracil (5FU) (FIG. 1B). CMA was maximally upregulated by day 8 after 5FU injection and returned to basal levels by day 16. Lysosomal protein degradation and levels of LAMP2A, but not other lysosomal markers, were also higher at day 8 post activation 53. *In*

vivo treatment with polyI:C to potentially induce HSC cell cycle entry, also upregulated CMA in HSC but not in myeloid progenitor cells

EXAMPLE 3. CMA PREVENTS ACTIVATED HSC DEPLETION

[0102] We eliminated CMA selectively in hematopoietic cells by crossing LAMP2A^{fl/f} mice with ^{Vav-i}Cre mice (Vav-iCre-LAMP2A^{fl/f} mice, hereafter termed L2AKO). Young L2AKO and control mice showed similar number of hematopoietic cells in the bone marrow (BM) and frequencies of T (CD3ε+), B (B220+), mature myeloid (CD11b+ Gr1+) or of lineage-negative (Lin-) progenitor cells. However, the frequency and number of stem cell enriched (Lin-Sca-1+cKit+, LSK) and rigorously defined phenotypical HSC (Lin-Sca-1+cKit+CD150+CD48-) populations was lower in L2AKO mice at steady-state and differences with control became more pronounced upon 5FU-mediated myeloablation, when expansion of the HSC pool is required to restore mature hematopoietic cells (FIG. 1C).

[0103] L2AKO mice died of pre-mature bone marrow failure after constitutive acute HSC 68 activation by serial 5FU injections (Fig. 1D,E). Furthermore, serial transplantation and competitive BM repopulation, a more chronic paradigm of HSC activation (Fig. 1F), showed first a 50% reduction in the number of L2AKO donor-derived BM HSC, but not Lin- cells, by 16 weeks after first transplantation and then, persistent reduced reconstitution of all peripheral blood lineages after secondary competitive BM transplantation compared with recipients of control cells, thus demonstrating functional stem cell loss (Fig. 1G,H). *Ex vivo* studies also showed impaired self-renewal of L2AKO HSC (Fig. 1I,J).

[0104] We conclude that CMA is dispensable for multilineage blood cell differentiation, but it is necessary to maintain functional HSC during steady state and upon activation.

EXAMPLE 4. CMA DEFICIENCY IMPAIRS HSC ACTIVATION

[0105] Reduced BM repopulation by CMA-deficient HSC is in part due to their delay in cycling, as more than 50% of L2AKO HSC (vs. 15% of control HSC) were in G0 8 days after 5FU injection and remained cycling after control HSC had returned to quiescence, whereas cycling of control and L2AKO myeloid progenitor cells was comparable (Fig. 2A).

[0106] L2AKO HSC, but not myeloid progenitor cells, showed lower ATP and higher ROS levels in response to 5FU than control cells (Fig. 2B,C). Higher ROS levels - proven to impair HSC self-renewal- were also observed in L2AKO BM-derived HSC upon transplantation (FIG. 2D). Although HSC ROS also increase upon macroautophagy blockage, we confirmed that macroautophagy flux, its transcriptional program and autophagic compartments were unchanged in L2AKO HSC and that the higher proliferation rates and

pronounced mitochondrial alterations of macroautophagy-deficient HSC were absent in L2AKO HSC.

[0107] In support that the poor energetic status and elevated ROS may be behind the delayed cycling and compromised self-renewal capacity of L2AKO HSC, we identified 405 genes (related with *metabolism, motility, cell cycle and proliferation*) at steady-state and 855 genes (related to 94 *cell-to-cell signaling, cellular movement and proliferation*) during activation differentially expressed between control and L2AKO HSC (Fig. 2E,F). Quantitative proteomics confirmed higher abundance of proteins related to *metabolic pathways* in L2AKO LSK and identified 300+8 proteins as potential CMA substrates that upon activation decrease in control HSC but that accumulate in L2AKO cells (Fig. 2G,H). Most differences in enzyme abundance between activated control and L2AKO HSC occurred only at the protein and not at the transcriptional level, supporting that CMA may facilitate their direct degradation for metabolic adaptation during activation.

EXAMPLE 5. REDUCED GLYCOLYSIS IN CMA-DEFICIENT HSC

[0108] Metabolomics analyses revealed impaired glycolysis and glucose-alanine pathways, critical for HSC quiescence in steady-state L2AKO HSC (FIG. 2I-K). Direct measurement confirmed that decreased glycolysis upon CMA blockage is specific to stem cells (FIG. 2L,M). Steady-state L2AKO stem cells displayed lower enzymatic activities of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK) - known CMA substrates- (FIG. 2N) likely due to their higher content of oxidized residues (FIG. 2O). Failure to degrade oxidized non-functional glycolytic enzymes may promote their accumulation and reduce the ability of L2AKO HSC to accommodate the metabolic requirements of activation.

[0109] L2AKO HSC display a general loss of proteostasis detected as higher levels of carbonylated peptides, oxidized proteins and protein inclusions (FIG. 2P-R; note lack of changes in lipid peroxidation products in agreement with CMA exclusively degrading proteins). Oxidized proteins bearing CMA-targeting motifs accumulating in L2AKO HSC included proteins involved in *cellular energetics, cytosolic and ER protein quality control* – which could explain L2AKO HSC dilated ER - *cell cycle and cellular mobilization*.

EXAMPLE 6. CMA REGULATES HSC LIPID METABOLISM

[0110] Upon HSC activation, reduced glycolysis and less active oxidized glycolytic enzymes were still evident in L2AKO LSK cells (FIG. 2P), but the most noticeable metabolic differences with control cells were in *linoleic acid metabolism, fatty acid biosynthesis* and

beta-oxidation (FIG. 3A-D). Flux through linoleic and α -linolenic acid metabolism was upregulated in control LSK upon activation but, not in L2AKO LSK that instead displayed accumulation of the two precursors (α -linolenic and linoleic acid) (FIG. 3B-D). Inhibition of fatty acid desaturase 2 (FADS2), rate limiting enzyme in linoleic acid metabolism, reduced self-renewal ability of control cells (FIG. 3E). Conversely, adding γ -linolenic acid (GLA), the direct product of FADS2, restored L2AKO HSC self-renewal capacity (FIG. 3E) supporting that blockage in α -linolenic and linoleic acid metabolism is behind their loss of function.

[0111] Although blockage in linoleic acid metabolism in L2AKO HSC occurred at the step catalyzed by FADS2, we found similar increase in FADS2 levels upon activation in control and L2AKO LSK cells. We investigated next possible differences in FADS2 posttranslational modifications that could explain its lower activity and focused on acetylation because i) FADS2 would acquire a CMA-targeting motif if K⁴² becomes acetylated (³⁹VIDRK⁴² changes to VIDRQ-like) (FIG. 3G); ii) putative acetylation-generated CMA-targeting 1motifs were enriched in proteins decreasing in control cells but accumulating in L2AKO HSC upon activation; iii) we detected a trend towards higher acetylation in that protein group; and iv) proteins with higher acetylation in L2AKO LSK and those that accumulate in L2AKO HSC and in HSC from old people, were often involved in *cellular metabolism*, including *lipid metabolism*. Mass spectrometry analysis identified higher K⁴² acetylation of FADS2 in L2AKO LSK cells upon activation (FIG. 3G). An acetylation mimetic mutant of FADS2 (FADS2^{VIDRQ}) was rapidly degraded in lysosomes in a L2A-dependent manner in HSC, whereas the acetylation-resistant mutant FADS2^{VIFRA} was no longer degraded (FIG. 3H,I). We concluded that, as described for other enzymes, acetylation triggers CMA removal of inactive FADS2 and that, by changing active/inactive enzyme ratios, CMA positively regulates FADS2 activity and increases fatty acid metabolism during HSC activation.

[0112] Failure to upregulate linoleic acid metabolism, the subsequent lower mitochondrial fatty acid β -oxidation (FIG. 3J,K) and overall lower ATP levels (FIG. 2B) reduced activated L2AKO HSC self-renewal ability because, *ex vivo* supplementation with methyl-pyruvate (to bypass reduced β -oxidation) significantly increased L2AKO HSC function (FIG. 3L). Addition of a ROS scavenger also restored L2AKO HSC long-term colony initiation abilities (FIG. 3M), suggesting that the toxic effect of unmetabolized linoleic and α -linolenic fatty acids, observed as higher ROS levels (FIG. 2C,D) and dilated ER, contributed to reduced function.

EXAMPLE 7. CMA ACTIVATION KEEPS OLD HSC FUNCTIONAL

[0113] CMA blockage in young mice HSC partially phenocopies the proteome changes in old HSC, with half of the proteins that accumulated in HSC from 25m old mice also elevated in 4m L2AKO mice HSC. Similarly, HSC from old mice revealed changes in glycolysis and free fatty acids metabolites consistent with those observed in HSC from young L2AKO mice, low ratios of GLA to LA (metabolite and precursor in the step catalyzed by FADS2) (FIG. 3N) and elevated acetylation of FADS2 at the K⁴² residue (FIG. 3O). Furthermore, daily *in vivo* GLA supplementation of old mice improved their HSC function (FIG. 3P) and *ex vivo* GLA supplementation of CD34+ cells derived from old (>65 years) patients also increased the number of functional human stem cells in long-term cultures (FIG. 3Q). These findings support the contribution of defective linoleic and α -linolenic fatty acid metabolism to loss of HSC function during aging and highlight a similar mechanism between aging and our CMA-deficient model.

[0114] As L2AKO mice age, we observed reduced numbers of phenotypical HSC, further decreased HSC reconstitution ability and more pronounced age-dependent increase in intracellular ROS levels than in wild type littermates, supporting that 173 depletion of functional HSC in absence of CMA accentuates with age. To place CMA changes with age (FIG. 1A) in the context of the well-known functional heterogeneity of aged HSC12, we calculated the absolute number of KFERQ-Dendra+ puncta per cell and identified two groups of old HSC with different CMA activity. About 45% of old cells had very high levels of cytosolic KFERQ-Dendra protein, low number of fluorescent puncta and reduced KFERQ-Dendra degradation, whereas CMA flux in the rest of old HSC was indistinguishable from young HSC. Old HSC with low CMA activity also had high levels of 180 ROS and oxidized proteins, and reduced GAPDH activity, further supporting contribution of CMA decline with age to loss of HSC proteostasis.

[0115] To test whether preventing CMA decline in old mice preserves HSC function, we used ER^{Cre}-hL2A mice (hereafter hL2A^{OE}) in which expression of human L2A was induced from 12 months of age onward to compensate for reduced mouse L2A levels. This intervention preserved CMA flux in old HSC and prevented appearance of the low-CMA HSC population.

[0116] Aging associates with a well-characterized expansion of the stem cell population but of restricted functionality. In contrast, compared to control old littermates, the frequency of HSC in old hL2A^{OE} BM was significantly lower (FIG. 4A) and they showed lower intracellular ROS levels (FIG. 4B,C), improved PK and GAPDH activities (FIG. 4D,E),

higher glycolysis rates (FIG. 4F) and higher levels of FADS2-generated polyunsaturated fatty acids (FIG. 4G). Better proteostasis and preserved glucose and fatty acid metabolism in HSC from old hL2A^{OE} mice is probably responsible for their improved functionality (FIG. 4H shows higher reconstitution in BM transplantation). Activating hL2A expression in young hL2A^{OE} mice did not change HSC and LTC-IC frequency, ROS levels or PK activity, supporting maximal beneficial effect of CMA upregulation in conditions with an underlying CMA defect, such as aging.

EXAMPLE 8. RESTORATION OF CMA REJUVENATES AGED HSC

[0117] We found that upregulation of CMA in already old HSC is a viable intervention to improve their function. Oral administration of a well-characterized pharmacological CMA activator (CA) optimized for *in vivo use* activated CMA in HSC from old mice, reduced oxidized proteins levels, restored GAPDH activity and increased glycolytic flux (FIG. 4I-K, which all likely contributed to the observed improved HSC function (FIG. 4L). Functional improvement was due to direct activation of CMA in HSC and independent of other systemic effects of the drug, because *in vitro* exposure old HSC to CA resulted in a similar increase in LTC-IC number and higher generation of viable mature cells of various lineages (FIG. 4M-O). Addition of CA to CD34+ hematopoietic stem and progenitor cells (HSPC) from old donors (>59 years) markedly increased multi-lineage potent HSPC and sustained overall cell output upon long term culture (FIG. 4P,Q), supporting that pharmacological activation of CMA could be effective in improving and restoring aged HSC function.

CLAIMS

1. A method of increasing the activity of a population of hematopoietic (blood-forming) stem cells (HSCs) in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a Chaperone-mediated Autophagy (CMA) activating compound to increase the number of blood cells produced by the HSCs in a time period relative to the number of blood cells produced by the HSCs in the same time period prior to being contacted with the CMA.

2. A method of treating or reducing the risk of a blood cancer or treating or reducing the risk of myelodysplastic syndrome in a mammal comprising administering a therapeutically effective amount of a CMA activator to the mammal.

3. The method of claim 1 or 2 wherein the mammal is a human, aged 65 years or more, aged 70 years or more, or aged 75 years or more.

4. The method of claim 1 or 2, wherein the mammal is a companion animal (such as a dog or cat) or a livestock animal (such as cow, pig, sheep, goat, or horse).

5. The method of any of the preceding claims wherein the mammal is suffering from myeloid leukemia.

6. The method of any of preceding claim wherein the mammal has a blood cancer and the blood cancer is acute myeloid leukemia.

7. The method of any one of claims 1 to 6, wherein the mammal has a myelodysplastic syndrome or is at risk for myelodysplastic syndrome.

8. The method of claim 7, where the myelodysplastic syndrome, is a syndrome of low red blood cell count, such as anemia, a syndrome of low white cell count, such as neutropenia, or a syndrome of low platelet count such as thrombocytopenia.

9. The method of claim 1, wherein the blood cells are red blood cells.

10. The method of claim 1, wherein the blood cells are white blood cells, such as neutrophils.

11. The method of claim 1, wherein the blood cells are platelets.

12. The method of any one of claims 1 to 11, wherein the CMA activator is a compound of Formula I, Formula II, or Formula III as disclosed in the specification.

13. The method of any one of claims 1 to 11, wherein the CMA activator is a compound of Table 1, Table 2, of a specific compound of Formula III as disclosed in the specification, or a salt of a compound of Table 1, Table 2, of a salt of a specific compound of Formula III as disclosed in the specification.

14. A method of improving the proteostasis of a population of hematopoietic (blood-forming) stem cells (HSCs) in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a Chaperone-mediated Autophagy (CMA) activating compound to sufficient to improve a marker of proteostasis in the population of HSCs relative to the marker of proteostasis in the population of HSCs prior to being contacted with the CMA.

15. The method of claim 14, wherein the marker of proteostasis glycolysis, fatty acid metabolism, compound, polyunsaturated fatty acid level, or number of activated T cells, each of which is increased in HSCs contacted with the CMA activating compound.

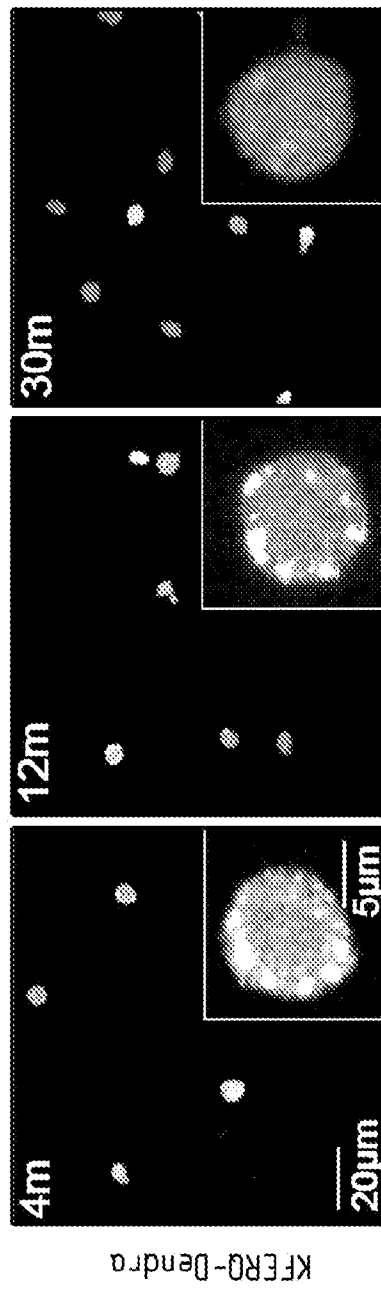
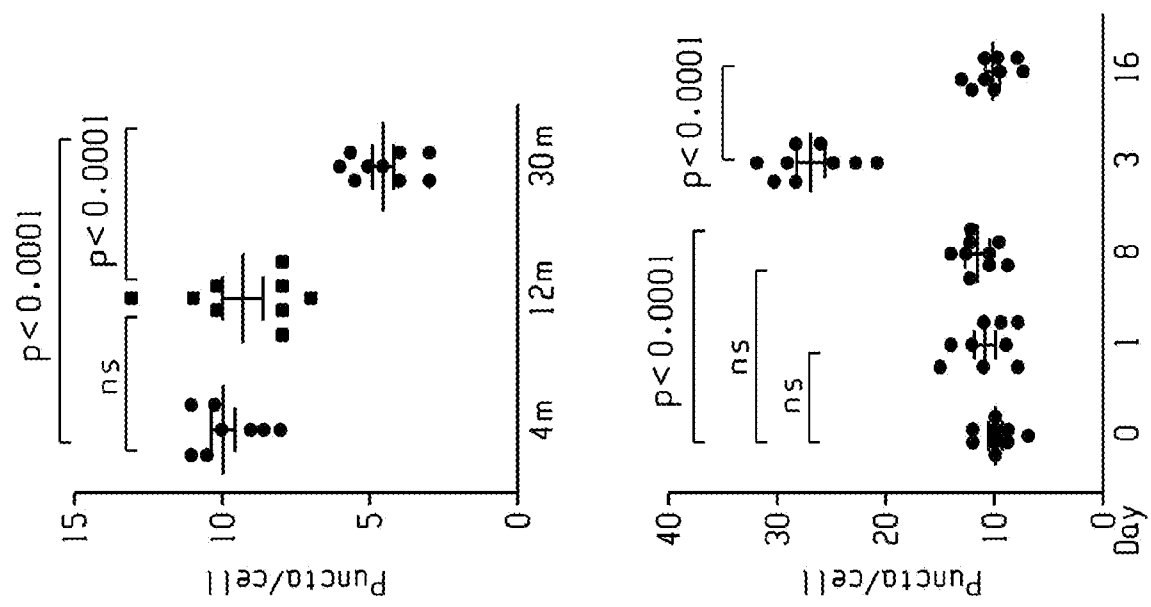


Fig. 1A

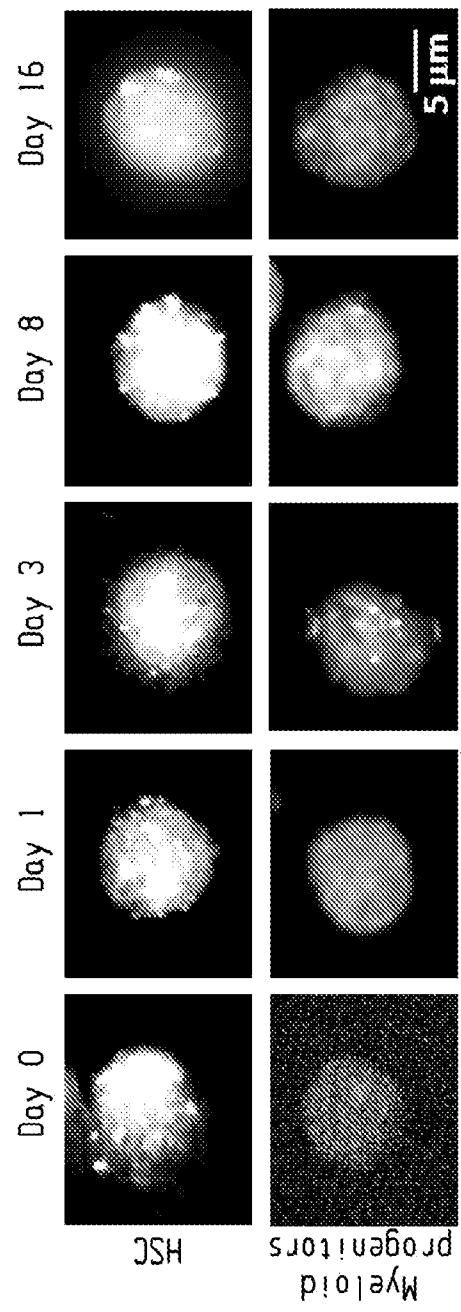
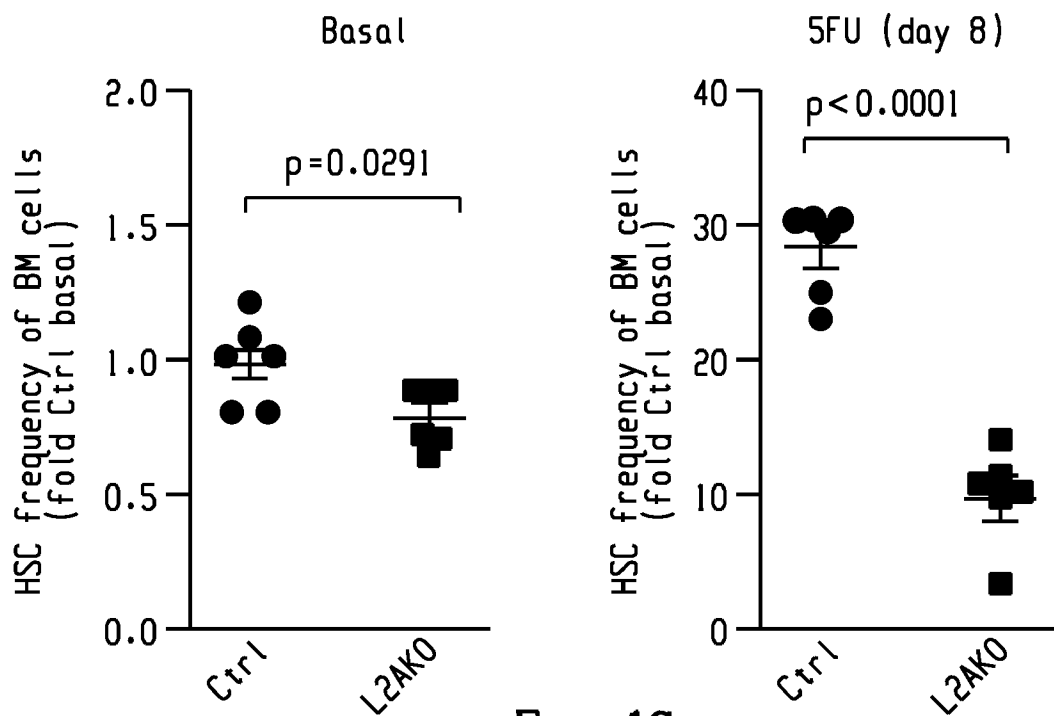
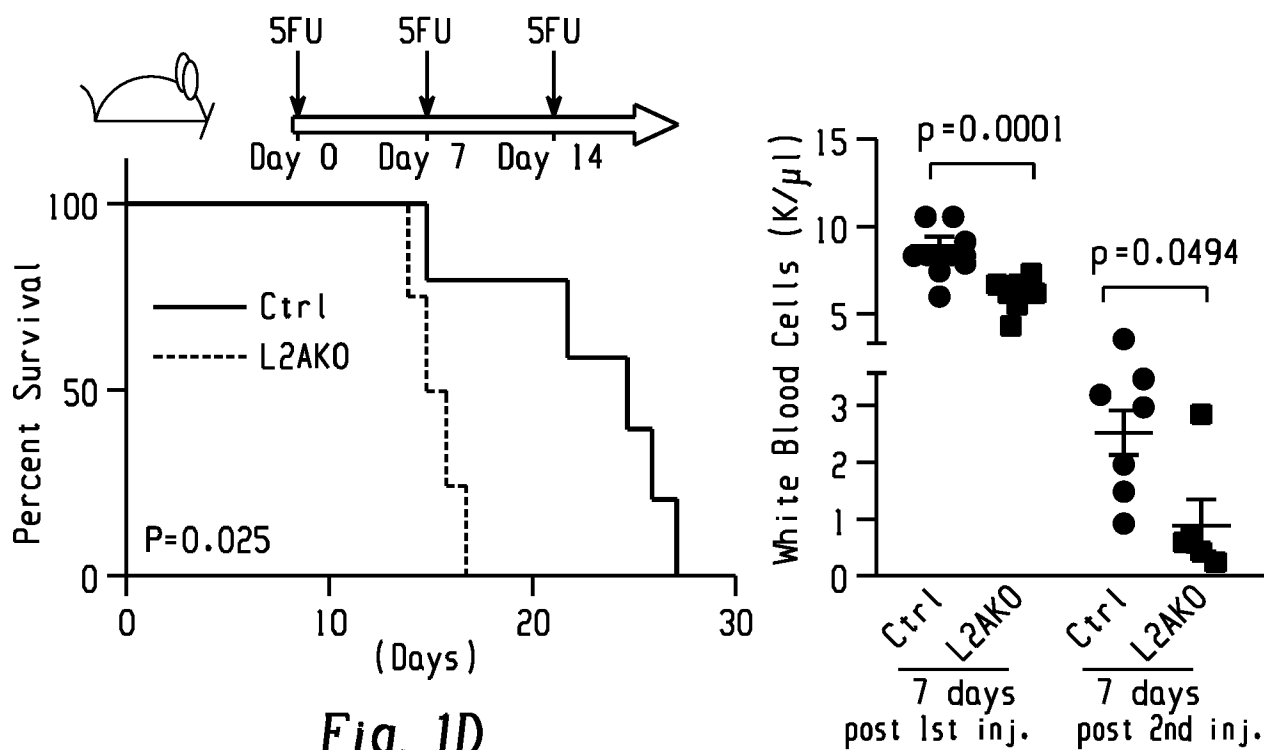


Fig. 1B

*Fig. 1C**Fig. 1D**Fig. 1E*

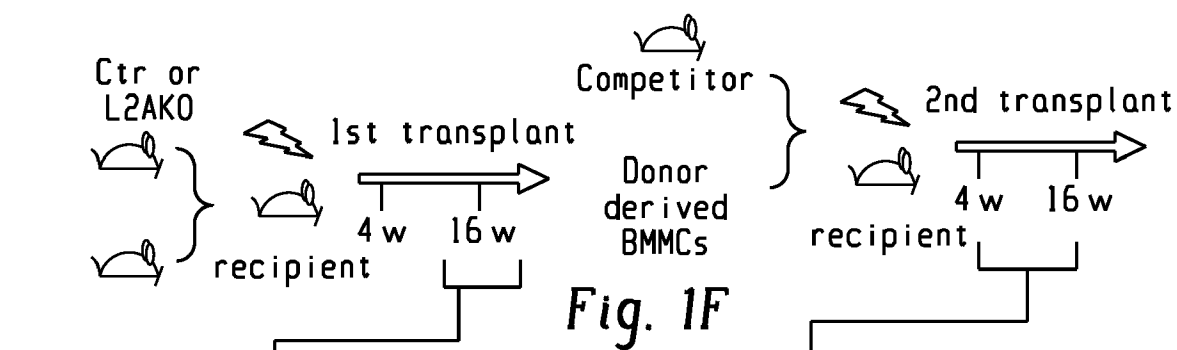


Fig. 1F

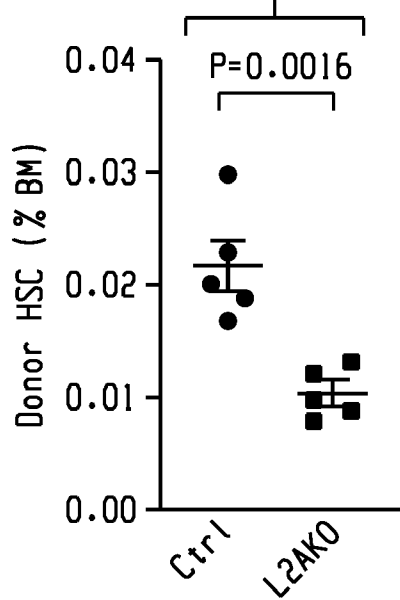


Fig. 1G

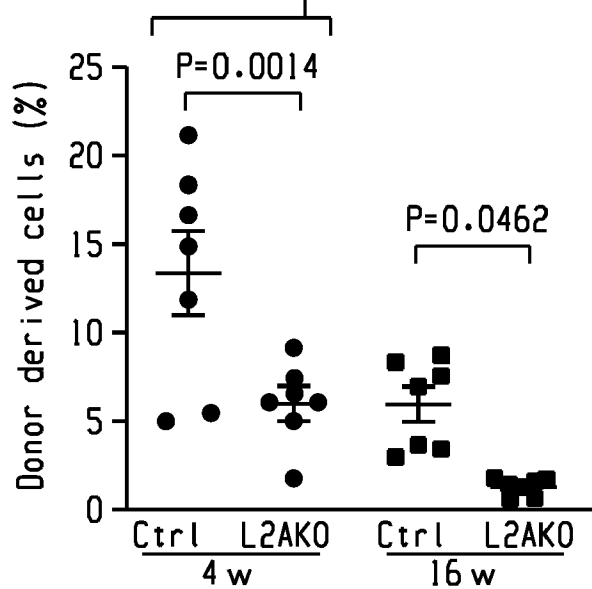


Fig. 1H

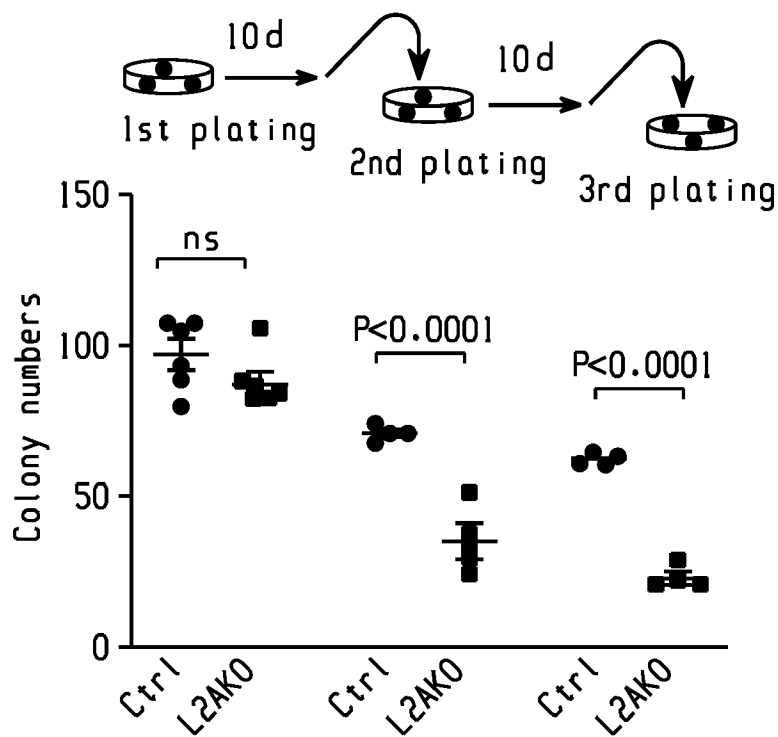


Fig. 1I

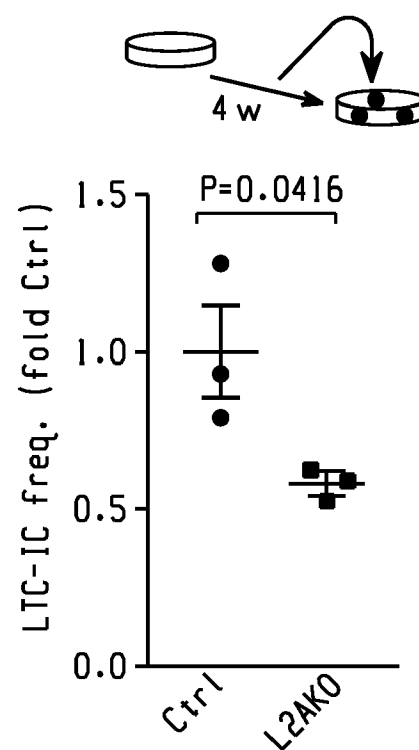


Fig. 1J

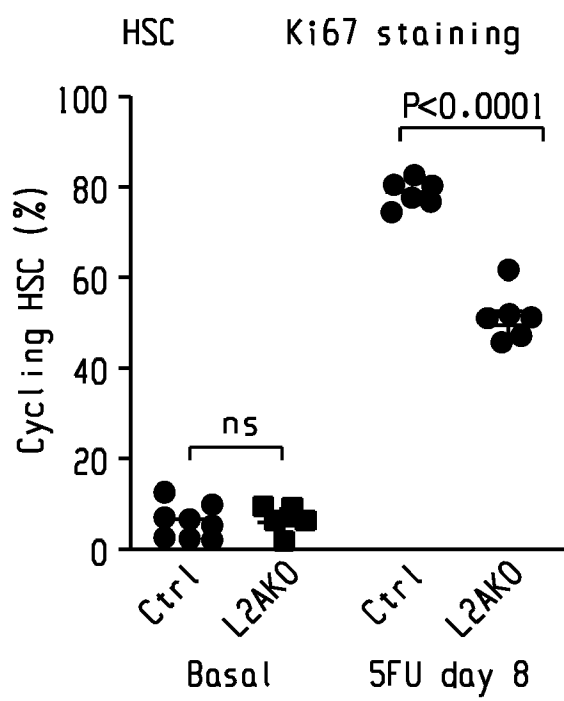


Fig. 2A

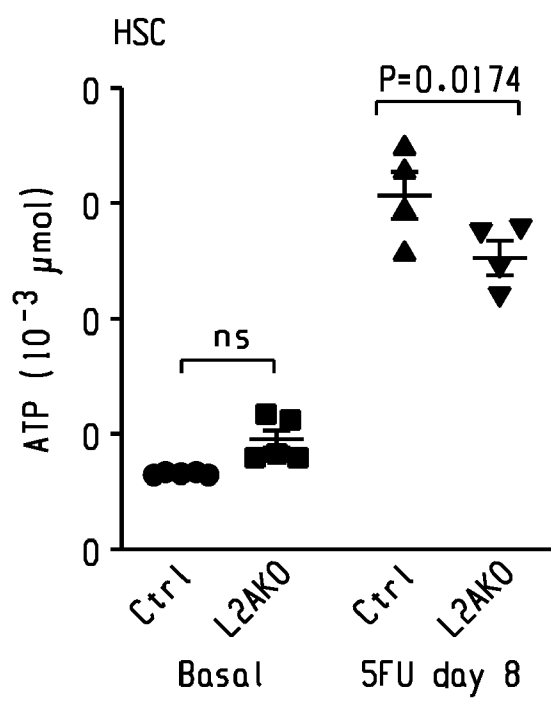


Fig. 2B

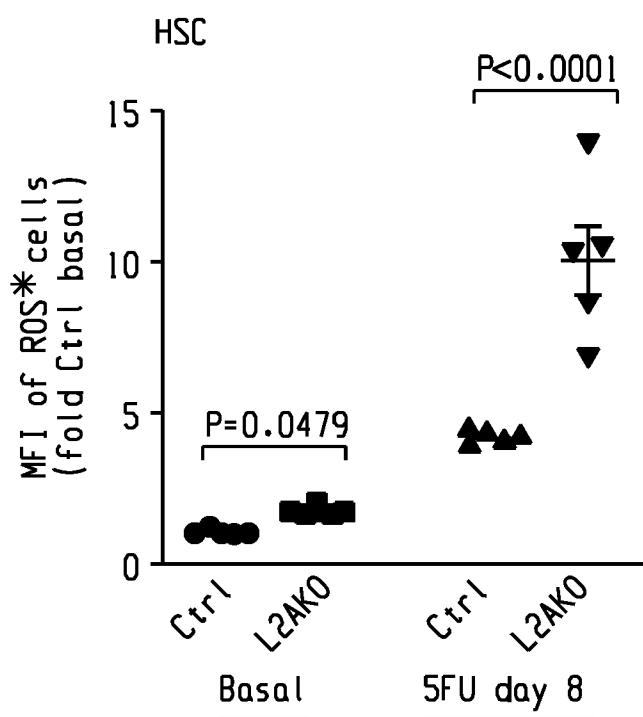


Fig. 2C

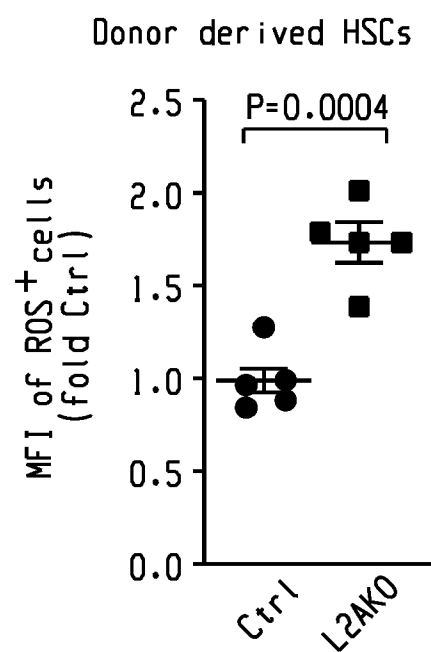


Fig. 2D

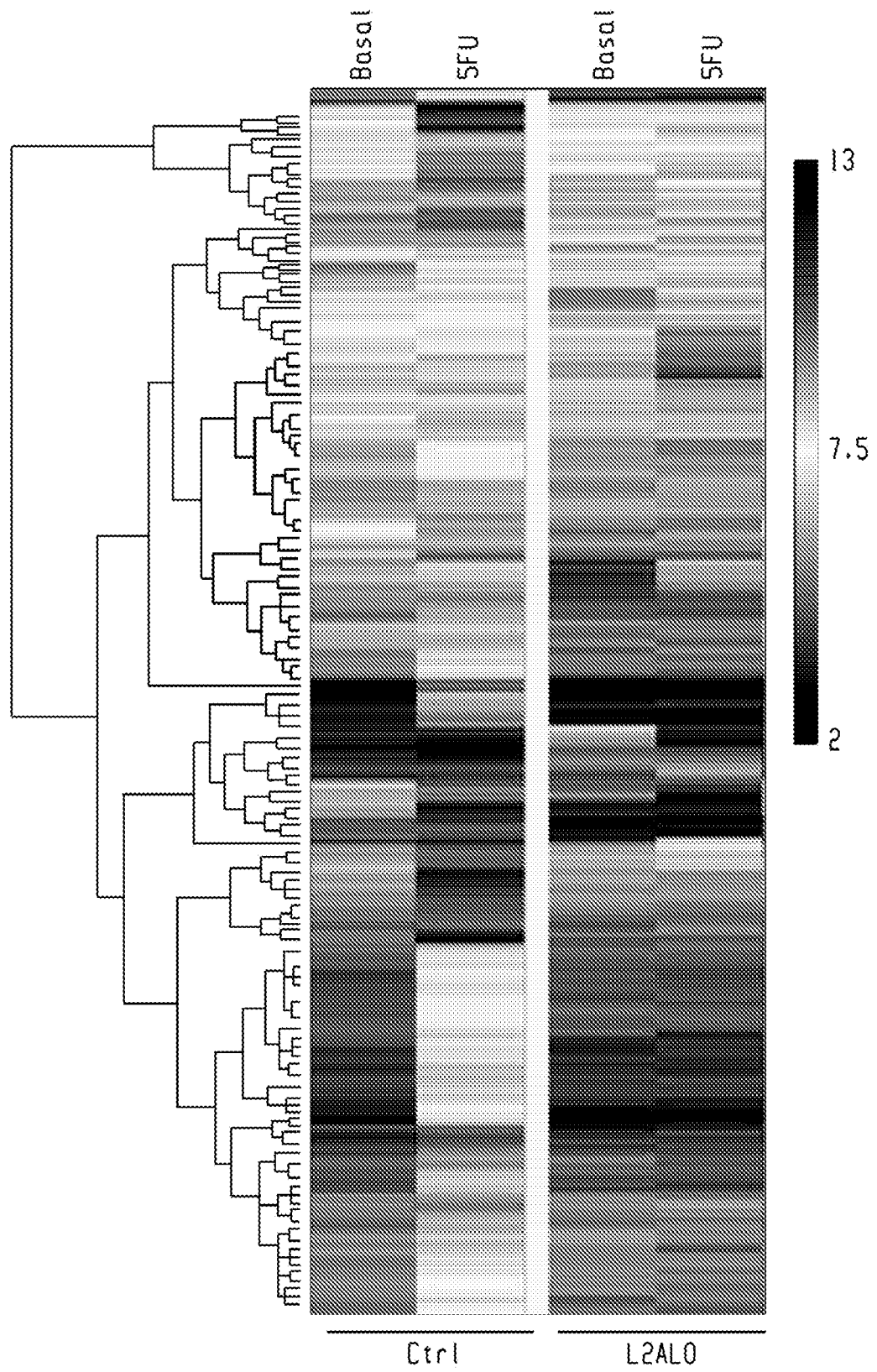
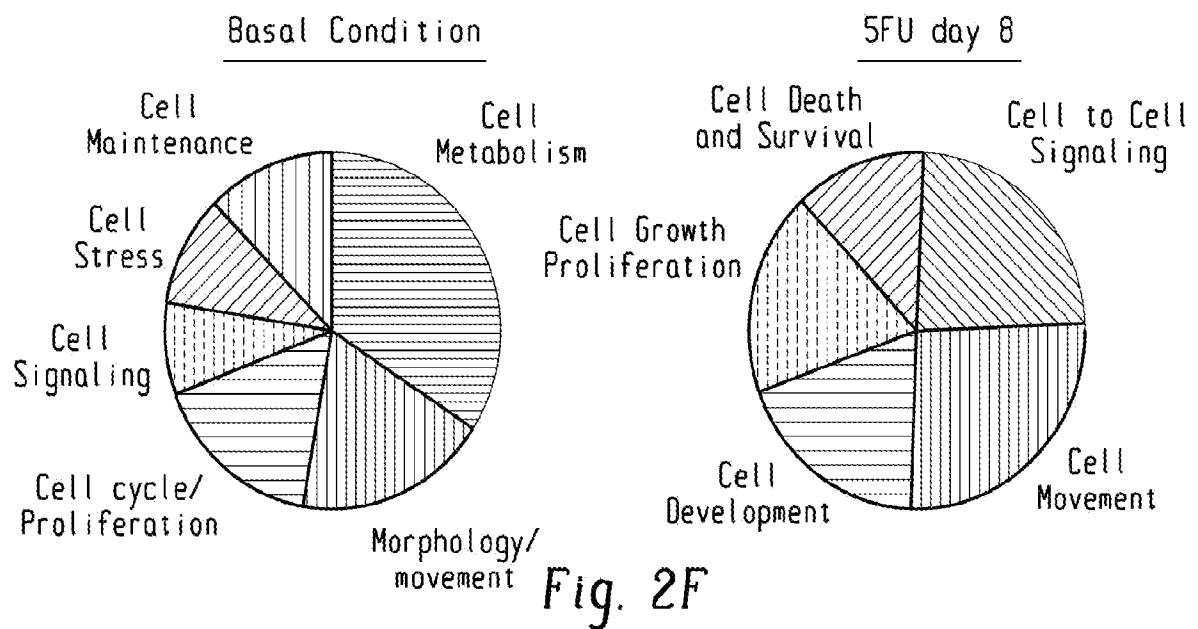
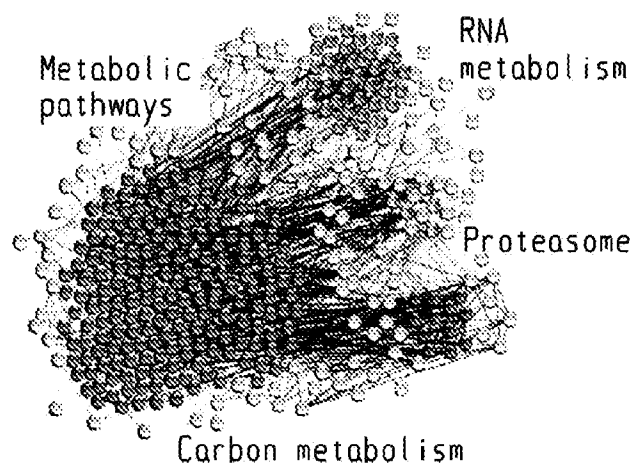


Fig. 2E



Reduced in Ctrl upon activation



5FU L2AKO > 5FU Ctrl

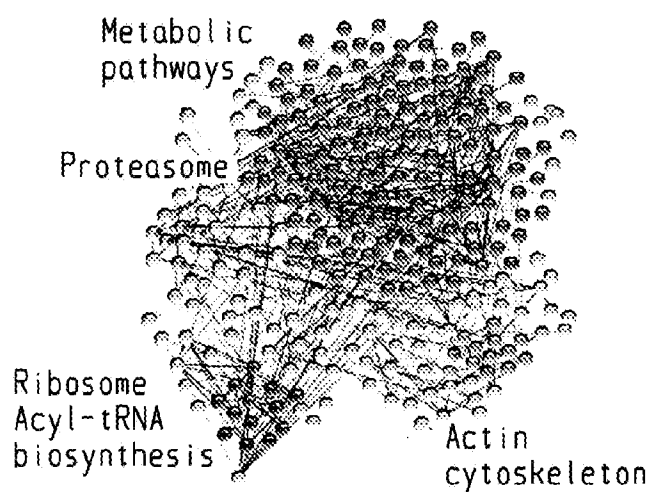


Fig. 2G

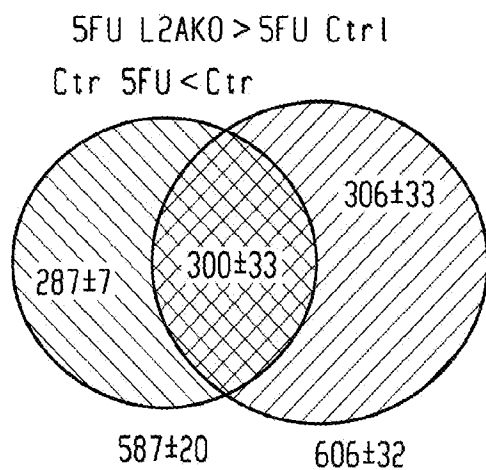


Fig. 2H

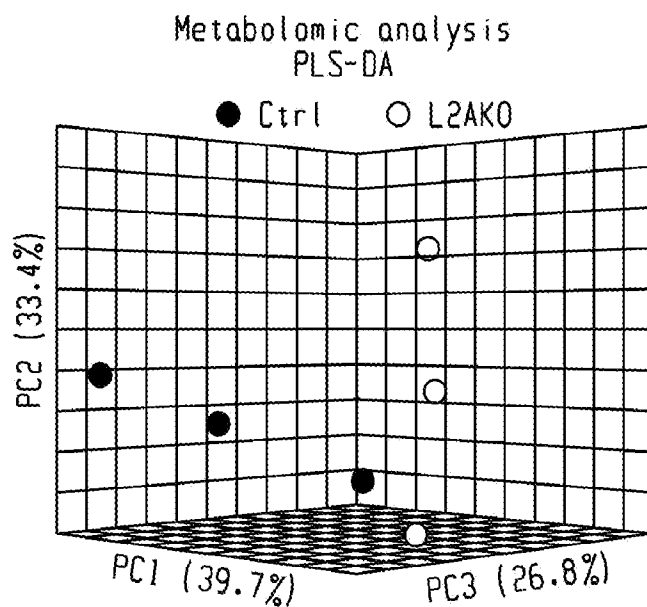
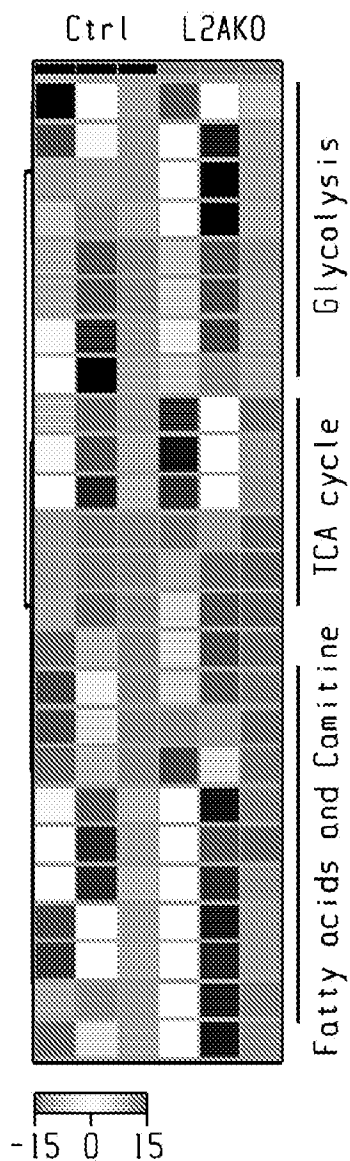


Fig. 2I

Fig. 2J



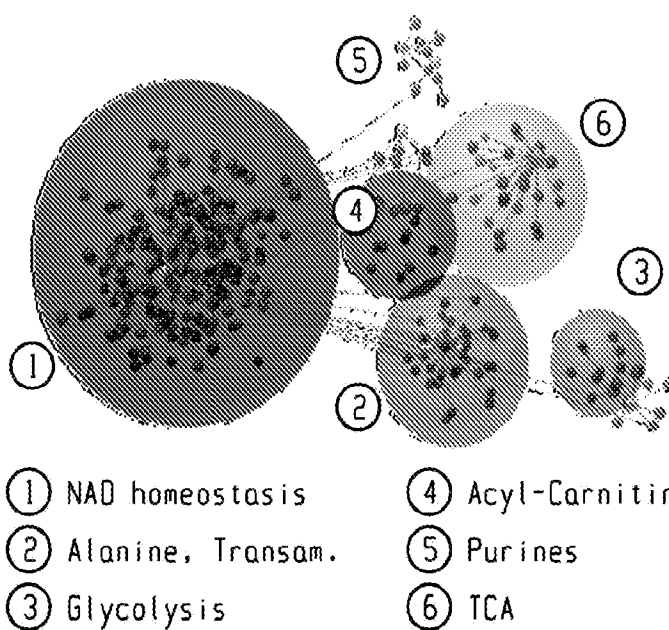


Fig. 2K

Fig. 2L

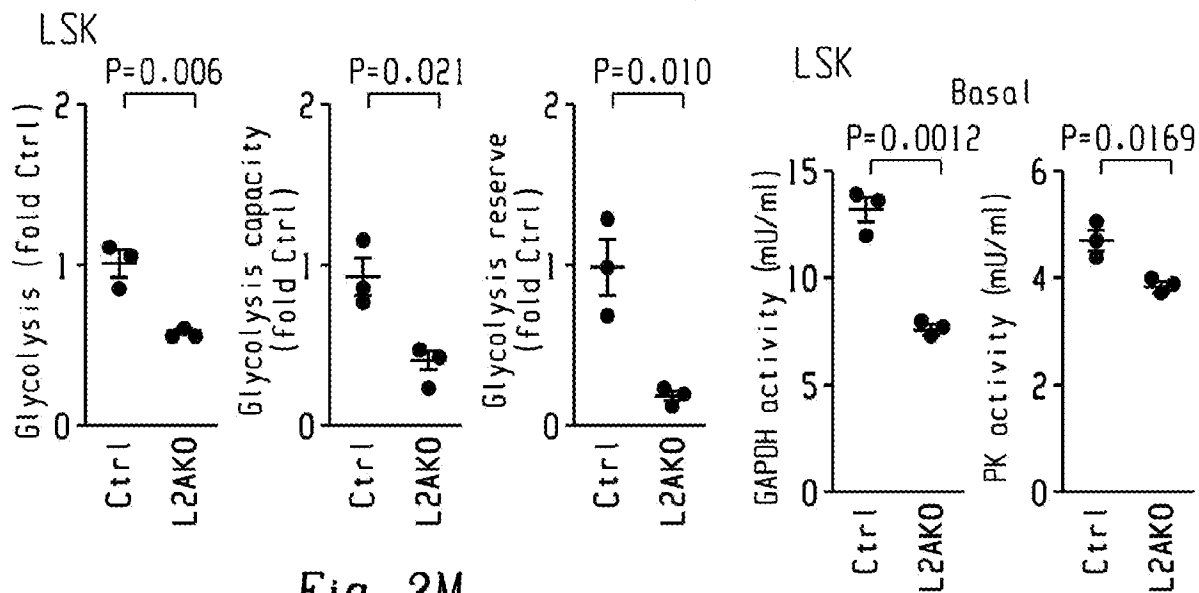
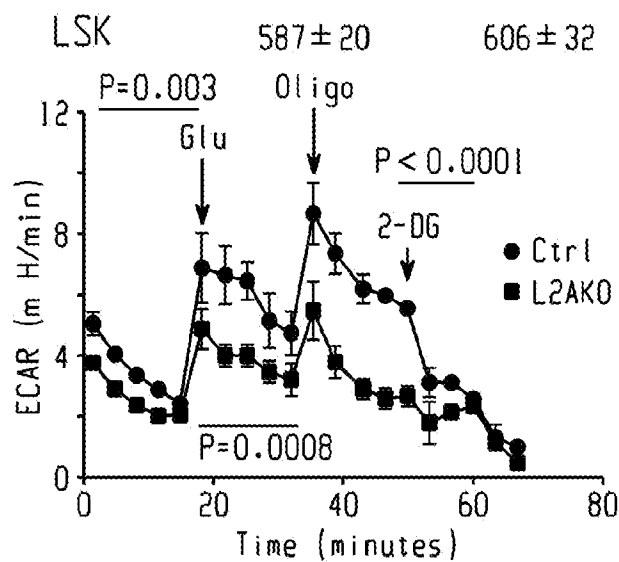
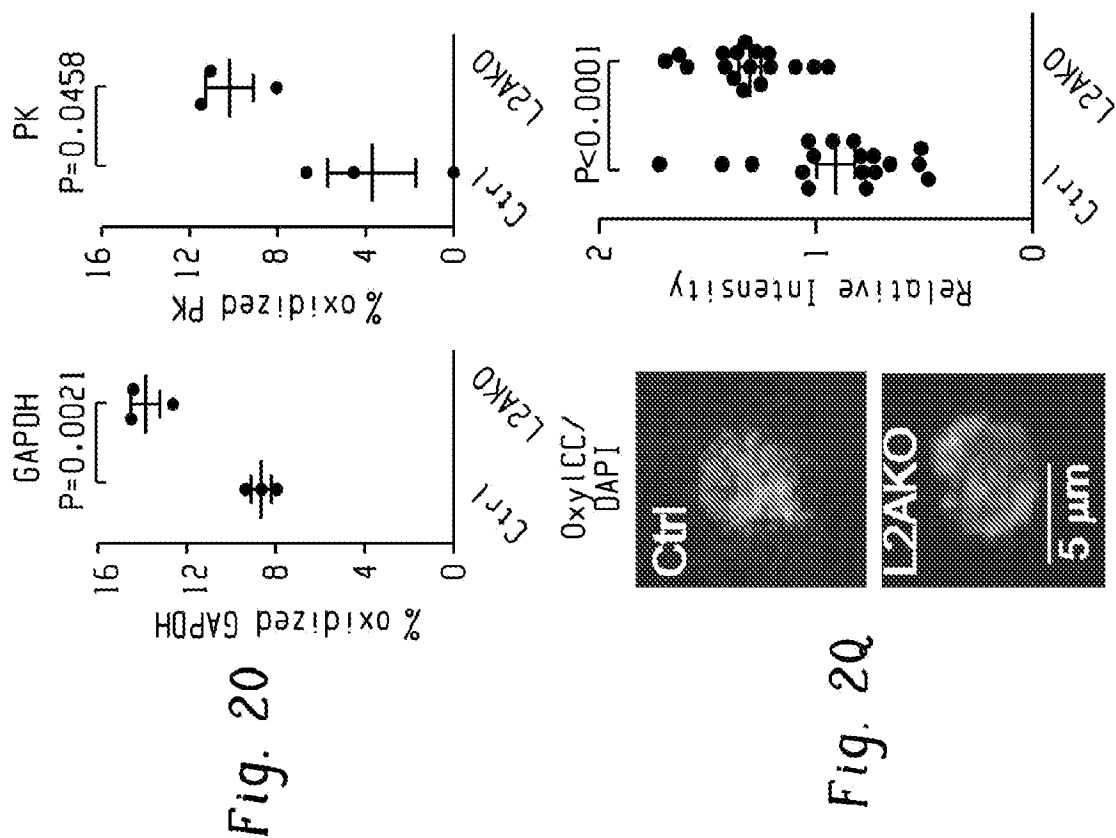
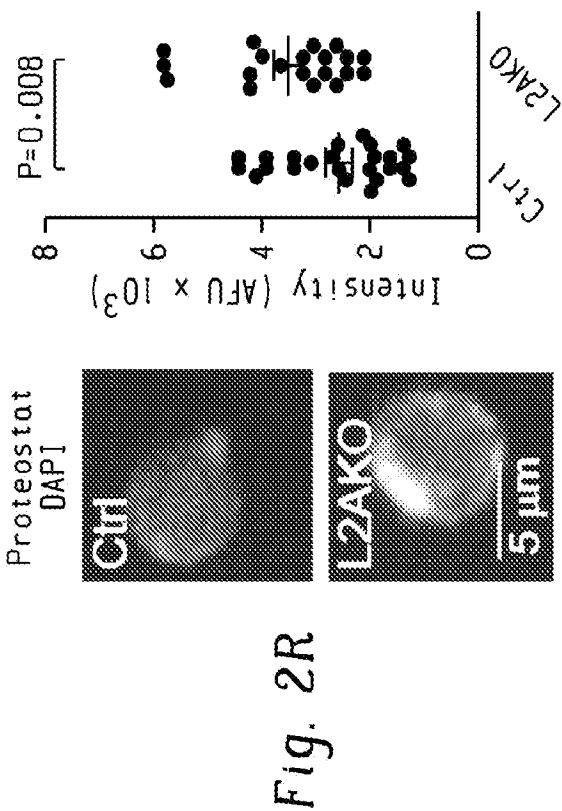
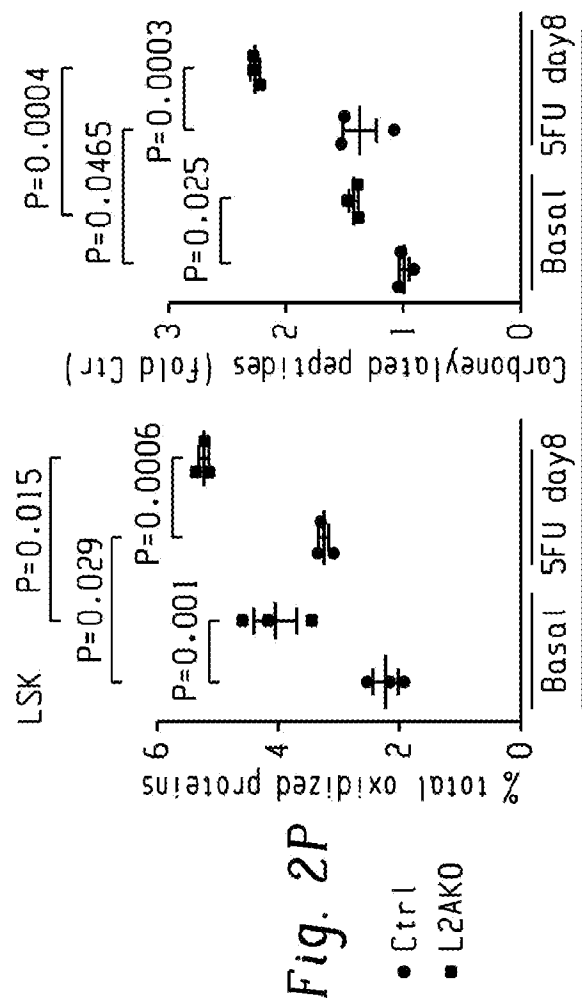


Fig. 2M

Fig. 2N



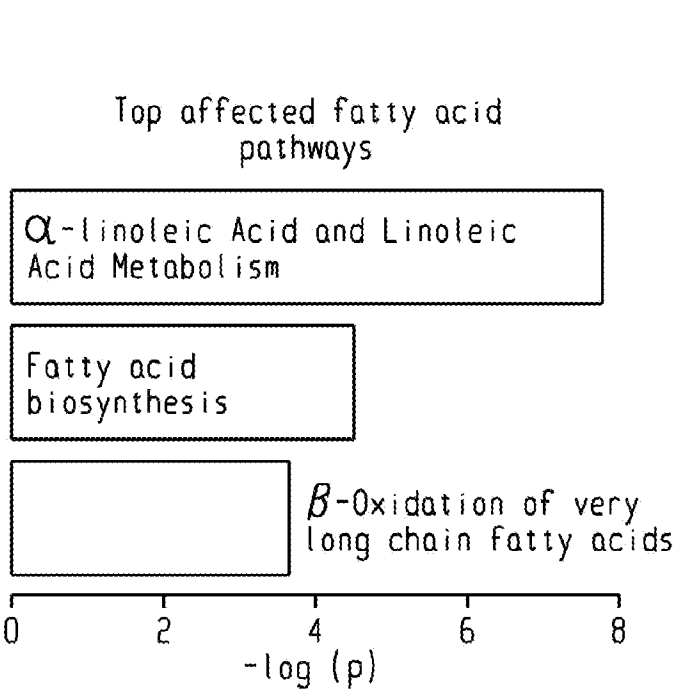


Fig. 3A

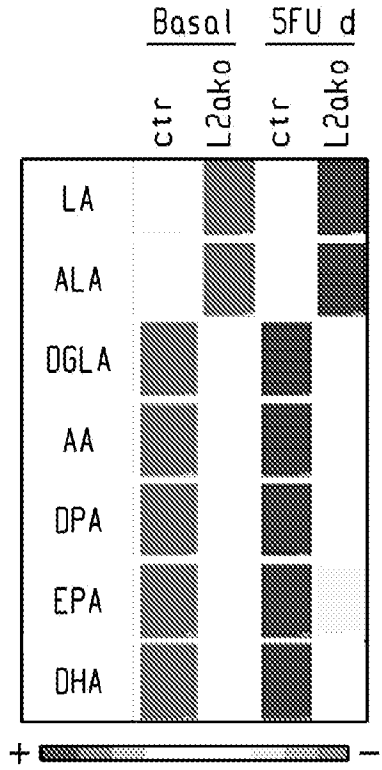


Fig. 3B

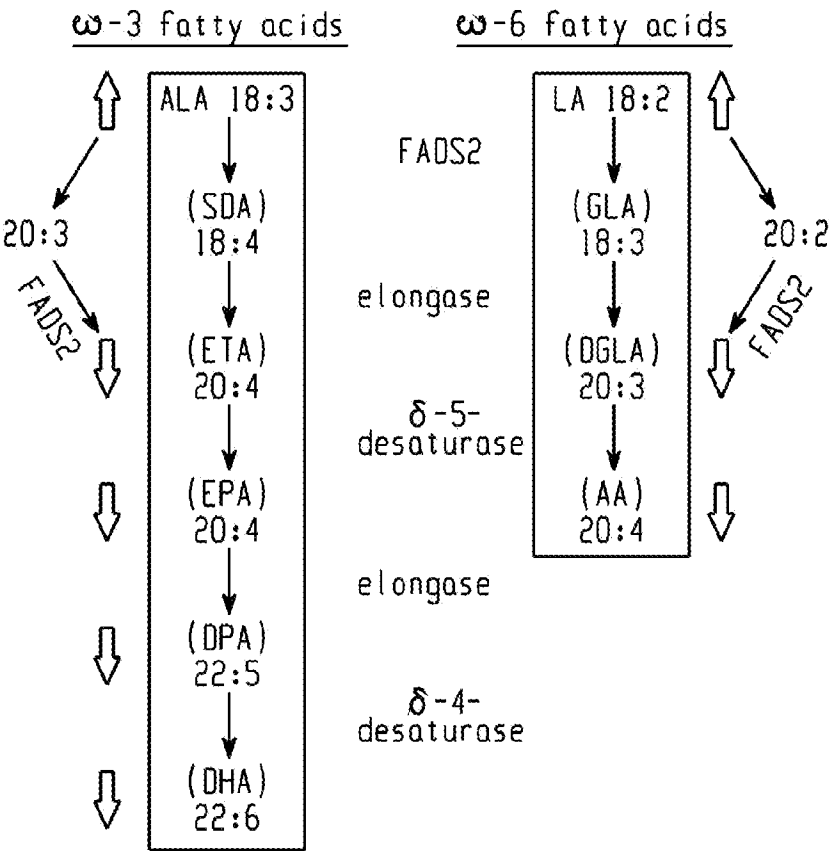


Fig. 3C

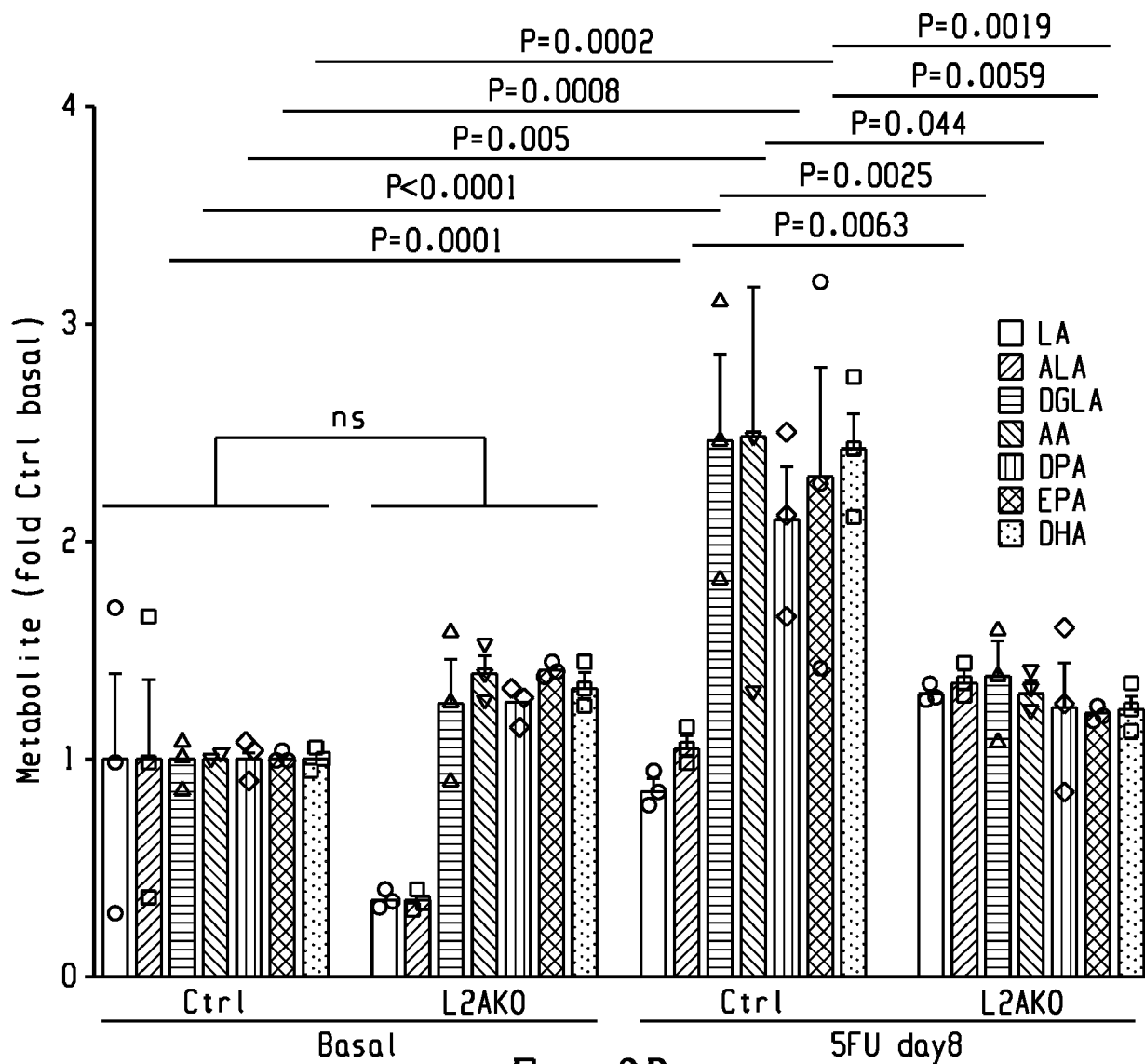


Fig. 3D

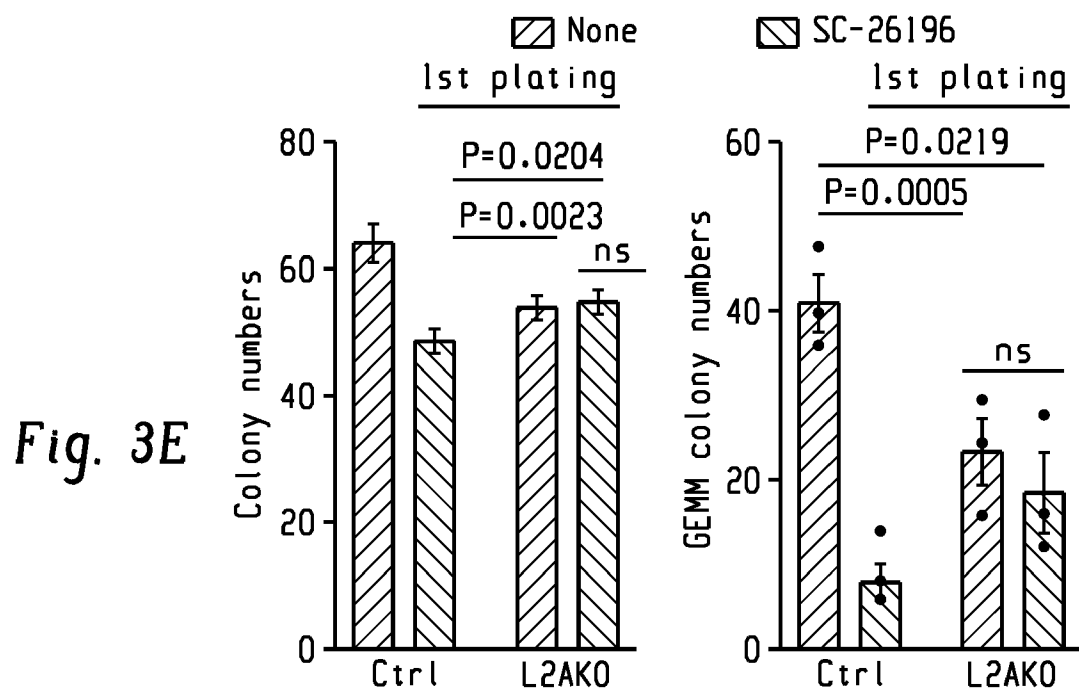


Fig. 3E

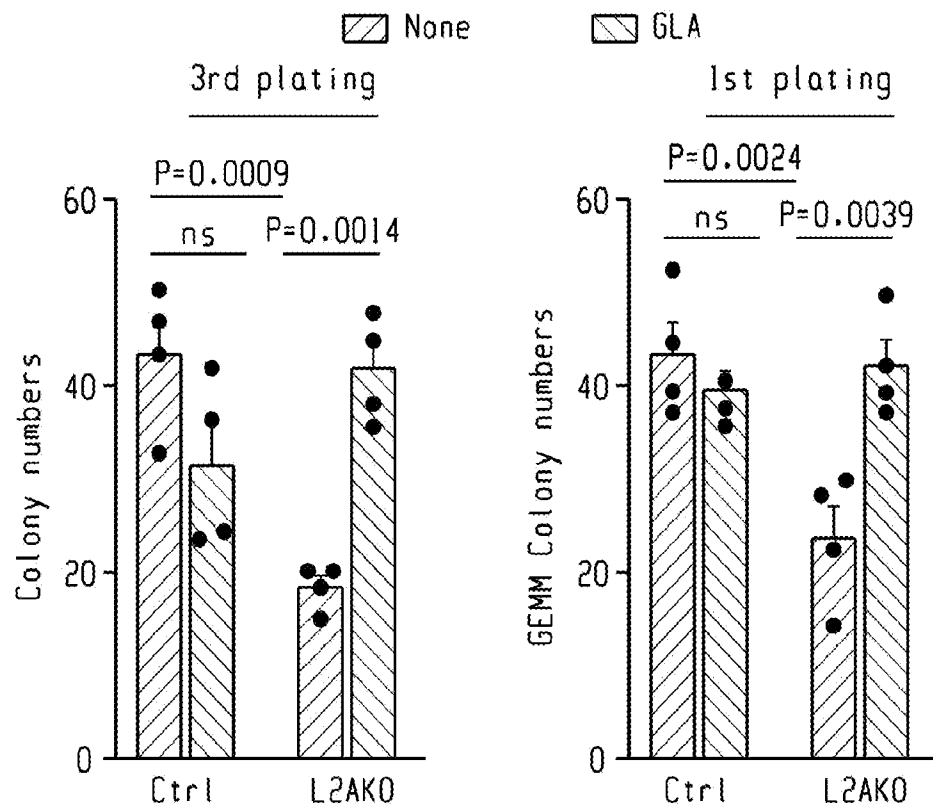


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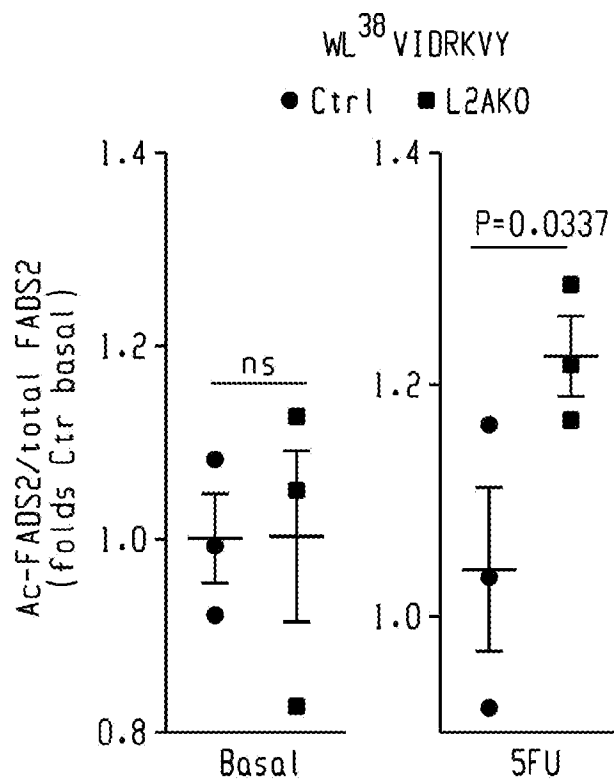


Fig. 3G

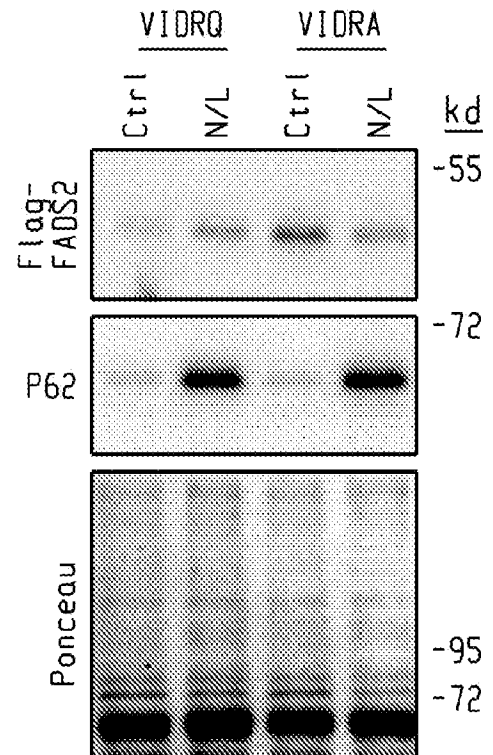


Fig. 3H

Fig. 3I

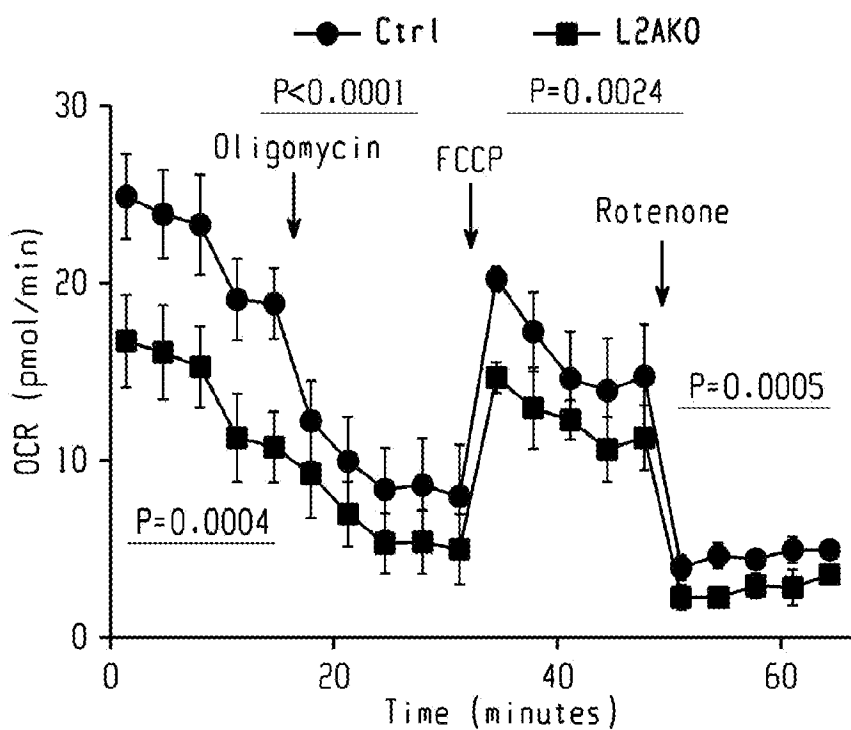
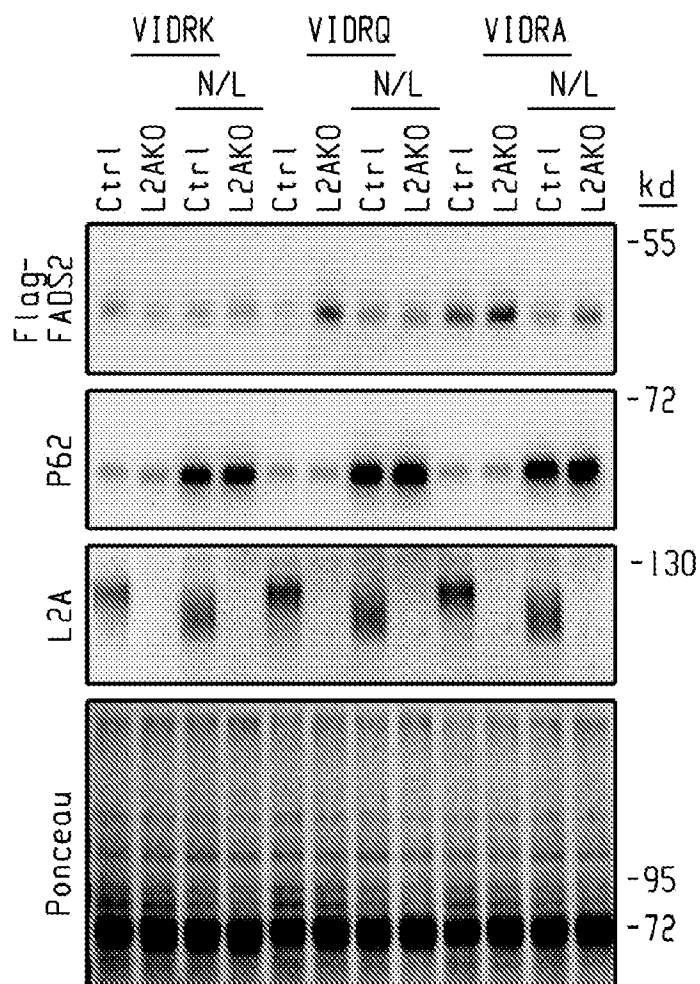


Fig. 3J

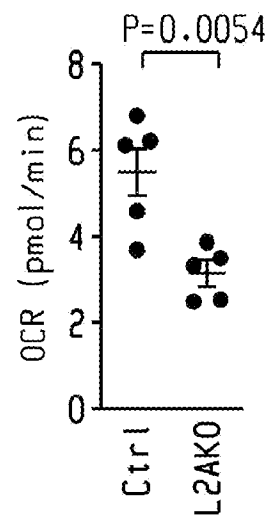
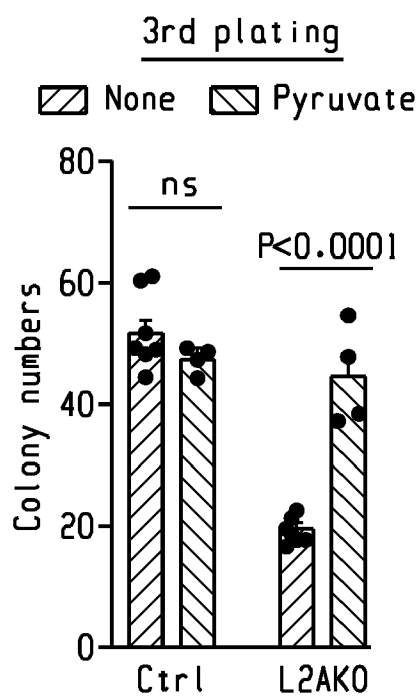
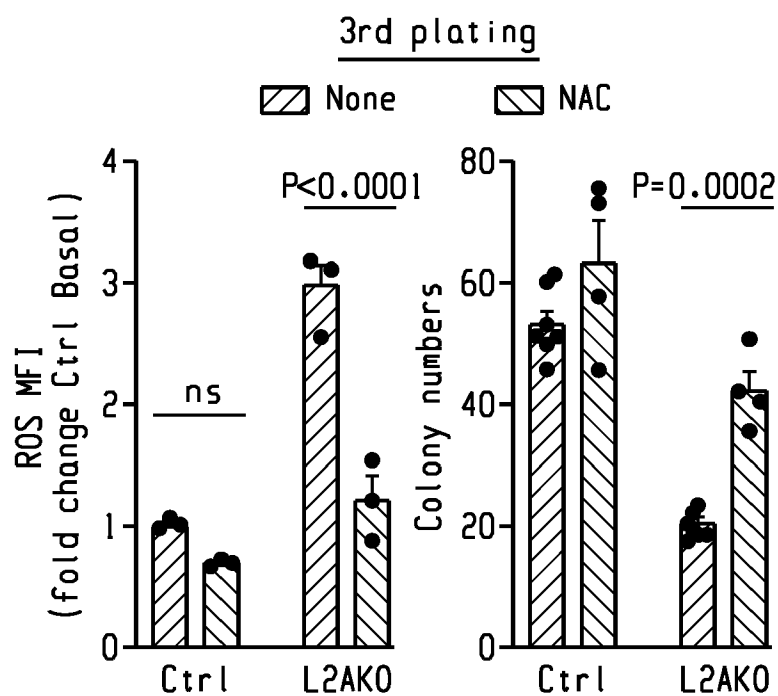
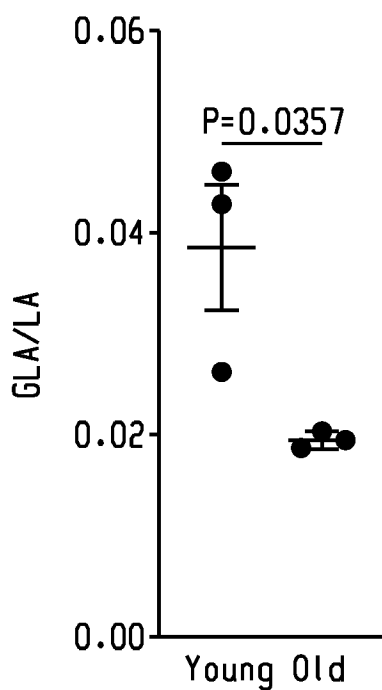
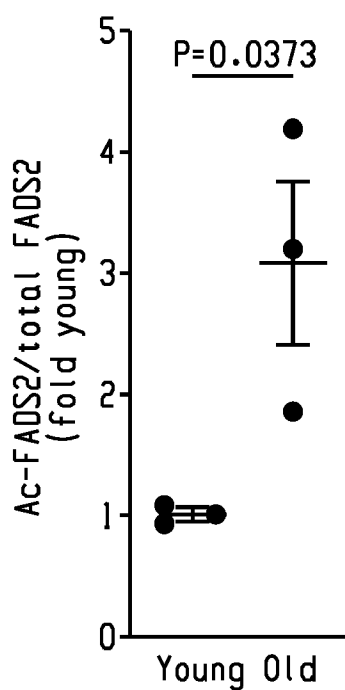
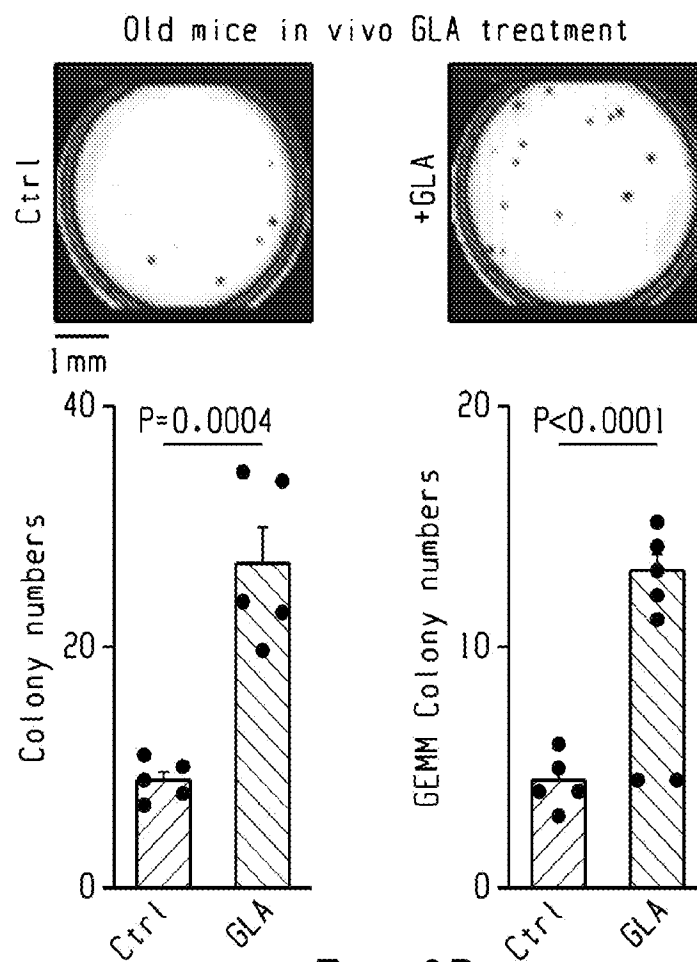
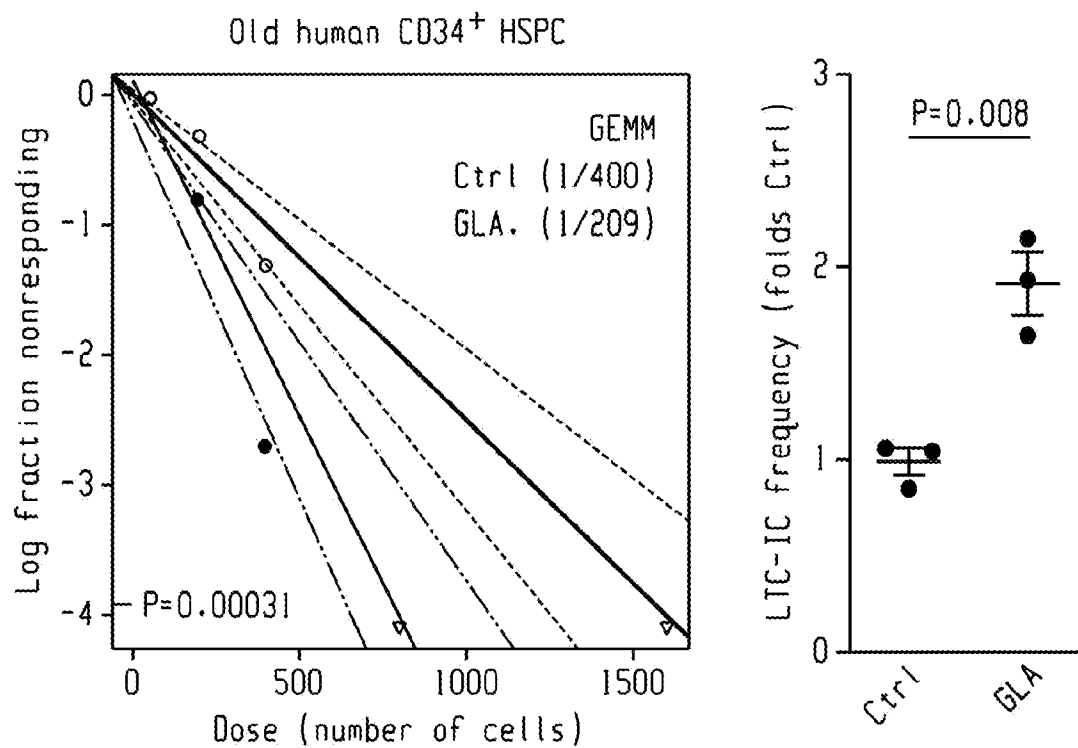


Fig. 3K

*Fig. 3L**Fig. 3M**Fig. 3N**Fig. 3O*

*Fig. 3P**Fig. 3Q*

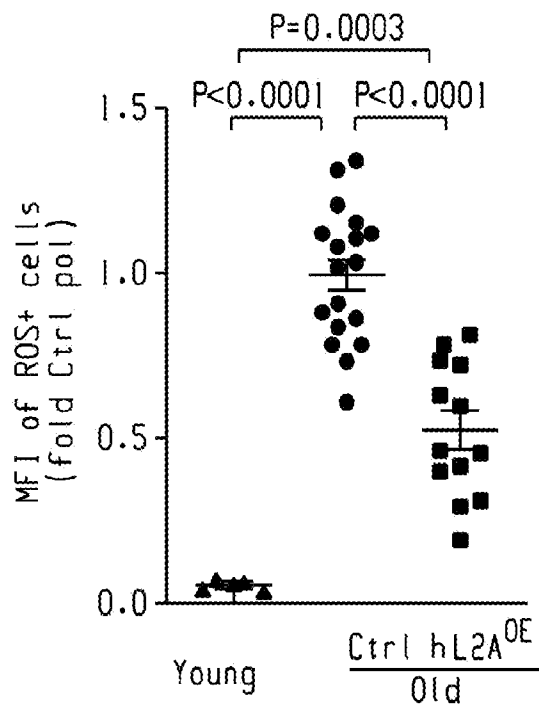


Fig. 4A

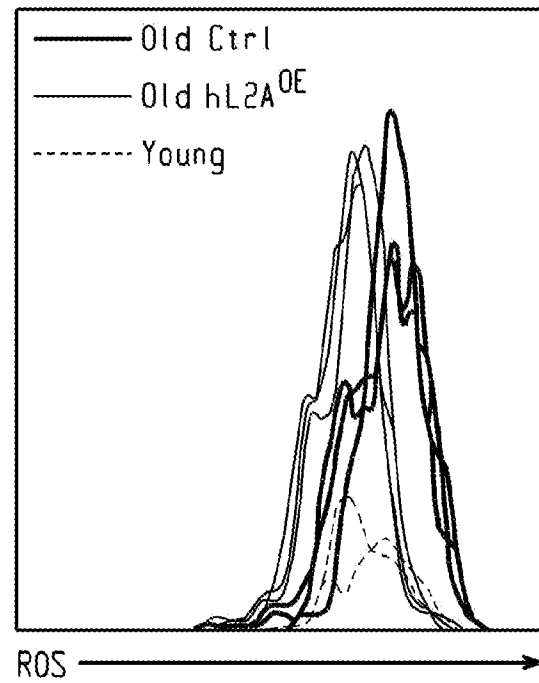


Fig. 4B

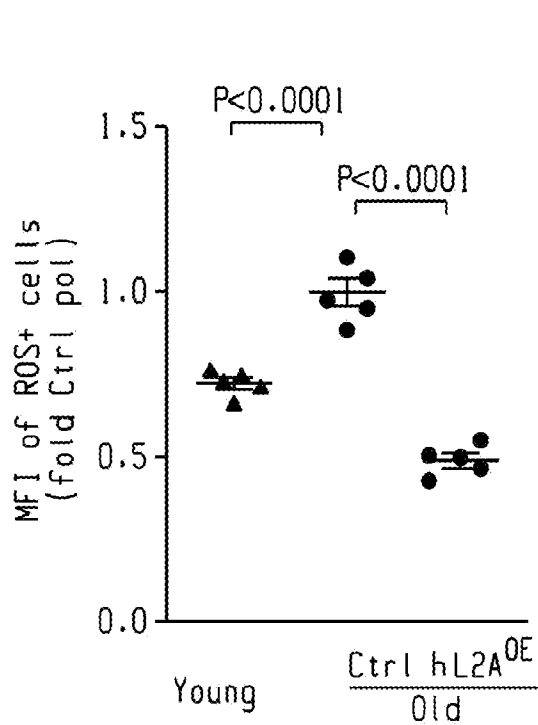


Fig. 4C

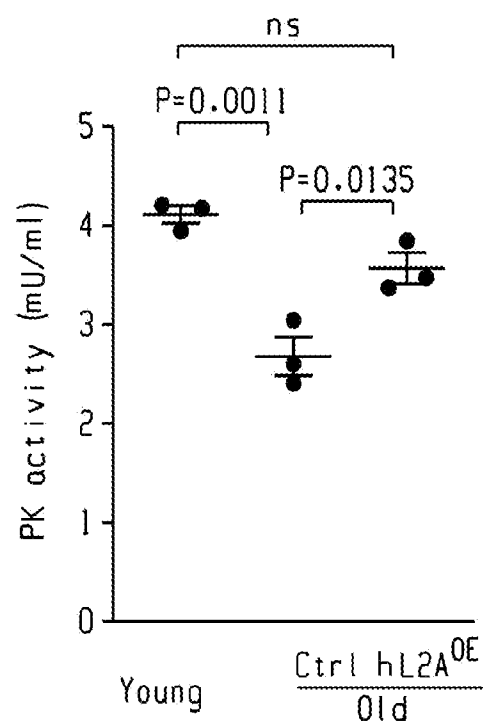
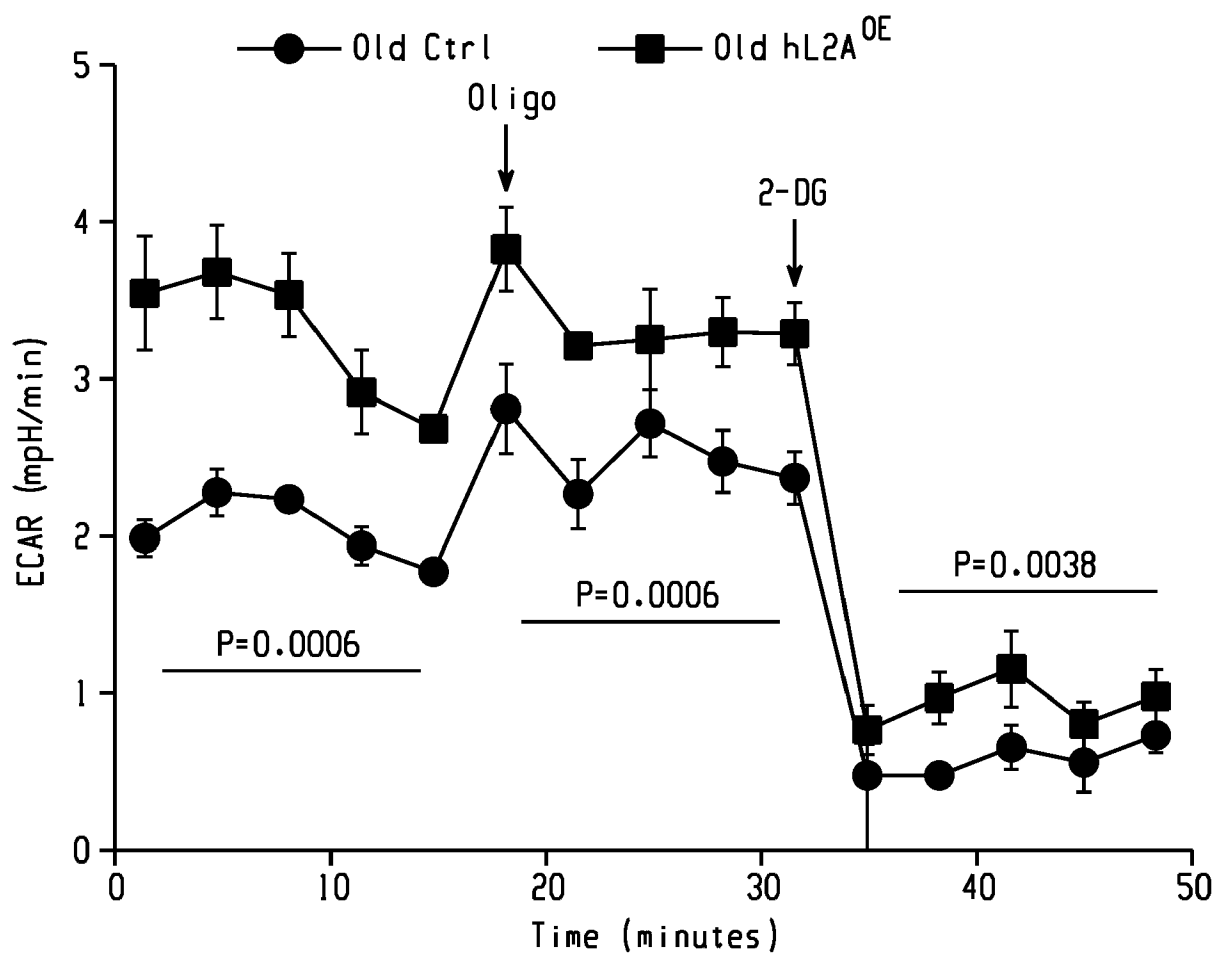
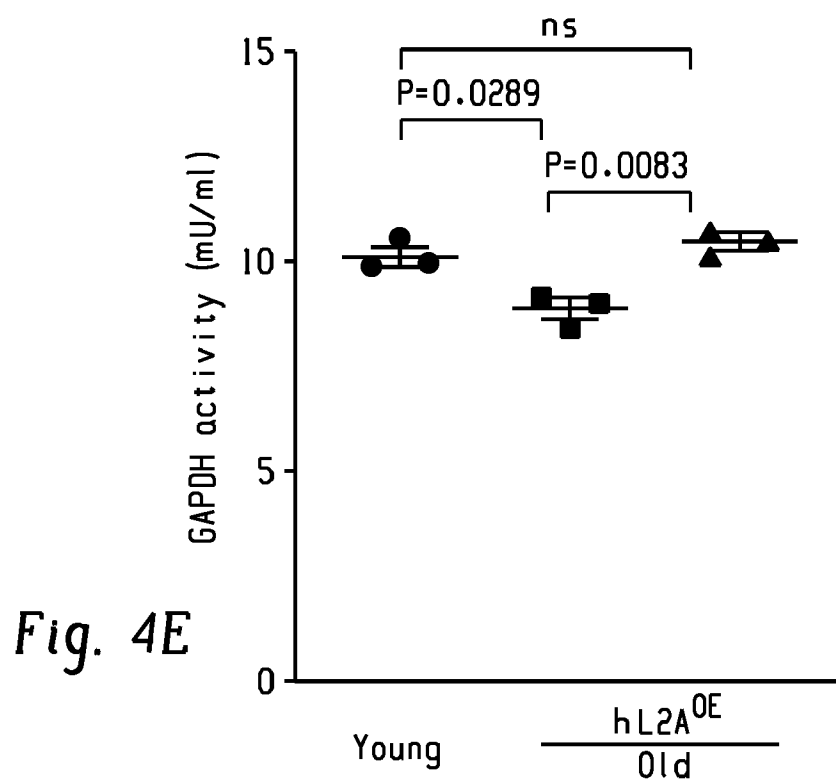


Fig. 4D



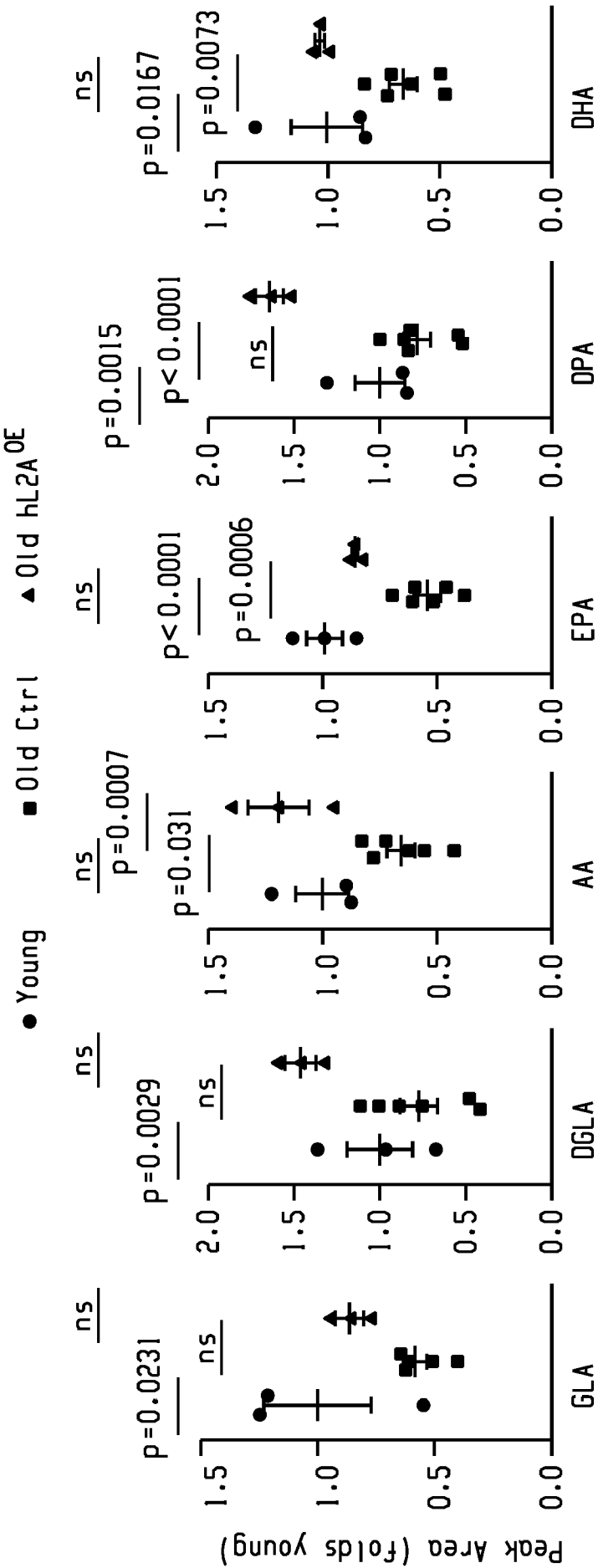


Fig. 4C

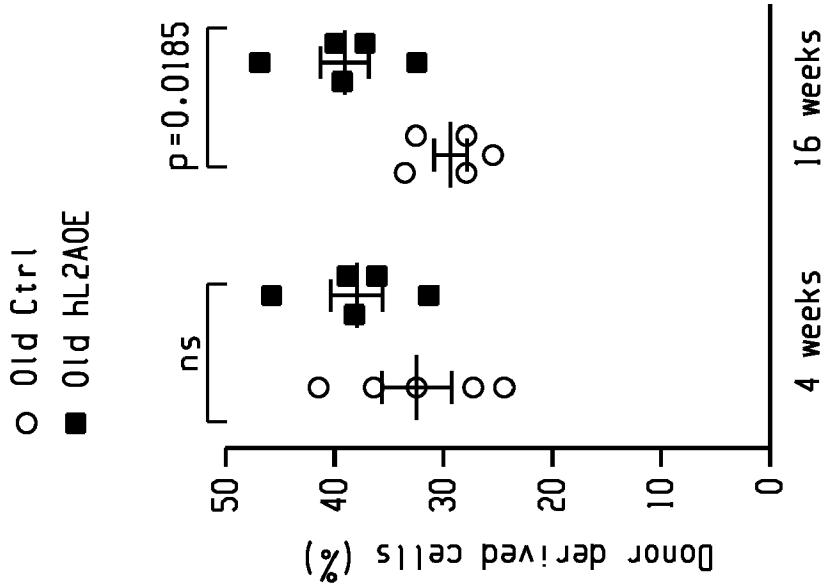


Fig. 4H

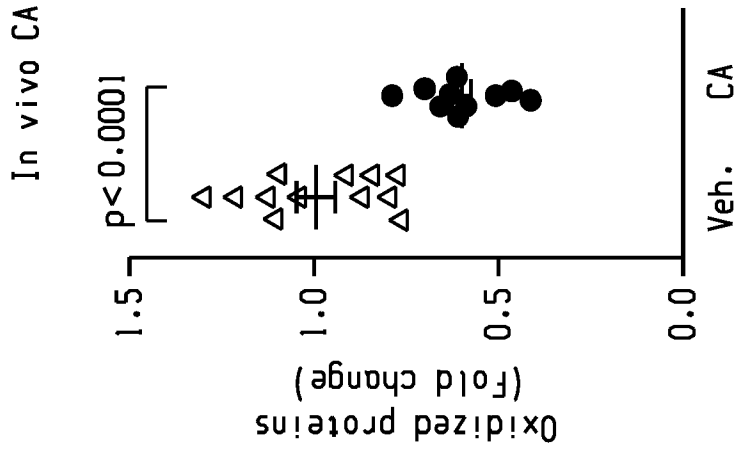


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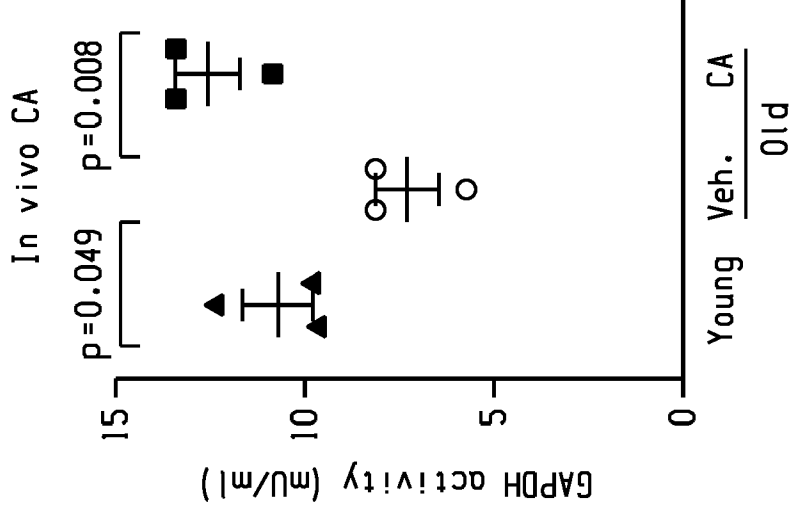


Fig. 4J

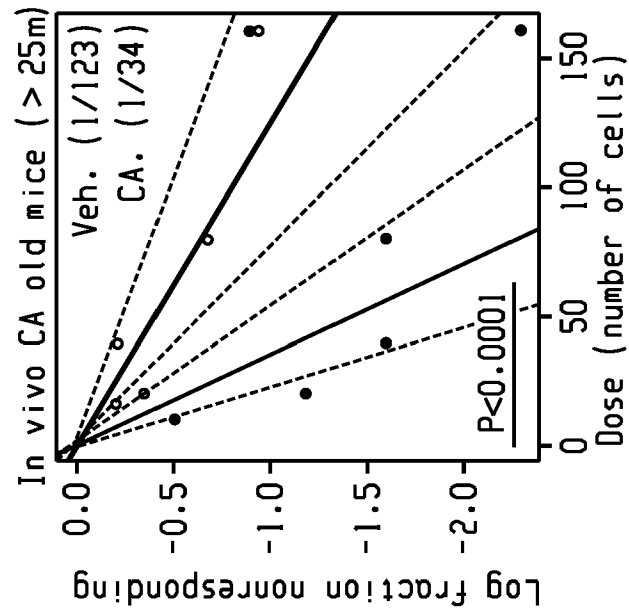


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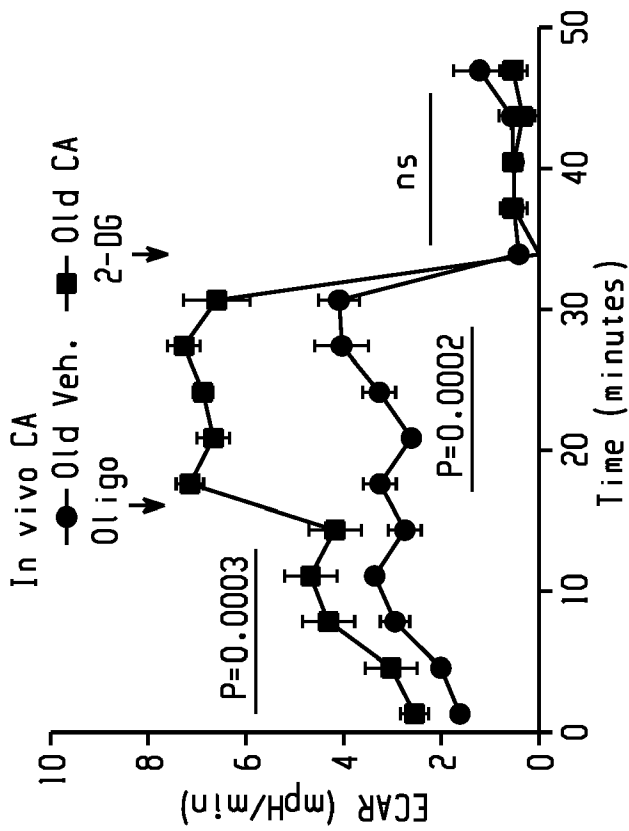


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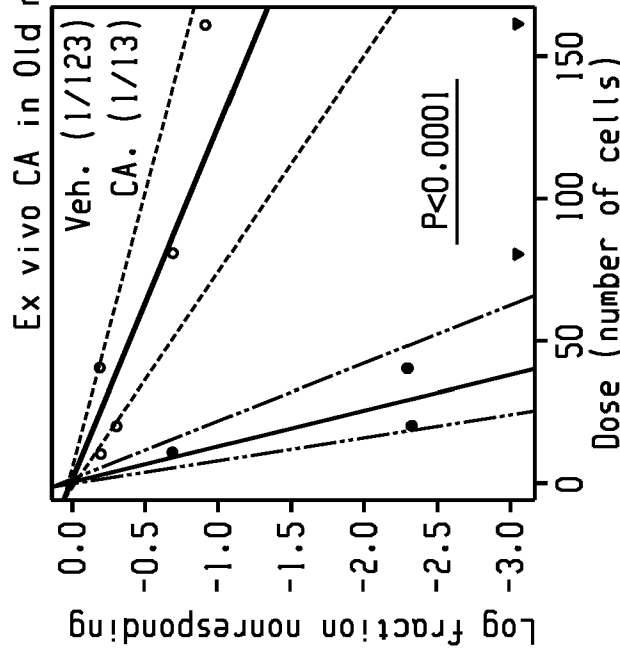
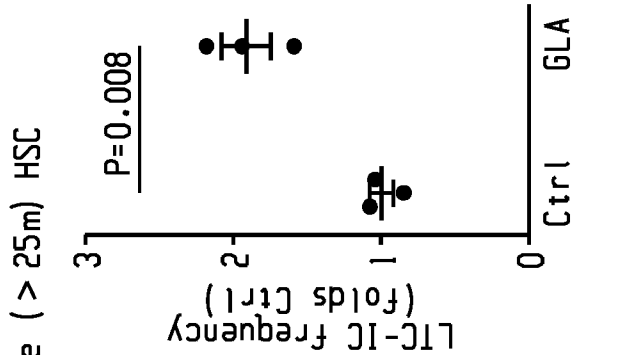


Fig. 4M

Ex vivo CA in Old mice (>25m) HSC

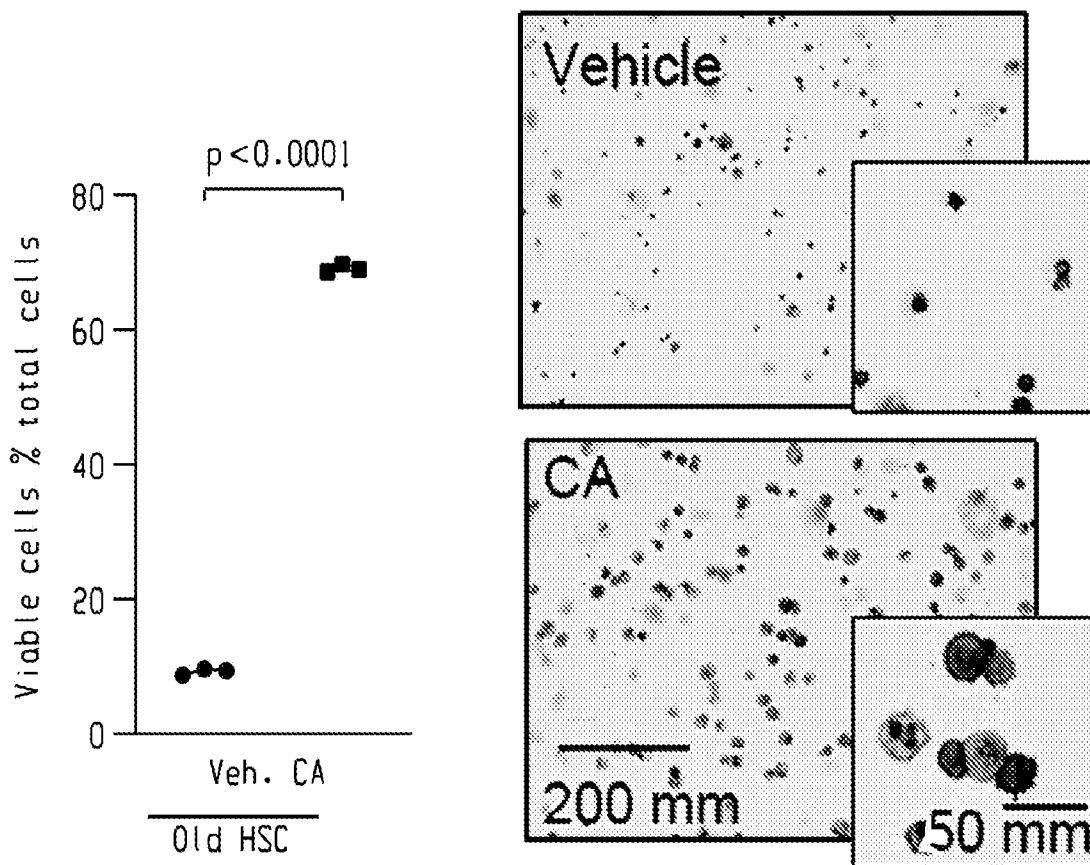


Fig. 4N

Fig. 4O

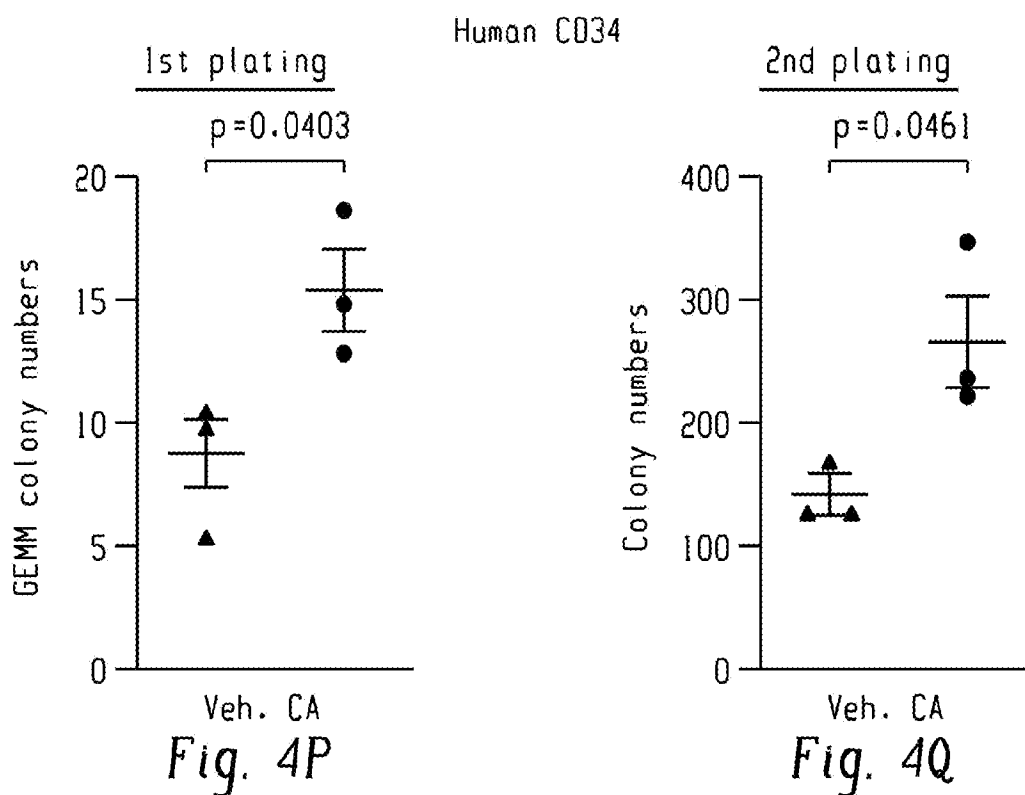


Fig. 4P

Fig. 4Q

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/12309

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 35/28, C12N 5/074, C12N 5/078, C12N 5/071, A61K 45/06, A61P 37/04 (2022.01)

CPC - A61K 38/18, A61P37/04, A61K31/422, A61K31/423, A61K31/497, C07D 263/56, C07D 263/57, C07D 413/10, C07D 413/12, A61P 43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	✓ DONG et al. Chaperone-Mediated Autophagy Ensures Hematopoietic Stem Cell Maintenance. Blood, 13 November 2019, Vol. 134, Supplement 1, Abstract 272; in entirety	1, 14 ----- (3,4)/1, 9, 15
Y	WO 2020/077024 A1 (ALBERT EINSTEIN COLLEGE OF MEDICINE) 16 April 2020 (16.04.2020) claims 19, 20, 23, 24; para [0005], [0132], [0133], [0085], [0086]	(3,4)/1
Y	✓ KUHIKAR et al. Transforming growth factor b1 accelerates and enhances in vitro red blood cell formation from hematopoietic stem cells by stimulating mitophagy. Stem Cell Research & Therapy, 19 February 2020, Vol. 11, Article 71; Abstract, pg 2, col 2 to pg 6, col 1	9
Y	✓ NAKAMURA-ISHIZU et al. Hematopoietic Stem Cell Metabolism during Development and Aging. Dev Cell. 11 August 2020, Vol. 54, No. 2, pg 239-255; Abstract, pg 2 [according to the document posted], pg 12, pg 29, Fig 3 and its legend	15

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

06 April 2022

Date of mailing of the international search report

MAY 17 2022

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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Authorized officer

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/12309

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

a. ☒ forming part of the international application as filed:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 22/12309

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-8, 12, 13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

***** See Supplemental Sheet to continue *****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, (3,4)/1, 9, 14-15

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

In Continuation of
Box III. Observations where unity of invention is lacking:

Group I+, claims 1, (3,4)/1, 9-11, 14-15, directed to a method of increasing the activity/improving proteostasis of a population of hematopoietic stem cells (HSCs) in vivo or in vitro comprising contacting the population of HSCs with a Chaperone-mediated Autophagy (CMA) activating compound. The method will be searched to the extent that the resulting blood cells encompass red blood cells. It is believed that claims 1, (3,4)/1, 9, 14-15 encompass this first named invention, and thus these claims will be searched without fee to the extent that the resulting blood cells encompass red blood cells. Additional produced blood cell(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected produced blood cell(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be produced white blood cells, i.e., claims 1, (3,4)/1, 10, 14-15.

Group II, claims 2, (3,4)/2, directed to a method of treating a blood cancer or myelodysplastic syndrome in a mammal comprising administering a therapeutically effective amount of a CMA activator to the mammal.

The inventions listed as Group I+ and Group II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The special technical feature of inventions of Group I+ is a method of increasing the activity/improving proteostasis of a population of HSCs that is not required by Group II.

The special technical feature of inventions of Group II is a method of treating a blood cancer or myelodysplastic syndrome in a mammal that is not required by Group I+.

The special technical feature of some inventions of Group I+ is a specific blood cell recited therein.

The inventions of Group I+ and Group II share the technical feature of a method comprising contacting a population of cells with/ administering to a subject a sufficient concentration of an CMA activator. However, this shared technical feature does not represent an improvement over prior art as being anticipated by WO 2020/077024 A1 to ALBERT EINSTEIN COLLEGE OF MEDICINE (16 April 2020) (hereinafter "AECM").

AECM discloses a method comprising administering to a subject a sufficient concentration of an CMA activator (claim 23, "A method of selectively activating chaperone-mediated autophagy in a subject in need thereof comprising administering an effective amount of a compound of any one of claims 1 to 20 to the subject"; pg 26, para [0132], [0133], "EXAMPLE 161. MEASUREMENT OF CMA ACTIVITY IN VITRO").

As the technical feature was known in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the inventions.

The inventions of Group I+ share the technical feature of a method of increasing the activity of a population of HSCs in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a CMA activating compound to increase the number of blood cells produced by the HSCs in a time period relative to the number of blood cells produced by the HSCs in the same time period prior to being contacted with the CMA [activating compound] (claim 1). Some inventions of Group I+ share the technical feature of a method of improving the proteostasis of a population of HSCs in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a CMA activating compound sufficient to improve a marker of proteostasis in the population of HSCs relative to the marker of proteostasis in the population of HSCs prior to being contacted with the CMA [activating compound]. However, these shared technical features do not represent an improvement over prior art as being anticipated by a publication titled "Chaperone-Mediated Autophagy Ensures Hematopoietic Stem Cell Maintenance" by Dong, et al. (Blood 2019, Vol. 134, Supplement 1, Abstract 272) (hereinafter "Dong").

Dong discloses a method of increasing the activity/ improving the proteostasis of a population of HSCs in vivo or in vitro comprising contacting the population of HSCs with a sufficient concentration of a CMA activating compound to increase the number of blood cells produced by the HSCs in a time period relative to the number of blood cells produced by the HSCs in the same time period prior to being contacted with the CMA activator or to improve a marker of proteostasis in the population of HSCs relative to the marker of proteostasis in the population of HSCs prior to being contacted with the CMA activator (pg 1 to pg 2, "Using novel mouse models that allow for CMA tracking (KFERQ-Dendra mice) and for selective depletion of LAMP-2A in hematopoietic cells..., we have investigated the physiological role of CMA in HSCs during steady-state and upon activation? the basal CMA activity detected in quiescent HSC under steady-state conditions is significantly stimulated upon HSC activation following 5-fluorouracil (5-FU) in vivo exposure. This upregulation of CMA is necessary to ensure HSCs persistence during activation because, upon serial 5-FU injections, CMA-deficient HSCs had a significantly reduced multilineage reconstitution ability with premature bone marrow failure... Using comparative transcriptomics and metabolomics on HSCs from control and LAMP-2A-deficient mice, we found evidence for metabolic alterations and dysfunctional redox signaling. We confirmed that CMA-deficient HSCs have reduced rates of glycolysis, lower ATP production and higher reactive oxygen species levels than control cells. Deficient cellular energetics and increased oxidative stress are important consequences of CMA failure in HSC, since supplementation with pyruvate or treatment with anti-oxidant agents (i.e. N-acetyl-cysteine) was sufficient to restore CMA-deficient HSC function, as measured by serial colony formation. Proteomic analysis of CMA-deficient LinSca1+c-Kit+ (LSK) cells revealed an overall increase of acetylated and oxidized proteins, including key metabolic enzymes and proteins required for the cellular response to oxidative stress. We propose that, upon CMA failure, the inability of HSC cells to timely turning over these regulatory proteins, favors accumulation of unwanted post-translational modifications that interfere with their normal functioning... our findings suggest that CMA upregulation during HSCs activation [by 5-FU] is required for the proteome remodeling that facilitates transition from quiescent to activated cells. By assuring timely turnover of selected proteins, CMA sustains the metabolic adaptation required to meet these cells' energetic needs and assures an efficient cellular response to stress").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the inventions.

Therefore, inventions of Group I+ lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.