Concept Paper

Toward systemic lipofuscin removal

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Toward Systemic Lipofuscin Removal

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Abstract: Lipofuscin is indigestible garbage that accumulates in the autophagic vesicles and cytosol of post-mitotic cells with age. Drs. Brunk and Terman postulated that lipofuscin accumulation is the main or at least a major driving factor in aging. They even posited that the evolution of memory is the reason why we get lipofuscin at all, as stable synaptic connections must be maintained over time, meaning that the somas of neurons must also remain in the same locale. In other words, they cannot dilute out their garbage over time through cell division. Mechanistically, their position certainly makes sense given that rendering a large percentage of a post-mitotic cell's lysosomes useless must almost certainly negatively affect that cell and the surrounding microenvironment. It may be the case that lipofuscin accumulation is the main issue with regard to current age-related disease. Degradation *in situ* may be an insurmountable task currently. However, a method of systemic lipofuscin removal is discussed herein.

Keywords:: anti-aging; lipofuscin; *Hydra vulgaris*; TFEB; secretory autophagy; and tissue-resident macrophages

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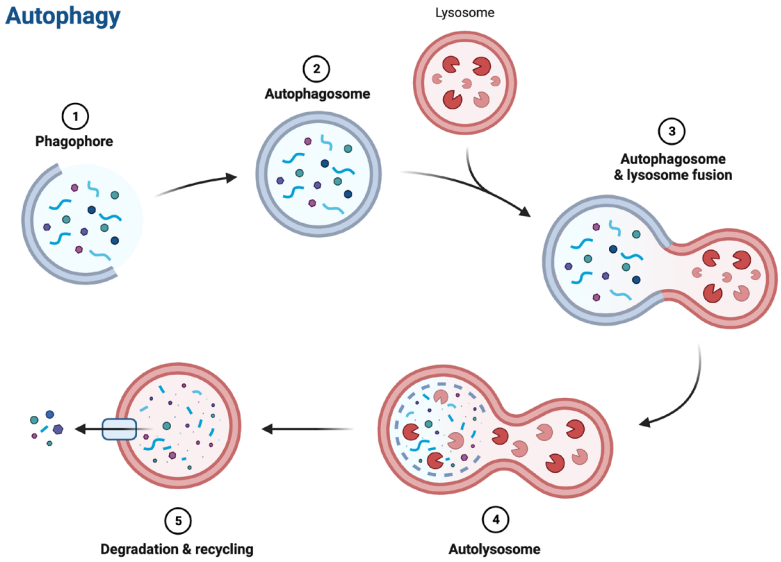
Graphical abstract:

A.



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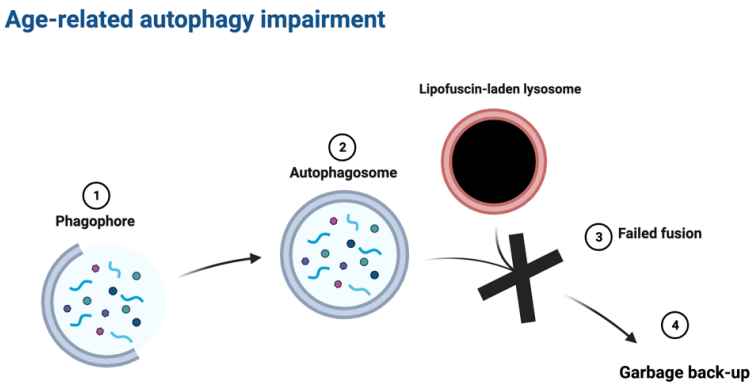
B.



A. Normal autophagy in a young, healthy cell. B. Autophagy impairment with age due to lipofuscin accumulation.

Introduction:

Biological aging is a complex molecular process that takes place over time in all organisms. However, organisms have evolved mechanisms to repair various forms of age-related damage. For example, DNA repair enzymes exist that can fix damage in nuclear DNA. Mitophagy enables the degradation of damaged mitochondria. The immune system, when one is young at least, eliminates at least some proportion of one's senescent cells.

There are many theories about why we age, but one stands out as being the most plausible based on the evolutionary and mechanistic evidence. That is the "garbage catastrophe theory of aging." Drs. Brunk and Terman postulated years ago that the problem of aging can essentially be summed up as a "garbage disposal issue [1]." The main idea is that basically old molecules are sometimes damaged in ways that prevent the lysosomes from breaking them down properly, and over time these damaged, old molecules accumulate inside the lysosomes. Eventually, the lysosomes become full of this indigestible garbage, i.e., "lipofuscin", and cannot perform their normal function - then there is a garbage back-up and the cell starts to decline health-wise.

There are two arguments for lipofuscin removal being the most important goal of anti-aging science currently. One is evolutionary and one is mechanistic.

Evolutionary argument:

In nature, there are only a handful of organisms that can be said to be essentially biological immortal. *Hydra vulgaris* (i.e., *magnipapillata*) is one of such organisms, and the reason for this might be that its indigestible garbage is essentially released from its body over time. It has three cell lineages - ectodermal epithelial, interstitial, and endodermal epithelial. All the epithelial cells in its body column are stem cells that continuously divide - displacing cells toward its extremities. The cells at the extremities slough off eventually [2,3]. In terms of the interstitial lineage, the differentiated cells that its stem cells produce are closely associated with epithelial cells and so are continuously displaced as well. This is a convenient way to dispose of lipofuscin - i.e., through dilution and cell shedding. However, continuous replacement of neurons may not allow for the stable inter-neuronal interactions required for long-term memory [4].

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Additionally, *H. vulgaris* displaces and sheds extracellular matrix (ECM), i.e., mesoglea, associated with its epithelial cells [5]. However, mesoglea in the head region remains stationary. Mesoglea in this region appears to be turned over instead, and rather slowly at that.

Lobsters continually grow throughout life; their fully differentiated cells express telomerase, allowing them to keep dividing as needed [6]. This includes the cells of their central nervous system, which allows for adult neurogenesis [7]. They also shed their shells periodically. Thus, the same logic appears to apply to them. However, their growth process does not appear to be fast enough to prevent lipofuscin from accumulating over time [8]. Notably, lipofuscin accumulation in eyestalk ganglia [9] is used as a gauge of biological age in lobsters (and myocardial lipofuscin accumulation can be used a marker of chronological age in humans [10]). Lobsters can retain memories, but only for a short time span [11].

Naked mole rats are also very long lived, and it has been shown that they have unusually active autophagic systems [12,13]. They also have very effective anti-cancer defenses [14]. However, even with enhanced autophagy, naked mole rats still accumulate lipofuscin in their post-mitotic tissues [15].

It appears as though all animals that age, e.g., flies [16], worms [17,18], lobsters, naked mole rats, mice [19], non-human primates [20], and humans [21] accumulate lipofuscin in their post-mitotic tissues. None of the aforementioned organisms seem to possess any evolutionarily "built-in" ways of exporting the lipofuscin that accumulates in their post-mitotic cells from their bodies, presumably because that would be an unnecessary expenditure of energy in light of procreation.

Export from the post-mitotic cells themselves is possible through exocytosis [22], extracellular vesicle secretion, or secretory autophagy. That part is not too energetically costly. However, from *in vitro* studies, it does not seem as though lipofuscin is exported from post-mitotic cells very often [23]. It also has only rarely been observed *in vivo [24]*.

More importantly, when exported, there would ultimately be nowhere for the garbage to go except to be picked up by tissue-resident or circulating phagocytes, which themselves become bloated with lipofuscin. (Transfer of lipofuscin to tissue-resident phagocytes through tunneling nanotubes [TNTs] or partial cell fusion is also theoretically possible.)

Lipofuscin within aged tissue-resident macrophages is perhaps mostly derived from damaged molecules generated by their own, internal metabolic processes - rather than the phagocytosis of extracellular, lipid-saturated debris or efferocytosis in the context of aged, lipofuscin-laden cells [25].

Crucially, there is no evidence in the literature that tissue-resident or circulating phagocytes efficiently leave the body through migration to the gastrointestinal tract, urogenital tract, skin, or lungs, except possibly when there are infections in those areas.

Mechanistic argument:

Lipofuscin is broadly a complex amalgam of highly oxidized cross-linked macromolecules, including proteins, lipids, sugars, and metal cations. It varies in composition between species, individuals, cell types, and plausibly even cells of the same type [21,26–28].

While it was originally widely believed that lipofuscin is inert, it may in fact permeabilize or otherwise destabilize lysosomes and promote apoptosis or necrosis [21,29–32]. Even if the damaged molecules are mostly sequestered within lysosomes and are not actively harmful to the cell, the fact that many lysosomes become full of garbage and therefore are almost surely unable to perform their normal functions nearly as well just logically seems as though it must be a major problem for the cell. If a critical threshold is reached in enough cells in a tissue, e.g., the brain, it clearly would be problematic. The cells may try to produce more lysosomes - but will eventually reach capacity.

Along these lines, lipofuscin accumulation decreases the ability of cells to adapt to amino acid starvation [33] and increases their susceptibility to oxidative stress [34]. Increases in the dietary intake of metal cations such as Fe^{2+}, which plays a key role in the formation of lipofuscin, augments lipofuscin accumulation [35–37] and speeds up aging [38]. Manganese acts similarly [39]. Furthermore, artificial lipofuscin loading into human cells results in a significant loss of cellular

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viability [40,41]. Another study showed that the dietary intake of artificial lipofuscin shortens the lifespan of *Drosophila melanogaster [42]*.

With regard to humans, it has been demonstrated that multiple neuronal cell subtypes become densely packed with lipofuscin granules with age [43–45]. In large motor neurons of centenarians, lipofuscin constitutes up to 75% of total cytoplasmic volume [46]. Other post-mitotic cell types also accumulate substantial amounts of lipofuscin [10,47,48]. Lipofuscin-laden lysosomes are often much larger than typical lysosomes. The typical size of a lysosome in a fed, unaged cell is ~100 nm-500 nm in diameter [49]. In contrast, lipofuscin granules are generally 1-5 microns in diameter [46].

Finally, lipofuscin accumulation also explains the downward spiral of functionality that is seen in aging - i.e., the rapid acceleration in decline starting around 60-70 years of age [50]. That is because the decline in autophagy probably increases the rate at which lipofuscin is formed by allowing aggregates to stay around longer and develop further oxidative damage. Also, lipofuscin accumulation may lead to more free radical production [51], thus accelerating its accumulation as lipofuscin is heavily comprised of oxidatively damaged biomolecules. Furthermore, eventually, when autophagy levels have decreased substantially in many cells in a tissue, the rate of accumulation of other forms of age-related damage probably accelerates as well, which could further accelerate lipofuscin accumulation.

Clearance of Undigested Rubbish via Extraction (CURE):

I propose a strategy for systemic lipofuscin removal called "Clearance of Undigested Rubbish via Extraction" (CURE). As formulated currently, CURE involves three steps. The first step is to replace a patient's endogenous tissue-resident macrophages (TRMs) with edited variants. The second step is to administer a small molecule that induces these edited TRMs to transiently become hyper-motile and export molecular instructions for the secretion of mixed-age lysosomes from target cells. The third step is for them each to phagocytose an experimentally determined number of those lysosomes and then asymmetrically divide, wherein one progeny cell inherits all the phagosomes containing exported lysosomes and then migrates to an extraction point in the body.

There appear to be three TRM subsets: one is completely self-renewing, another is partially self-renewing, and the last is completely reseeded by HSCs. However, this was shown to be the case in a parabiotic system with a lack of prior depletion [52]. Under continuous endogenous depletion conditions, those subsets may be reseeded entirely by HSC-derived TRMs. For example, when TRMs were cleared in mice using a CD45 antibody-drug conjugate (ADC), they were replaced by HSC-derived monocytes [53]. Notably, HSCs were recently shown to contribute to TRM population maintenance over time in non-human primates at all sites tested even in the absence of conditioning [54]. Thus, HSC-mediated TRM replacement may be a viable option. If so, patient HSCs could be harvested and edited *ex vivo* or edited *in vivo* using a dual adenovirus system to produce certain genetic alterations and install the necessary therapeutic genes under a macrophage-specific promoter [55,56].

Systemic depletion of TRMs could then potentially be effected via small molecule CSF1R inhibitors in combination with anti-CSF1R and anti-CD45 ADCs [53,57–65]. If the HSCs are edited *ex vivo*, the CD45 ADC would also suffice as non-genotoxic conditioning for subsequent transplant [66]. If the HSCs are edited *in vivo*, their *cd45* gene could be altered to preclude recognition of the corresponding protein by the CD45 ADC, thus allowing for *in vivo* selection of the edited HSCs [67].

While the FDA-approved small molecule CSF1R inhibitor PLX3397 can deplete peripheral TRMs as well as microglia, it may not do so as efficiently for certain subtypes [62,63]. However, the *csf1r* gene in patient HSCs could be edited to confer resistance to PLX3397, allowing for the continuous depletion of peripheral TRMs over a longer period of time [61]. Transplanted HSCs could encode a diphtheria toxin-resistance marker [68]. That way, progeny TRMs could secrete a CD11b or CD64 immunotoxin within their host tissues after arriving at their niches, helping to eliminate any remaining endogenous TRMs.

After systemic TRM replacement, a small molecule could be administered to induce hyper-motility and the production of an RNA delivery vector. This vector would induce lipofuscin export.

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While lysosomal exocytosis is a possibility for cellular ejection of lipofuscin, this would release it in a bare form, which may break apart and be more difficult for TRMs to phagocytose than lysosomes. They could also not count the number of phagocytosis events without binding to a specified ligand. A better option may be to enact secretory autophagy of lipofuscin-laden lysosomes [69]. TFEB overexpression would generate new lysosomes in the cell to compensate for some of the remaining unladen or partially lysosomes being exported via secretory autophagy. (TFEB-mediated lysosomal exocytosis should be inhibited, however.)

TRM hyper-motility could be induced via a self-generated chemokine gradient and a synthetic gene circuit like a noisy repressilator [70,71]. Alternatively, for microglia, hyper-motility could potentially be effected via inducible LRRK2 inhibition [72]. LRRK2 inhibition may have the opposite effect on peripheral TRMs as it does on microglia, however [73]. Thus, for peripheral TRMs at least, Bmal1 inhibition may be more suitable [74]. Bmal1 inhibition may increase microglial motility as well.

COURIER is a cell-to-cell communication system that was recently developed. It involves the secretion of mRNA that is packaged in protein nanocages [75]. The packaging capacity of COURIER is at least 9.8 kb. Alternative protein nanocage architectures could theoretically allow for larger cargo capacities if necessary. A polyprotein could be expressed that includes the tobacco etch virus protease, which would cleave it apart after translation. 2A sequences could also be used to express multiple proteins.

The cargo mRNA could encode a nanobody targeting LAMP-1 with a fused domain that interacts with Sec22b - as well as an LC3-interaction region (LIR) [76]. FKBP51 overexpression may also be important [77]. If more packaging space is available, overexpression of Sec22b, Stx3/4, and SNAP23/29 may also help [69]. Furthermore, autophagosome-lysosome fusion could be inhibited via a dominant-negative STX17 variant [[78]].

The next step would be for the hyper-motile TRMs to phagocytose the mixed-age lysosomes. Notably, mitochondria secreted via secretory autophagy seem to lack an encapsulating membrane after export [79]; thus, in the case of lysosomes, a lysosomal membrane protein, e.g., LAMP-1, could be targeted by TRMs [80]. The final step would be for the garbage-disposal cells to migrate to an extraction point in the body when full.

The edited TRMs could also employ a synthetic gene circuit to count the number of lysosomes they phagocytose [81,82]. In this context, the phagosomes containing said lysosomes should be unable to fuse with TRM lysosomes. Once an experimentally determined number of lysosomes have been phagocytosed, the given TRM would asymmetrically divide; one progeny cell would inherit all of the aforementioned phagosomes [83,84]. That progeny cell would also irreversibly express a receptor mediating its chemoattraction to the gastrointestinal lumen by responding to a blood-brain barrier (BBB)-permeable small molecule produced by the patient's gut microbiome bacteria [85]. Alternatively, if necessary, a device could be placed in the peritoneal space that slowly releases a chemoattractant for the TRMs - allowing for non-invasive, albeit manually effected egress [86–88].

While in most tissues TRMs make up ~10–15% of the constituent cells, in some tissues they are less prevalent [89,90]. Induced replication of edited TRMs may be necessary in some tissues, which could be achieved via small molecule administration in combination with tuned TRM subset-specific promoters or miRNAs. It will be important to ensure that this does not cause excessive inflammation. M1 macrophages are typically considered inflammatory, while M2 macrophages are anti-inflammatory. Thus, it may be best to skew TRM polarity towards M2.

Random motility should only be induced when periodic treatment is required. Otherwise, it may impair the ability of the TRMs to fight infection.

Some of the edited TRMs may wander outside of their host tissues, but perhaps they would assume the identity of whatever tissue they end up in.

Eliminating the TRMs of elderly patients initially may release a large quantity of lipofuscin into tissues. In this case, asymmetric division of the edited TRMs could initially involve lysosomal inheritance rather than phagosomal inheritance.

Elderly patients will likely need an extensive first lipofuscin removal session. Once the garbage back-up is cleared, oxidatively damaged mitochondria would then be mitophagocytosed and

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cytoplasmic lipofuscin granules would be engulfed by autophagosomes and delivered to new lysosomes. Thus, new lysosomes might be quickly overwhelmed again - and they should be exported as well. Aged cells may also have autophagosomes that have been present for too long in the cytoplasm. Their outer membranes may have become "corrupted" by oxidatively warped lipids; thus, they may not even be able to fuse with new lysosomes. They might have to be exported as well. Also, if ECM turnover efficiency is restored to youthful levels, cross-linked proteins could be excised from the surrounding ECM and taken up; at least some cross-links may be indigestible by lysosomal enzymes [91].

Ideally, whole-body lipofuscin removal would perhaps be effected once every decade - starting at ~30 years of age.

Testing CURE in animal models:

Proof-of-concept for lipofuscin removal could potentially be undertaken in an animal model with a multitude of transgenes installed in a genomic safe harbor locus [55] once secretory autophagy of lysosomes can be reliably induced in a wide variety of target cell types.

The mouse TRMs could inducibly become hyper-motile and secrete a peptide that activates a synthetic receptor in target cells [92], triggering transient TFEB overexpression and mixed-age lysosome secretion.

In short-lived species, like mice and rats, lipofuscin may not have enough time to accumulate to pathological levels before they die of cancer. It is estimated that 50-90% of aged mice die of cancer [93]. Even still, it was shown that in the cerebral cortex neurons of lamina Vb in 630-700-day old rats, lipofuscin occupied 23% of the soma volume [94,95]. This could still certainly have a negative physiological effect. Unsurprisingly, we do see a cognitive decline in mice with age [96]. However, even the oldest mice do not develop age-related neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis [97]. Ostensibly, they simply do not live long enough for sufficient build-up of lipofuscin in their neurons. Other conditions like age-related macular degeneration and sarcopenia do occur in mice, but whether their most severe cases are as bad as the most severe human cases is unclear to me. It is also possible that some mouse tissues accumulate lipofuscin more rapidly than others due to cell type differences in metabolic rates, etc. Some types of human neurons, for example, have not accumulated much lipofuscin by the time others are nearly full[98].

One way to test CURE would be to utilize a mouse model with a Cre-inducible, neuron-specific lysosomal storage disorder in combination with genetically-altered microglial variants [99].

Also, extraction from the peritoneal space would perhaps be easiest initially, as deschloroclozapine [87,88] is BBB-permeable and can be slowly released from biodegradable beads placed there laparoscopically [86,87].

For age-related proof-of-concept, non-human primates would be the best model organisms. However, perhaps pigs, cats, or dogs would be useful model organisms in this context, as well.

Discussion:

Another possible option for removing lipofuscin in the future involves TRM partial cell fusion with post-mitotic cells and acquisition of aged lysosomes.

Fixing nuclear DNA mutations and damage in stem cells and long-lived post-mitotic cells on a fundamental level may be the most difficult challenge we face with aging. Notably, it has become clear that nuclear DNA mutations damage accumulate with age in post-mitotic cells as well as stem cells [100].

There may only be only feasible strategy for addressing this on fundamental level in the relatively near future - "whole-body induced cell turnover" [101]. Edited TRMs could eliminate adult stem cells via immunotoxins. They could also asymmetrically divide, wherein one progeny cell dedifferentiates into an iPSC via Yamanaka factor expression. The empty niche could potentially then guide the iPSC into engrafting and differentiating into the appropriate adult stem cell type. Then, the new, edited adult stem cell could be induced to kill tissue-resident cells and divide to repopulate the

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tissue. This could be a viable strategy even in the brain, if done slowly over time - and it may be necessary in the short-term. The telomeres of the edited, adult stem cells could be elongated via hTERT overexpression to enable more rapid repopulation of tissues than is typical.

However, iPSCs can form teratomas *in vivo*, so this may not be appropriate. The progeny cell of the TRMs that is for reseeding adult stem cells may need to more directly convert to the given stem cell type instead.

Dr. Aubrey de Grey and Ben Zealley have suggested allotopic expression as a means of addressing mtDNA mutations with age [102]. An intercellular communication system involving DNA transfer may be possible in the future. Alternatively, bacteria can conjugate with mitochondria, although whether second strand synthesis would naturally occur after DNA transfer is unclear [103].

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