 doi:10.20944/preprints202208.0229.v1

Review

Lipofuscin as the Main Driving Force of Current Age-Related Disease: Justification and Strategies for Removal

Michael Renteln

Molecular Genetics and Biochemistry from the University of Southern California
* Correspondence: mrenteln@gmail.com

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. Here, I explore the possibility that the accumulation of lipofuscin to some extent underlies all other categories of age-related damage as defined by Dr. Aubrey de Grey. I do not think that lipofuscin removal will reverse/prevent all forms of aging, just the major component facing us currently. It may suffice for the next few hundred years by itself. In this piece, I will review what is known about lipofuscin accumulation from evolutionary and mechanistic standpoints and discuss ways of removing it from post-mitotic cells (and then the body).

Keywords: Anti-aging therapy; lipofuscin; SENS; TFEB; intracellular microbe; and synthetic chemotaxis

---

Introduction:
Biological aging is a complex molecular processes 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 enzyme exist to 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.

Years ago, Dr. Aubrey de Grey posited that there are seven types of damage that accumulate with age in the human body. These include extracellular aggregates, senescent cells, extracellular matrix stiffening, intracellular aggregates, mitochondrial mutations, cancerous cells, and cell loss/tissue atrophy. He devised a strategy to eliminate each form of damage from the body, called Strategies for Engineered Negligible Senescence (SENS)[i]. He is of the belief that all seven categories must be addressed before substantial life extension can be attained.

However, I believe that one of his categories, i.e., intracellular aggregate accumulation, actually underlies many of the other categories of damage - and that the removal of indigestible lysosomal garbage (lipofuscin) could, by itself, substantially extend the human lifespan. It is very difficult to guess by how much, but as a truly tentative guess, I suggest that it could suffice to grant at least 300 more years of healthy life. It could serve to substantially reverse aging and also prevent it (if the therapy were applied perhaps every decade or so to individuals starting at an age of ~30).

---

 doi:10.20944/preprints202208.0229.v1

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 are one of such organisms, and the reason for this might be that all of their cells turn over, including those of their central nervous system - except cells at their extremities (which are presumably shed over time)[ii]. This is a convenient way to dispose of lipofuscin - i.e., through dilution and cell shedding. However, it is quite plausible that replicating neurons do not allow for long-term memory at least - as this process would conceivably prevent the stable maintenance of synaptic connections[iii]. Lobsters continually grow throughout life; their fully differentiated cells express telomerase, allowing them to keep dividing as needed[iv]. This includes the cells of their central nervous system, which allows for adult neurogenesis[v]. 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[vi]. Notably, lipofuscin accumulation in eyestalk ganglia[vii] is used as a gauge of biological age in lobsters (and myocardial lipofuscin accumulation can be used a marker of chronological age in humans[viii]). Lobsters can retain memories, but only for a short time span[ix]. Naked mole rats are also very long lived, and it has been shown that they have unusually active autophagic systems[x,xi] (in addition to better anti-cancer defenses[xii]). Even with better autophagy, naked mole rats still do accumulate lipofuscin in their post-mitotic tissues[xiii].

It appears as though all animals that age, e.g., flies[xiv], worms[xv,xvi], lobsters, naked mole rats, mice[xvii], non-human primates[xviii], and humans[xix] 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, exosome/microvesicle 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[xx]. It also has never been observed *in vivo*[xxi]. More importantly, if exported *en masse*, there would be nowhere for the garbage to go except to be picked up by tissue-resident phagocytes, which themselves become bloated with lipofuscin - probably mostly from damaged molecules deriving from their own metabolic processes[xxii,xxiii]. (Transfer of lipofuscin to tissue-resident phagocytes through tunneling nanotubes or partial cell fusion is also theoretically possible.) Finally, I have seen no evidence in the literature that tissue-resident phagocytes - full of lipofuscin or not - are able to efficiently leave the body through migration to the gastrointestinal tract, skin, or lungs.

Mechanistic argument:

Lipofuscin can eventually occupy a large portion of the cytoplasm in certain cell types. If it reaches a critical level that negatively affects cellular metabolism in a large enough number of cells in a tissue, it could not only cause pathology, but also cause a downward spiral of functionality due to an exponential increase in the whole-body accumulation of the other six categories of age-related damage as defined by Dr. Aubrey de Grey. For example, damaged mitochondria will not be mitophagized as rapidly and may then start to accumulate. Beta-amyloid that is normally degraded may sit around for longer in the extracellular space and begin to form plaques. Senescent cells may start to accumulate if the tissue-resident immune cells are rendered inert either by phagocytosis of ejected lipofuscin or more likely, the general non-functionality of the parenchymal cells around them leading to dilapidation of the extracellular matrix. Cancer will be more likely to form if many microenvironments throughout the body are corrupted. Similarly, stem cell niche corruption may prevent them

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 August 2022 doi:10.20944/preprints202208.0229.v1

from replicating efficiently to replenish tissues. And, it is clear that with the extracellular matrix not being tended to properly by its resident cells, stiffening due to cross-links may occur more frequently than they can be cleaved.

It has been argued that lipofuscin is inert. Recently, more researchers are starting to look into whether it may in fact play a pathological role inside a given cell[xix,xxiv]. 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.

Along these lines, in the cerebral cortex neurons of lamina Vb in 630-700-day old rats, lipofuscin occupies 23% of the soma volume[xxv,xxvi]. For humans, it was shown that multiple neuronal cell subtypes become densely packed with lipofuscin granules with age[xxvii]. Other post-mitotic cell types also accumulate substantial amounts of lipofuscin[xxviii,xxix]. Sometimes lysosomes containing large quantities of lipofuscin or lipofuscin granules in the cytoplasm can be quite sizeable - exceeding 3 microns in diameter[xviii]. In comparison, the typical size of a lysosome in a fed, unaged cell is ~100 nm-500 nm in diameter[xxx].

Also, it should be noted that many studies of lipofuscin have relied upon autofluorescence measurements[xxxi], but lipofuscin is also very heterogeneous with regard to its structure and composition between different species, tissues, perhaps even cells of the same type, and when found at different concentrations[xix]. Thus, it seems as though relying on a specific wavelength range to visualize a cell's lipofuscin deposits may not be prudent.

If one could remove the lysosomes from laden macrophages stuck at plaques in artery walls, the plaques may eventually regress, given a healthy diet and sufficient exercise as well[xxxii]. Similarly, with regard to extracellular plaques like beta-amyloid plaque, Le Chatelier's principle may apply. If there are fewer of the soluble protein monomers in solution in that area, the solid mass may dissolve back into solution. Parenchymal cells devoid of lipofuscin could endocytose monomers; as they are degraded, the extracellular plaques could start to dissolve and be taken up as monomers as well - for subsequent degradation. Tau tangles would be autophagocytosed and degraded as well. For the elderly, multiple rounds of lysosomal clearance may be necessary to achieve full results - because, for example, defective mitochondria could have accumulated that will be mitophagized after the initial lipofuscin clearance, which may then become lipofuscin inside the lysosomes due to extensive damage of the mitochondrial membranes by free radicals.

Removal strategies *in vivo* (on a cellular basis):

The simplest way to prevent lipofuscin accumulation is to prevent the excessive uptake of biomolecules from external sources via caloric restriction[xxxiii]. Caloric restriction triggers upregulated autophagy[xxxiv], meaning that reversal and prevention are possible to some degree through this method. Exercise also increases the degradative capacity of our cells[xxxv]. But, of course, these two methods alone are not enough to reverse/prevent aging in a truly substantial manner.

TFEB is the master regulator of autophagy, and its overexpression can increase lysosomal acidity and boost lysosomal hydrolase levels in lysosomes, which could help to shrink existing lipofuscin deposits in lysosomes. Here, it is important to draw a distinction between "false" and "true" lipofuscin. False lipofuscin would be any intracellular garbage that can be digested by cellular machinery if said machinery is maximally expressed. True lipofuscin is that which is indigestible by cells no matter how they are transcriptionally manipulated. But TFEB (i.e., HLH-30) overexpression only grants a 15-20% increase in lifespan for *C. elegans*[xxxvi]. Presumably this is because lipofuscin still accumulates in the intestinal cells over

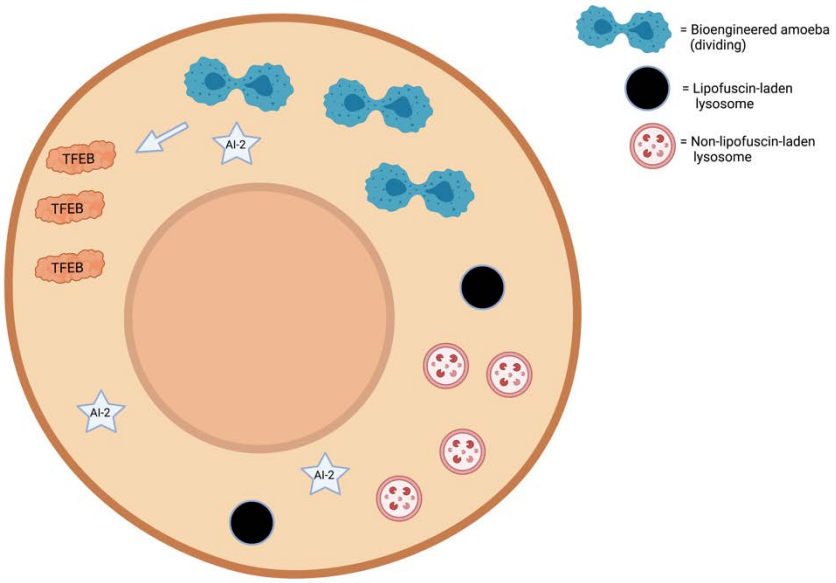
Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 August 2022 doi:10.20944/preprints202208.0229.v1

time, even with HLH-30 overexpression. For this truly indigestible garbage, TFEB could still be of use. It also induces the exocytosis of lysosomal contents through fusion of the lysosomal and plasma membranes.

One problem with boosting endogenous exocytosis processes is that they may lose potency with age; oxidatively warped lipids from lipofuscin may insert themselves into the membranes of the lysosomes that contain them and prevent efficient fusion with the plasma membrane. The main problem, however, is that the two most well-known inducers of lysosomal exocytosis, Ca^{2+} and TFEB, do not induce a substantial amount of exocytosis - even in youthful cells[xxxvii,xxxviii]. Perhaps over time, TFEB could work well enough to exocytose all a post-mitotic cell's lipofuscin, but other cellular mechanisms may need to be combined with TFEB for truly effective exocytosis - and there may be cell type differences in what machinery is required.

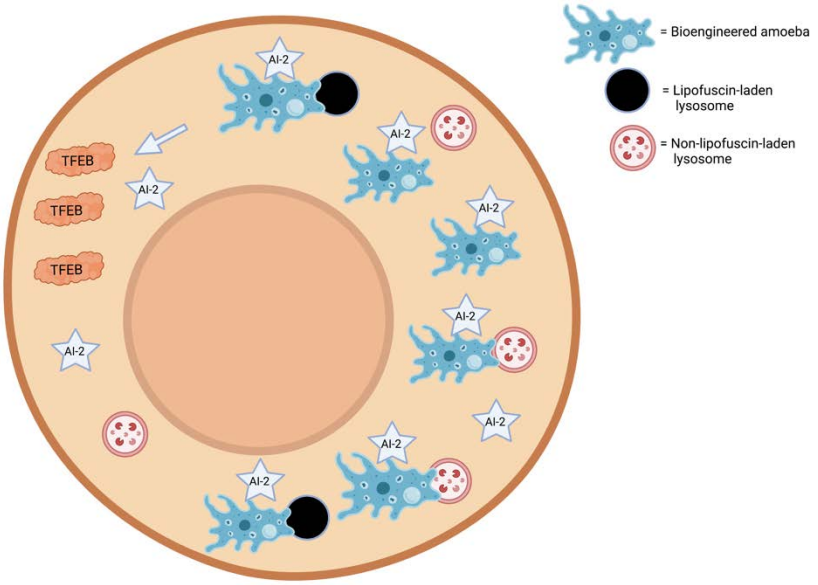
Instead of inducing lysosomal exocytosis, lipofuscin could be exported from post-mitotic cells through microvesicular secretion of lysosomes or by transferring lysosomes through tunneling nanotubes (TNTs) to bioengineered macrophages delivered to their locale. (However, the diameter of some lipofuscin-laden lysosomes may be too great for them to be transferred through TNTs.) Secretory autophagy may also be relevant here[xxxix,xl].

A.



B.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 August 2022 doi:10.20944/preprints202208.0229.v1



C.

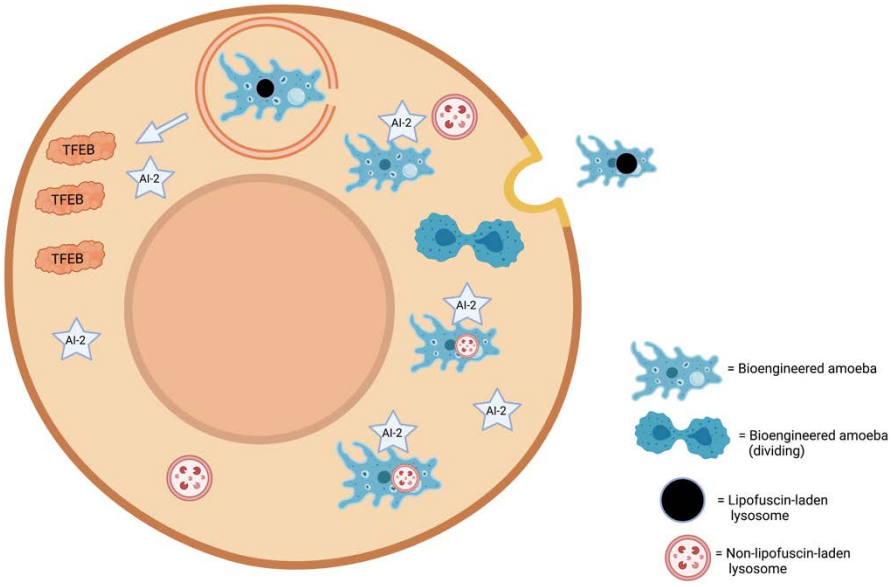


Figure 1: A) Amoebae enter the target post-mitotic cell and replicate up to a particular copy number restrained by quorum sensing using autoinducer 2 (AI-2). They secrete TFEB to ensure that cytoplasmic aggregates are engulfed by autophagosomes that will then fuse with lysosomes for subsequent pickup. Also, more lysosomes are generated via TFEB secretion in order to replenish the cell's supply of these organelles and maintain its health. B) They reach the appropriate copy number level and then begin to engulf lysosomes. C) Amoebae that have picked up indigestible lysosomal cargo re-enter a vesicle and are ejected from the cell via exocytosis. Remaining amoebae replicate to reach the appropriate copy number level again. Over time, through random chance, even with new lysosomes being generated, many if not most of the garbage-filled lysosomes will be removed.

 doi:10.20944/preprints202208.0229.v1

Instead of trying to determine which combination of proteins would promote each post-mitotic cell type to efficiently rid itself of its lipofuscin, an intracellular microbe could be harnessed to enter post-mitotic cells and engulf lipofuscin-laden lysosomes directly, then escape the cell. The smallest white blood cell with phagocytic properties has a diameter of 12-15 microns (neutrophils). Some post-mitotic cells have a diameter of around 10 microns. Thus, instead of trying to bioengineer a leukocyte to be intracellular, it might be best to use a micro-organism with a diameter of ~3 microns so that they can enter the cell and engulf lysosomes that have grown to a relatively large size due to lipofuscin accumulation. If the micro-organism could expand (i.e., if it had a ruffled membrane) to accommodate even larger cargo, that would be ideal. One micro-organism of the appropriate size and with phagocytotic properties is an amoeba known as *Massisteria voersi*[xli].

Only two genes are required to change a normally extracellular bacterium to an intracellular bacterium, an invasin gene and the listeriolysin O gene from *Listeria monocytogenes*[xlii]. Perhaps those two could be utilized here as well for an amoeba. Moreover, ActA could potentially be utilized to promote intracellular movement of the amoebae[xliii]. The phagocytic receptors on the amoebae would be redirected to LAMP-1, a lysosomal transmembrane protein. The amoebae could also secrete Bcl-2 to prevent stress-induced apoptosis of host cells - in case its presence inside the cell is excessively detrimental to the metabolism of that cell[xliv]. However, further bioengineering may certainly be necessary to prevent host cell cytotoxicity.

Once an amoebae has entered a target cell and become motile (to help avoid xenophagy and facilitate eventual wholesale lysosome pick-up) - it could theoretically be allowed to replicate to a low-medium copy number inside the host cell, restrained by the quorum sensing signal AI-2[xlv]. Then, once the copy number limit is reached, in a system resembling competence induction in *B. subtilis*[xlvi], some small number of the amoebae could be induced to engulf some of the cell's lysosomes and subsequently exit the cell, leaving the remaining ones to then divide and replenish the amoeba population and repeat the process.

The amoebae could be bioengineered to phagocytose lysosomes only, perhaps via selective recognition of targets heavily decorated with LAMP-1[xlvii]. (If LAMP-1 is too non-specific in terms of its localization on other autophagic/endocytic vesicles, an AND-gated receptor system could certainly be developed - similar to SynNotch[xlviii].) After an amoeba binds its target, there could then be a pause using a synthetic gene circuit like a temporal promoter cascade, allowing for the engulfment process to proceed and be completed. Then, the amoeba could express TRIM16 on its surface to allow for its selective encapsulation into an autophagosome destined for secretory autophagy[xlix]. The efficiency of secretory autophagy for these vesicles specifically could potentially be enhanced by engineering the surface-displayed TRIM16 to specifically bind to a modified Sec22b protein with very high affinity. The modified Sec22b protein would be secreted at high levels by the other amoebae. Other downstream effectors like Stx3/4 and SNAP23/29 could be engineered to specifically interact with the modified Sec22b with very high affinity as well. Notably, re-entry into an autophagic vesicle after venturing around the cytoplasm is already done by the intracellular bacterium *F. tularensis*[l]. (Of course, prior to an amoeba engulfing a lysosome, autophagosomal engulfment must be prevented. ActA would help - through motility-dependent and motility-independent mechanisms[li]. Also, expression of USP30[lii,liii] on the surface of the amoebae could help. Production of at least the former protein should be halted when the amoeba has engulfed its target and needs to be autophagocytosed.)

Figure 1 depicts a single-membraned vesicle forming around the amoebae. For *in vivo* purposes, this may be more ideal - as will be discussed in the **Systemic delivery of lipofuscin removal machinery and garbage pick-up issues (*in vivo*) -**

 doi:10.20944/preprints202208.0229.v1

Strategy 1 section. However, I am not aware of any known pathways to form large, single-membraned vesicles around cytoplasmic entities[liv].

The amoeba could even be altered potentially through rational design and/or directed evolution to have digestive vacuoles with interiors that are maintained at exceedingly low pH to facilitate breakdown of engulfed cargo. Thus, rather than the act of simply engulfing a target lysosome leading to an amoeba leaving a target cell - it could patrol intracellularly and continue eating lysosomes, digesting all it can before becoming full of material that it truly cannot, even with the low pH of its vacuole(s), digest - and subsequently leaving the target cell. To determine when it is full of indigestible garbage, the amoeba's vacuole(s) could be bioengineered to employ chaperone-mediated autophagy (CMA) as a sensor. When CMA efficiency drops below a certain level, it would be indicative of a garbage back-up within itself[lv]. CMA may decline after a large "meal", even if it can eventually be digested. To account for this, one could employ a second synthetic gene circuit following the threshold effect circuit - e.g., a temporal promoter cascade wherein it can be cancelled at any time before the latest promoter time-point is reached if CMA efficiency increases above the chosen threshold level.

Testing this theory *in vitro* now:

In order to test the theory that lipofuscin is the main issue with regard to age-related disease perhaps for the next few hundred years at least, it appears that we must either figure out how to induce a particular post-mitotic cell type (e.g., a neuron derived from an iPSC) to export its garbage using a gene vector or remove its lipofuscin-laden lysosomes with host cell-autonomous means. Then we could see how long it lives in culture as opposed to normal cells of the same type. However, while primary cell cultures often die after a few days-weeks[lvi], the cause of death may not be related to normal aging. It may instead be related to infection or hyperosmolality due to evaporation of the media[lvii]. It was shown, however, that at ~day 25, neurons *in vitro* have an aged phenotype, including dysfunctional mitochondria, higher reactive oxygen species production, and at least one feature of senescence[lviii]. It was also shown that defective autophagy was linked to this senescence-like phenotype - and that aged neurons in culture accumulate lipofuscin[lix]. Thus, if the lipofuscin were removed from the cells and new, functional lysosomes were introduced exogenously or generated through TFEB, their aged phenotype may reverse.

Another approach would be to study aged skeletal muscle cells that contain large quantities of lipofuscin taken from biopsies of elderly humans[lx]. Their lipofuscin-laden skeletal muscle cells may also eventually die from other causes than normal aging due to culture conditions. But by removing the lipofuscin from those cells, one could at least see if markers of health are restored in the cells; i.e., defective mitochondria could be mitophagized, any cytoplasmic lipofuscin granules could eventually be autophagocytosed and delivered to lysosomes, and a senescence-like phenotype could eventually be reversed.

To get a cell to export its garbage, perhaps one could force lysosomes out to the periphery via JIP4 inhibition[lxi], and then administer Ca^{2+} to the cell to induce fusion of the lysosomes with the plasma membrane[xxxvii]. TFEB would subsequently be induced from another AAV vector genome in the nucleus via small molecule to induce lysosomal biogenesis[lxii]. This approach would clearly be applicable to many cells in culture.

Two host cell-autonomous means occur to me. Both involve a FluidFM-based approach[lxiii]. Every few days or so, a single cell could be injected with large iron particles conjugated with antibodies against LAMP-1. Then, a magnetic field could be applied to cluster the lysosomes to one small area at the plasma membrane. They would then be removed by the needle. TFEB would subsequently be induced from an AAV vector genome in the nucleus via small molecule to replenish the

 doi:10.20944/preprints202208.0229.v1

intracellular supply of functional lysosomes. The second means would involve the bioengineered amoebae described before. Amoebae containing large quantities of magnetic nanoparticles could infect or be injected into host cells. They could then engulf the target lysosomes. After giving them some time to do so, a magnetic field could be applied near the cell to cluster them in one small area at the plasma membrane. Then, they could be withdrawn via needle. TFEB again would be utilized here.

Systemic delivery of lipofuscin removal machinery and garbage pick-up issues (*in vivo*) - Strategy 1:
Provided the *in vitro* experiments indicate that lipofuscin removal is very positive for the longevity of post-mitotic cells, one could deliver a gene vector to at least the vast majority of post-mitotic cells in order to force efficient lipofuscin export. Then, once the lipofuscin is exported from the cell, the trash must be picked up somehow.

With regard to gene delivery, an adeno-associated viral (AAV) vector could be utilized that encodes a small-molecule (e.g., riboswitch)-inducible TFEB module, but even though certain AAV vectors can extravasate to a relatively large extent, have broad tropism, and are able to transduce a variety of cells efficiently, a single vector is unlikely to be able to extensively transduce the majority of cells of the central nervous system (CNS) and most peripheral organs after IV injection[lxiv,lxv,lxvi]. A combination of AAV vectors could be used with differing tropisms, but it starts to get a little too involved and ridiculous. It is possible that the M-CREATE method[lxvii] will help in this regard. However, as mentioned before, a set of proteins may be required to effectively force lipofuscin expulsion in a given post-mitotic cell type - and the sets may differ between different post-mitotic cell types.

Instead, one could solve both the delivery problem and the pickup problem by using bioengineered macrophages. Specifically, one could deliver bioengineered macrophages to the locale of the post-mitotic cells, induce those target cells to release their lipofuscin and stimulate lysosomal biogenesis through exosome or tunneling nanotube-based communication - and then migrate about randomly until full of lipofuscin. Their marker of fullness could be based on CMA, as mentioned before, or clinicians could simply wait for an experimentally-determined period of time before withdrawing the bioengineered macrophages. Withdrawal could be effected by implanting small biodegradable beads that slowly release a chemoattractant molecule[lxviii] inside small devices that serve as a trap for the bioengineered macrophages, perhaps via conjugation of antibodies to the interior of the trap that target a certain surface protein of the bioengineered macrophages.

If the post-mitotic cells' lysosomes are expelled wholesale through microvesicles or secretory autophagy, bioengineered macrophages could recognize a synthetic protein present in high quantities on the microvesicular or inner autophagosomal membrane via a T-cell receptor[lxix]-type strategy, and that could trigger migration to an extraction point. (If single-membraned vesicles can be induced to form around the lysosomes, they could be delivered bare to the extracellular space, at which point recognition would be simple - through LAMP-1.) However, picking up a single lysosome per macrophage would not be ideal. Perhaps the recognition process would trigger a counting mechanism[lxx], and when perhaps ~10 lysosomes had been bound and subsequently engulfed - the bioengineered macrophages could be induced to migrate to an extraction point.

As a huge number of intraparenchymal injections of bioengineered macrophages into all regions containing post-mitotic cells is not really feasible, an intravenous route of delivery to all post-mitotic cells would be ideal. To achieve this, we may need to effect a more involved strategy than we would like. However, it may be the only option. Clinicians could infect all or the majority of a patient's vascular endothelial cells[lxxi] with a herpes simplex virus type 1 (HSV-1) vector. (Different

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 August 2022 doi:10.20944/preprints202208.0229.v1

pseudotypes of HSV-1 or bi-specific antibodies may be necessary to ensure that all vascular endothelial cells throughout the body are transduced; one marker may not be enough - as the gene expression pattern of vascular endothelial cells in different anatomical locales are different[lxxii].) They would then inducibly express from the viral genomes (upon small molecule administration) synthetic, luminal adherence proteins and chemokines[lxxiii]. These would allow for the attachment and transmigration of bioengineered macrophages. After transmigration, perhaps chemorepellants could be secreted abluminally to direct them away from the vasculature towards the interstitial matrix/parenchymal post-mitotic cells. The macrophages could then randomly migrate around until binding to a cell type of interest, at which point they could directionally secrete exosomes containing proteins that induce lipofuscin export and stimulate lysosomal biogenesis - or transfer such proteins to their target cells via tunneling nanotubes.

There are two important considerations here. One is that clinicians should perhaps slowly infuse the patient with bioengineered macrophages after inducing the synthetic luminal adherence proteins and chemokines, so as not to create too much stress on the vasculature. The other is that one may need to utilize superinfection exclusion (SIE) in terms of the HSV-1 vector to make sure that all or the majority of vascular endothelial cells are transduced - but without ending up with too high of a vector copy number per given cell. SIE would ideally be on the level of intracellular capsid trafficking. For example, the degradation system cited here[lxxiv] could ensure the degradation of incoming capsids before they transfer their DNA to the nucleus[lxxv]. A synthetic gene circuit imbuing network-dosage compensation could also be of use in this situation potentially[lxxvi]. Given that the average EC lifespan is >1 year[lxxvii], there is a clear therapeutic window here if the vasculature can be thoroughly transduced first. The extent of vascular endothelial transduction could be visualized via the HSV-1 thymidine kinase. And a kill switch could be included to destroy the vector genomes (based on CRISPR, for example) after lipofuscin removal treatment.

Notably, if lysosomal exocytosis is enacted, the contents of the lysosomes would spill out into the extracellular environment, and pieces of the lipofuscin granules could theoretically break off and get stuck in various locales. This might lead to some extracellular matrix dilapidation. Or, perhaps more likely, those pieces could be endocytosed by neighboring post-mitotic cells, requiring exocytosis to be enacted again. Finally, if lysosomal exocytosis is induced *en masse* throughout a tissue, the resulting acidification of the extracellular matrix could also be a problem. (Of course, the *en masse* exocytosis process could simply be promoted a bit more gradually.) A gene vector could also be delivered by the bioengineered macrophages that can inducibly express TFEB alone upon small molecule administration[lxxviii]. If TFEB expression is periodically induced, it may decrease the frequency with which we must remove lipofuscin - as some of it will almost certainly be "false" lipofuscin, as mentioned earlier.

Systemic delivery of lipofuscin removal machinery and garbage pick-up issues (*in vivo*) - Strategy 2:
Instead of trying to figure out how to induce lysosomal export using only cellular machinery, one could deliver amoebae to post-mitotic cells to engulf their lysosomes. The delivery would be effected in the same way as mentioned above - through vascular endothelial transduction with HSV-1, for example, and the small molecule-mediated induction of transmigration of bioengineered macrophages containing amoebae. The amoebae could be kept at a tolerable copy number inside the bioengineered macrophages and be donated through microvesicles or shed via secretory autophagy, at which point they could enter host cells.

That way, a single macrophage could "infect" many post-mitotic cells. It would potentially allow one to avoid copy number maintenance of the amoebae in post-mitotic cells in the patient's body, as conditions may vary from cell-to-cell and almost

 doi:10.20944/preprints202208.0229.v1

certainly from cell type-to-cell type. One would not want the amoebae replication to become excessive within a large number of the patient's post-mitotic cells. Instead, they could simply be continuously donated by the bioengineered macrophages; they could enter target cells, engulf their lysosomes, and escape via microvesicle secretion or secretory autophagy. More bioengineered macrophages or perhaps neutrophils could then pick them up; honing in on a chemoattractant molecule produced by the (full) amoebae. Amoebae can probably migrate through the extracellular matrix on their own to some extent - but would also potentially cause a lot of damage. To amplify the chemoattraction signal from individual amoebae, one could bioengineer them to express a pro-drug activating enzyme, so that an exogenous small molecule could be converted into a chemoattractant signal.

Both the first and second strategy for *in vivo* treatment will likely require immunosuppression during the duration of the treatment. Perhaps immunologically "stealthed" amoebae can eventually be developed through bioengineering, however.

Conclusion:

It is worth noting that as I was finishing the writing process for this article, I found the paper referenced here[lxxix] about phagocytic bacteria. Perhaps phagocytic bacteria (~4-5 microns in diameter as described in that paper) would be easier to work with than amoebae in terms of having them survive inside host cells and not damage said cells.

I envision that this treatment may be needed once every decade starting from 25 years of age. As mentioned earlier, for elderly patients, the first treatment may take longer - or perhaps it should be repeated a certain number of times in quick succession (e.g., within the same year) to allow for cytoplasmic lipofuscin granules and defective mitochondria to be engulfed by autophagosomes and delivered to lysosomes. Any resulting lipofuscin accumulation in lysosomes could then be dealt with by further wholesale removal of lysosomes. (With the amoeba approach, it may be prudent to simply target autophagosomes as well as lysosomes - at least in the elderly, as they may contain indigestible garbage as well and as a result not be able to fuse efficiently with new, functional lysosomes that the cell subsequently generates after lipofuscin-laden lysosome removal.)

Additionally, if lipofuscin removal is indeed such a potent rejuvenation therapy, our risk of getting cancer would decrease to the level of younger (perhaps 25-year-old) individuals. However, younger individuals can still get cancer, although much more rarely (e.g., through essentially random metabolic errors or excessive sunlight exposure). Thus, being able to effectively treat cancer is also paramount to anti-aging. I have written two articles on this subject about a novel approach, which I call Oncolytic Vector Efficient Replication Contingent on Omnipresent Mutation Engagement (OVERCOME)[lxxx,lxxxi]. If this doesn't work, perhaps computerized microrobots and/or nanorobots will end up being necessary to truly prevent/eliminate cancer.

If lipofuscin accumulates in a dividing cell rapidly, even with dilution through division, it could be a problem. Thus, we may need to treat dividing cells as well, especially *slowly*-dividing cells perhaps like stem cells in tissues like the brain and heart. Becoming bloated with lipofuscin may lead to such cells entering a reversibly senescent state.

Together, I call both *in vivo* lipofuscin removal approaches, "Clearance of Undigested Rubbish by Encapsulation" (CURE). That is because regardless of how the lipofuscin is ejected from post-mitotic cells, it ends up in a bioengineered leukocyte that will at some point migrate to an extraction point.

The two-step transmigration delivery system with vascular endothelial transduction and bioengineered leukocytes also applies to treating other human health conditions like genetic disease. The bioengineered leukocytes could also continuously export copies of a viral vector while wandering around within a target tissue. (One of the proteins the virus normally encodes that is essential for the replication of its DNA would be deleted and expressed by the bioengineered leukocyte. Thus, the viral DNA would be able to replicate exclusively in the nucleus of the leukocyte. It would do so up to a particular copy number - restrained by a plasmid copy number control mechanism[lxxxii] - thus providing new genomes for packaging in subsequently secreted virion particles while preventing the leukocyte from being overwhelmed.) The viral vector could deliver CRISPR components to target cells. Additionally, the leukocytes could protect our cells against viral infections by secreting a gene vector encoding double-stranded RNA-activated caspase oligomerizer (DRACO)[lxxxiii] - or they could be used for treatment and simply secrete the DRACOs themselves. SIE and maybe a synthetic gene circuit for network-dosage compensation should be employed when continuous gene vector export is desired.

Acknowledgements:
I would like to thank Dr. Aubrey de Grey - as well as Michael Rae - for their work on anti-aging. Through Dr. Aubrey de Grey's 2005 TED talk and his book with Michael Rae, I was introduced to the concept of lipofuscin. Thank you to my family and Sid Verma for very helpful conversations. The figure in this piece was created with BioRender.com.

Competing interests:
The author declares no competing interests.

Funding:
Funding not received for the study.

References:

[i] Zealley B, de Grey ADNJ. Strategies for Engineered Negligible Senescence. *Gerontology* (2013) 59:183–189. doi:10.1159/000342197

[ii] Siebert S, Farrell JA, Cazet JF, Abeykoon Y, Primack AS, Schnitzler CE, Juliano CE. Stem cell differentiation trajectories in Hydra resolved at single-cell resolution. *Science* (2019) 365:eaav9314. doi:10.1126/science.aav9314

[iii] Terman A, Brunk UT. Is aging the price for memory? *Biogerontology* (2005) 6:205–210. doi:10.1007/s10522-005-7956-3

[iv] Klapper W, Kühne K, Singh KK, Heidorn K, Parwaresch R, Krupp G. Longevity of lobsters is linked to ubiquitous telomerase expression. *FEBS Letters* (1998) **439**:143–146. doi: 10.1016/S0014-5793(98)01357-X

[v] Beltz BS, Sandeman DC. Regulation of life-long neurogenesis in the decapod crustacean brain. *Arthropod Structure & Development* (2003) **32**:39–60. doi: 10.1016/S1467-8039(03)00038-0

[vi] Peregrim I. Why we age — a new evolutionary view. *Biologia* (2017) **72**:475–485. doi: 10.1515/biolog-2017-0064

[vii] Sheehy M, Shelton P, Wickins J, Belchier M, Gaten E. Ageing the European lobster Homarus gammarus by the lipofuscin in its eyestalk ganglia. *Mar Ecol Prog Ser* (1996) **143**:99–111. doi: 10.3354/meps143099

[viii] Kakimoto Y, Okada C, Kawabe N, Sasaki A, Tsukamoto H, Nagao R, Osawa M. Myocardial lipofuscin accumulation in ageing and sudden cardiac death. *Sci Rep* (2019) 9:3304. doi:10.1038/s41598-019-40250-0

[ix] Karavanich C, Atema J. Individual recognition and memory in lobster dominance. *Animal Behaviour* (1998) 56:1553–1560. doi:10.1006/anbe.1998.0914

[x] Zhao S, Lin L, Kan G, Xu C, Tang Q, Yu C, Sun W, Cai L, Xu C, Cui S. High autophagy in the naked mole rat may play a significant role in maintaining good health. *Cell Physiol Biochem* (2014) **33**:321–332. doi: 10.1159/000356672

[xi] Triplett JC, Tramutola A, Swomley A, Kirk J, Grimes K, Lewis K, Orr M, Rodriguez K, Cai J, Klein JB, et al. Age-related changes in the proteostasis network in the brain of the naked mole-rat: Implications promoting healthy longevity. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* (2015) **1852**:2213–2224. doi: 10.1016/j.bbadis.2015.08.002

[xii] Hadi F, Kulaberoglu Y, Lazarus KA, Bach K, Ugur R, Beattie P, Smith ESJ, Khaled WT. Transformation of naked mole-rat cells. *Nature* (2020) **583**:E1–E7. doi: 10.1038/s41586-020-2410-x

[xiii] Edrey YH, Hanes M, Pinto M, Mele J, Buffenstein R. Successful aging and sustained good health in the naked mole rat: a long-lived mammalian model for biogerontology and biomedical research. *ILAR J* (2011) **52**:41–53. doi: 10.1093/ilar.52.1.41

[xiv] Panno JP, Nair KK. Effects of increased lifespan on chromatin condensation in the adult male housefly. *Mech Ageing Dev* (1986) **35**:31–38. doi: 10.1016/0047-6374(86)90063-1

[xv] Clokey GV, Jacobson LA. The autofluorescent "lipofuscin granules" in the intestinal cells of Caenorhabditis elegans are secondary lysosomes. *Mech Ageing Dev* (1986) **35**:79–94. doi: 10.1016/0047-6374(86)90068-0

[xvi] Houthoofd K, Braeckman BP, Lenaerts I, Brys K, De Vreese A, Van Eygen S, Vanfleteren JR. Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of C. elegans. *Exp Gerontol* (2002) **37**:1015–1021. doi: 10.1016/s0531-5565(02)00063-3

[xvii] Goyal VK. Lipofuscin pigment accumulation in the central nervous system of the mouse during aging. *Exp Gerontol* (1982) **17**:89–94. doi: 10.1016/0531-5565(82)90041-9

[xviii] Gilissen EP, Staneva-Dobrovski L. Distinct Types of Lipofuscin Pigment in the Hippocampus and Cerebellum of Aged Cheirogaleid Primates. *The Anatomical Record* (2013) **296**:1895–1906. doi: 10.1002/ar.22809

[xix] Moreno-García A, Kun A, Calero O, Medina M, Calero M. An Overview of the Role of Lipofuscin in Age-Related Neurodegeneration. *Frontiers in Neuroscience* (2018) **12**: https://www.frontiersin.org/article/10.3389/fnins.2018.00464 [Accessed March 31, 2022]

[xx] Terman A, Brunk UT. Is Lipofuscin Eliminated from Cells? *Investigative Ophthalmology & Visual Science* (1999) **40**:2463–2464.

[xxi] Julien S, Schraermeyer U. Lipofuscin can be eliminated from the retinal pigment epithelium of monkeys. *Neurobiol Aging* (2012) **33**:2390–2397. doi: 10.1016/j.neurobiolaging.2011.12.009

[xxii] O'Neil SM, Witcher KG, McKim DB, Godbout JP. Forced turnover of aged microglia induces an intermediate phenotype but does not rebalance CNS environmental cues driving priming to immune challenge. *Acta Neuropathologica Communications* (2018) **6**:129. doi: 10.1186/s40478-018-0636-8

[xxiii] Burns JC, Cotleur B, Walther DM, Bajrami B, Rubino SJ, Wei R, Franchimont N, Cotman SL, Ransohoff RM, Mingueneau M. Differential accumulation of storage bodies with aging defines discrete subsets of microglia in the healthy brain. *eLife* (2020) **9**:e57495. doi: 10.7554/eLife.57495

[xxiv] Brunk UT, Terman A. Lipofuscin: mechanisms of age-related accumulation and influence on cell function. *Free Radic Biol Med* (2002) 33:611–619. doi:10.1016/s0891-5849(02)00959-0

[xxv] Samorajski T, Ordy JM, Rady-Reimer P. Lipofuscin pigment accumulation in the nervous system of aging mice. *The Anatomical Record* (1968) **160**:555–573. doi: 10.1002/ar.1091600305

[xxvi] Brizzee KR, Johnson FA. Depth distribution of lipofuscin pigment in cerebral cortex of albino rat. *Acta Neuropathol* (1970) **16**:205–219. doi: 10.1007/BF00687360

[xxvii] Mann DM, Yates PO, Stamp JE. The relationship between lipofuscin pigment and ageing in the human nervous system. *J Neurol Sci* (1978) **37**:83–93. doi: 10.1016/0022-510x(78)90229-0

[xxviii] Dayan D, Abrahami I, Buchner A, Gorsky M, Chimovitz N. Lipid pigment (lipofuscin) in human perioral muscles with aging. *Experimental Gerontology* (1988) **23**:97–102. doi: 10.1016/0531-5565(88)90074-5

[xxix] Wing GL, Blanchard GC, Weiter JJ. The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. *Investigative Ophthalmology & Visual Science* (1978) **17**:601–607.

[xxx] Xu H, Ren D. Lysosomal Physiology. *Annu Rev Physiol* (2015) **77**:57–80. doi: 10.1146/annurev-physiol-021014-071649

[xxxi] Kakimoto Y, Okada C, Kawabe N, Sasaki A, Tsukamoto H, Nagao R, Osawa M. Myocardial lipofuscin accumulation in ageing and sudden cardiac death. *Sci Rep* (2019) **9**:3304. doi: 10.1038/s41598-019-40250-0

[xxxii] Jaffer FA, Blankstein R. Getting Down With Diet and Exercise for Coronary Artery Disease Treatment. *JACC: Cardiovascular Imaging* (2021) **14**:1203–1205. doi: 10.1016/j.jcmg.2021.01.003

[xxxiii] Idrobo F, Nandy K, Mostofsky DI, Blatt L, Nandy L. Dietary restriction: effects on radial maze learning and lipofuscin pigment deposition in the hippocampus and frontal cortex. *Archives of Gerontology and Geriatrics* (1987) **6**:355–362. doi: 10.1016/0167-4943(87)90014-8

[xxxiv] Chung KW, Chung HY. The Effects of Calorie Restriction on Autophagy: Role on Aging Intervention. *Nutrients* (2019) **11**:2923. doi: 10.3390/nu11122923

[xxxv] Subramanyam MVV. Dietary vitamin E and physical exercise: II. Antioxidant status and lipofuscin-like substances in aging rat heart. https://core.ac.uk/reader/72797312?utm_source=linkout [Accessed April 5, 2022]

[xxxvi] Lapierre LR, De Magalhaes Filho CD, McQuary PR, Chu C-C, Visvikis O, Chang JT, Gelino S, Ong B, Davis AE, Irazoqui JE, et al. The TFEB orthologue HLH-30 regulates autophagy and modulates longevity in Caenorhabditis elegans. *Nat Commun* (2013) **4**:2267. doi: 10.1038/ncomms3267

[xxxvii] Jaiswal JK, Andrews NW, Simon SM. Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells. *Journal of Cell Biology* (2002) **159**:625–635. doi: 10.1083/jcb.200208154

[xxxviii] Medina DL, Fraldi A, Bouche V, Annunziata F, Mansueto G, Spampanato C, Puri C, Pignata A, Martina JA, Sardiello M, et al. Transcriptional Activation of Lysosomal Exocytosis Promotes Cellular Clearance. *Dev Cell* (2011) **21**:421–430. doi: 10.1016/j.devcel.2011.07.016

[xxxix] Autophagy sequesters damaged lysosomes to control lysosomal biogenesis and kidney injury. *The EMBO Journal* (2013) **32**:2336–2347. doi: 10.1038/emboj.2013.171

[xl] Martinelli S, Anderzhanova EA, Bajaj T, Wiechmann S, Dethloff F, Weckmann K, Heinz DE, Ebert T, Hartmann J, Geiger TM, et al. Stress-primed secretory autophagy promotes extracellular BDNF maturation by enhancing MMP9 secretion. *Nat Commun* (2021) **12**:4643. doi: 10.1038/s41467-021-24810-5

[xli] Mylnikov AP, Weber F, Jürgens K, Wylezich C. Massisteria marina has a sister: Massisteria voersi sp. nov., a rare species isolated from coastal waters of the Baltic Sea. *Eur J Protistol* (2015) 51:299–310. doi:10.1016/j.ejop.2015.05.002

[xlii] Grillot-Courvalin C, Goussard S, Huetz F, Ojcius DM, Courvalin P. Functional gene transfer from intracellular bacteria to mammalian cells. *Nat Biotechnol* (1998) 16:862–866. doi:10.1038/nbt0998-862

[xliii] Yi J, Schmidt J, Chien A, Montemagno CD. Engineering an artificial amoeba propelled by nanoparticle-triggered actin polymerization. *Nanotechnology* (2009) **20**:085101. doi: 10.1088/0957-4484/20/8/085101

[xliv] Bilbao G, Contreras JL, Zhang H-G, Pike MJ, Overturf K, Mikheeva G, Krasnykh V, Curiel DT. Adenovirus-mediated gene expression in vivo is enhanced by the antiapoptotic bcl-2 gene. *Journal of virology* (1999) **73**:6992–7000.

[xlv] Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, Hughson FM. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. *Mol Cell* (2004) **15**:677–687. doi: 10.1016/j.molcel.2004.07.020

[xlvi] Mirouze N, Dubnau D. Chance and Necessity in Bacillus subtilis Development. *Microbiol Spectr* (2013) **1**:10.1128/microbiolspectrum.TBS-0004–2012. doi: 10.1128/microbiolspectrum.TBS-0004-2012

[xlvii] Xu X, Pan M, Jin T. How Phagocytes Acquired the Capability of Hunting and Removing Pathogens From a Human Body: Lessons Learned From Chemotaxis and Phagocytosis of Dictyostelium discoideum (Review). *Frontiers in Cell and Developmental Biology* (2021) **9**: https://www.frontiersin.org/article/10.3389/fcell.2021.724940 [Accessed April 15, 2022]

[xlviii] Morsut L, Roybal KT, Xiong X, Gordley RM, Coyle SM, Thomson M, Lim WA. Engineering Customized Cell Sensing and Response Behaviors Using Synthetic Notch Receptors. *Cell* (2016) **164**:780–791. doi: 10.1016/j.cell.2016.01.012

[xlix] Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. *The EMBO Journal* (2017) **36**:42–60. doi: 10.15252/embj.201695081

[l] Checroun C, Wehrly TD, Fischer ER, Hayes SF, Celli J. Autophagy-mediated reentry of Francisella tularensis into the endocytic compartment after cytoplasmic replication. *Proceedings of the National Academy of Sciences* (2006) **103**:14578–14583. doi: 10.1073/pnas.0601838103

[li] Yoshikawa Y, Ogawa M, Hain T, Yoshida M, Fukumatsu M, Kim M, Mimuro H, Nakagawa I, Yanagawa T, Ishii T, et al. Listeria monocytogenes ActA-mediated escape from autophagic recognition. *Nat Cell Biol* (2009) 11:1233–1240. doi:10.1038/ncb1967

[lii] Manzanillo PS, Ayres JS, Watson RO, Collins AC, Souza G, Rae CS, Schneider DS, Nakamura K, Shiloh MU, Cox JS. The ubiquitin ligase parkin mediates resistance to intracellular pathogens. *Nature* (2013) 501:512–516. doi:10.1038/nature12566

[liii] Bingol B, Tea JS, Phu L, Reichelt M, Bakalarski CE, Song Q, Foreman O, Kirkpatrick DS, Sheng M. The mitochondrial deubiquitinase USP30 opposes parkin-mediated mitophagy. *Nature* (2014) 510:370–375. doi:10.1038/nature13418

[liv] Florey O, Kim SE, Sandoval CP, Haynes CM, Overholtzer M. Autophagy machinery mediates macroendocytic processing and entotic cell death by targeting single membranes. *Nat Cell Biol* (2011) **13**:1335–1343. doi: 10.1038/ncb2363

[lv] Hsu C-Y, Pan Z-M, Hu R-H, Chang C-C, Cheng H-C, Lin C, Chen B-S. Systematic Biological Filter Design with a Desired I/O Filtering Response Based on Promoter-RBS Libraries. *IEEE/ACM Trans Comput Biol and Bioinf* (2015) **12**:712–725. doi: 10.1109/TCBB.2014.2372790

[lvi] Li Z. "5.43 - In Vitro Micro-Tissue and -Organ Models for Toxicity Testing.," In: Moo-Young M, editor. *Comprehensive Biotechnology (Second Edition)*. Burlington: Academic Press (2011). p. 551–563 doi: 10.1016/B978-0-08-088504-9.00503-1

[lvii] Potter SM, DeMarse TB. A new approach to neural cell culture for long-term studies. *Journal of Neuroscience Methods* (2001) **110**:17–24. doi: 10.1016/S0165-0270(01)00412-5

[lviii] Dong W, Cheng S, Huang F, Fan W, Chen Y, Shi H, He H. Mitochondrial dysfunction in long-term neuronal cultures mimics changes with aging. *Med Sci Monit* (2011) **17**:BR91–BR96. doi: 10.12659/MSM.881706

[lix] Moreno-Blas D, Gorostieta-Salas E, Pommer-Alba A, Muciño-Hernández G, Gerónimo-Olvera C, Maciel-Barón LA, Konigsberg M, Massieu L, Castro-Obregón S. Cortical neurons develop a senescence-like phenotype promoted by dysfunctional autophagy. *Aging (Albany NY)* (2019) **11**:6175–6198. doi: 10.18632/aging.102181

[lx] Triolo M, Hood DA. Manifestations of Age on Autophagy, Mitophagy and Lysosomes in Skeletal Muscle. *Cells* (2021) **10**:1054. doi: 10.3390/cells10051054

[lxi] Willett R, Martina JA, Zewe JP, Wills R, Hammond GRV, Puertollano R. TFEB regulates lysosomal positioning by modulating TMEM55B expression and JIP4 recruitment to lysosomes. *Nat Commun* (2017) **8**:1580. doi: 10.1038/s41467-017-01871-z

[lxii] Wurmthaler LA, Sack M, Gense K, Hartig JS, Gamerdinger M. A tetracycline-dependent ribozyme switch allows conditional induction of gene expression in Caenorhabditis elegans. *Nat Commun* (2019) **10**:491. doi: 10.1038/s41467-019-08412-w

[lxiii] Gäbelein CG, Feng Q, Sarajlic E, Zambelli T, Guillaume-Gentil O, Kornmann B, Vorholt JA. Mitochondria transplantation between living cells. *PLOS Biology* (2022) **20**:e3001576. doi: 10.1371/journal.pbio.3001576

[lxiv] Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther* (2011) 19:1058–1069. doi:10.1038/mt.2011.72

[lxv] Bevan AK, Duque S, Foust KD, Morales PR, Braun L, Schmelzer L, Chan CM, McCrate M, Chicoine LG, Coley BD, et al. Systemic gene delivery in large species for targeting spinal cord, brain, and peripheral tissues for pediatric disorders. *Mol Ther* (2011) 19:1971–1980. doi:10.1038/mt.2011.157

[lxvi] Hordeaux J, Wang Q, Katz N, Buza EL, Bell P, Wilson JM. The Neurotropic Properties of AAV-PHP.B Are Limited to C57BL/6J Mice. *Mol Ther* (2018) 26:664–668. doi:10.1016/j.ymthe.2018.01.018

[lxvii] Ravindra Kumar S, Miles TF, Chen X, Brown D, Dobreva T, Huang Q, Ding X, Luo Y, Einarsson PH, Greenbaum A, et al. Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. *Nature Methods* (2020) **17**:541–550. doi: 10.1038/s41592-020-0799-7

[lxviii] Park JS, Rhau B, Hermann A, McNally KA, Zhou C, Gong D, Weiner OD, Conklin BR, Onuffer J, Lim WA. Synthetic control of mammalian-cell motility by engineering chemotaxis to an orthogonal bioinert chemical signal. *Proc Natl Acad Sci USA* (2014) **111**:5896–5901. doi: 10.1073/pnas.1402087111

[lxix] Morrissey MA, Williamson AP, Steinbach AM, Roberts EW, Kern N, Headley MB, Vale RD. Chimeric antigen receptors that trigger phagocytosis. *eLife* (2018) **7**:e36688. doi: 10.7554/eLife.36688

[lxx] Friedland AE, Lu TK, Wang X, Shi D, Church G, Collins JJ. Synthetic Gene Networks that Count. *Science* (2009) **324**:1199–1202. doi: 10.1126/science.1172005

[lxxi] Krolak T, Chan KY, Kaplan L, Huang Q, Wu J, Zheng Q, Kozareva V, Beddow T, Tobey IG, Pacouret S, et al. A high-efficiency AAV for endothelial cell transduction throughout the central nervous system. *Nat Cardiovasc Res* (2022) **1**:389–400. doi: 10.1038/s44161-022-00046-4

[lxxii] Chi J-T, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, et al. Endothelial cell diversity revealed by global expression profiling. *Proceedings of the National Academy of Sciences* (2003) **100**:10623–10628. doi: 10.1073/pnas.1434429100

[lxxiii] van Buul JD, Hordijk PL. Signaling in Leukocyte Transendothelial Migration. *Arteriosclerosis, Thrombosis, and Vascular Biology* (2004) **24**:824–833. doi: 10.1161/01.ATV.0000122854.76267.5c

[lxxiv] Caussinus E, Kanca O, Affolter M. Fluorescent fusion protein knockout mediated by anti-GFP nanobody. *Nature Structural & Molecular Biology* (2012) **19**:117–121. doi: 10.1038/nsmb.2180

[lxxv] Horan KA, Hansen K, Jakobsen MR, Holm CK, Søby S, Unterholzner L, Thompson M, West JA, Iversen MB, Rasmussen SB, et al. Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. *J Immunol* (2013) **190**:2311–2319. doi: 10.4049/jimmunol.1202749

[lxxvi] Acar M, Pando BF, Arnold FH, Elowitz MB, van Oudenaarden A. A general mechanism for network-dosage compensation in gene circuits. *Science* (2010) **329**:1656–1660. doi: 10.1126/science.1190544

[lxxvii] Montezano, Augusto C., Karla B. Neves, Rheure A. M. Lopes, and Francisco Rios. "Isolation and Culture of Endothelial Cells from Large Vessels." In *Hypertension: Methods and Protocols*, edited by Rhian M. Touyz and Ernesto L. Schiffrin, 345–48. Methods in Molecular Biology. New York, NY: Springer, 2017. https://doi.org/10.1007/978-1-4939-6625-7_26.

[lxxviii] Torra A, Parent A, Cuadros T, Rodríguez-Galván B, Ruiz-Bronchal E, Ballabio A, Bortolozzi A, Vila M, Bové J. Overexpression of TFEB Drives a Pleiotropic Neurotrophic Effect and Prevents Parkinson's Disease-Related Neurodegeneration. *Mol Ther* (2018) **26**:1552–1567. doi: 10.1016/j.ymthe.2018.02.022

[lxxix] Shiratori T, Suzuki S, Kakizawa Y, Ishida K. Phagocytosis-like cell engulfment by a planctomycete bacterium. *Nat Commun* (2019) **10**:5529. doi: 10.1038/s41467-019-13499-2

[lxxx] Renteln M. Conditional replication of oncolytic viruses based on detection of oncogenic mRNA. *Gene Therapy* (2018) **25**:1–3. doi: 10.1038/gt.2017.99

[lxxxi] Renteln M. Molecular switches that can detect patient-specific ubiquitous mutations and promote oncolytic vector replication. *Medical Hypotheses* (Submitted: March, 2022)

[lxxxii] Okibe N, Suzuki N, Inui M, Yukawa H. Antisense-RNA-mediated plasmid copy number control in pCG1-family plasmids, pCGR2 and pCG1, in Corynebacterium glutamicum. *Microbiology* (2010) **156**:3609–3623. doi: 10.1099/mic.0.043745-0

[lxxxiii] Rider TH, Zook CE, Boettcher TL, Wick ST, Pancoast JS, Zusman BD. Broad-Spectrum Antiviral Therapeutics. *PLOS ONE* (2011) **6**:e22572. doi: 10.1371/journal.pone.0022572