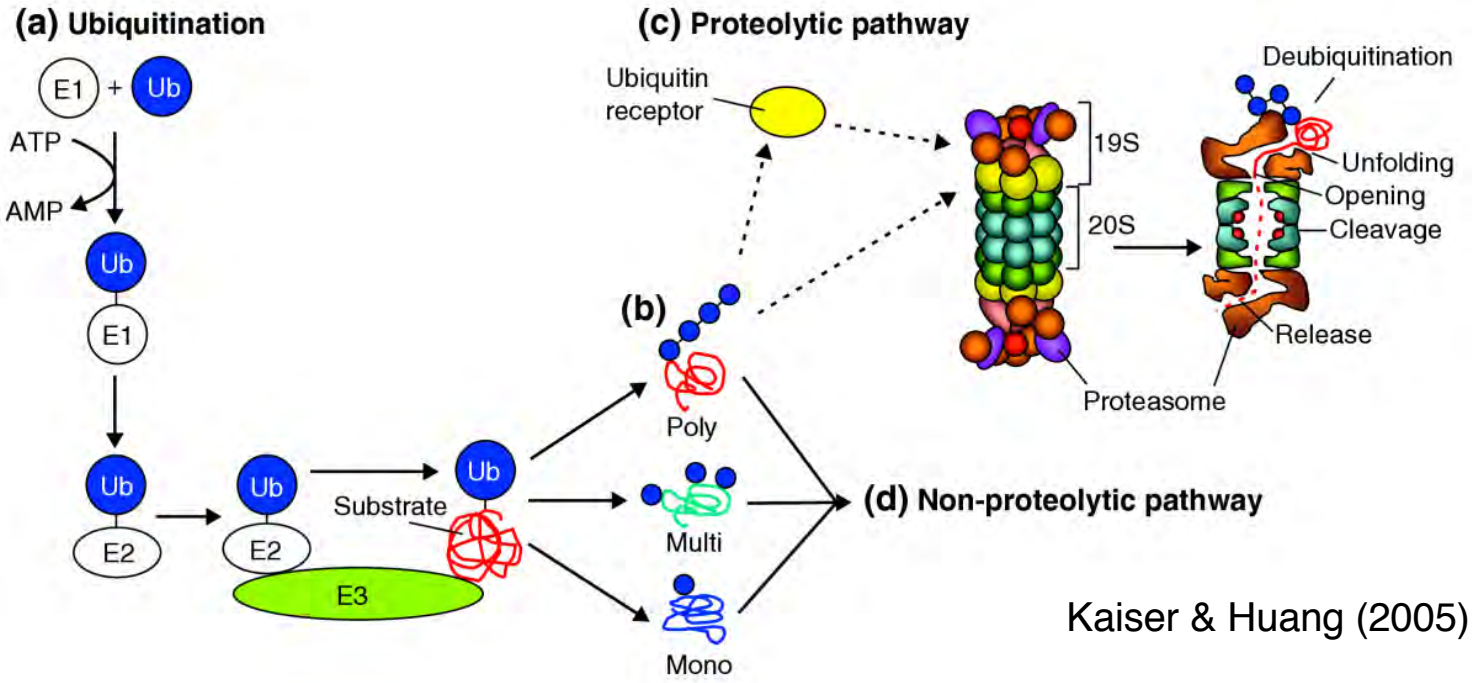
The 2 major disposal pathways in the cell

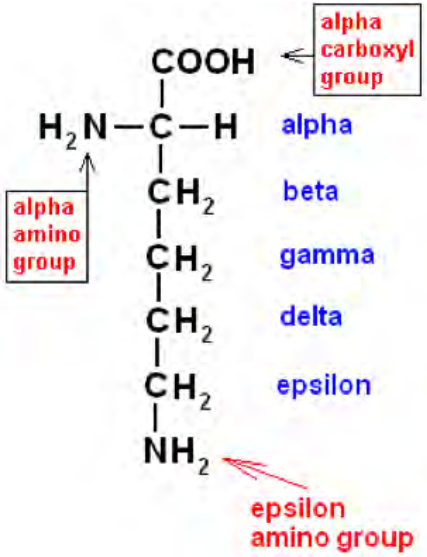
- The 26S protease
    - Ubiquitin-proteasome-system (UPS)—Ub is the tag
    - Degradation of short-lived proteins
- Autophagy

The ubiquitin proteasome system

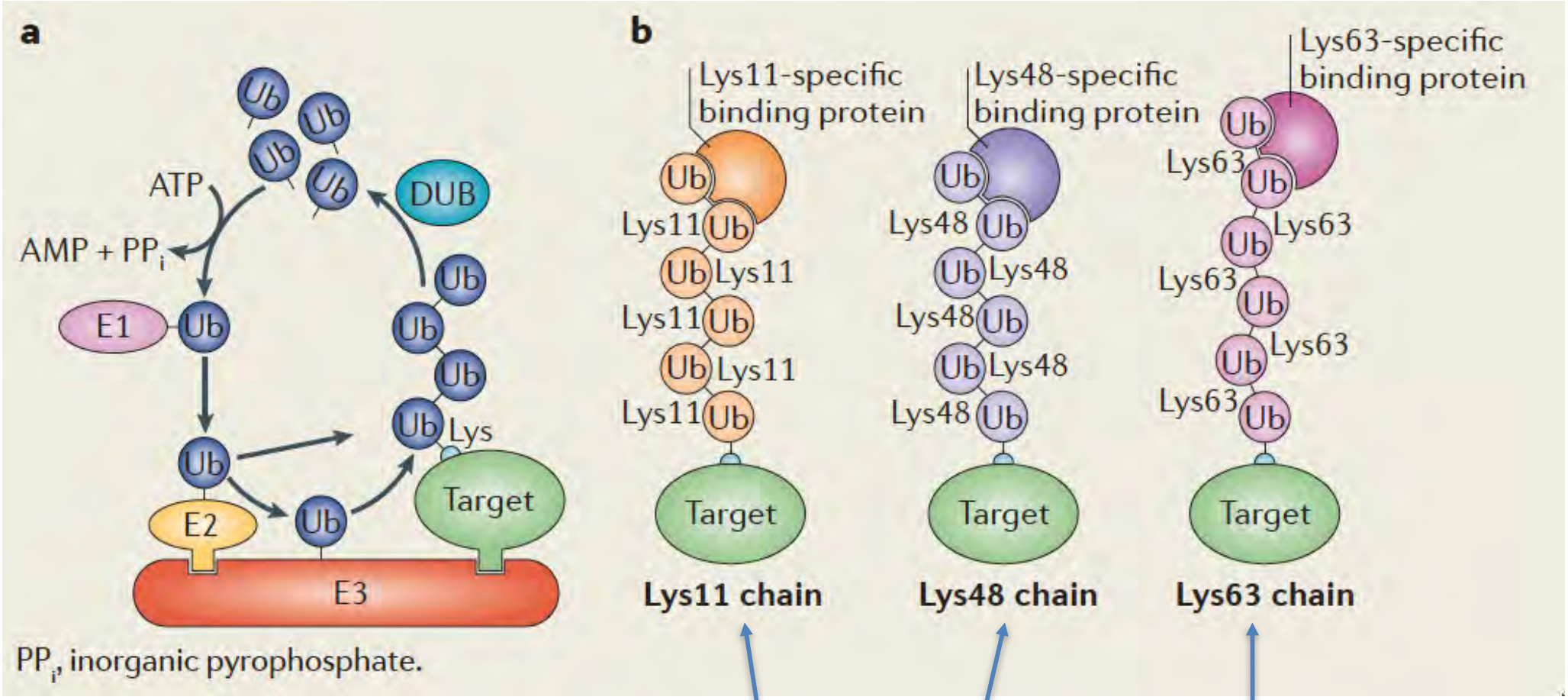


Kaiser & Huang (2005) Genome Biology

- Ub (76 aa protein) activated by E1 (thioester bond); Ub transferred to E2.
- E2 and E3 transfer Ub to lysine of substrate protein
- isopeptide bond formed: carboxylic acid group of Ub's terminal glycine with ε amino group of substrate's lysine
- Ub has 7 lysines (e.g., K11, K48, K63)
- UPS classically associated with K48 poly-Ub (4 or more)

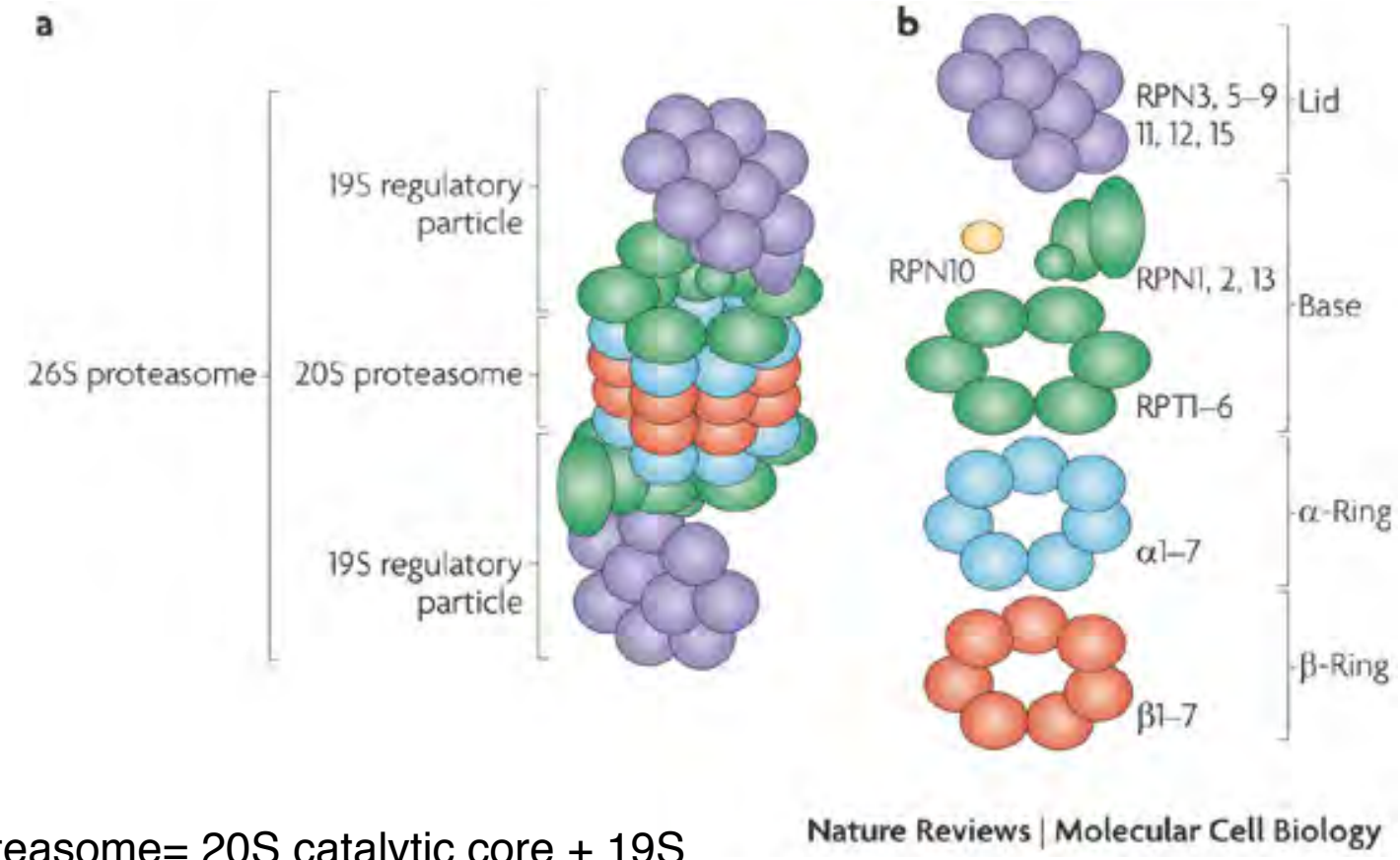


K48 polyubiquitin chains are the classic signal for protein degradation



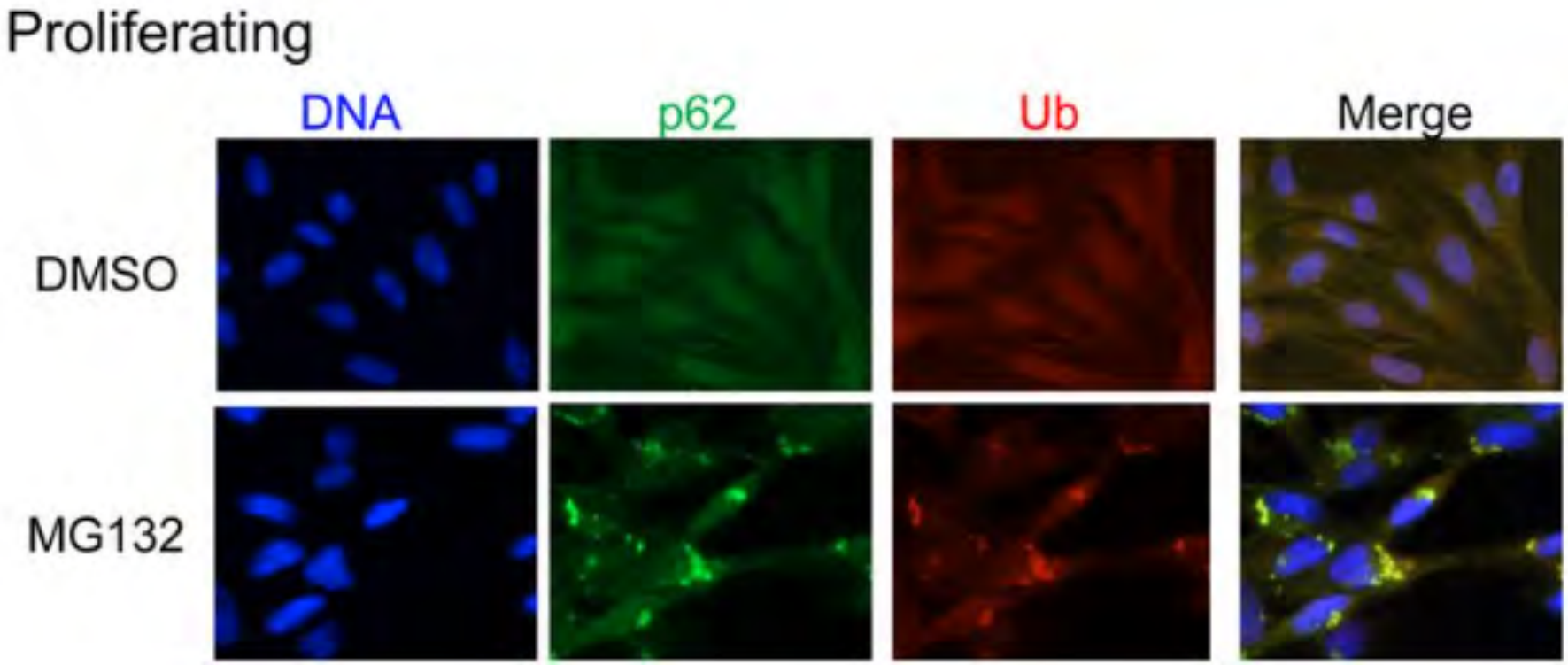
Iwai et al (2014) NRMCB

The 26S proteasome



- 26S proteasome= 20S catalytic core + 19S regulatory cap
- Cap contains lid that recognizes Ub'd substrates and base that unfolds substrates and interacts with core
- Core is a cylinder of 4 rings with proteolytic activity to degrade substrates into small peptides

Inhibition of the UPS results in accumulation of protein aggregates



- MG132: proteasome inhibitor
- Treatment of proliferating fibroblasts with MG132 causes accumulation of Ub-positive, perinuclear aggregates (24 hr)

Legesse-Miller et al. (2012) MBoC

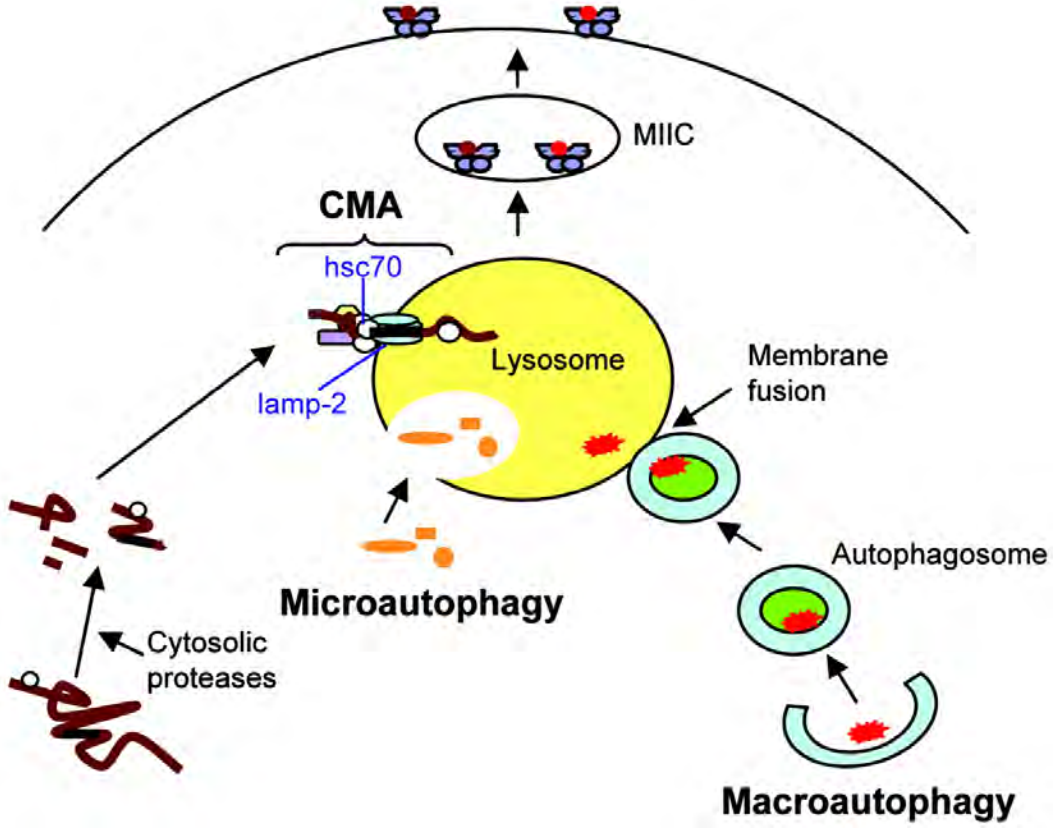
Modes of autophagy (subcellular "self-eating")

Autophagy: degradation systems involving delivery to lysosome

• **Macroautophagy**: formation of double-membraned autophagosome that fuses with lysosome; involves membrane biosynthesis. This pathway is what most people mean when the term “autophagy” is used.

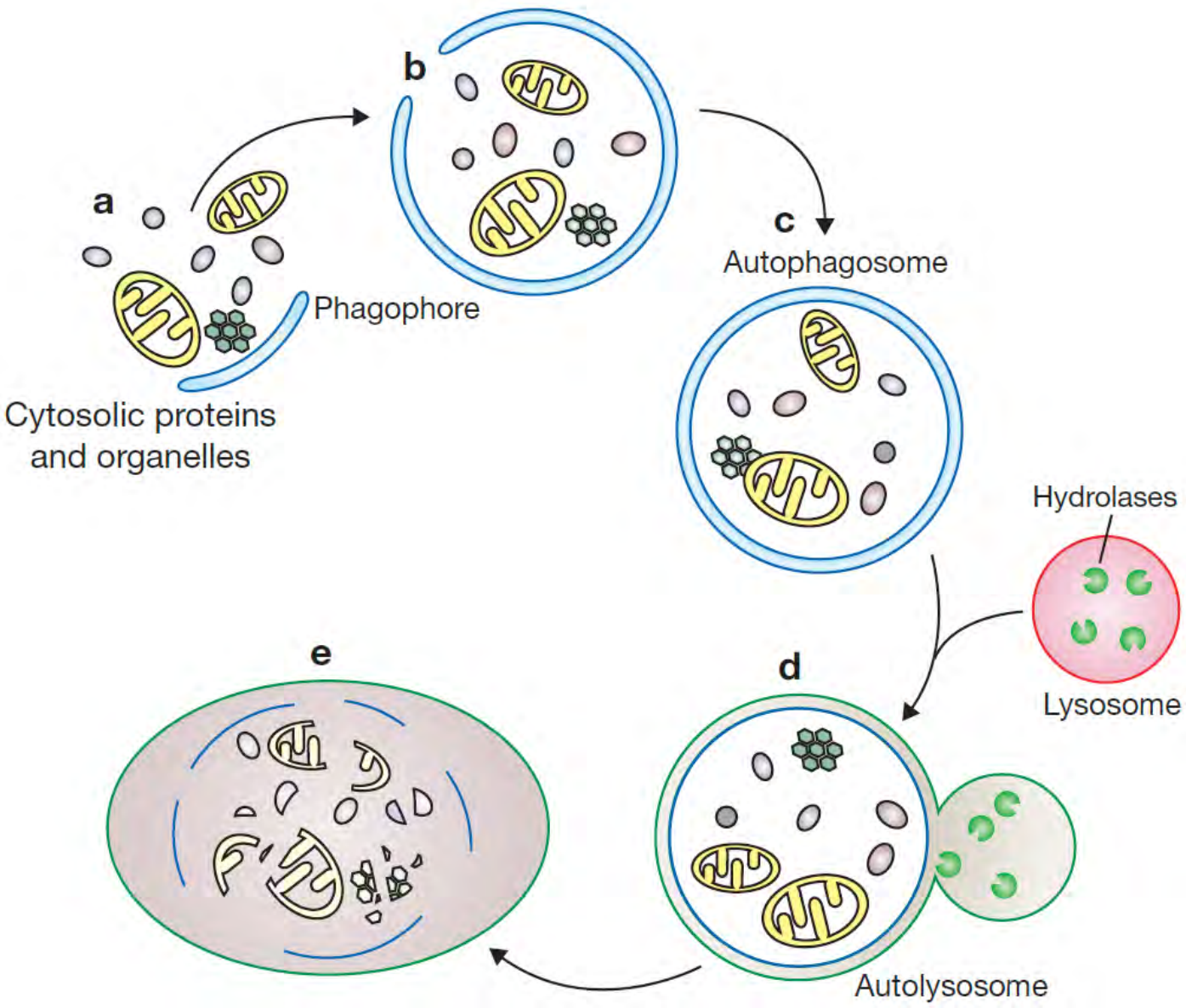
• **Microautophagy**: direct engulfment of cytoplasm by invaginations of the lysosome membrane; topologically similar to multi-vescular body formation in endosomes

• **Chaperone-mediated autophagy**: a mechanism to translocate unfolded, soluble proteins into the lysosome. Can be an important mechanism to degrade some proteins.



Crotzer & Blum (2005) PNAS

Macroautophagy



Xie & Klionsky (2007) NCB

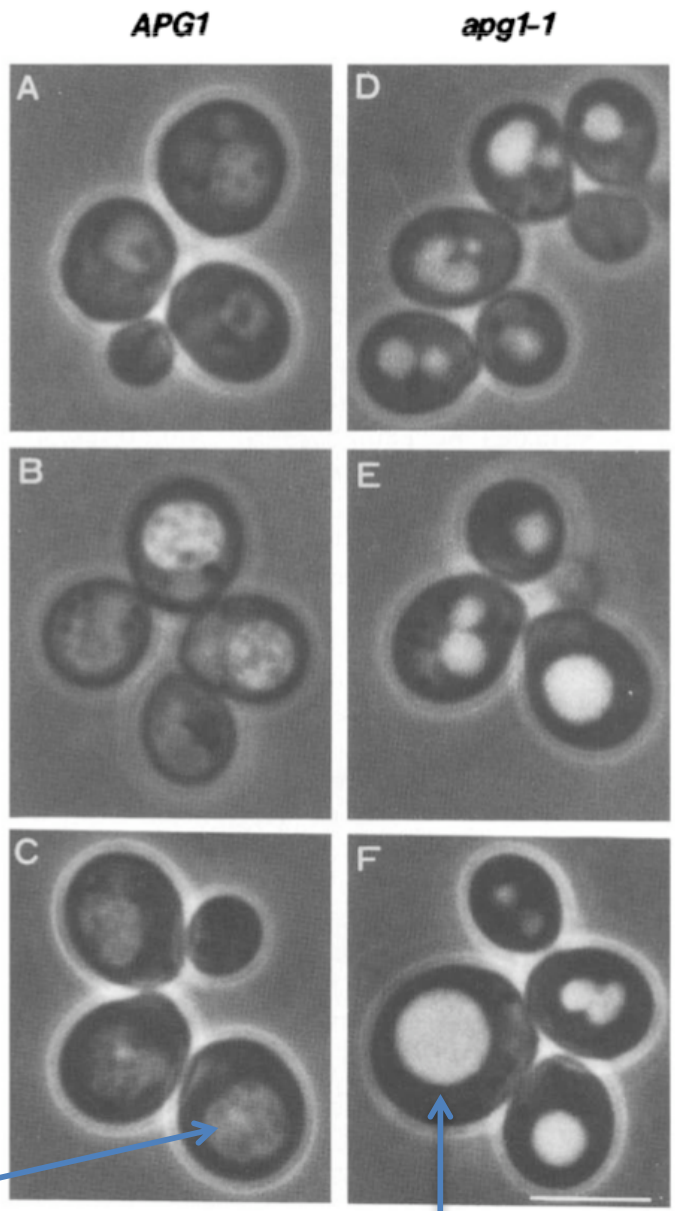
• Autophagy is activated by starvation, as a mechanism to generate amino acids.
• Starvation induced autophagy *nonspecifically* removes cytosol and organelles for nutrients.
• Autophagosome is double membrane organelle that fuses (SNARE-dependent) with lysosome for hydrolytic digestion of contents. The autophagosome forms from expansion of the isolation membrane, or phagophore (flattened membrane sac).

• Autophagy may also be important for turnover of excess/defective organelles, removal of protein aggregates, and long-lived proteins.

• Level can be *basal* or *induced*, in response to starvation, stress.

• Autophagy usually considered a protective cellular mechanism. But in some cases, autophagy is associated with programmed cell death.

Morphological screen identifies autophagy (ATG) genes



2 hr in nitrogen starvation containing PMSF

4 hr

8 hr

accumulation of autophagic bodies inside vacuole

Empty vacuoles

Autophagy induced by nutrient deprivation.

Yeast lacking vacuolar proteases (or incubated with the serine protease inhibitor PMSF) accumulate autophagic bodies inside vacuoles upon starvation. Without PMSF, WT yeast would rapidly degrade such bodies.

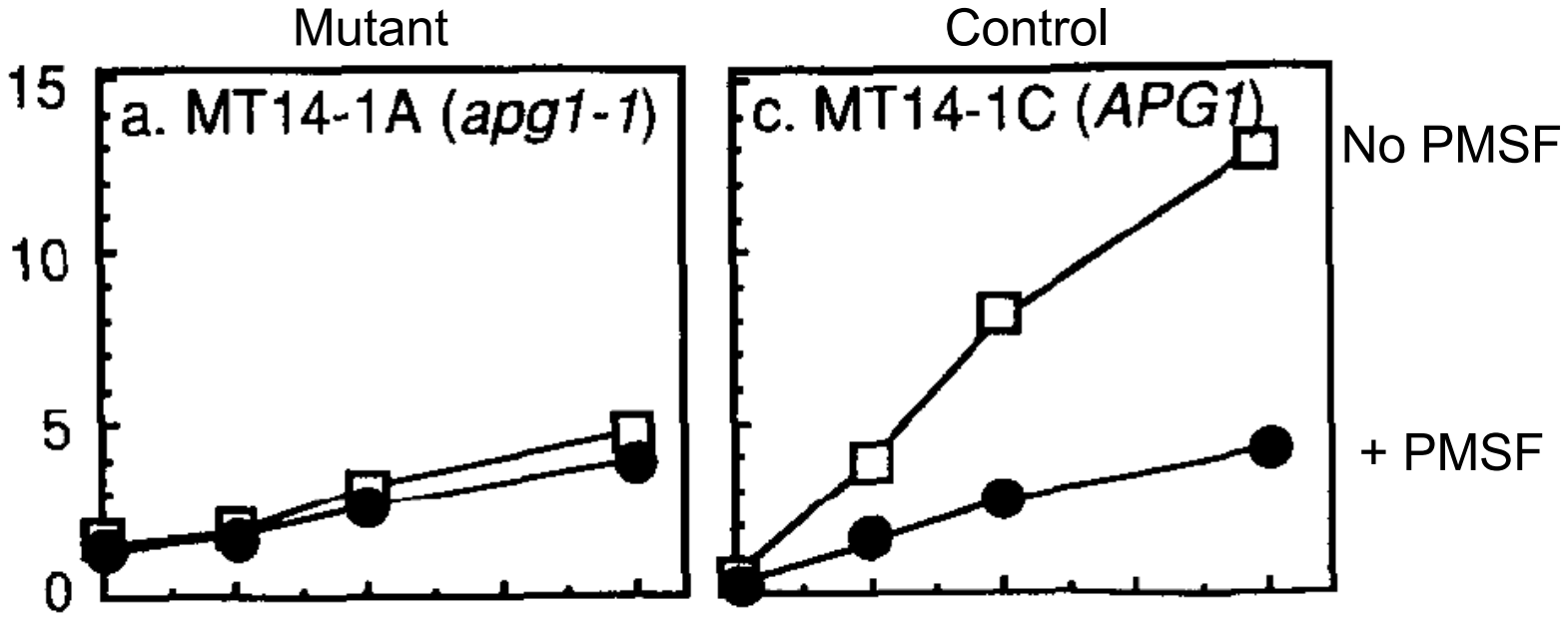
Isolate yeast deficient in autophagic bodies upon starvation (vacuoles appear empty).

Mutants show reduced protein turnover and amino acid deficiency upon starvation.

Mutants more sensitive to starvation (decreased viability with starvation).

Tsukada & Ohsumi (1993) FEBS Letters

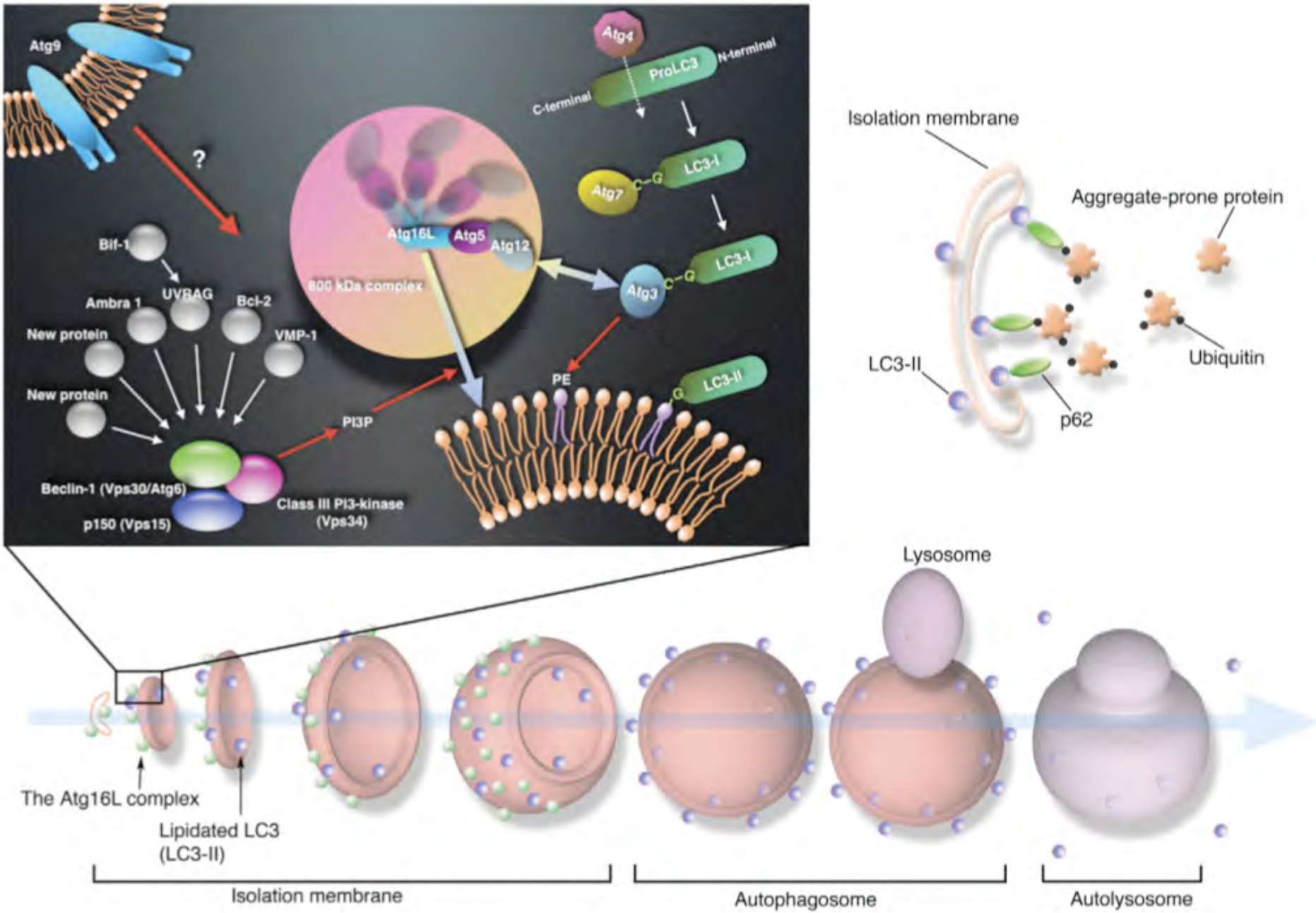
Autophagy mutant shows reduced protein degradation upon starvation



- Protein degradation was measured in autophagy mutant (apg1-1) under nitrogen starvation conditions (open squares). This degradation was inhibited by PMSF (filled circles).

Tsukada & Ohsumi (1993) FEBS Letters

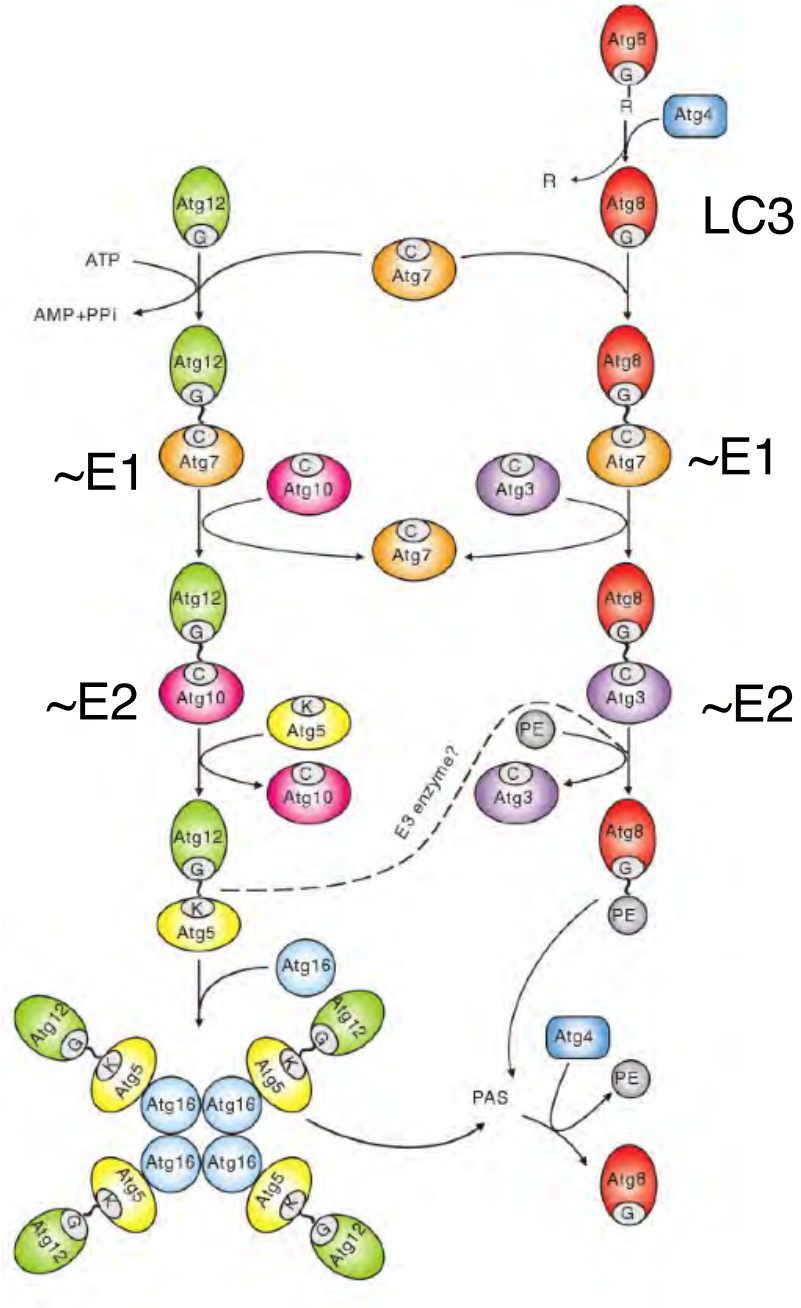
Some molecules involved in autophagy



The ATG12-ATG5/ATG16 complex decorates the outer side of the phagophore; LC3 is uniform.

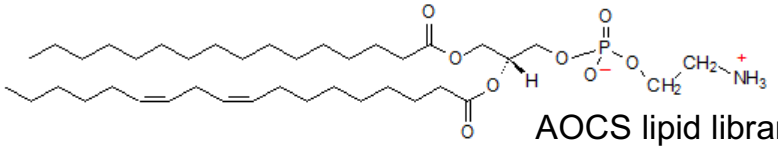
Yoshimori & Noda (2008) Curr. Opin. Cell Biol.

Two ubiquitin-like conjugation systems regulate phagophore expansion



- 2 proteins with ubiquitin-like folds—ATG12 and ATG8 (LC3)—are involved in formation of the autophagosome.
- ATG12 is conjugated to ATG5.
- ATG8 is conjugated to PE (phosphatidylethanolamine).

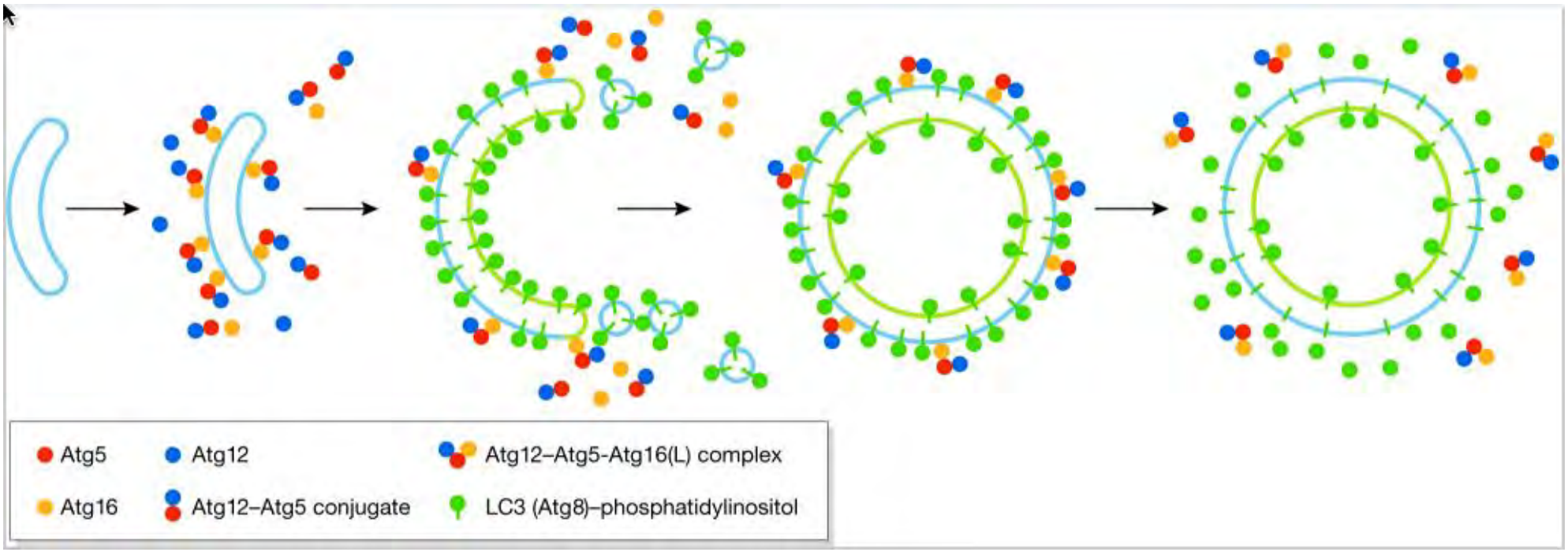
PE



AOCS lipid library

- ATG12 (glycine) is conjugated to ATG5 (on lysine) by ATG7 (~E1) and ATG10 (~E2). The end-product is assembled with a tetramer of ATG16 to form a high molecular weight complex (ATG5-ATG12/ATG16).
- ATG8 conjugated to the lipid phosphatidylethanolamine (PE) by ATG7 (~E1) and ATG3 (~E2). The end-product is PE-ATG8.
- Mammalian orthologs of ATG8 include LC3.
- The ATG5-ATG12 conjugate is required for ATG8-PE conjugation.

Sequential action of the two conjugation systems during phagophore expansion



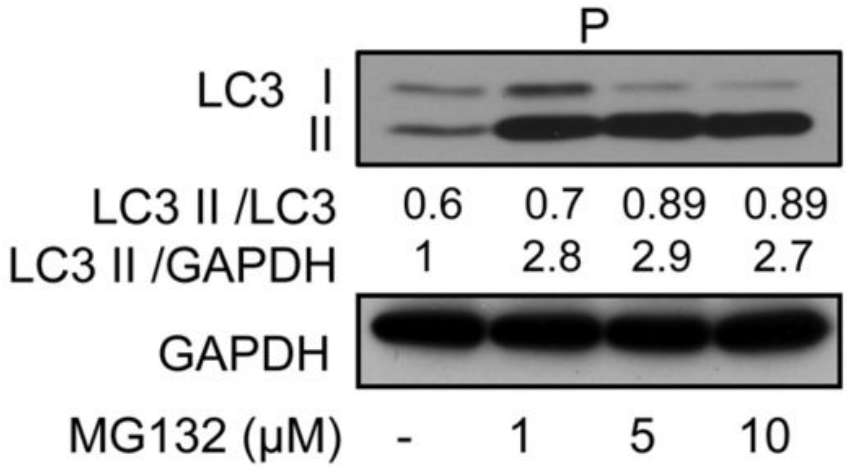
Ath12/Atg5/Atg16 is recruited to the early phagophore, ultimately concentrating on outer surface. Atg12/Atg5 is required for LC3 lipidation.

Atg8 localization to the PAS (phagophore-assembly site) is Atg12–Atg5-Atg16-dependent, but not vice versa.

Geng & Klionsky (2008) EMBO reports

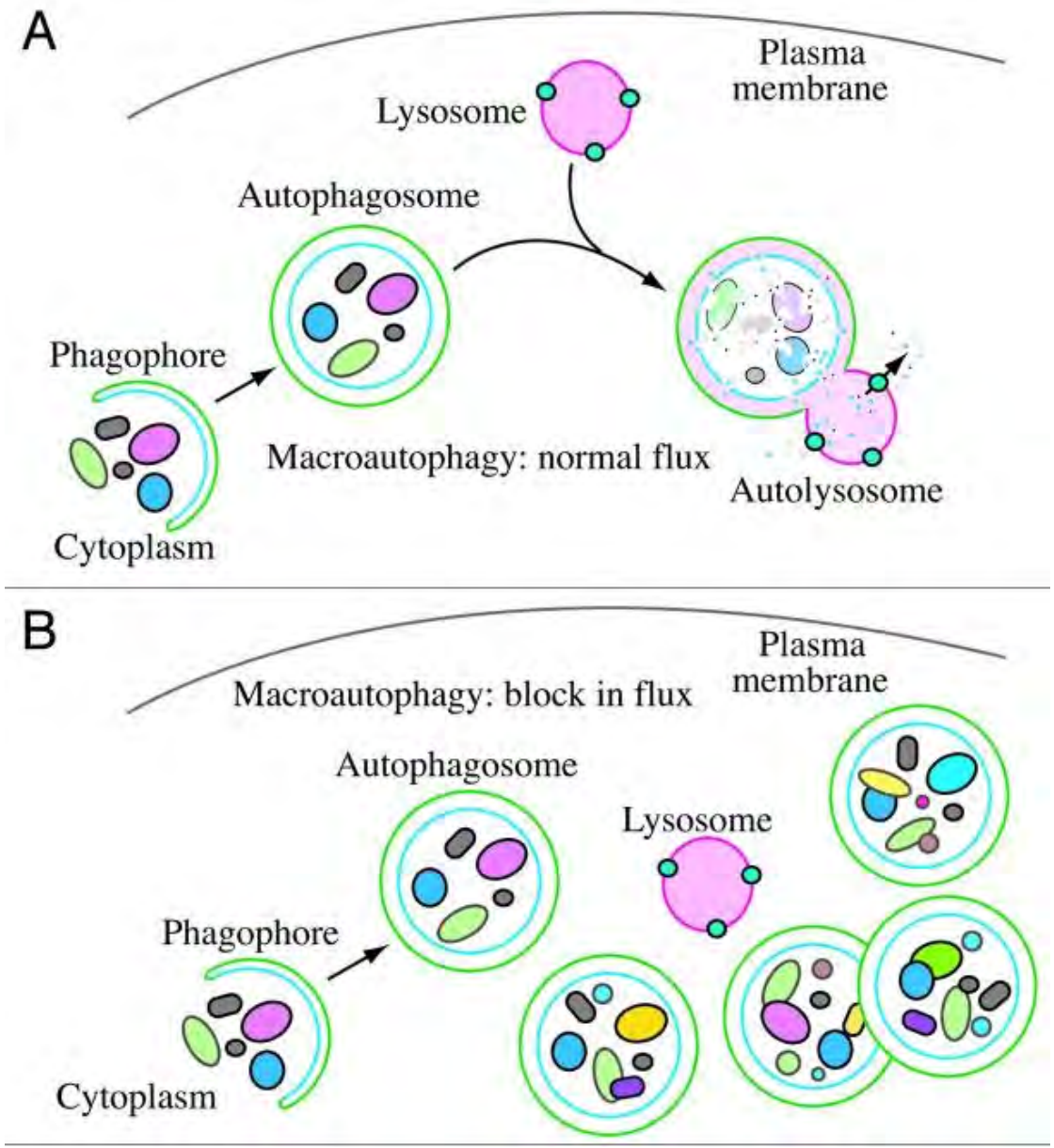
Inhibition of the UPS results in upregulation of autophagic flux

- Treatment of proliferating fibroblasts with MG132 causes increased processing of LC3
- nascent LC3 (proLC3) is hard to detect; it is processed by ATG4 protease to yield LC3-I
- LC3-I is cytosolic
- LC3-II=LC3-I conjugated with PE (larger molecular weight, but migrates faster)
- LC3-II is on isolation membranes and autophagosomes
- LC3-II levels can correlate with levels of autophagy

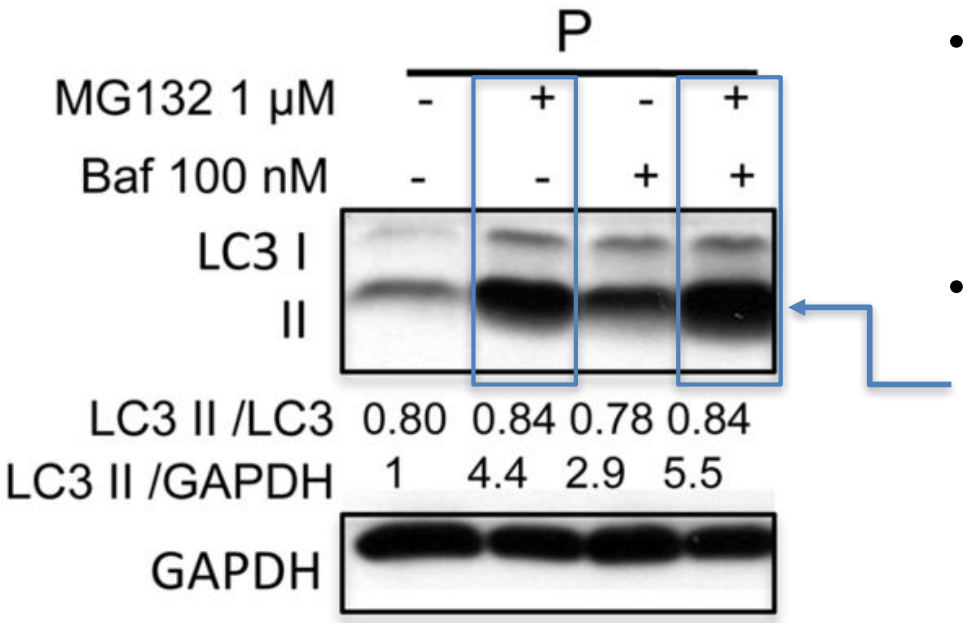


Legesse-Miller et al. (2012) MBoC

Quantification of autophagy can be complicated



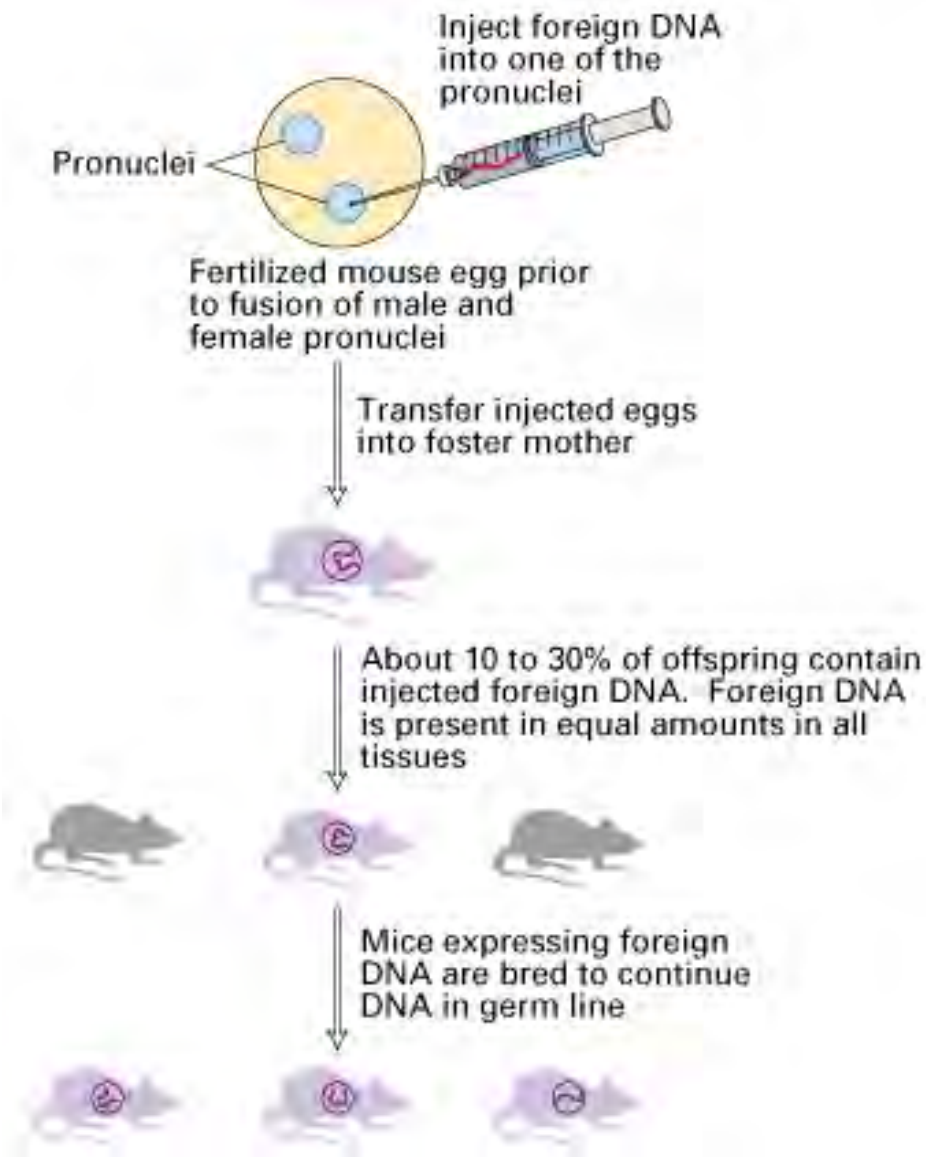
Inhibition of the UPS results in upregulation of autophagic flux



- Bafilomycin is a vacuolar H^+ pump inhibitor and inhibits fusion of autophagosomes and lysosomes
- Result (additional increase in LC3-II with bafilomycin treatment) is consistent with increased autophagic flux

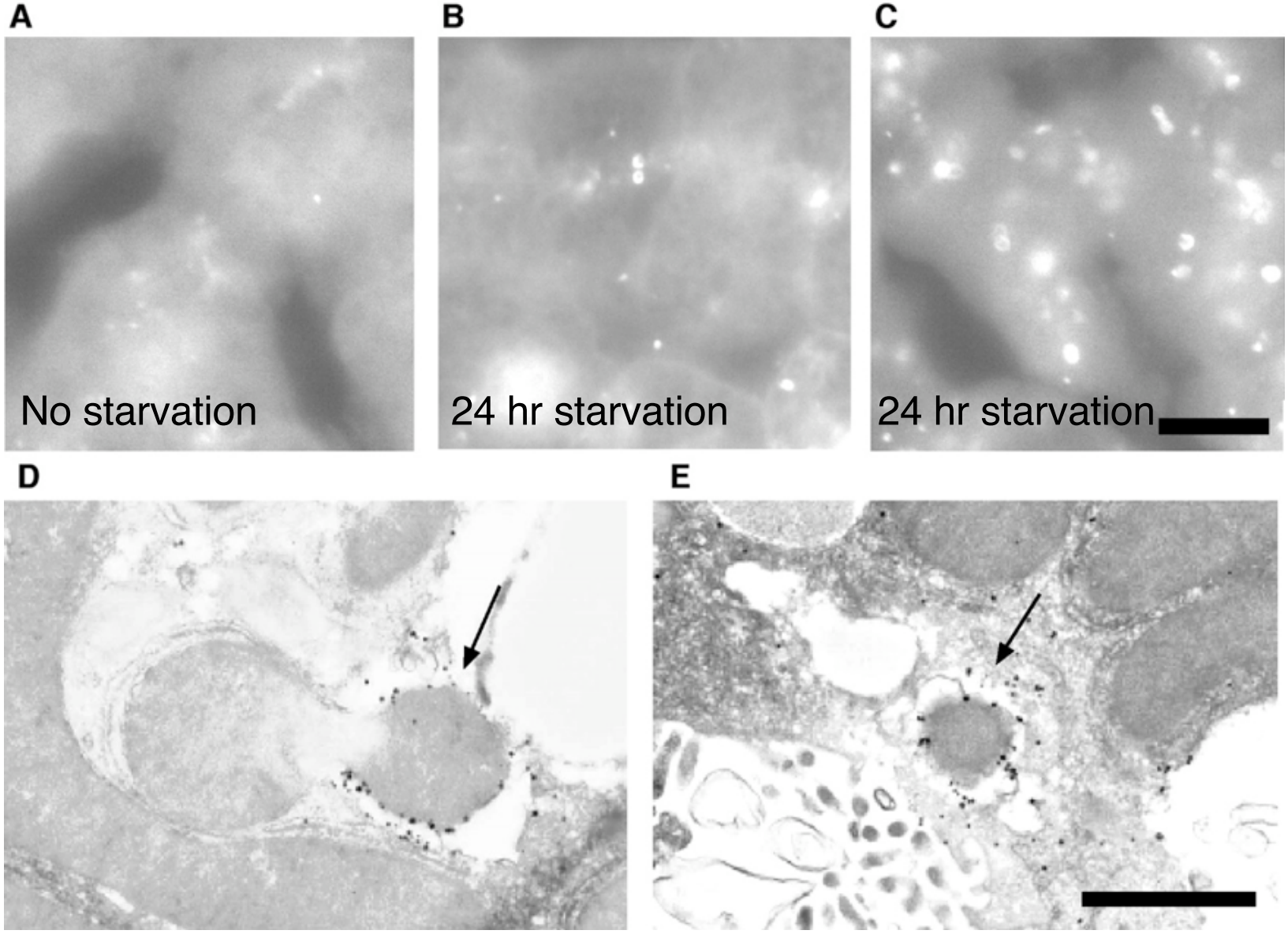
Legesse-Miller et al. (2012) MBoC

Production of transgenic mice



Lodish et al, 2000

Analysis of autophagy in GFP-LC3 transgenic mice



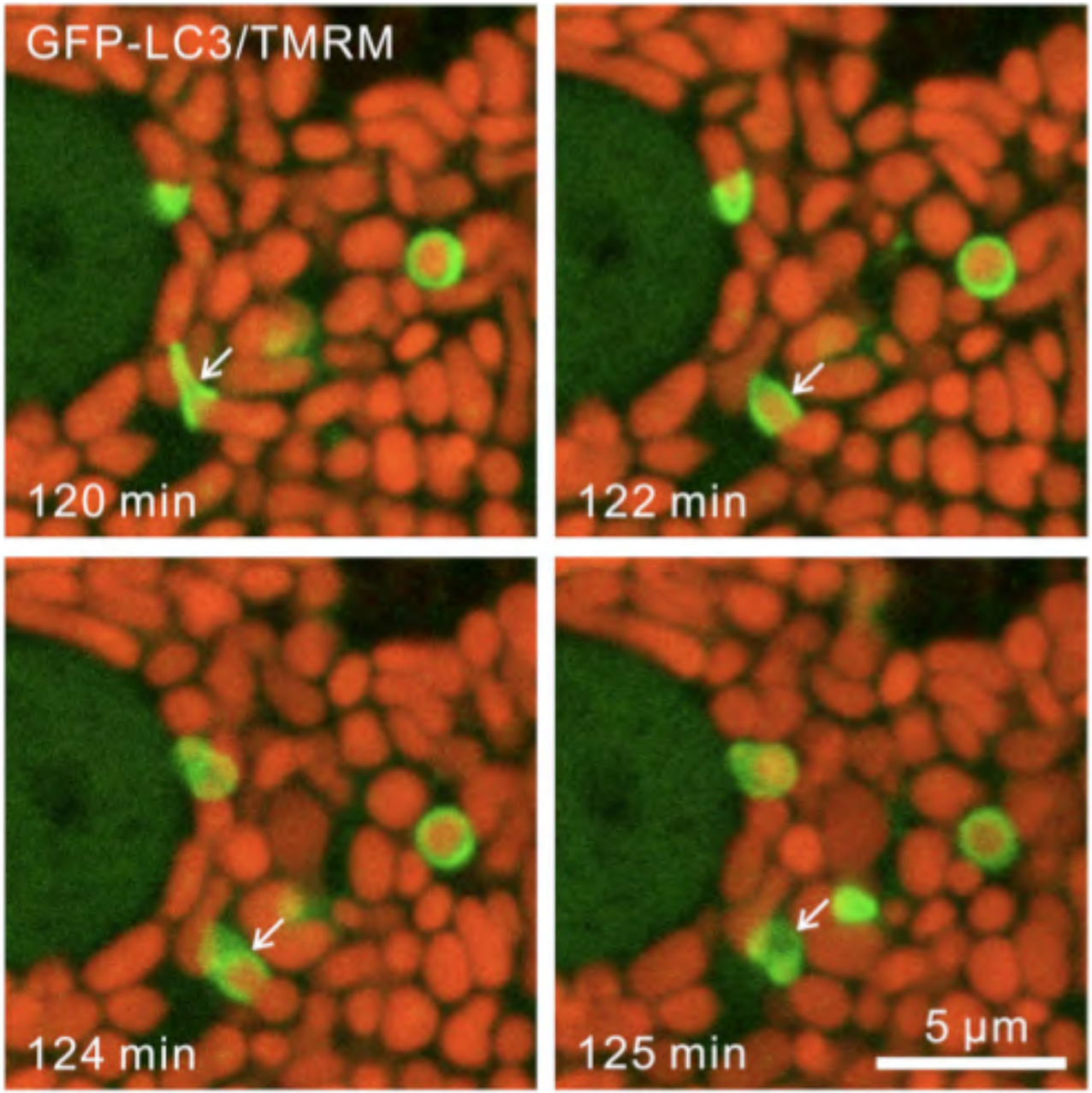
Transgenic mice carry GFP-LC3 driven by CMV immediate early enhancer/chicken β actin promoter

GFP fluorescence in liver cryosections
a) No starvation
b) 24 hr starvation
c) same

d) immunogold against GFP
e) same

Mizushima et al. (2003) MBC

Autophagy can turnover organelles like mitochondria

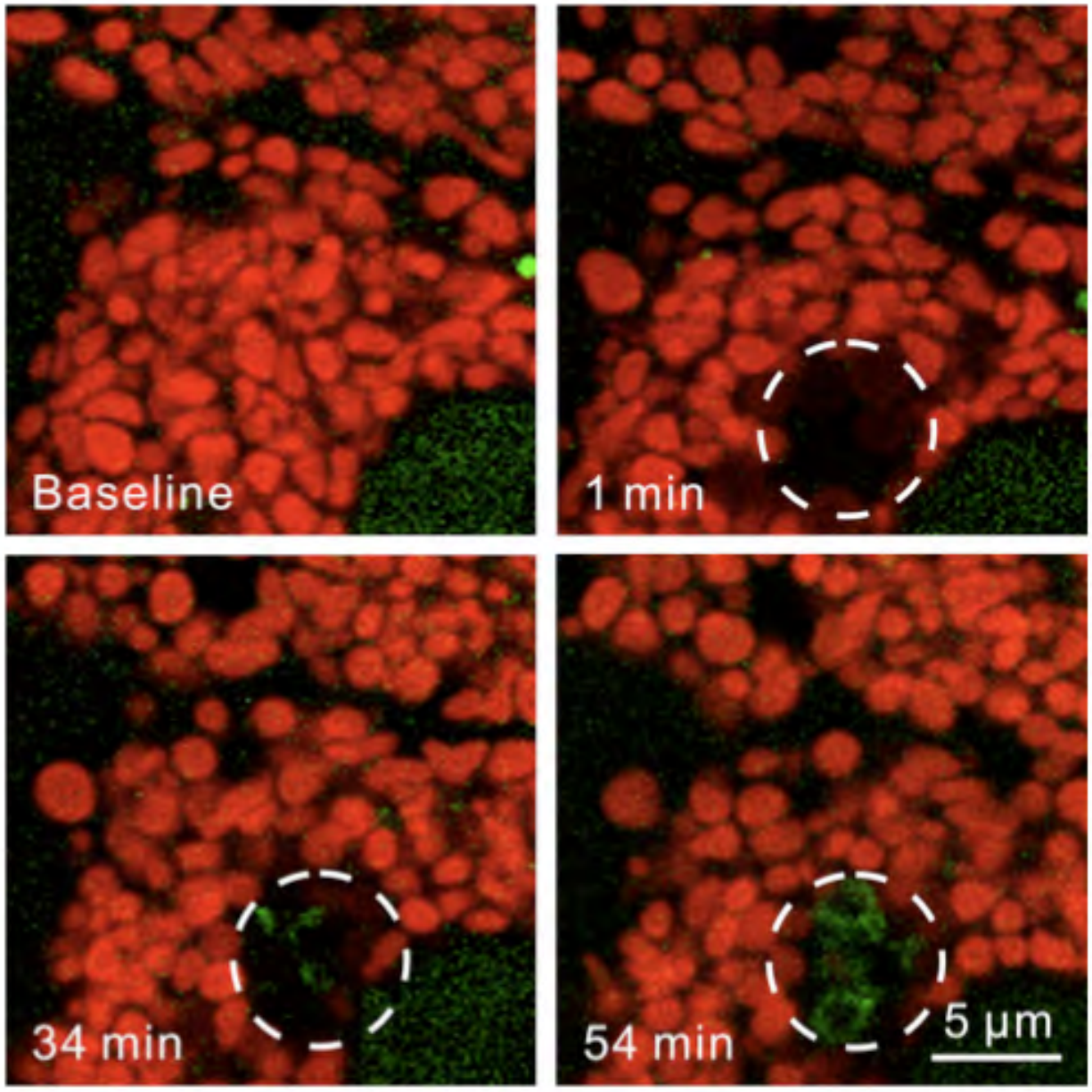


GFP-LC3 transgenic hepatocytes loaded with TMRM; cells placed in nutrient deprivation; GFP-LC3 spots appear, and then membrane depolarization occurs.

- In this case, mitophagy appears nonselective; results in degradation of mitochondria and loss of membrane potential.

Kim et al. (2007) Arch Biochem Biophy

Mitophagy may be selective

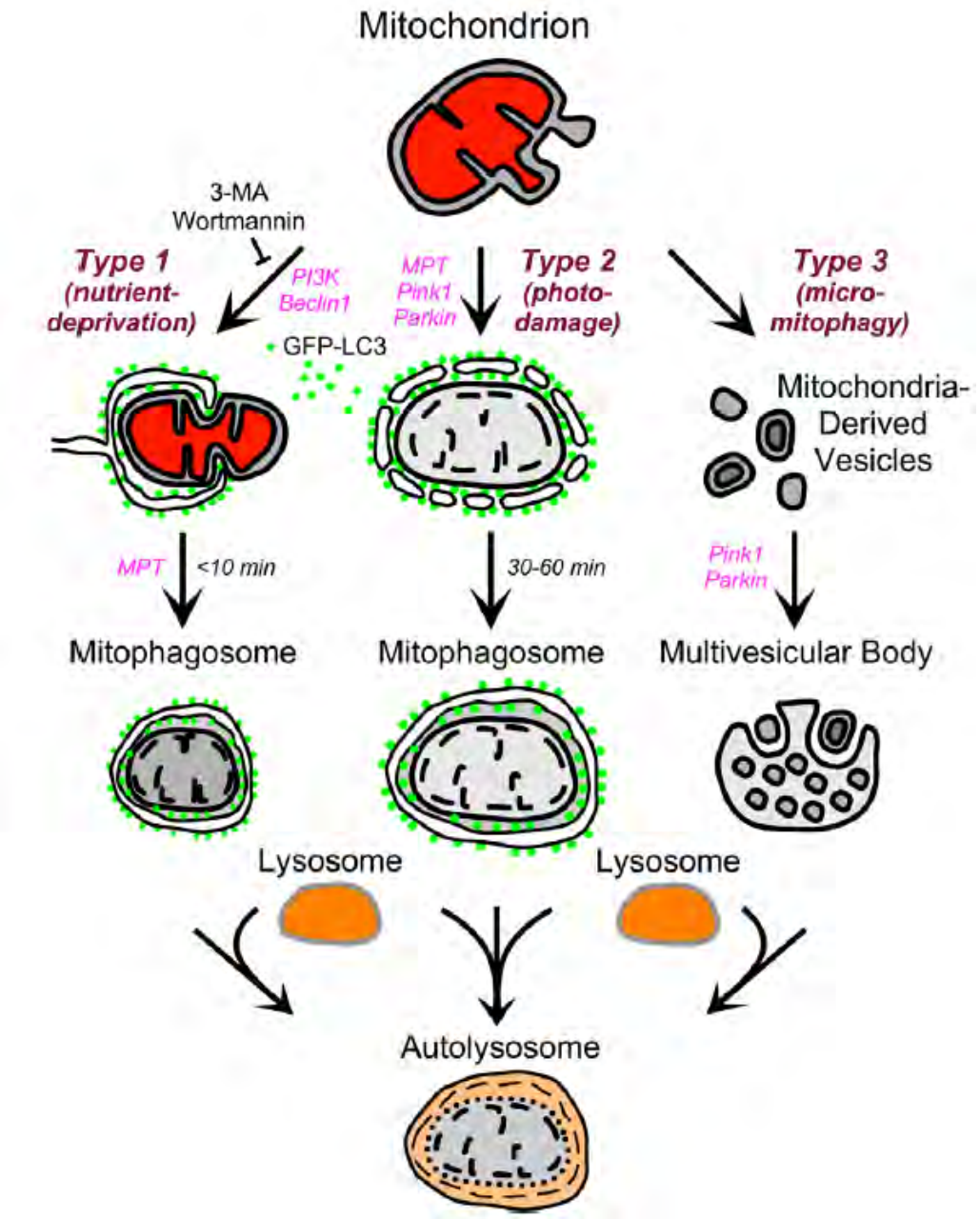


GFP-LC3 transgenic hepatocytes loaded with TMRM; 488 nm laser used to damage mitochondria within circle; GFP-LC3 appears within an hour.

- In this case, mitophagy is selectively activated on dysfunctional mitochondria.

Kim et al. (2007) Arch Biochem Biophy

Types of mitophagy in mouse hepatocytes



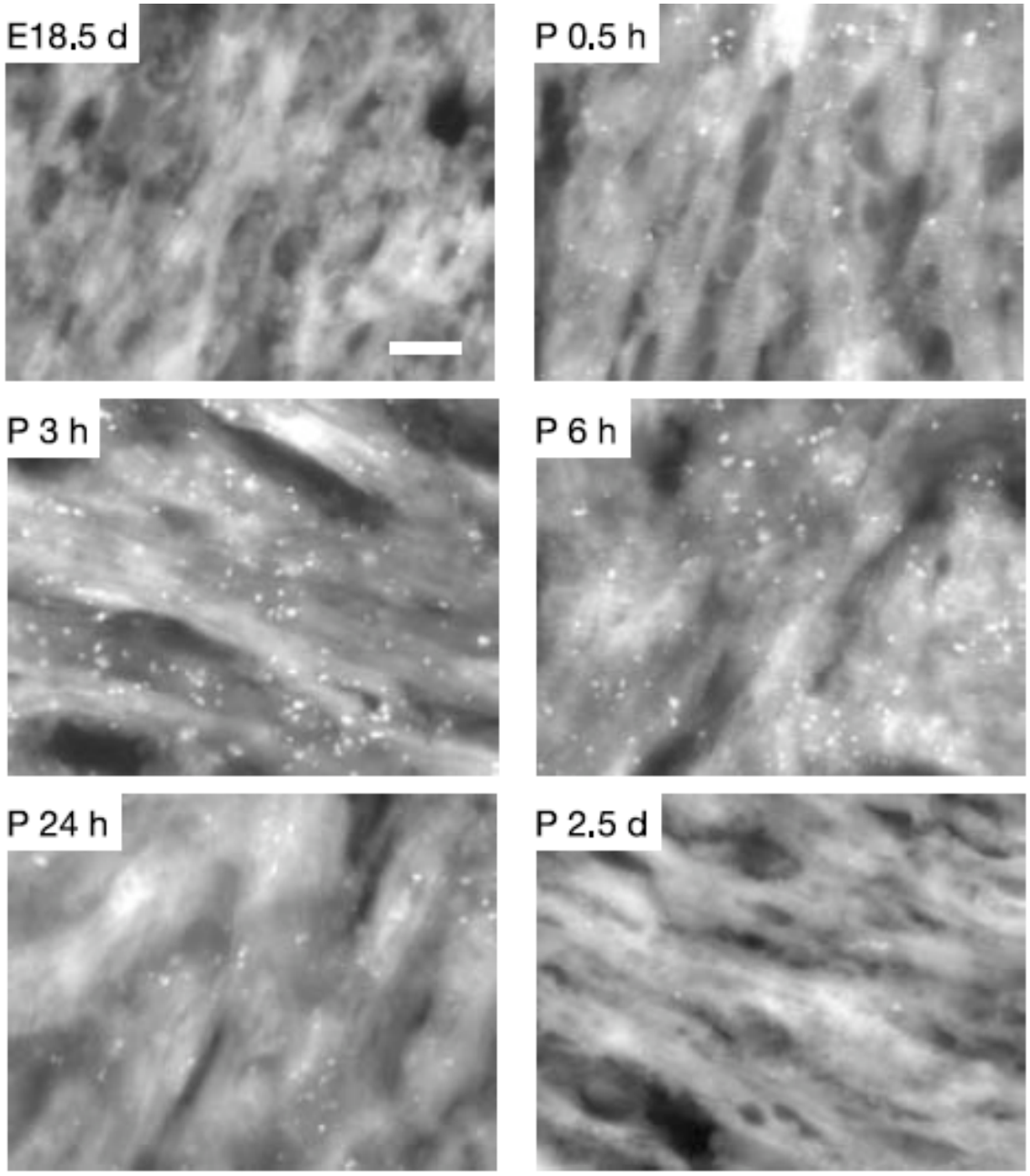
Type 1: Starvation-induced mitophagy

Type 2: Depolarization-induced mitophagy

Can be distinguished by pharmacological inhibition: 3-methyladenine and wortmanin are PI3 kinase inhibitors: inhibit autophagosome formation in type 1 but not type 2.

J. Lemasters (2014) Redox Biology

Autophagy is transiently induced immediately following birth

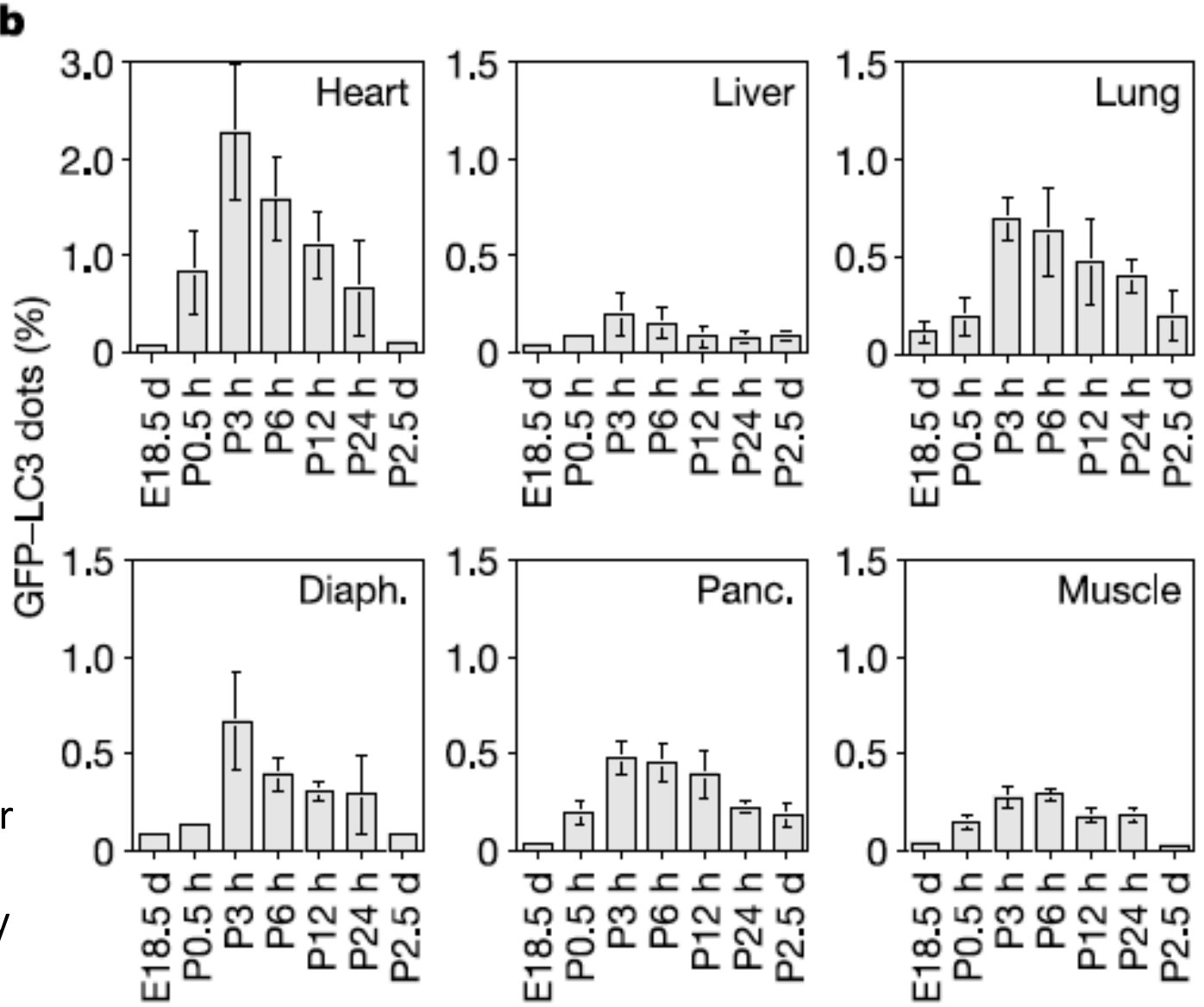


Autophagy monitored in heart muscle of embryonic and post-natal GFP-LC3 transgenic mice. Increase LC3 seen postnatally till ~24 hours.

Rationale: Embryo is nourished by the placenta. Upon birth, neonates suddenly face starvation until milk restores energy stores. Autophagy may be a method of self-nourishment.

Kuma et al. (2004) Nature

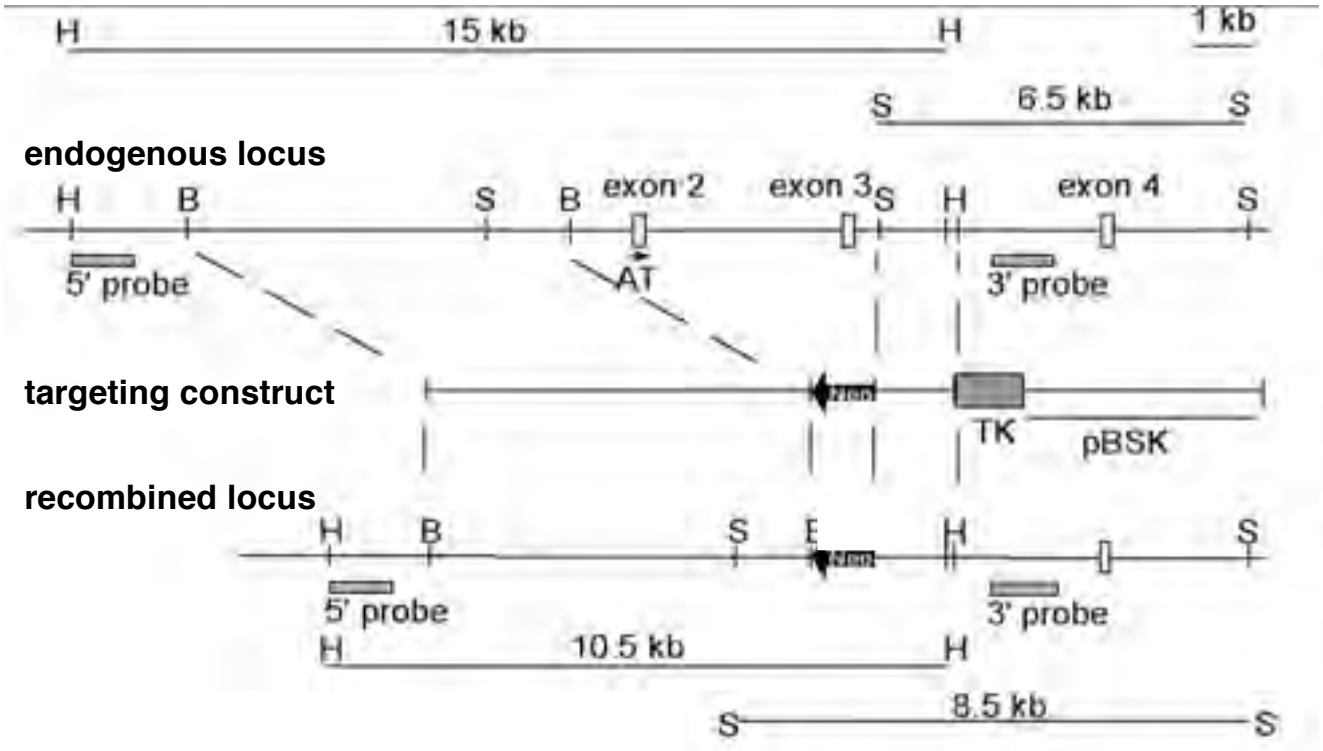
Autophagy is transiently induced immediately following birth

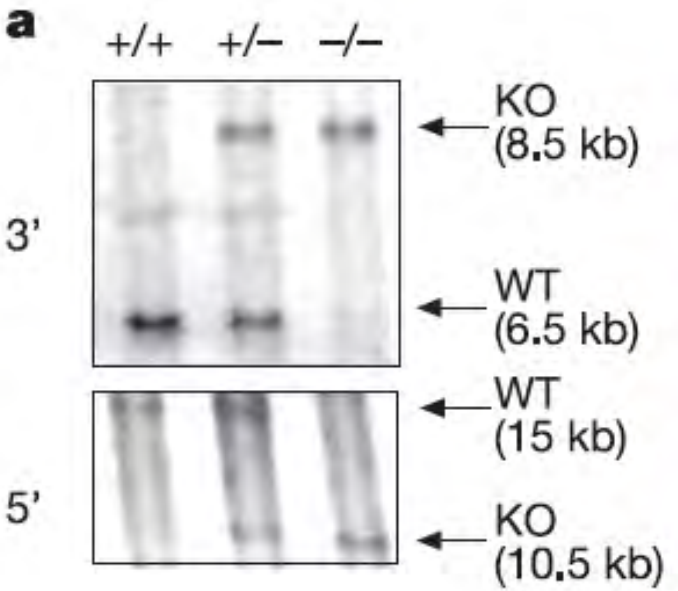


% of cellular surface occupied by GFP-LC3 spots.

Kuma et al. (2004) Nature

Targeted disruption of ATG5

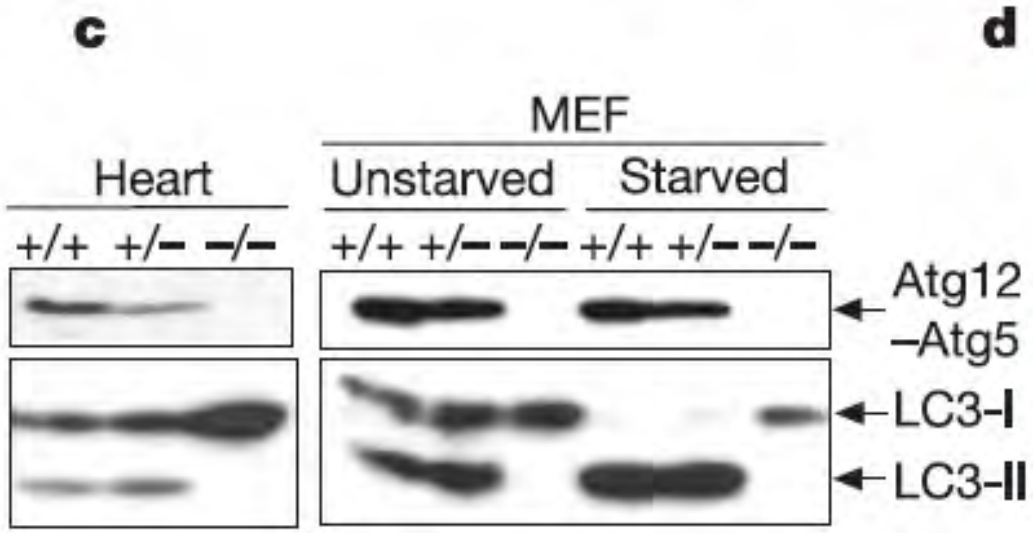




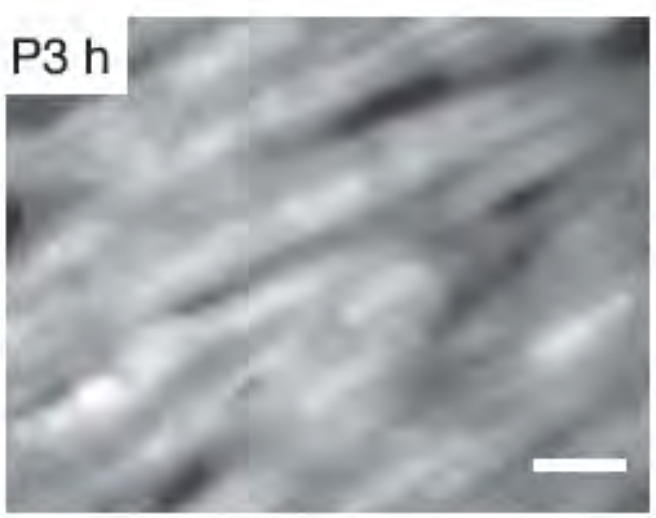
3’ probe: genomic DNA digested with SpeI
5’ probe: genomic DNA digested with HpaI

Kuma et al. (2004) Nature

ATG5 knockout mice fail to induce autophagy and die neonatally



No Atg12-Atg5 conjugate is found in mutants.



heart muscle from KO show no GFP-LC3 puncta.

ATG5 is conjugated with the ubiquitin-like molecule ATG12; conjugation necessary for progression of the autophagic isolation membrane.

- ATG5 mutants appear normal at birth, but die within 1 day.
- Little LC3-II was produced, whereas LC3-I is increased. No GFP-LC3 spots.
- 10 hours after delivery, amino acid concentration in plasma was low.

Kuma et al. (2004) Nature

Autophagy and neuronal function

ATG5 knockout causes neonatal lethality.

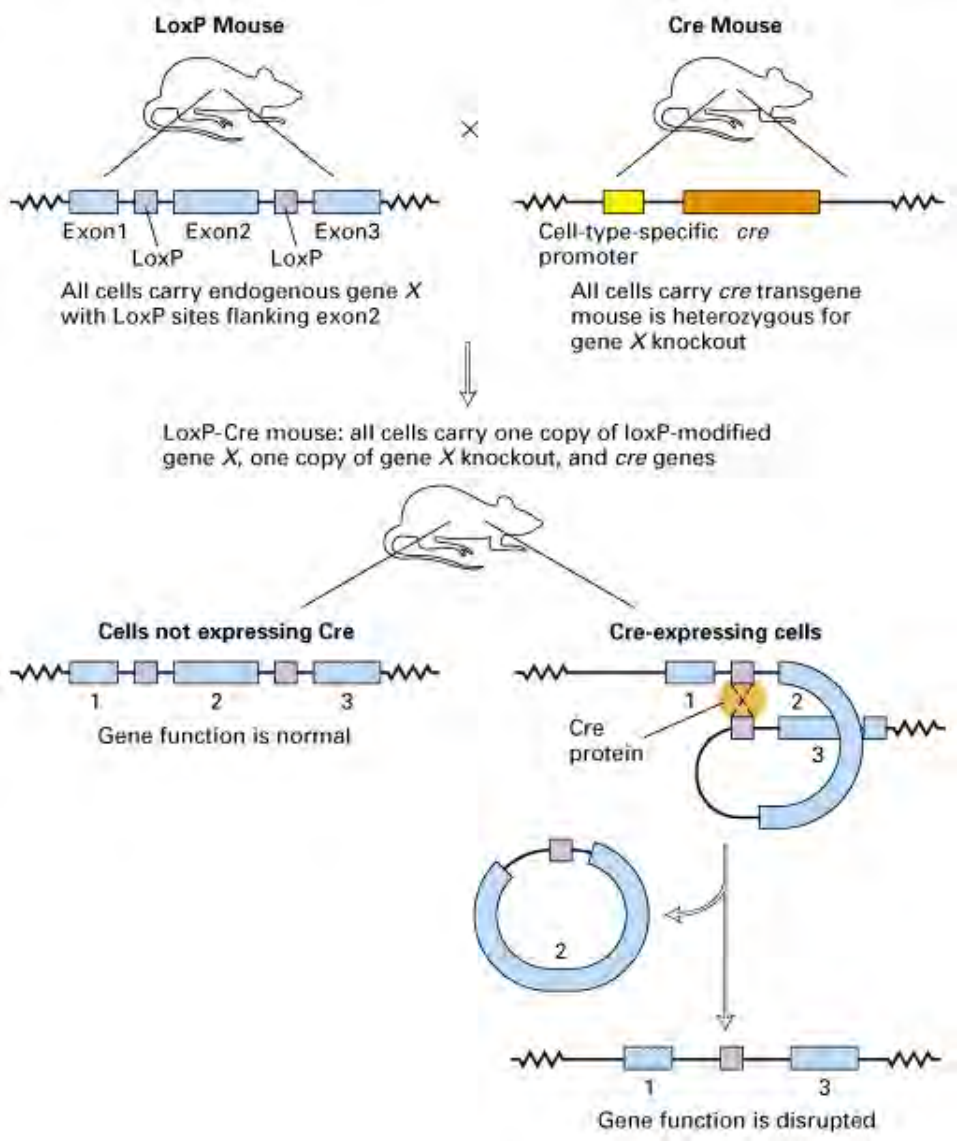
Cytoplasmic inclusions found in hepatocyte and neurons of ATG5 mutant neonates.

Macroautophagy thought to be primarily an *induced* response to starvation, a mechanism for producing amino acids within cells.

But *constitutive* autophagy may help to clear intracellular debris.

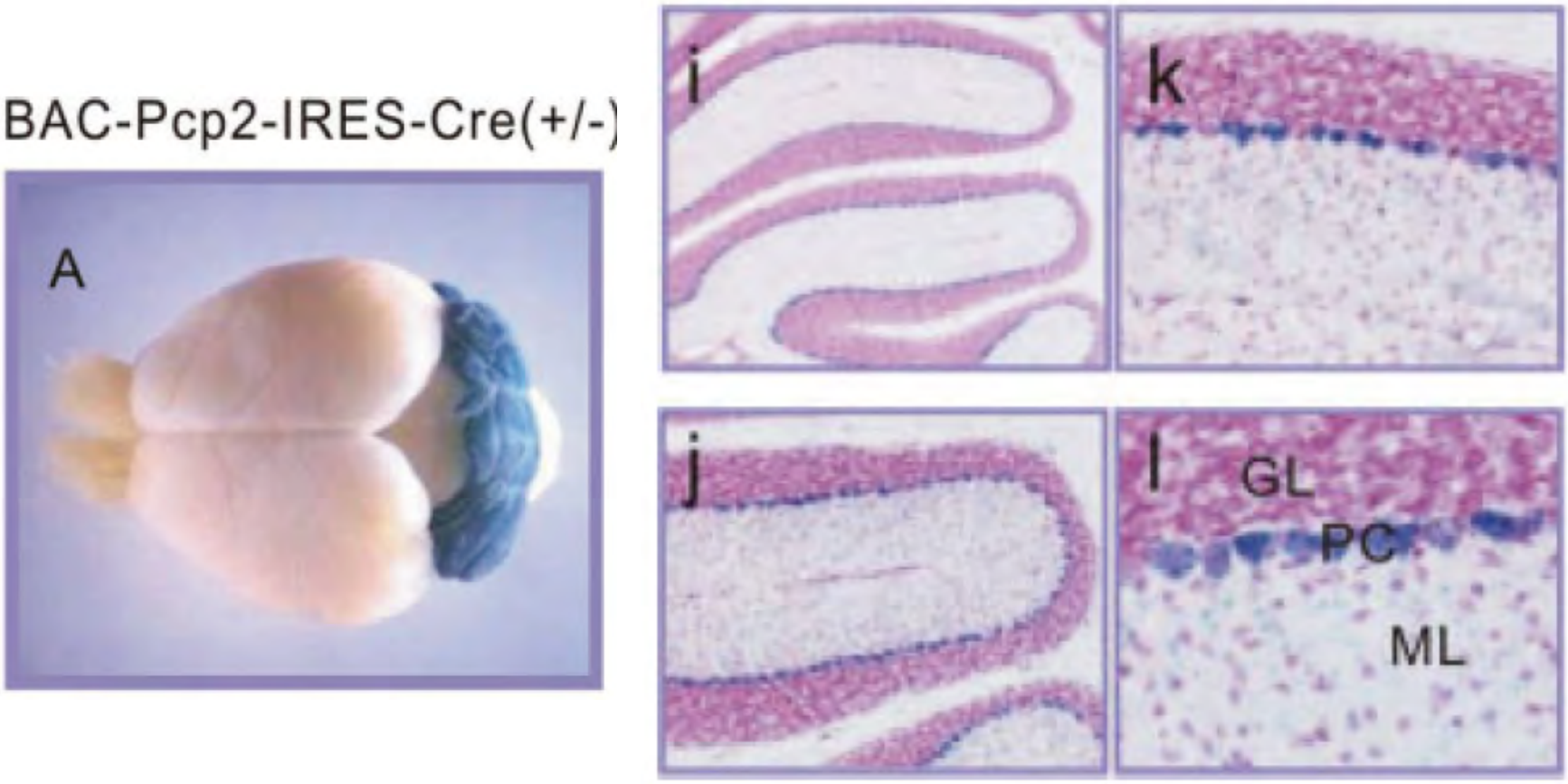
Intracellular inclusions are found in several neurodegenerative diseases.

Cell-type-specific (conditional) gene knockouts in mice



Lodish et al, 2000

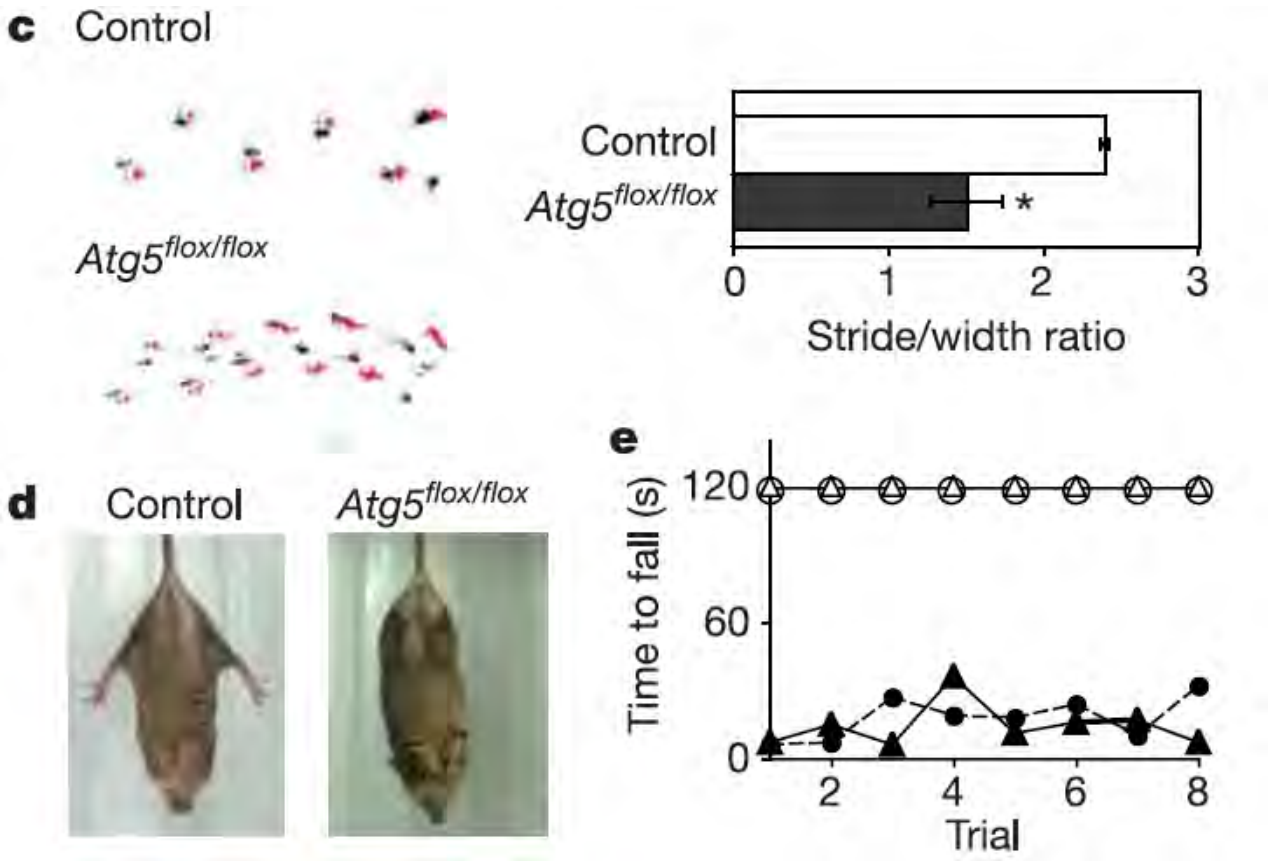
The *cre-loxP* system allows temporal and spatial control of gene disruption



specific expression of Cre recombinase in
Purkinje cells of cerebellum

Zhang et al (2004) *Genesis*

Neurological defects in mice lacking ATG5 in neurons

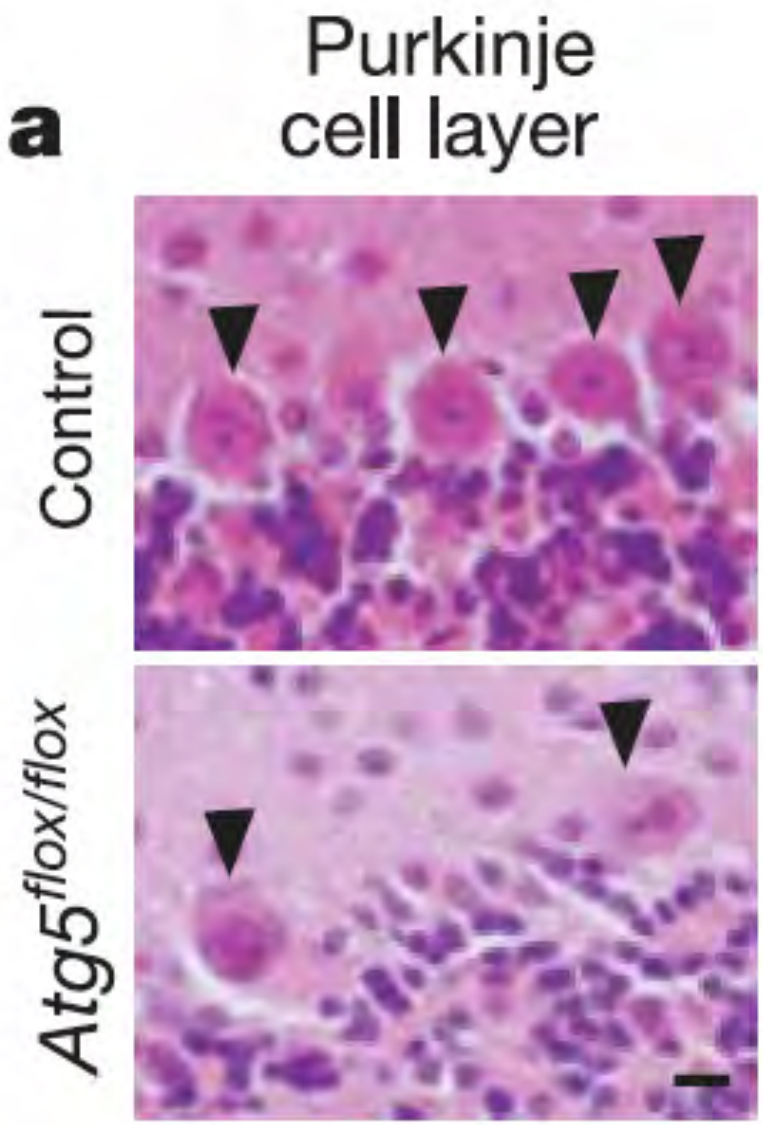


Floxed ATG5 allele was deleted by pan-neuronal Nestin-cre.
(c) Ataxic walking pattern with decrease stride length.
(d) Abnormal limb clasping reflex.
(e) Rotarod analysis. Example:
http://www.dnatube.com/video/586/SCA1-Mouse-on-Rotarod

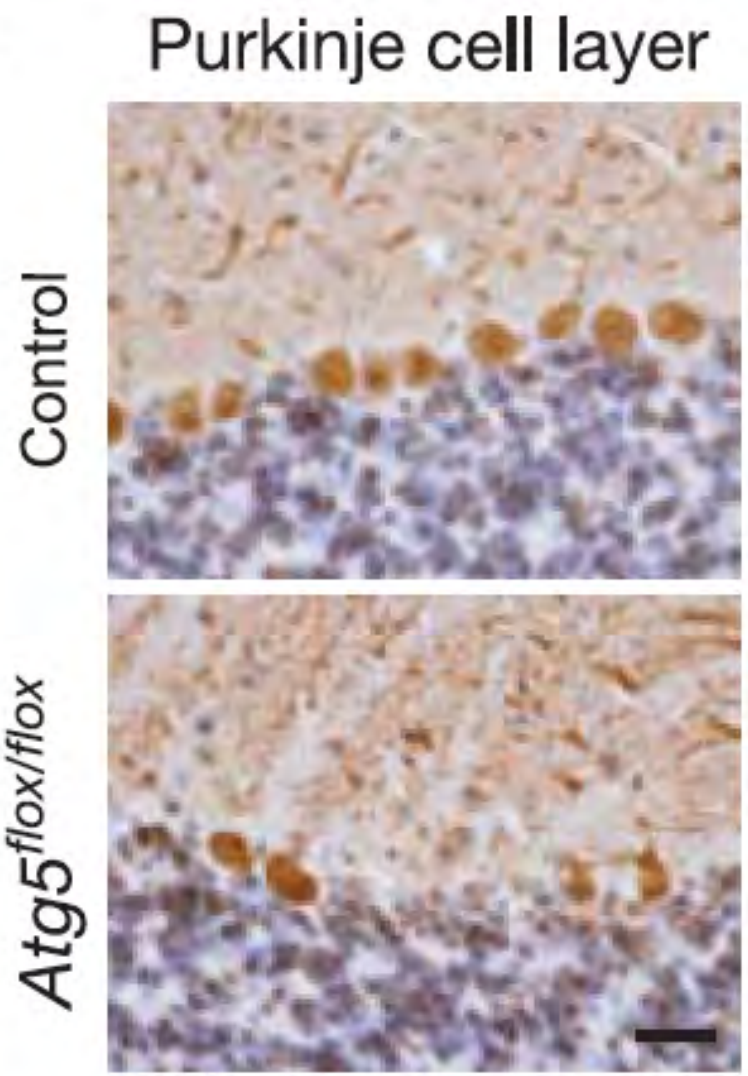
Other phenotypes: wire-hanging defect, tremor.

Hara et al. (2006) Nature

Purkinje cell defect



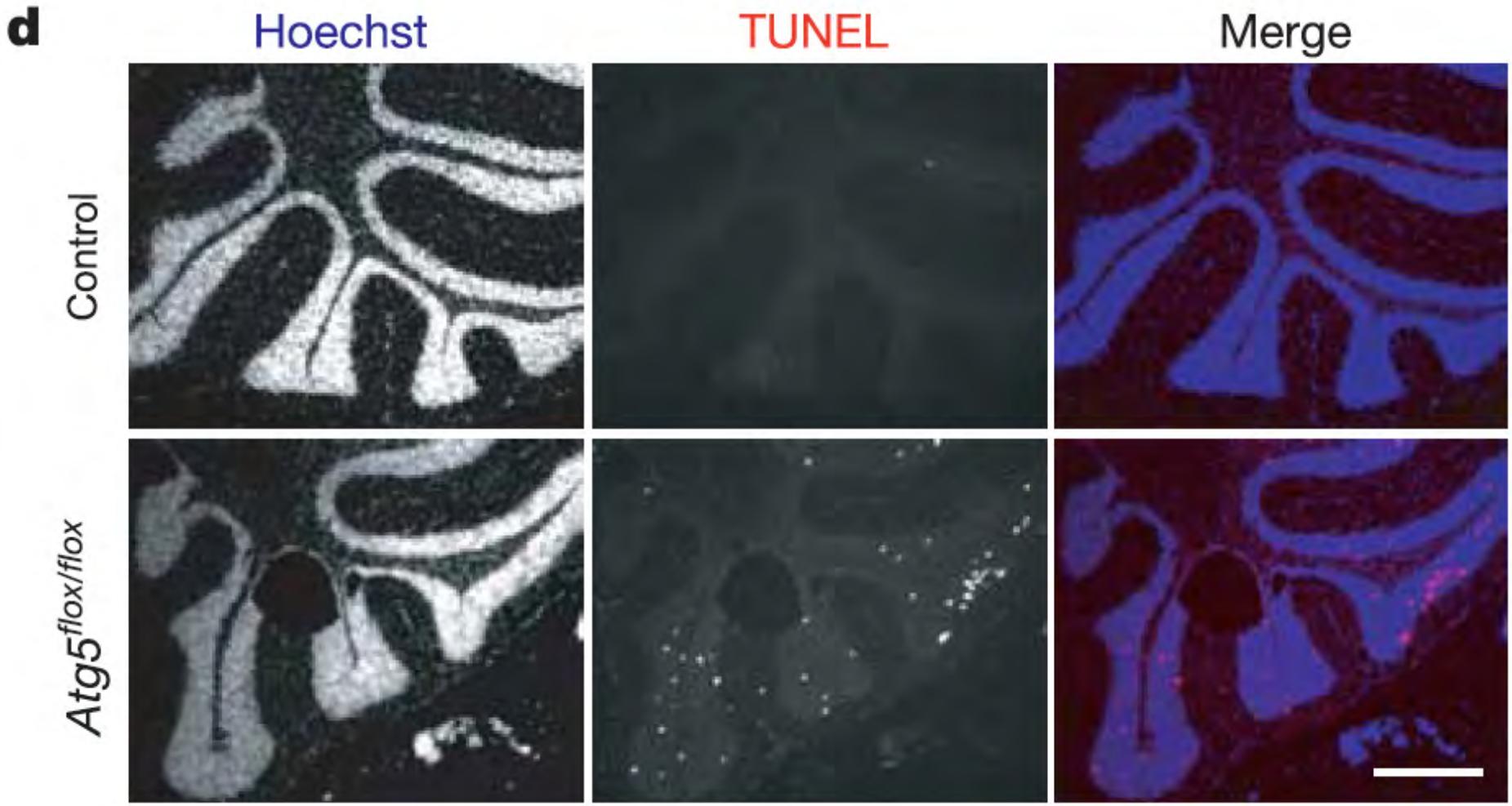
H&E



Calbindin as a marker to
specifically label Purkinje cells

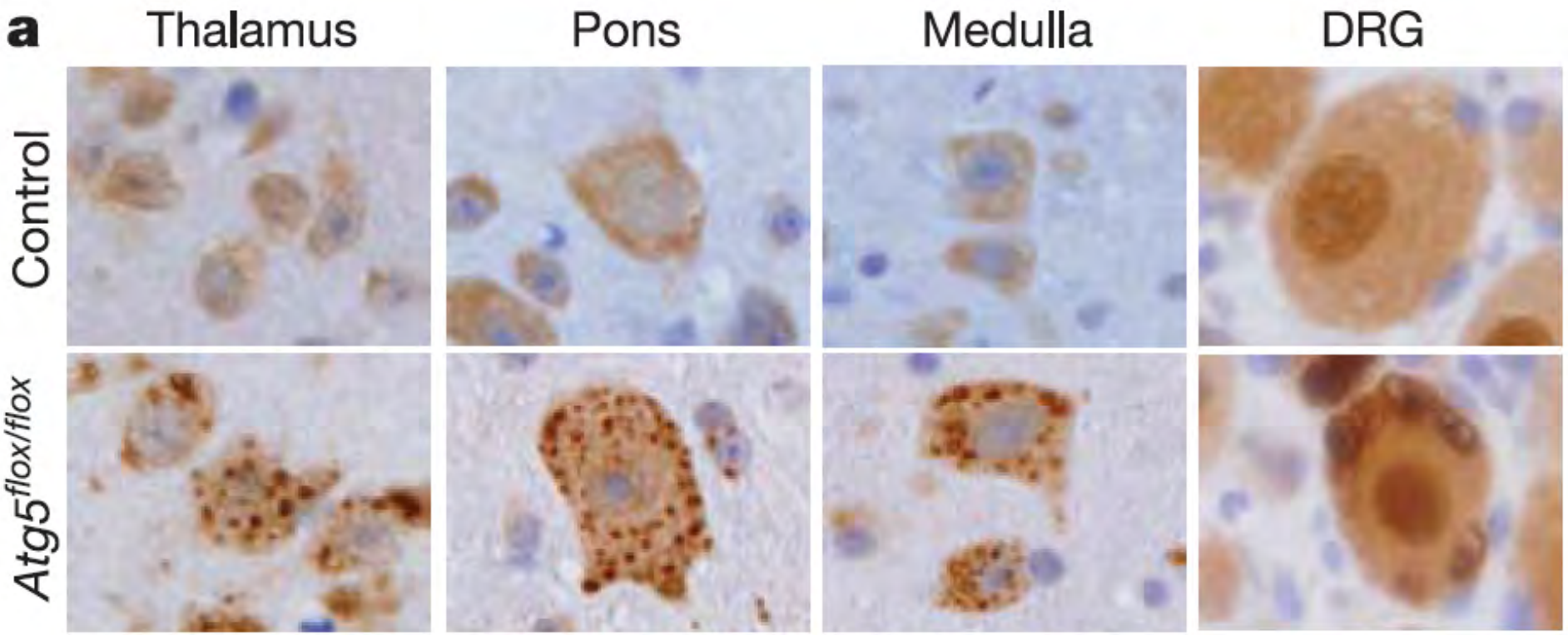
Hara et al. (2006) Nature

Granule cell defect



Hara et al. (2006) Nature

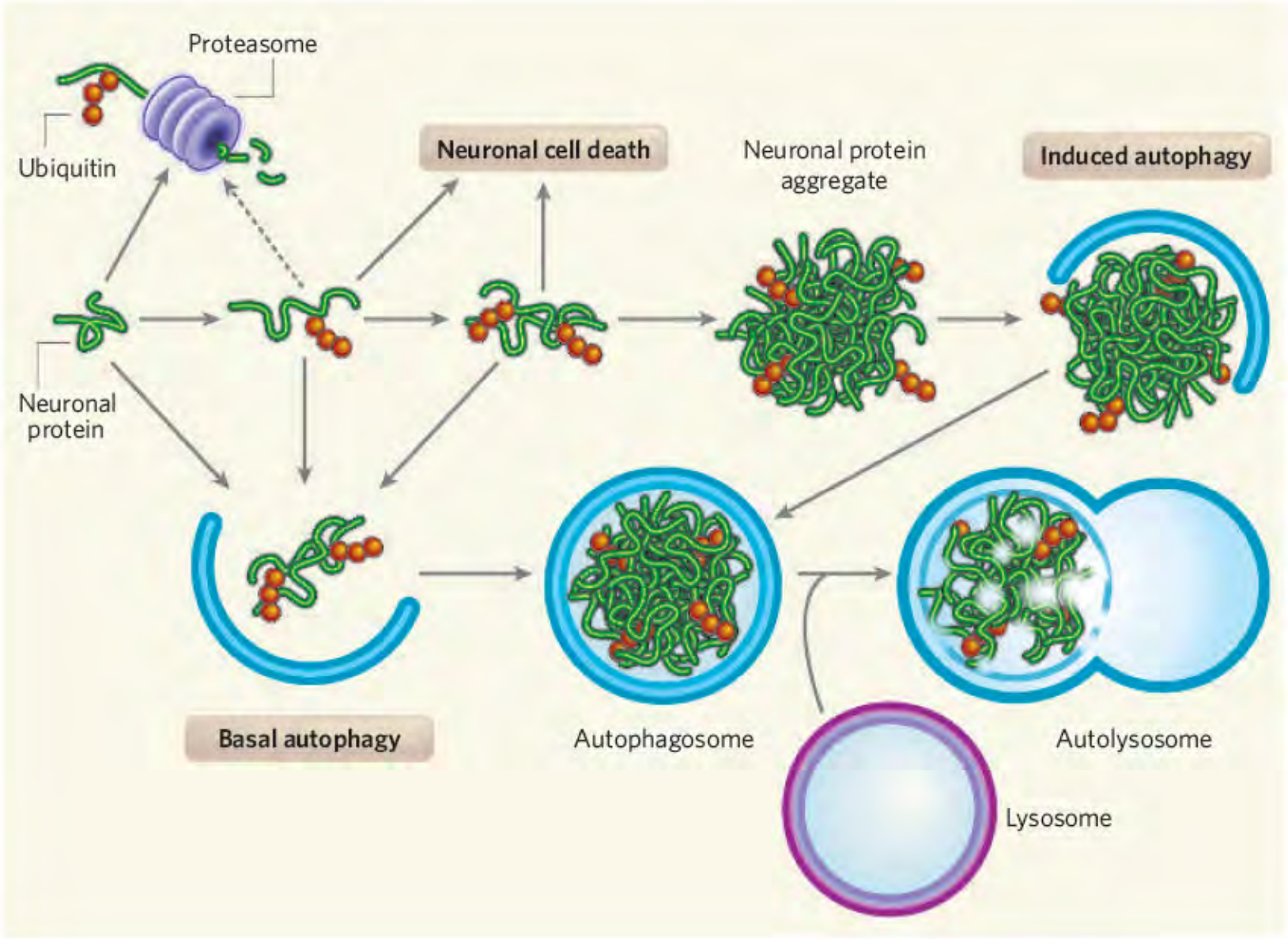
Ubiquitin-positive intracellular inclusions



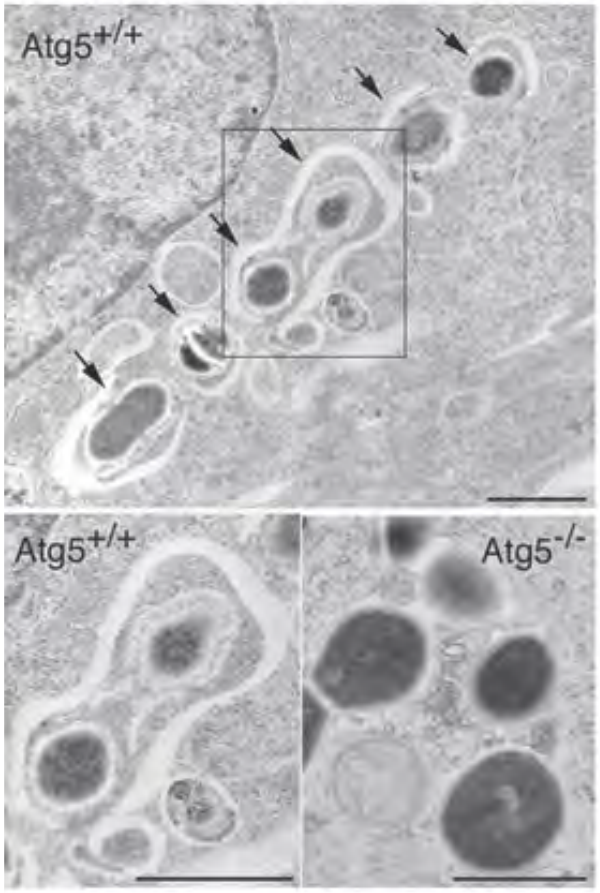
Brown stain: anti-Ub

Hara et al. (2006) Nature

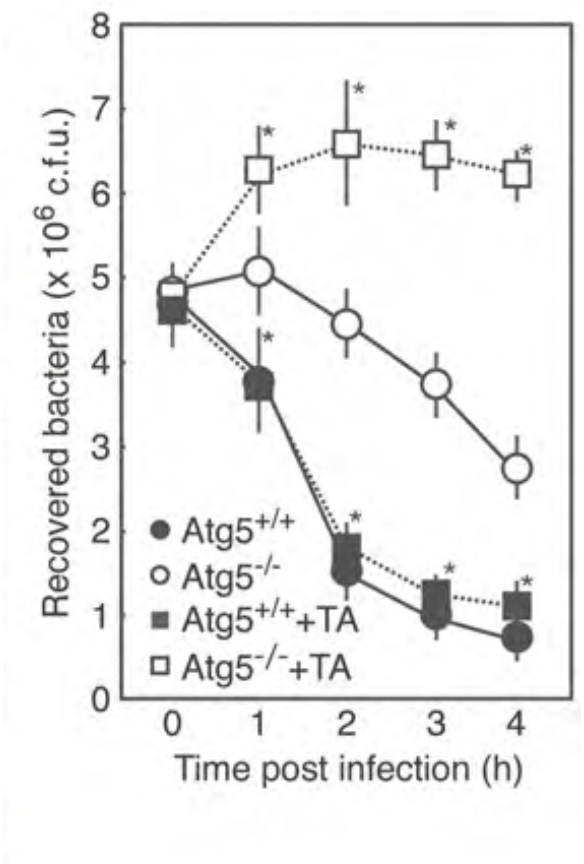
Autophagy as a protein clearance pathway



Atg5 deficiency allows group A streptococci to survive within host cells



WT MEFs form autophagosome-like vesicles around bacteria; Atg mutants do not

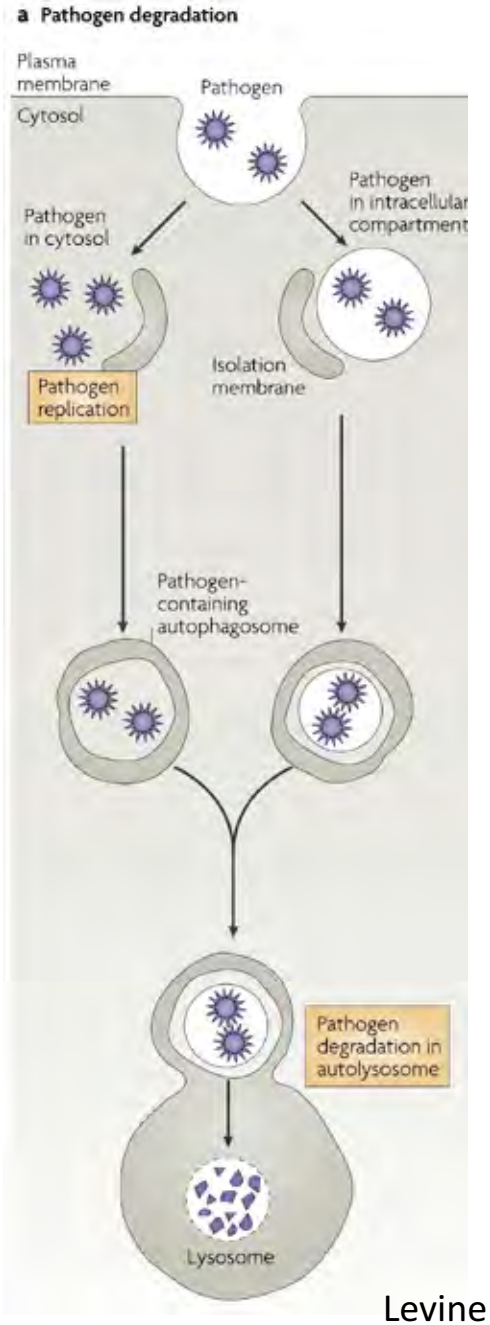


Increased numbers of viable bacteria in atg mutant:

TA=tannic acid, a cell-impermeable fixative that prevents fusion between secretory vesicles and plasma membrane (to prevent external escape of bacteria)

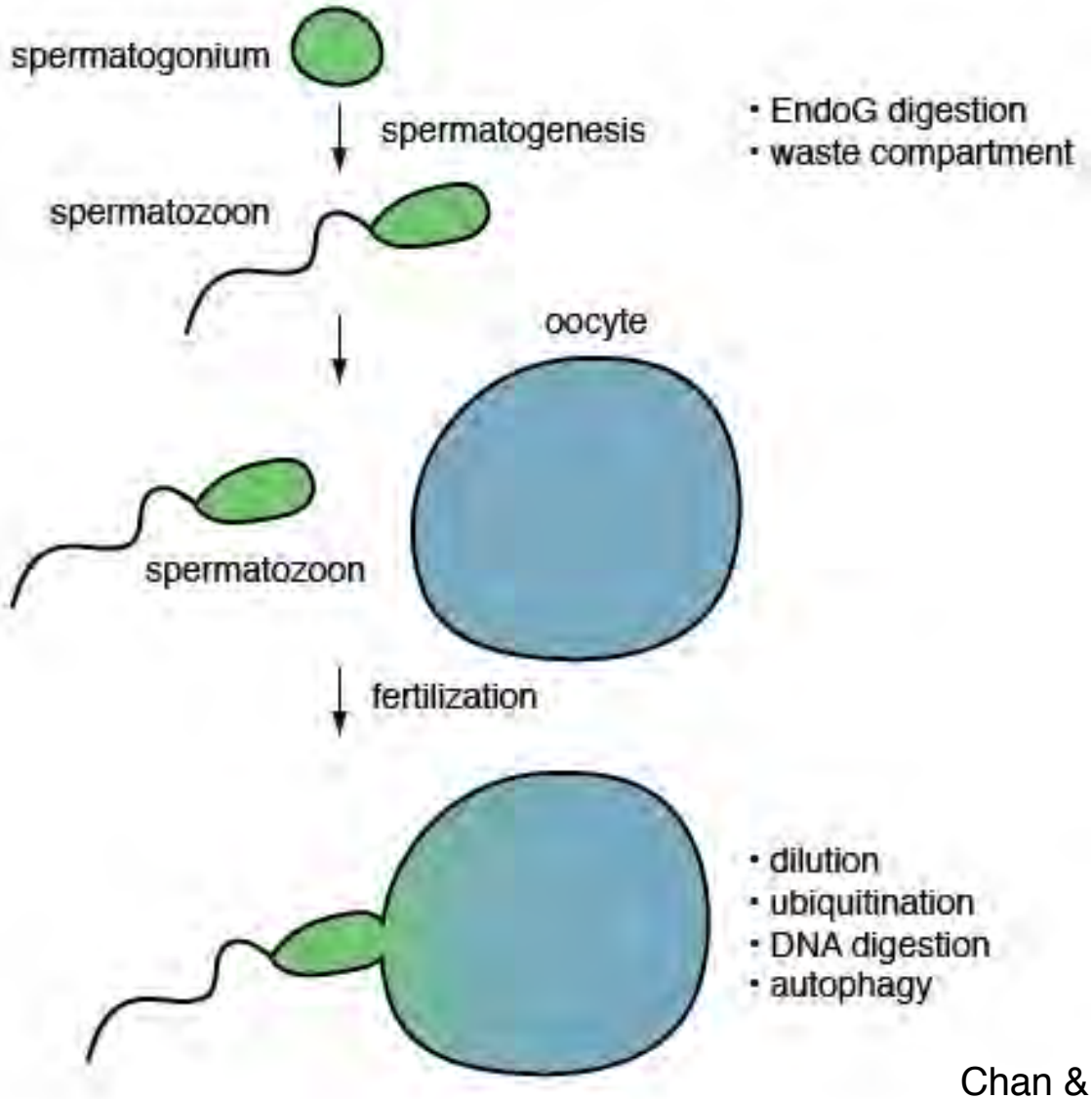
Nakagawa et al (2004) Science

Autophagy in bacterial infection



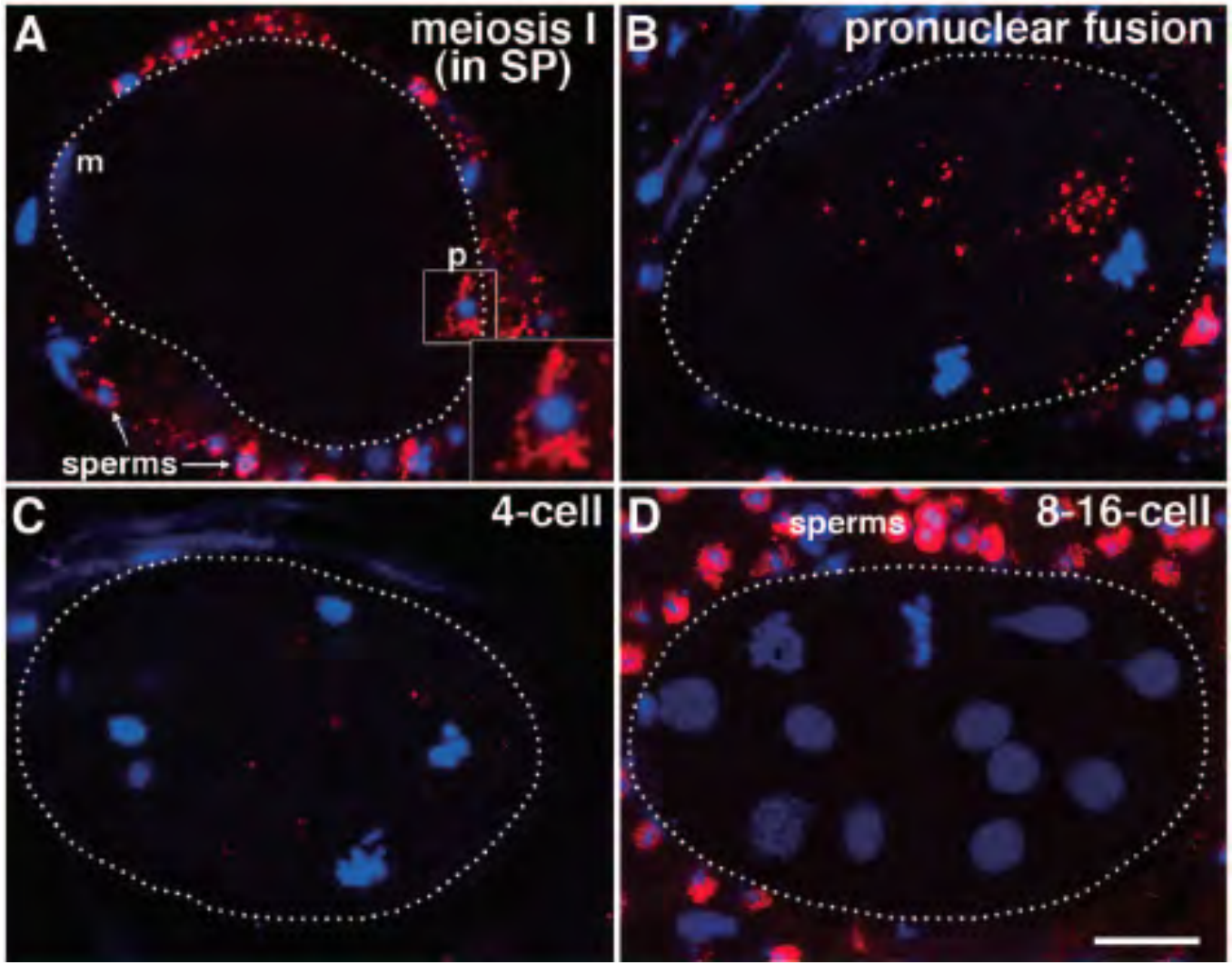
Levine & Deretic, Nature Reviews Immunology (2007)

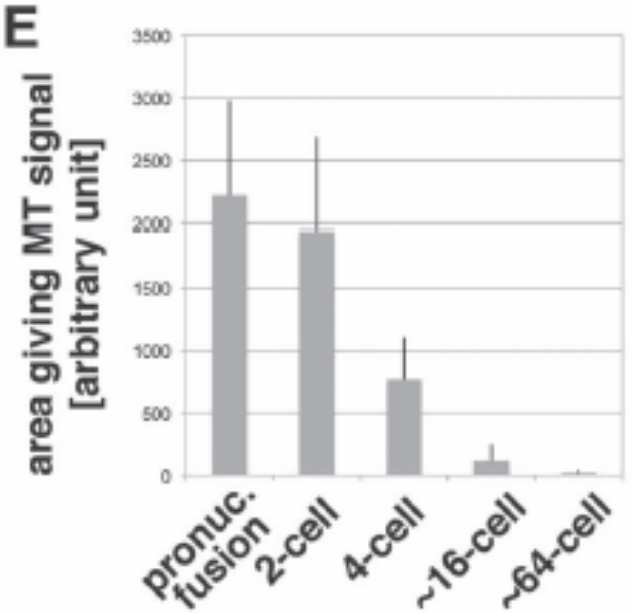
Multiple mechanism to ensure uniparental inheritance of mtDNA



Chan & Schon (2012) Dev Cell

Loss of paternal mitochondria in nematode embryos

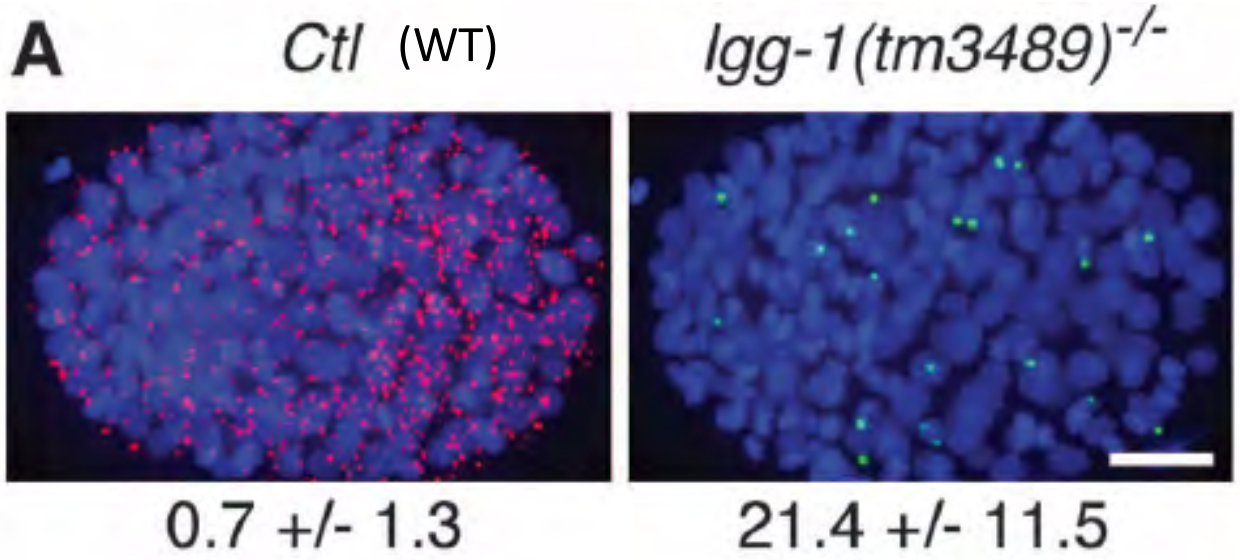




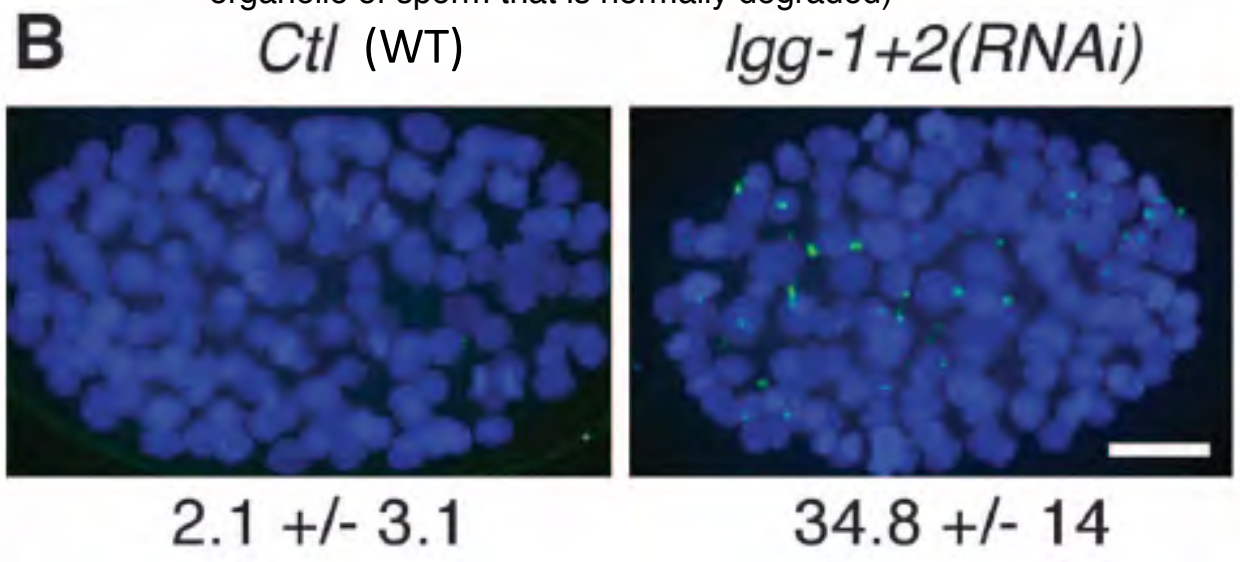
Hermaphrodites were mated to males labeled with MitoTracker Red. Embryos stained with DAPI (DNA). Red signal outside embryo (outlined) are free sperm.

Sato & Sato (2011) Science

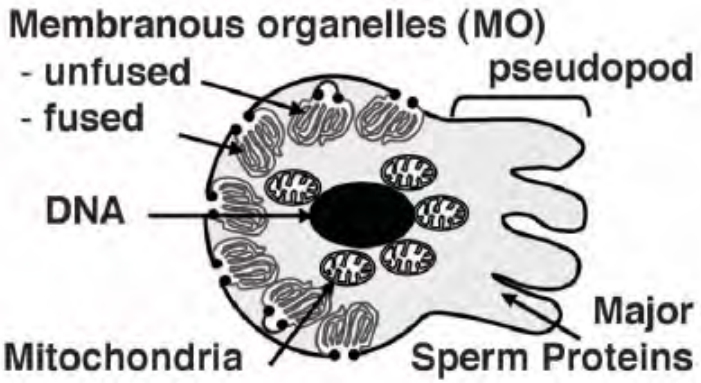
Autophagy necessary for removal of paternal sperm mitochondria in nematode embryos

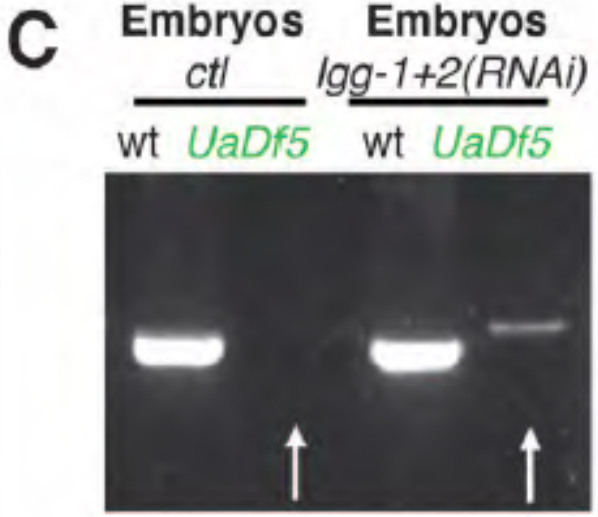


red=lgg-1 (LC3 homolog); green=MO, membranous organelle (an organelle of sperm that is normally degraded)



green=MitoTracker Green; 100/120 cell stage embryo

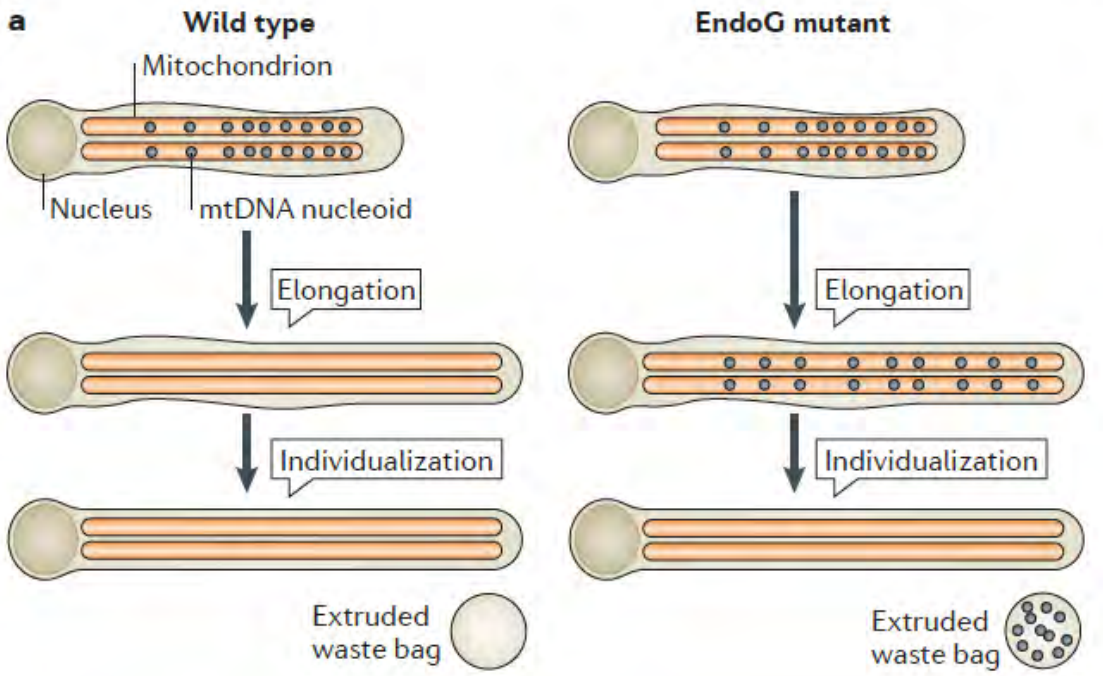




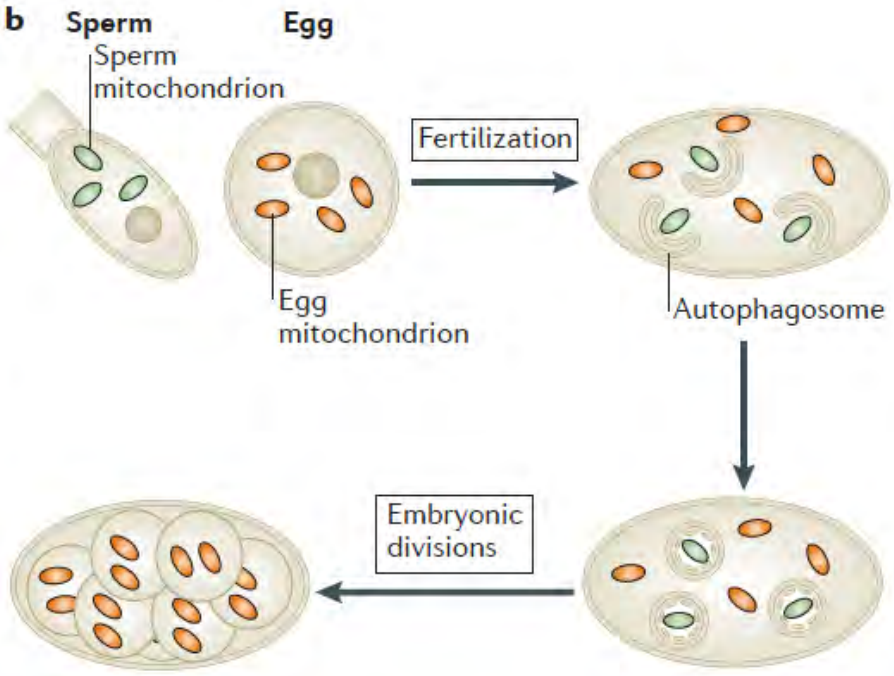
sperm carrying wt and UaDf5 mtDNA mated with hermaphrodites; UaDf5 mtDNA persists only in hermaphrodites lacking lgg1/2

Al Rawi et al (2011) Science

Pre- and post-fertilization mechanisms for paternal mtDNA degradation



Pre-fertilization mtDNA degradation in *D. melanogaster*



Post-fertilization mitophagy in *C. elegans*

Mishra & Chan (2014) Nat Rev Mol Cell Biol