ANAT 365

Cellular Trafficking

NTC Set **5**

Lectures **13** to **15**

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ANAT 365
Lecture 13 – Mitochondrial dynamics

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCs to us through e-mail: macss.academic@gmail.com

Announcements:
- MACSS Logo contest:
  - Submit designs to it.macss@gmail.com by Oct. 8 at 8pm to win Free NTC's
- MACSS Lab Recruitment Luncheon
  - Free lunch and research presentations Oct. 5 in 2/36 at 11:30am
- MACSS Wine and Cheese
  - Oct. 13 11am in Anatomy Reading Room

What's going on with the paper?
- Look for your number on the document posted in myCourses
- Papers listed are a starting point
- Read more! (And include these in your bibliography.)

Yoshinori Ohsumi won the Nobel Prize for Autophagy TODAY!!!!
- Last year he won the Gairdner Foundation International Award – the Canadian version/unofficial precursor to Nobel Prize
- First individual recipient of the Nobel Prize in Physiology or Medicine in a long time.
- Significant because he did most of his research alone and was super ahead of his time.
- Ohsumi's discoveries led to a new paradigm in our understanding of how the cell recycles its content. His discoveries opened the path to understanding the fundamental importance of autophagy in many physiological processes, such as in the adaptation to starvation or response to infection. Mutations in autophagy genes can cause disease, and the autophagic process is involved in several conditions including cancer and neurological disease.
- This is Wednesday's lecture topic

Mitochondrial Dynamics.
Already know: Mitochondria is the powerhouse of the cell!!! (Thank you, high school biology.)
Now we will learn the cell biology of the mitochondria.
- It is very important to for the generation of ATP.
- ~1000 reactions occur inside
- Any cell signaling decision also tells the mitochondria what to do
- It is the recipient of every cell signal (highly regulated).
- It has many tissue-specific functions.
- Mitochondria make fatty acids, lipids, steroids, heat, define cell fate (particularly cell death), calcium homeostasis, and citric acid cycle

- Mutations/errors cause aging? (Controversial)
- V important in living breathing creatures

Cyanobacteria: Origin of mitochondria and chloroplasts.

- Mitochondria were trapped inside host cell to create multicellularity.
- Host/parasite symbiosis occurred about 2100 million years ago.
- They lost most of their own DNA because they are trapped in host cells (entrapment).
- We know from their DNA that they are bacterial because many sequences are highly conserved, and the way they make proteins proteins/do other things is a bacterial system.
- Mitochondria still have 16kB of their own circular DNA in the matrix (in humans).
- Different mutations known to cause human diseases.

Mitochondrial DNA encodes:

- 13 proteins – all of which are central to the oxidative phosphorylation cycle for respiration
- 22 tRNA – encoded on circular DNA, needed in order to make these proteins
- 2 rRNA - encoded on circular DNA, needed in order to make these proteins
- NO INTRONS in humans; there are still some in yeast.

Other ~98% of 1500 proteins found in mitochondria encoded in nucleus and imported post-translationally.

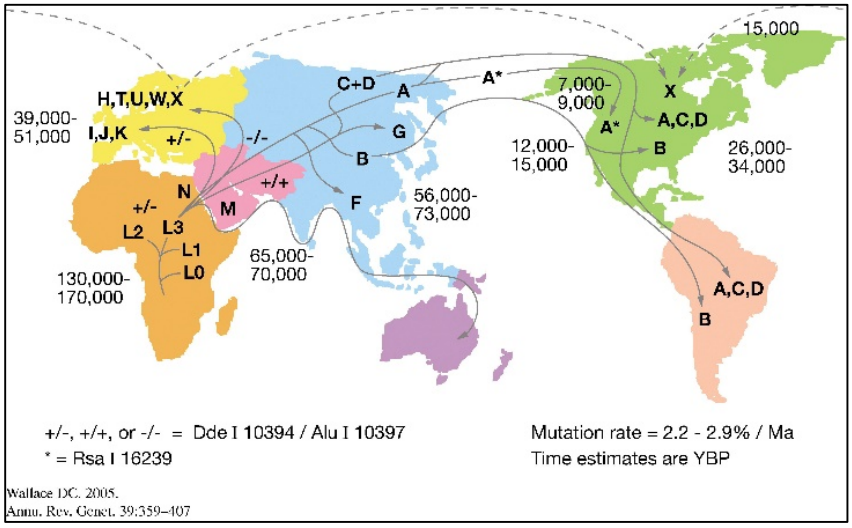
Inheritance of mitochondrial mutations is NOT Mendelian genetics.

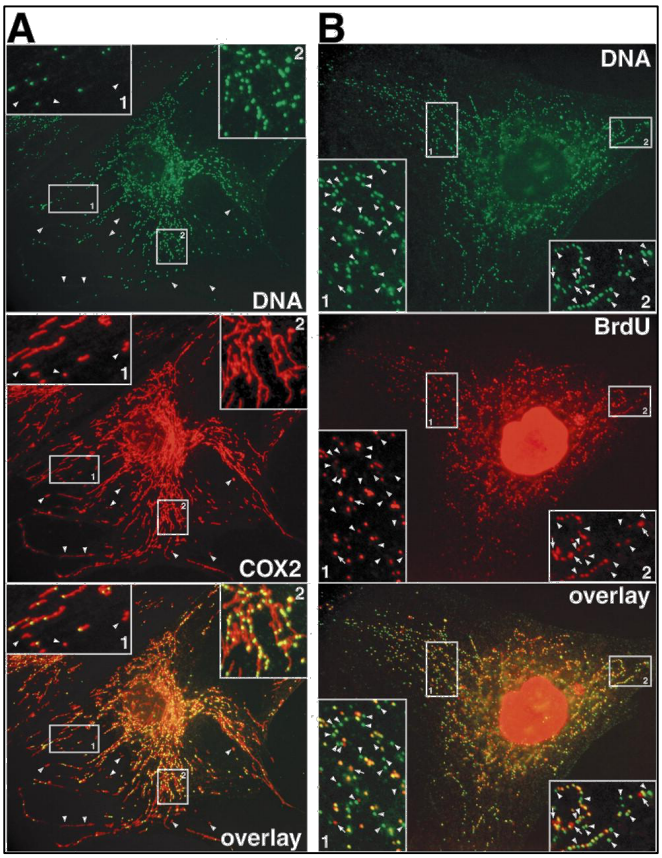
- Each mitochondria has 10-100 copies of this plasmid DNA.
- Therefore, each cell has 1000-10000 copies of these little genomes.

If one plasmid DNA makes an error, there will be thousands of other copies that make the right protein. Thus, the organism won't feel error until it is at least 70% heteroplasmy for a mutant genome.
Mitochondrial disease are difficult to trace because they can arise over time in somatic tissues, which is probably why people think it's related to ageing.
Mutations = disease
Diseases are dose dependent, not Mendelian.

Sequence variation in mtDNA

- Allow us to map human migration patterns from where humans began (Africa).
- Changes made could be 1 amino acid, silent, or correlate with functional changes in mitochondria.
- These changes are thought to coincide with diet and weather restrictions.
- They may predispose ethnic populations to diabetes, cancer, etc.





Figures A and B) Staining for mitochondrial DNA with mitochondrial protein marker.

Static kidney bean structure of mitochondria is completely wrong.

DNA plasmids make mitochondria appear very spotty because of their

Mitochondrial membranes are long and filamentous looking.

COX2 is labelling a component of the mitochondrial electron transport chain. COX2 is staining the electron transport chains on the inner membrane of the mitochondria, in this image you are just labeling the whole thing because the inner and outer membranes are very close.

Outer membrane: tight
Inner membrane: have cristae

If you overlay these two images then you can see DNA spots sitting on the membranes.

"Mitochondria are very long and beautiful."

Size and shape can vary with cell type:

- Muscle – quite small, fibroblasts
- Other cell types – up to 3 microns long.

The wavelength of light from the fluorophore that is conjugated to this tag is approximately 400 nm.

- Regular wide field microscopy image is fuzzy
- You can see it in better detail with electron microscopy.
- Superresolution microscopy is changing this because we can now look at individual compartments within mitochondria.

mtDNA is assembled in nucleoids.

DNA seen here is packaged - not just one plasmid DNA. The nucleoid proteome is published.

- Mitochondrial DNA are packaged similarly to cellular DNA in the nucleus except with different molecules.
- This glob of DNA and protein is anchored to the inner face of the IMM.
- Mechanisms to ensure DNA replication and segregation is still unknown.

Regulation of mtDNA replication is unknown

- No correlation between a tissues oxidative capacity and mtDNA copy number.

- No correlation between proliferation of mitochondria and mtDNA copy number.
- This means the mass/bulk of the mitochondria can grow, but mitochondrial genome number can stay the same.
- It is unclear how this actually works.

Transcriptional regulation of those 13 mitochondrial proteins is also unknown.

- The transcriptional regulation is thought to be pretty much all the time, which means we don't know what it is.
- Thought to be strictly regulated by expression of the nuclear encoded factors via PGC-1, etc. But no direct links.
- This is interesting because we've known about mitochondria for a long time, there have been Nobel prizes given about it, but some of the basic features about ow it works – especially the basic genetics – are still widely unknown.

If you have mitochondrial dysfunction, then the mitochondrial electron transport chain will spew off reactive oxidative species that will damage all kinds of things.

So if you have dysfunctional mitochondria, respiration is inefficient and you have increased Ras. Ras is also a good thing because it is a signaling mechanism for telling the nucleus to fix the mitochondria, but if you can't fix it because the genes are mutated in core components of the electron transport chain then you basically start to wear down and respiration becomes more inefficient.

There are 4 main complexes for oxidative phosphorylation.
Complex 5 (beautiful turbine) is the complex that takes the protons across the gradient to make ATP. It has many components - around 40 proteins and 2 might come from the mitochondrial genome. It's a very interesting question how you assemble these machines. You have stuff coming from the cytoplasm in linked with the assembly of the stuff coming from the mitochondria. Mutations in those 13 genes will create problems in individual components of the electron transport chain.

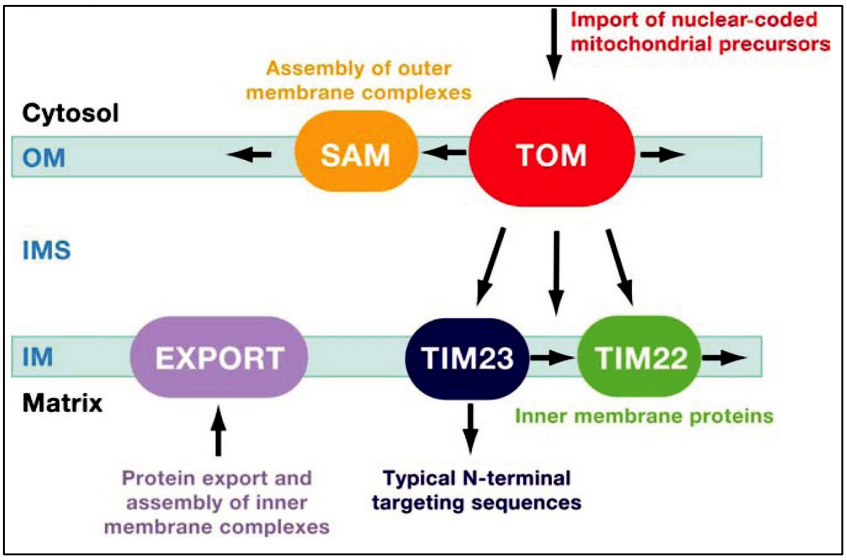
About 1500 proteins are encoded in the nuclear genome and post-translationally imported into the mitochondria.

- How these genes entered the nucleus is the subject of speculation.
- Many are obviously new additions to the mitochondrial proteome.
- Any new mitochondrial gene translocation would become a pseudogene since the Genetic code has drifted and they speak different languages

How do nuclear-encoded proteins get into the mitochondria?
Mitochondrial import Summary scheme.

- 99% of mitochondrial proteins are encoded in the nucleus and imported post-translationally.
- There are mechanisms that translocate polypeptide chains across bilayers. In mitochondria, we call them TIM and TOM.
- Topology of the protein will allow it to be recognized; not sequence-specific.
    - Positively charged helices are mitochondrial signals.
    - Amphipathic alpha helices also are mitochondrial signals

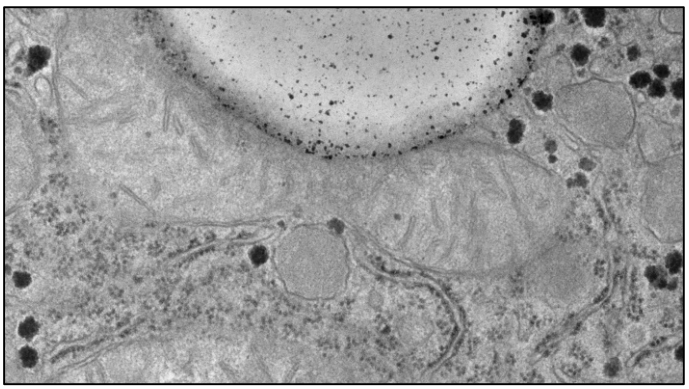
- Newly synthesized proteins are recognized by receptors on TOM complex, translocate across, and recognize the TIM complexes.
- TIM complexes will further translocate stuff across the matrix for assembly.
- Membrane-anchored proteins will go laterally.
- In both membranes there are specific kinds of machinery that will assemble beta barrels.
- This is the result of a huge amount of work that was done mostly in the 80's and 90's although there are a lot of interesting features still being discovered/added on today.



Mitochondrial research is "far behind" because they are very well-behaved by themselves.

- Biochemistry outlining respiration, Kreb's cycle, respiration, etc. was performed in a test tube over a period of 50-60 years, winning a few Nobel prizes.
- If you take mitochondria out of a liver or heart, put it in a test tube, and ask it to respire/break down fatty acids/etc. on its own, it will do it perfectly well.
- This led everyone to believe it didn't need anything else (own little factor) – Wrong.
- Concluded that mitochondria activity depended on metabolite concentrations. No more to discover, story complete…….

Adjacent figure) Electron microscope image of liver. The mitochondria is not by itself. The large round structure is a lipid droplet. The stringy structure with lumps is the endoplasmic reticulum. The small centrally-located structure is a peroxisome. Mitochondria are certainly not sitting by themselves.



When Dr. McBride finished her Ph.D. on mitochondrial import a lot of the mitochondria labs closed because they thought mitochondria research was complete. She couldn't do a postdoc in cellular biology on mitochondria because there were no labs working on mitochondria - unfunded, shut down. That's why she did her postdoc in vesicle trafficking so she could understand how membranes move with intent to come back to mitochondria. When she came back to mitochondria they realized they were very dynamic.

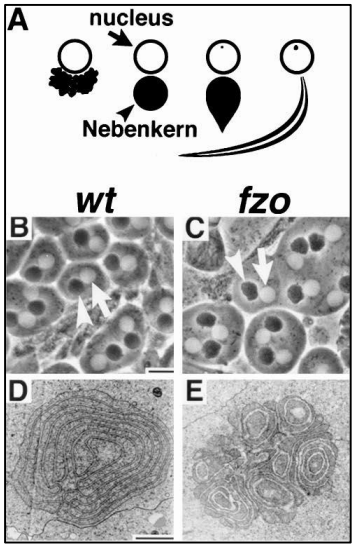
- Slide from Dr. McBride's lab.(Please see course slides.)
- It was discovered that mitochondria are highly dynamic. Morphology is regulated by the antagonistic forces of fission and fusion.

The center image is a single class 7 cell with the mitochondria labelled (bright spots).

- If you block fission, they will all fuse together into an interconnected tubular mass.
- If you block fusion, they will become hugely fragmented.
- Thus, they are not just "sitting there"; they have a means of "communication".

Mitochondrial dynamics in a single cultured COS7 cell .

- One image every 2 seconds, 100 frames
- YFP was targeted to the matrix compartment of the mitochondria
- Division event, Mitochondria is moving along cytoskeletal tracks, Fusion event.
- All the proteins imported from cytoplasm… No reason for them to do this?? (That we know of.)
- These things *should* all be identical yet they do this.
- Opportunity for further research: What are they doing? Why are they doing this? How did we miss this for 50 years?



How was mitochondrial fusion discovered?

Margaret Fuller: A genetic screen in fruit flies searching for genes leading to male infertility (1997). Came across a protein called Fuzzy onion (Fzo)

- Wild type of mitochondria: **All fuse** into one long mitochondria along the sperm tail.
  - Within the sperm tail, the mitochondria form a regular pattern along the shaft. After fertilization, these mitochondria cannot enter the egg (hence, maternal inheritance of mtDNA).
- Fzo mutant mitochondria: **No mitochondria fuse**. The sperm could no longer swim, leading to infertility
  - Loss of a new mitochondrial membrane-anchored GTPase they called Fzo led to an alteration in mitochondrial morphology within the sperm tail.
- This was the first study to show that mitochondria ever fused. But who cares about a sperm tail of a fly? Is this limited to the sperm tail?

Fzo is conserved in yeast (1998). So is mitochondrial fusion.

1997-98: This is while Dr. McBride is doing her postdoc and had run away from dynamics. Dr. Fuller had no idea about mitochondria because she was looking for infertility in flies. Dr. McBride was worrying about GTPases, Rab5, fusion, etc. When she read these papers she said, "Ahhh!! I have to get back (to mitochondria)!!"

Figure) A yeast mating assay mixes yeast containing red vs. green mitochondria. .(Please see course slides.)

If one yeast has green mitochondria and the other has red mitochondria, then they will normally fuse.

- Incubation with Fzo1 expressed leads to perfect colocalization/fusion of the mitochondria within the heterokaryon.

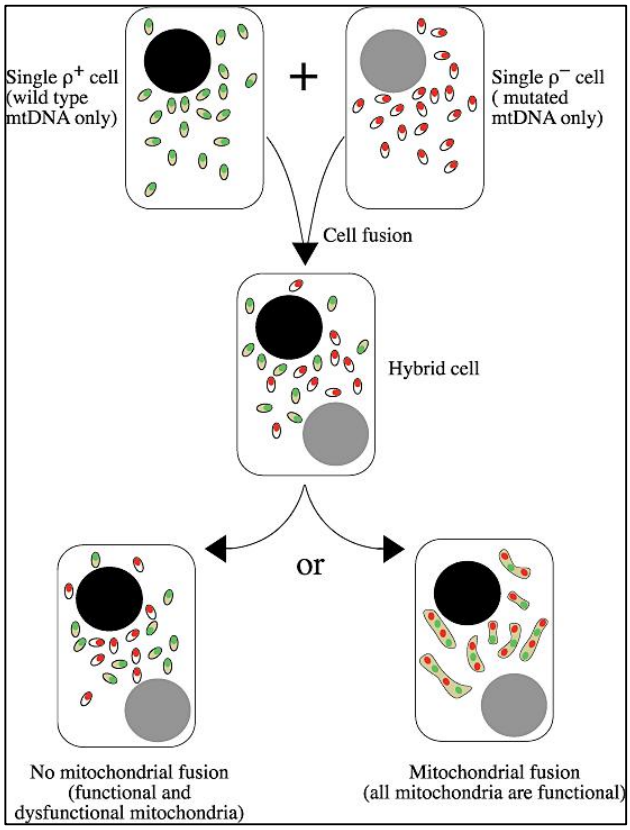
If you put a temperature-sensitive mutation on Fzo1 (Fzo gene orthologue/conserved gene), they stay separated.

- Incubation at temperatures that lead to the loss of Fzo1 (an Fzo1 -temperature-sensitive mutant strain) led to fragmented mitochondria that could not mix their contents.

We still don't know why they fuse at all.

In 2001 it was shown in mouse models that mitochondria fused in vivo.

- Genetic experiments in mammalian organisms show that mitochondria fuse in mammalian tissue as well.
- If you have a single mitochondria sitting by itself in a cell and it starts getting mutations in its genome, people originally though they fused to bring the good genes to the damaged one and rescue the mitochondria through complementation.
- Japanese scientists checked this: 2 different cell lines that had different specific mutations in mitochondria DNA, so that only if the cells fuse could they make a functional electron transport chain.



- Generated animals (mice) from heterokaryons expressing distinct mtDNA mutations.
- Only upon mitochondrial fusion and mixing of the matrices would the complete complement of mitochondrial encoded proteins be expressed to build the proper electron transport chain complexes.
- This occurred in all tissues examined. Every tissue rescued.
- These data confirm that fusion occurs, but didn't really confirm how often, or when.
- Still had to find all the machinery in mammalian systems (Fzo orthologues)….

Fusion requires membrane-anchored GTPases.

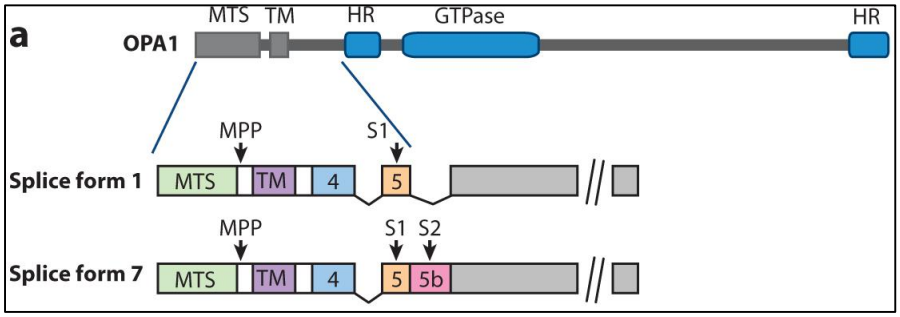
- Gene duplication in the Fzo gene: Mitofusin 1 and Mitofusin 2 are two genes (60% similar) in the outer membrane. Yeast have only **Fzo1**, the functional orthologue. Mfn2 is mutated in Charcott Marie Tooth Type 2A.
- **Mitofusins 1 and 2** each have a GTPase domain, HR1 peptide repeat (coiled coil domain), 2 TM (transmembrane) domains* that anchors them in the outer membrane, and HR2 another coiled coil.
- **Dr. McBride about to publish a paper that says there's only 1 TM domain.*
- Yeast also has **Mgm1** - another GTPase found in genetic screens (similar to Ohsumi's).

Humans have Opa1 (autosomal dominant optical atrophy) – another GTPase anchored at the N-terminus onto the inner mitochondrial membrane and that has coiled coil GTPase, and another coiled coil.

- If you knock any of these domains out in mammalian cells, the DNA are totally fragmented.
- If you knock any of these out in mice, then it is embryonically lethal.
- Mitochondria have to be able to fuse. (We still don't know why.)
- OPA1 is mutated in patients with a certain kind of blindness.
- Remember: mutations are not null – still part function in these genes.

- TM domains are thought to form antiparallel interactions, perhaps like the SNAREs.
- Known: GTP hydrolysis is required to drive fusion.
- Stress-induced disulfide bonds may break the coiled-coils, "priming" the mitofusins to bind in "trans" and drive fusion.
- Since these are large proteins, they are commonly considered to be like dynamins, and are sometimes all called **DRPs** (dynamin related proteins). I'm not so sure for Mitofusin 2 whose rates of GTP hydrolysis are very slow, more like Rabs…

Figure A) Opa1 is a larger protein found on the inner membrane with an MTS (mitochondrial targeting signal) and a TM domain (stop transfer transmembrane domain).



Opa1 has 8 splice variants!

- Mostly between exons 4 & 5.
- Nobody has done any work to understand the differences between splice variants.
- Opa1 is mutated in autosomal dominant optic atrophy (a form of late-onset blindness).
- Opa1 in the inner membrane has many variants, leading to changes near the N-terminal. Not clear why yet.
- Newest data shows that the longest, membrane anchored forms are required for fusion.

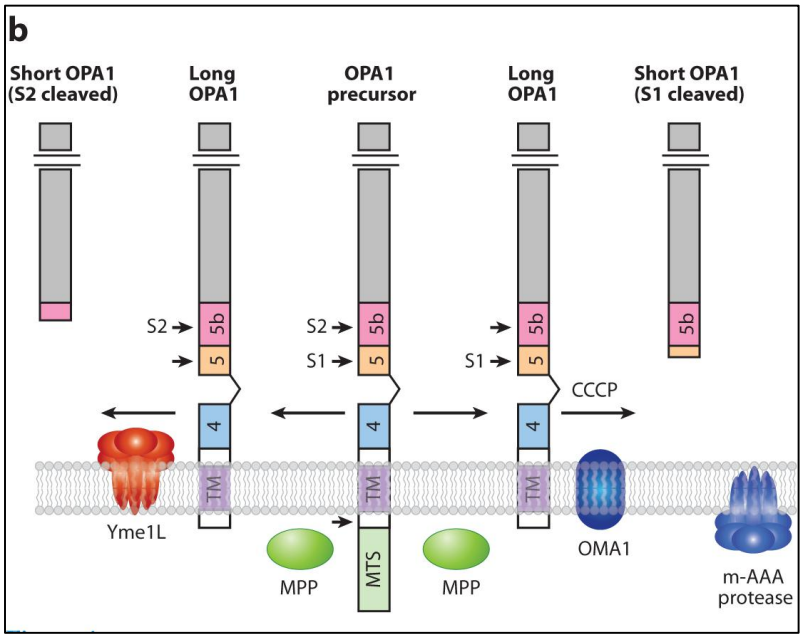


Figure B) Sequence:
Precursor comes in. Initial cleavage which always happens via the matrix metalloproteases that clip off the MTS (common for almost all mitochondrial imported proteins post-import). Left with little stub at TM domain. Different proteases then cleave at S1 and S2 sites.
There are a number of **regulated cleavage events that** allow it to be released into the inner membrane space.

- Constitutive (S2 site, Yme1)

- Induced (S1 site, Oma1)

In steady state:

- **Yme1** creates a mixture of short and long Opa1.

When mitochondrial electrochemical potential is lost aka they're not respiring anymore (addition of CCCP in lab):

- **Oma1** (another protease) becomes activated and cleaves all variants of Opa1 at S1 to the short form, soluble in the intermembrane space.
- No fusion occurs.
- This short form localizes to sites of mitochondrial division, promoting fragmentation. This makes them small so they can be eaten by the autophagic process.

- If a single mitochondria is totally messed and can't work at all, it will be excommunicated from the reticulum. It can't fuse back in, and it will be turned over by degradation by mitophagy.
- The cleavage of Opa1 is responsive to the function of the mitochondria to control when they will fuse and who's allowed to be part of the reticulum.

Inner membrane cristae are highly dynamic. Opa1 also regulates cristae assembly/ fusion.

- We have no idea how it mediates bilayer mixing. All we know is that if you knock it out, you don't have it.
- Inner mitochondrial membrane (IMM) is highly invaginated because that's where the electron transport chain (ETC) sits.

Please see course slides:

Figure A) The ETC forms **supercomplexes** in the IMM.

Figure B) The assembly at the neck (**cristae junction**) is highly complicated.

- A big complex called **MICOS** is required to anchor the cristae.
- Opa1 forms dynamin-like oligomers and is require to keep the cristae junction tight.
- Opa1 also involved in regulating dynamic cristae.

Figure C) Mitochondria control cell death (apoptosis).

- Opa1 oligomers are broken.
- Releasing **cytochrome c**, which is normally stuck up in the ETC, exits through pro-apoptotic **Bax/Bak** pores that are formed specifically during death.
- Cytosolic cytochrome c drives assembly of the cellular death machine, the **apoptosome**.

We don't know how cristae remodeling and dynamics is altered during mitochondrial fusion.

Drp1 mediate mitochondrial fission.

- Drp1 is essential or mitochondrial division, so if you don't have Drp1, then the mitochondria are wickedly hyperfused.
- Steady state: Mitochondria always fusing and dividing.

Drp1 is heavily modified by post-translational modifications, coupling fission with cell signaling.

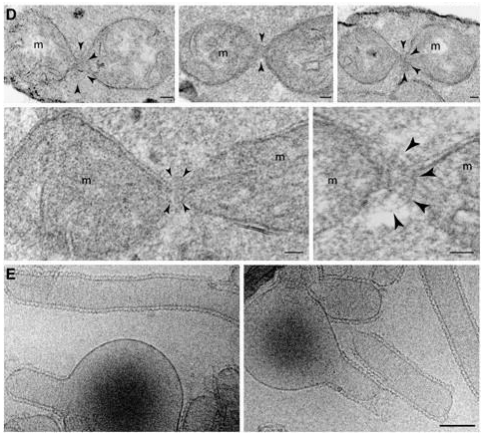
- Mitochondria are not dividing just for biogenesis.
- Mitochondria divide in response to signals (Ca, apoptosis, ubiquitination, etc.)

- Drp1 is a cytosolic protein that comes on and off the membrane, assembles into oligomeric rings (crystal structure to be published soon),

- **Interestingly:** Drp1 has no PH domain like other dynamins (ie; currently no PIP involvement – we have paper in revision….). Initial recruitment to the membrane unclear for a long time.
- Instead there are receptors. Mff is a membrane anchored adaptor for Drp1 recruitment. Fis1 and the MIDs support DRP1 activity, but function of regulation/recruitment still unclear. Mff was shown to be phosphorylated by AMP kinase, which is sensitive to nutrient status.

Unlike other dynamins, Drp1 is a different size.

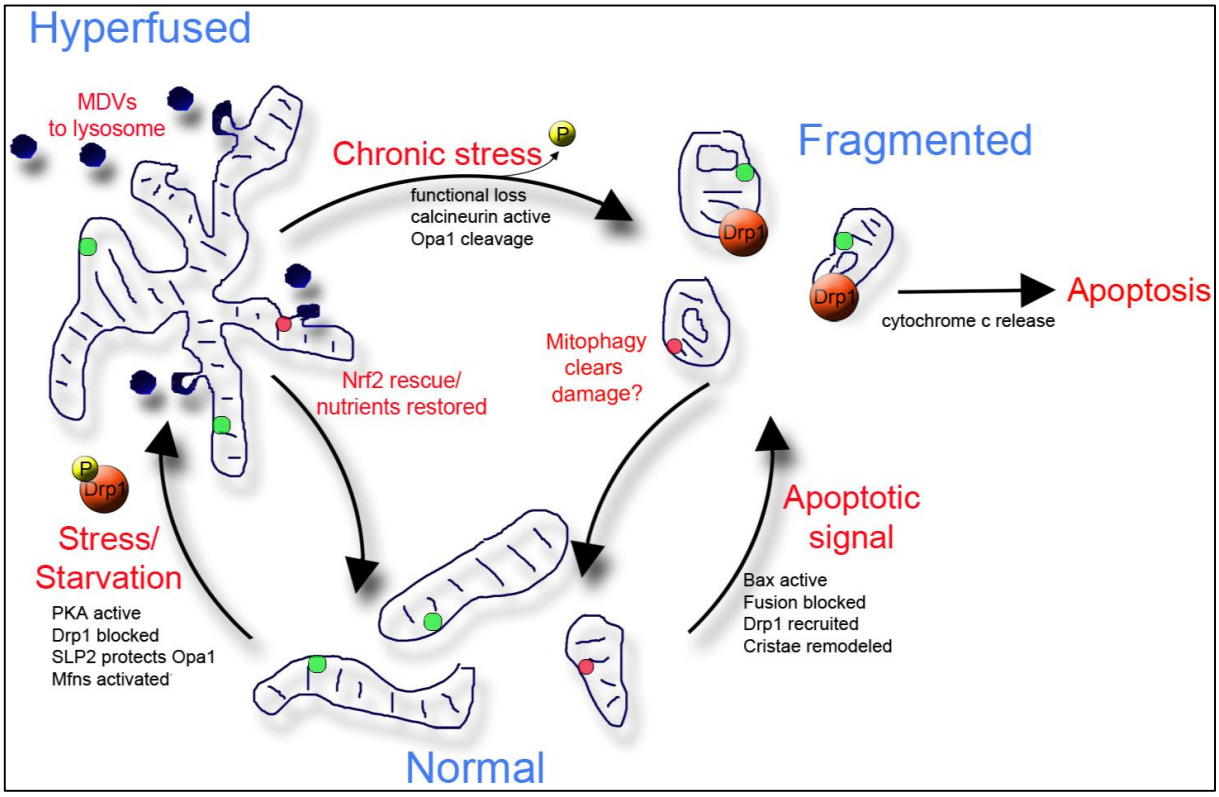
Cell surface Dynamin spirals are ~20nm. Clathrin coated vesicles ~100nm and come to a narrow neck of ~20nm.



Mitochondria are *big, huge beasts* that need to be shrunken down small enough to be divided. They can be ~200-500nm across.
Figure E) Dnm1 (yeast Drp1) spirals in vitro in liposomes are ~120nm.
Figure D) Necks of mitochondria under electron microscope. They can be 120nm, but can go smaller.

- How to get from 500 to 120? To get Drp1 there must already be sculpting event occurring.
- If Drp1 is so large, how to get from 120 to 0? Something needs to come do the final pinching.
- What happens on the IMM? What are the fission GTPase on the IMM? Cleaved Opa? Unclear.

Overall, mitochondrial plasticity is critical during cellular stress. = *"protect the collective"*

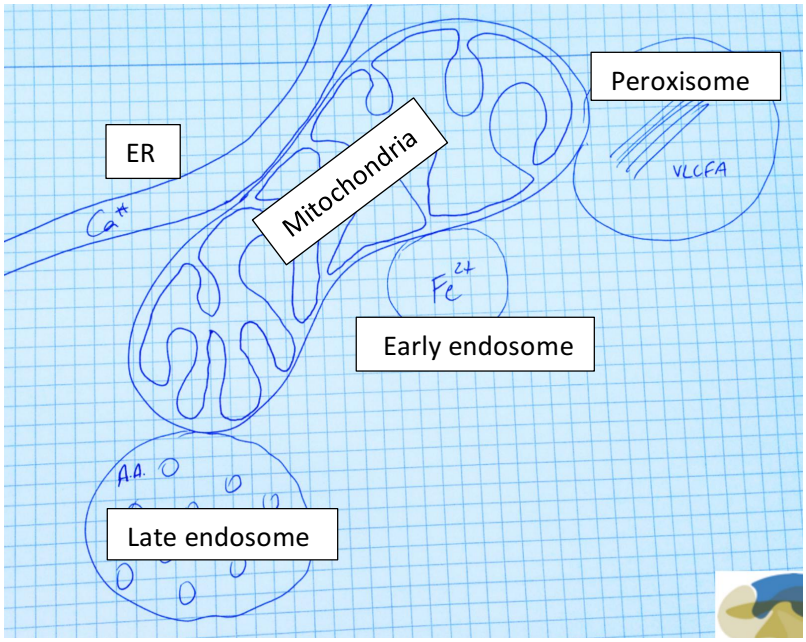


- Start with **normal** mitochondria with good DNA.
- Under stress or starvation, mitochondria **hyperfuse** into huge interconnected network.
    - Some machinery known: stress-induced protein kinases to phosphorylate Drp1 to stop it from coming onto the membrane, Mitofusins activated by Ras.
    - Vesicles bud and carry away selective cargo.
    - They can't drive cell death. They can't drive cytochrome c release.
    - They can't be eaten by autophagy. Too big to be engulfed by autophagosome.
- Under normal stress conditions this is reversible.
    - If this is just part of the feed/fast cycle then you'll get nutrients and cells will return to normal.
    - If this is a "Ras thing" the chromosomes will start to express stress response genes that will fix everything.
- Under chronic stress,
    - Functional loss of mitochondria.
    - Opa1 will be cleaved, and the mitochondria will be fragmented.
- Fragmented mitochondria
    - Ripe for apoptosis.
    - Can be selectively engulfed by mitophagy.
- Mitochondria with a death signal goes directly from the normal state to fragmented state.
    - Drp1 is recruited. Cristae remodeled. Directly to apoptosis.

So far: Homotypic mitochondrial actions. Showed electron micrograph video of mitochondria going through black space. No black space in reality. Black space is full of other stuff.

Dr. McBride thinks the motivation of mitochondrial dynamics has got to be metabolic. What is the "Cell Biology" behind metabolic flux?

The field is exploding with research into intraorganellar contacts with mitochondria.



- **Endoplasmic reticulum** contact is a large focus because it's the oldest thing we know a lot about. ER/mitochondria connection is important for Ca and lipid flux.
- Direct contact with **peroxisome**, which is breaking down very long chain fatty acids and sending them back to the mitochondria to burn the rest.
- Direct contact with the **early endosome**. Transferrin brings in iron to the cell. All the iron must go to the mitochondria.
    - JCB paper this month that discussed early endosome/mitochondria iron flux.

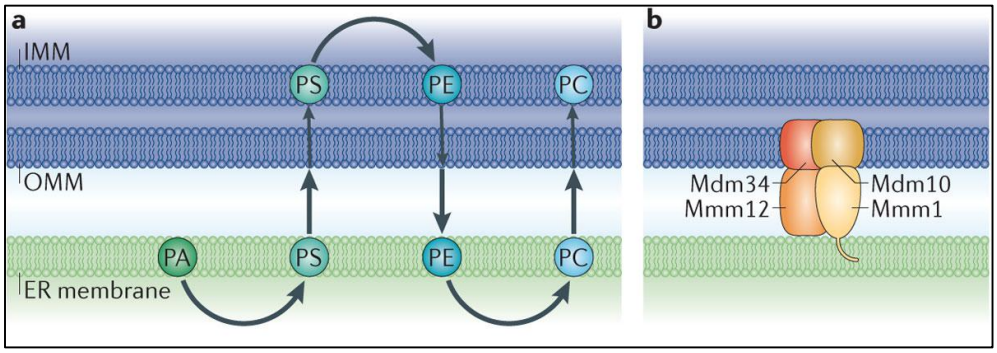
- Most cell biology things will leave you at the **lysosome**. But, in fact, the cargo coming in (e.g. lipids to be degraded) are all happening at the mitochondria. These contacts are much more direct for metabolic flux.
- The **late endosome** contact and **multi-vesicular bodies** are also emerging a lot fr amino acid metabolism and other things.
    - Last year, 2 groups just identified tether that will tether late endosome to mitochondria.

Mitochondria are in intimate contact with other intracellular organelles.

Video shown that illustrated:

- Contacts with ER.
- Contacts with early endosome.
- Molecular contacts with late endosome just described in yeast within the last years.
- "Feeding off the trough"
- Not random event, but nothing know about how/why this happens.

ER-Mitochondrial contacts are essential for lipid biosynthesis.



- Mitochondria is critical for making PE (phosphatidyl-ethanolamine).
- PE is not a PIP; it's just a regular phospholipid in the bilayer.
- In the ER membrane, the lipid phosphatidylserine is transported through the mitochondrial outer membrane into the mitochondrial inner membrane to be decarboxylated to phosphatidylethanolamine.
- Phosphatidylethanolamine is transported back to the ER membrane and used to make phosphatidylcholine, which is transported throughout the cell, including back into the mitochondria.
- These organelles are totally linked for the synthesis of lipids, which are used by the whole cell.
- The lipid flux between the organelles requires protein machineries to generate the contact site, here called the ERMES complex (ER/ Mitochondria encounter structure). These contact sites were first found in yeast 4 years ago. The mitochondrial morphology mutant proteins are not really conserved in humans and mammals, so Will Prince (first paper author) identified a mammalian complex that regulates lipid flux.

ER/Mitochondrial contacts are essential for calcium flux and buffering.

- Calcium exits the ER channel in a regulated manner of **inositol receptors** (addition of caffeine, or upon neuronal depolarization, for example).
- This calcium then crosses the open **VDAC channel** (voltage-dependent ion channel) on the OMM that allows all metabolites to cross the mitochondrial outer membrane.

  - 30% of OMM surface is VDAC pores (called porin in yeast).
  - Metabolites cross easily, while IMM is much more impermeable, needs more transporters.
- Tethers come forward (e.g. Mitofusin 2).
- The affinity of **MCU** (mitochondrial calcium uniporter) on the IMM is REALLY low, so contact sites are critical to keep the ion highly concentrated.
  - Need 1 microM of Ca to get in; cytoplasm never has this.
  - Contact sites are very important to keep concentration high.
  - *Although, people knew mitochondria buffered calcium for 60 years, MCU only identified about 3 years ago.*
- The identity of the tethering machinery is still being established.

Yeast high pressure frozen EM tomography reveals examples of ER encircling the mitos.

Figure A) Tomographic reconstructions, modeled here to see mitochondria (dark) and ER (light). (Please see course slides.)

- When mitochondria divide, they always divide at site where ER was in contact.
- By cryoelectron microscopy, we can see a site of division exact where the ER is wrapping around it.
- Within 2 cells, they quantified the types of contacts (less than 30nm), as seen in the circles.

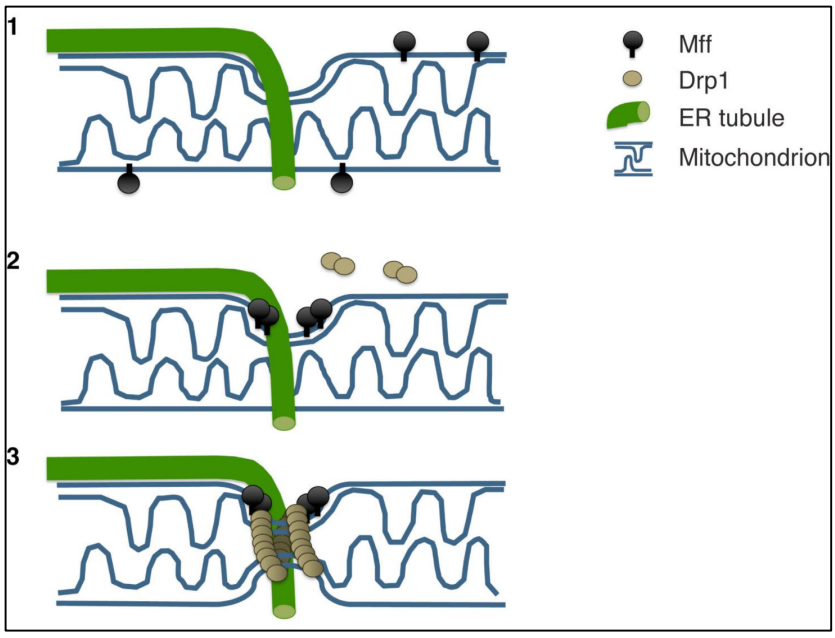
The more the ER wraps mitochondria, the more constricted the mitochondria become (to ~140nm).

- This is almost to the point where Drp1 could come in to constrict mitochondria.

Unproven, but idea is that the ER is needed to contact the mitochondria and perform some sort of lipid flipping/fluxing to constrict it. Calcium pulsing possible.

The same is true in mammalian cells.

- **Figures A, B, C, D)** Labeled mitochondria and ER with different fluorophores and image in 3D over time. This shows 4 examples of ER at the site of mitochondrial fission. Another example of intraorganellar contacts playing critical roles in the morphology of each other. *Same thing shown for endosome division.* (Please see course slides- for color)
- Unknown machinery initiates the ER contacts for fission. But ER contact responsible for early constriction, recruiting other things.

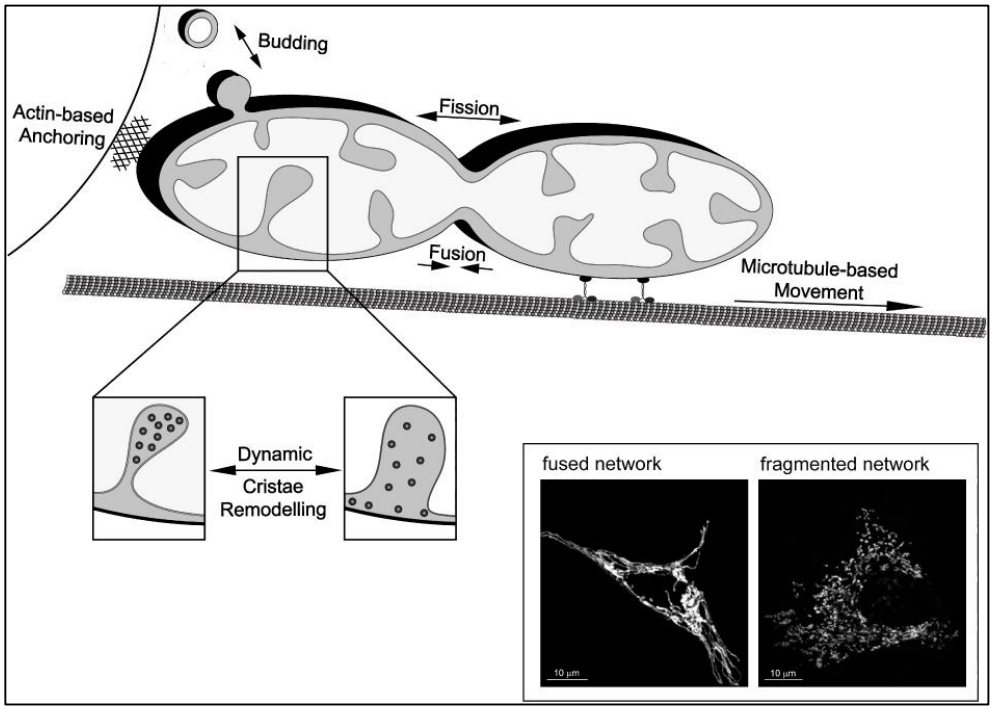


- Mff all over surface but enriched at ER contact site. Mff is required for Drp1 assembly, but not for the ER contacts.
- Drp1 assembles at the site of ER contact.
- New study in yeast showed mtDNA nucleoids also sit just beside division sites - likely some mechanism to ensure both new mitochondria contain mtDNA. (Just observation.)

Genetic screens in yeast identified proteins required to maintain mitochondrial shape.

- These mutants were named "**mdm**" for mitochondrial distribution and morphology.
- The last decade has seen many labs attempt to uncover the function of these proteins, and to characterize potential human orthologues of these proteins.

Figure) Image from Dr. McBride's lab summarizing today's lecture.



Current Summary of Dynamic Mitochondrial Processes.

- Interactions with cytoskeleton position the organelle: motors that bind it to microtubules.
- Fission is required to maintain mitochondrial numbers, and is important in cell death (apoptosis).
- Fusion protects against cell death and being eaten by autophagy (next lecture), is a stress response.
- Cristae remodeling is highly regulated at very dynamic. Constriction can help to concentrate metabolites and drive respiration. Opening cause respiration to be more relaxed. There is overlap in the machineries for fusion and cristae assembly.
- Mitochondria can also bud small vesicles that carry selected cargo. We discovered this new aspect
- Not always clear how morphology and inter-organellar contacts are linked to the multitude of mitochondrial functions (ATP synthesis, TCA cycle, amino acid production, steroid production, lipid generation, etc., etc., etc.)

ANAT 365
Lecture 14 – Autophagy
Dr. Heidi McBride – October 5, 2016

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text contained in the lecture slides will be reproduced here.
Please send any comments or questions about NTCS to us through e-mail: macss.academic@gmail.com

Announcements:
- Clothing orders are due Oct 10 (you can find the catalog and order form on the new MACSS website: macssmcgill.github.io/services.html)
- Submit a new logo for MACSS on Oct. 8 by 8pm
- NTCs are available for this course and other anatomy courses (but you already knew that)

Autophagy

- For the midterm, Dr. McBride said she is not one too excessive on full protein names (more about concepts)
- Autophagy means Self-eating in Greek
- Three kinds of autophagy
  - **Macroautophagy** – subject of today's lecture
    - Membranes will wrap around cellular compartments and large protein aggregations to be targeted for lysosome degradation
    - Can get lipid droplets, mitochondria, ribosomes and protein aggregation degradation
  - Microautophagy
    - The smaller contents from cytoplasm can be internalized
  - Chaperone-mediated autophagy
    - Some proteins are unfolded and translocated across channels to go into lysosome on protein by protein basis
- Autophagy ensures the removal of damaged cellular content
- Autophagy recycles cellular components, amino acids, lipids, ions, etc.

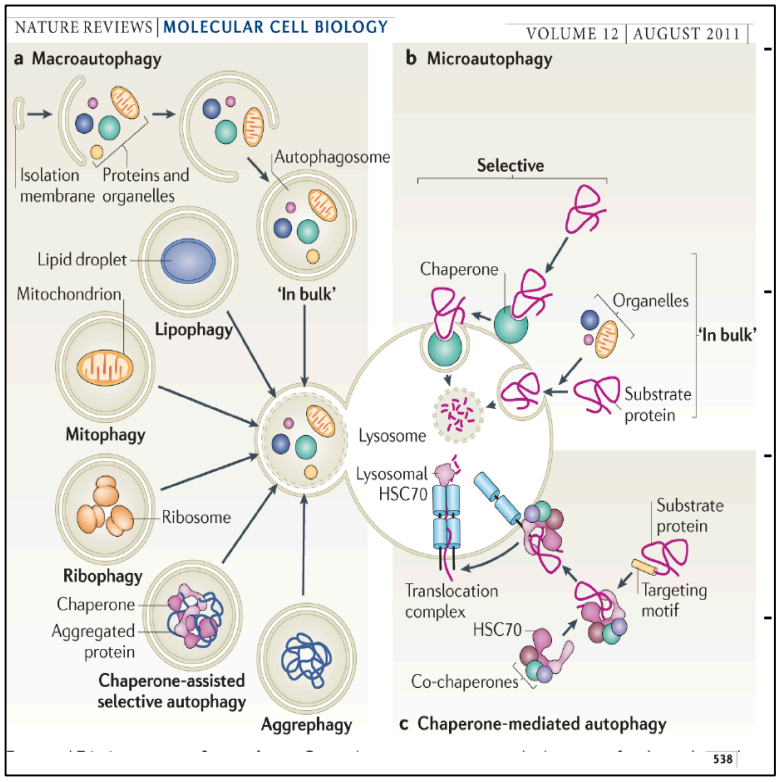


Figure 1: Visual of the different types of autophagy – Macro-autophagy, Micro-autophagy, and Chaperone-mediated autophagy

- Especially during starvation.

- Autophagy prevents cancers, neurodegeneration, etc.
  - Errors in autophagy are linked to many diseases, and even lifespan
  - The importance of autophagy wasn't first realized when it was being studied in yeast
- Ohsumi (won the Nobel Prize for his work on autophagy!) was able to recognize autophagy in yeast in early 1990s, and he considered it similar to old mammalian observations
  - Normally yeast vacuoles look empty
  - Most people in cell biology ignored autophagy in 1990s – they thought it was a sub-process of vacuole or lysosome, and there was more emphasis on endocytosis

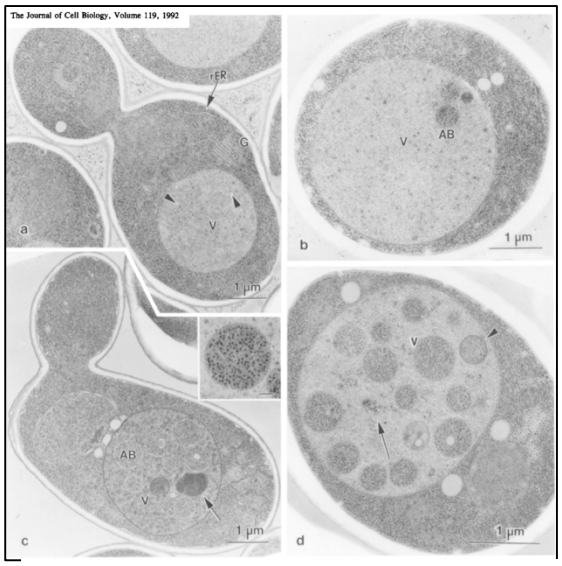


Figure 2: Ohsumi observed the formation of autophagic bodies in the vacuoles of yeast

- Ohsumi saw that if you took yeast cells and starved them, you will start to see structures in vacuoles he called **'autophagic bodies'** (Figure 2)
  - Ohsumi generated a yeast strain with mutations in vacuole proteases that would stabilize these structures (and prevent their degradation) and allow him to do a genetic screen for factors required for autophagy
  - By using yeast, Ohsumi could identify molecules for this process that would not have been done at the time in a mammalian system
- The results of the screen were published in 1993 in FEBs Letter
  - Published in 'low-impact journal' ("Classic for Nobel prizes")
  - We are looking at yeast strains that have wildtype system that, if you starve them, you will see "dotty things" (see Figure 3) below the microscope
  - If knocked out gene called **Apg1**, the vacuole just appears white and doesn't accumulate any structures inside
  - These genes were first called 'Apg' genes, but this was later changed to Atg ("for some irrelevant reasons")
  - The first paper showed 15 genes required (good for a first screen) for autophagy
  - These 15 genes were the basis of biochemical and genetic experiments for the next 20 years of work in yeast and mammalian systems

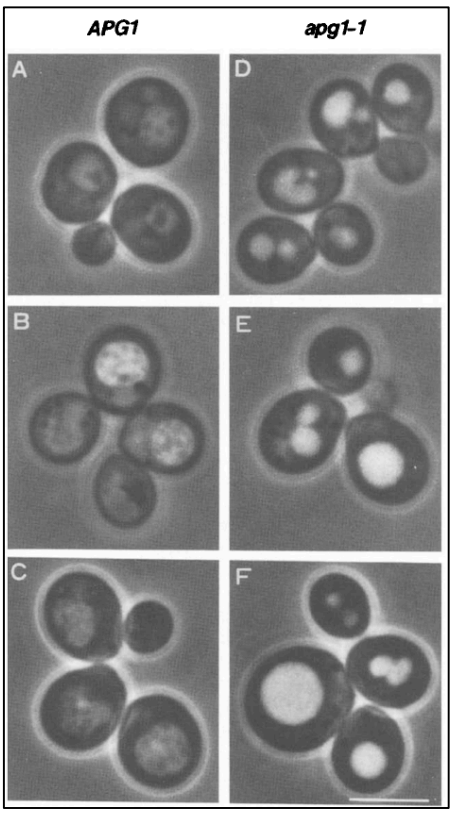


Figure 3: Knockouts of Apg1 led to stable vacuoles that did not undergo autophagy

- The yeast strains that did not have autophagy (knockouts of Apg1) would eventually get sick and die
  - Ohsumi was looking at survival and phenotypes in the initial screen

- There was an explosion of publication in the topic of autophagy in the last 20 years
  - Apoptosis is another subject that has exploded in cell biology before autophagy
- At this point…
  - The impact of autophagy on disease is tremendous, which is why Ohsumi won the Nobel Prize
  - The study of autophagy and cellular protein turnover led to a new understanding of the 'lysosomal network'
    - It is not just material coming in from the Golgi for lysosomal biogenesis → there are other functions of lysosomes for maintaining the cell
  - Autophagy is overall a protective process and is tightly linked to cell death
- Today: we will dissect autophagy into 3 steps
  - **Initiation**
  - **Elongation**
  - **Degradation**
- Autophagy is a very protective process
  - In 2000, Hanahan and Weinburg generated a scheme of processes that were required for metastatic transformation (these are typically referred to as the Hallmarks of Cancer)
  - Ex: oxidative stress, avoiding apoptosis, insensitivity to antigrowth signals, **disabled autophagy**
  - This shows how ongoing autophagy can clear damage, reduce oxidative stress, and inflammation, thereby stopping cancer

Autophagy: Initiation

- The classic place to start for autophagy is when the cell is in starvation, when the cell is low in energy
  - Like cancer, when you want to start to eat yourself, the process is not limited to one step or process
    - There have to be a couple of steps in place before autophagy can begin

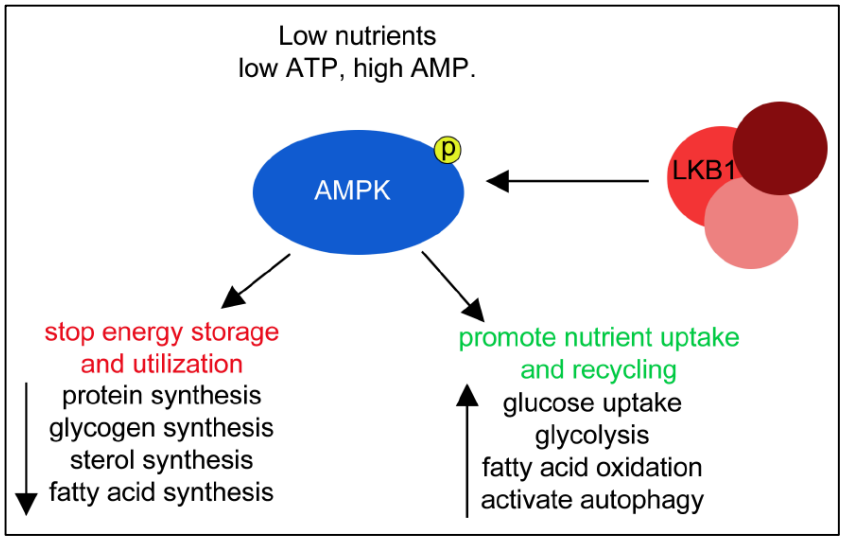


Figure 4: AMPK activation requires high AMP levels and phosphorylation by LKB1

- **AMPK** – AMP Kinase is activated when levels of **AMP are high**
  - The fuel of the cell is ATP – as ATP is consumed, we end up with ADP and then AMP (if the cell is really starving)
    - AMP binds to AMPK and activates it to become a kinase to do downstream actions
  - AMPK is not really active, even with high levels of AMP → it also activated by phosphorylation by **LKB1**
    - LKB1 is downstream from nutrient receptors

- So AMPK is looking at ATP levels and also changes in nutrients (when nutrients are low)

- When nutrients (amino acids) and energy (ATP) are low, then many processes are shut down, including protein translation
  - AMPK stops energy storage and utilization, including the following activities:
    - Protein synthesis,
    - Glycogen synthesis (in the liver) – want to burn the sugar for energy
    - Sterol synthesis
    - Fatty acid synthesis
  - AMPK promotes nutrient uptake and recycling, such as:
    - Glucose uptake,
    - Glycolysis,
    - Fatty acid oxidation,
    - Activating autophagy - to recycle more amino acid from what the cell is going to degrade
- A primary cellular sensor of nutrient status is AMPK (activated by AMP) as well as phosphorylation by LKB1
  - There are many downstream targets of AMPK that are summarized in the image (see below)
  - 2 required conditions for AMPK activity:
    - High AMP levels
    - LKB1 phosphorylation
      - AMPK will not be active without LKB1 phosphorylation

TOR Pathway

- Antagonizing AMPK is the **TOR pathway**
  - TOR pathway is required for growth (opposite of the AMPK pathway)
  - Upon hormone binding and in high amino acid concentrations, **mTOR** (target of rapamycin) becomes active and phosphorylates a number of substrates
    - mTOR is a multi-subunit complex (ex: TORC1, TORC2, TSC, and Ragulator complexes)
    - These different complexes respond to upstream signals and to amino acids
  - The effect of TOR activation is to promote:
    - Cell growth,
    - Metabolism,
    - Protein translation
    - Division
  - TOR also inhibits
    - Autophagy
    - Cell death (TOR pathway protects against cell death)
  - So it has the opposite effect as AMPK ("everything is shut down")

- Molecular basis not always known, but TOR is required for cellular effects

- Regulation of TOR activation
  - **TORC1** is regulated by small GTPase called **Rheb** (Ras homologue enriched in brain)
    - Rheb is localized onto the lysosomal membrane
    - When in **GTP-bound form**, Rheb will bind and **activate TORC1** and recruit it to the lysosome
    - It is not known what is the GEF is in this pathway
    - Rheb has other effectors that lead to increased phosphatidic acid (change in lipid composition) → promotes TORC1 activity as well (likely by recruitment to the lysosomal membrane)

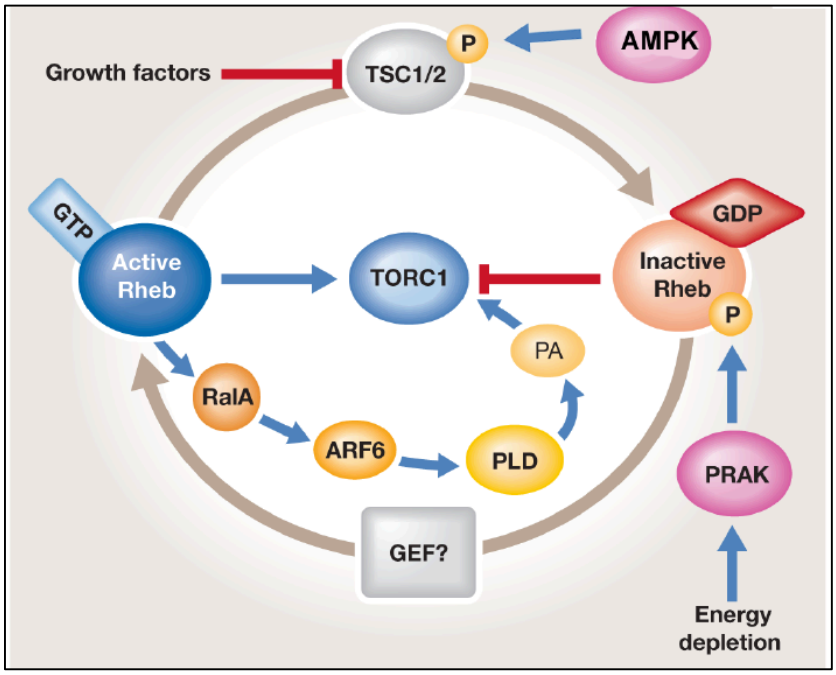


Figure 5: Regulation of TORC1 by GTPase Rheb

  - The GAP is **TSC1/2** (Tuberin Sclerosis Complex 1 and 2 - named after it was identified in cancers)
    - This molecule can be phosphorylated and activated by **AMPK**
    - One of the AMPK downstream targets is the GAP for Rheb → it shuts down the Rheb's activity and turning off the TOR pathway
    - TSC1/2 is also inhibited by growth factors, which would favor Rheb and TORC1 activity
  - Upon energy depletion, another kinase called **PRAK** phosphorylates **GDP-bound form** of Rheb and stabilizes it so Rheb is not active

- mTOR (mTORC1) is recruited to lysosomes in presence of amino acids
  - Under conditions of growth, mTOR is recruited to lysosome
    - There is massive recruitment of TOR to lysosomes in presence of amino acids
    - This leads to shutting down AMPK pathways
  - When amino acid levels are low, mTOR is primarily cytosolic
  - Question: how does it sense amino acids? By being on the lysosomal surface, we get the clue that there is an amino acid pool there from degraded proteins
    - mTOR is sitting on lysosome when amino acid concentrations are high – so there is some effect of amino acid concentrations
    - There could be a transporter on the lysosome membrane that is contributing to this sensor activity

- TORC1 recruitment to lysosome is regulated by amino acids
  - TORC1 goes to lysosomal surface when amino acids are added
    - Rheb is on the lysosomal membrane

- There is a complex on the lysosome called the **Ragulator**, and it binds to small **GTPases RagD and RagB**
- The Ragulator somehow 'senses' amino acids, but it is unclear

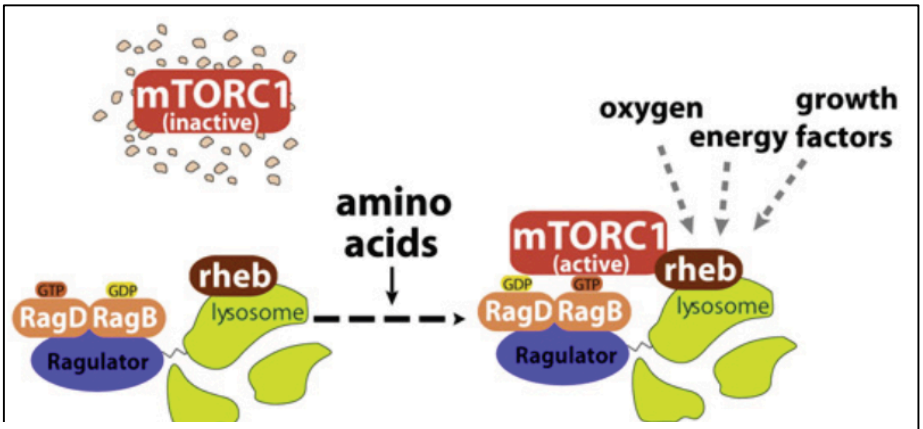


Figure 6: TORC1 activity and recruitment is regulated by amino acid concentrations

  - RagD and RagB don't have lipid modification at the C-terminus to mediate membrane binding → not going into the bilayer
  - They are obligate dimers: they can exist where one is GTP and other GDP bound → the Ragulator functions as a GEF
  - When RagB is GDP bound, it does not bind mTORC1;
  - When RagB is GTP bound (amino acid concentrations are high), it binds mTORC1
    - For now, RagB is the best published data that GTP-bound form will recruit TOR with Rheb
- When AA is high, nucleotide changes to GTP-bound, and Rheb is active
  - Bring mTOR to the surface

- **Summary: Activation of mTOR pathways – and cell growth – requires a combination of events**
  - Rheb GTPase must be active on the lysosome (GTP-bound form)
    - GAP (TSC1/2) therefore has to be inactive
  - Rheb effectors lead to increase in phospholipase D activity, and lipid modification to increase PA
    - There is a lot of lipid specificity
  - The Rag GTPases change their nucleotide state based on the level of amino acids
    - Sensor seems to be Ragulator – complex that contains GEF activity for the Rag GTPases
  - These three things (Rheb activation, underlying lipid architecture with high levels of PA, and Rags in right nucleotide state will keep mTORC complex on the lysosome
    - mTORC1 can then act as kinase and activate translation, cell growth and proliferation
- **Inactivation of mTOR pathway leads to a block in translation, growth and active autophagy**
  - When TORC1 is active, it regulates:
    - Metabolism (feedback loop on insulin growth),
    - Activates Serine-6-Kinase 1,
    - Drive lipid synthesis
    - Reduce lysosome biogenesis
    - Stops pathways that are related to autophagy

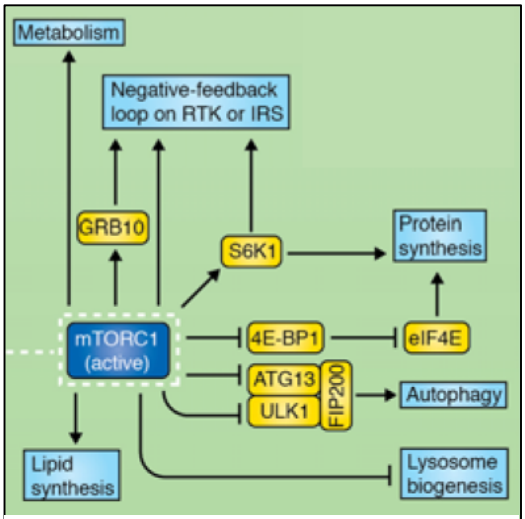


Figure 7: When active, mTORC1 activates and inhibits different pathways in promoting cell growth

- In low energy, AMPK activates and phosphorylates TSC1/2, GAP for Rheb – shuts down and release mTORC1 from lysosome
  - TCS1/2 GAP regulated by many other growth factor signaling pathways
- Active mTORC1 will block autophagy through direct phosphorylation of number of players
  - Therefore, inactivation of mTORC1 will relieve inhibition of autophagy initiating complexes and go forward
  - Rapamycin (a drug) also binds and inhibits mTORC1 → this is how to start autophagy in the lab
- **ATG13, ULK1, and FIP200** are initiators of autophagy (seen in Figure 7)

- mTORC1 phosphorylation on Atg13 and Ulk1/2 are inhibitory for their activity, <u>stopping</u> autophagy in high nutrients
  - In low nutrient, AMPK blocks mTORC via TSC1/2 and by direct phosphorylation
    - AMPK then phosphorylates Atg13 and Ulk1/2 on different sites and thereby activating autophagy
  - AMPK-phosphorylated Ulk1/2 complex is recruited to initiating Pre-autophagosomal structure (PAS) membrane
    - We are now telling the cell that we need to recycle substances for more amino acids in order to survive
    - This means we need to make a huge membrane to wrap around all the content to be degraded
  - ULK1/2 is also a kinase that phosphorylates a complex that includes a PI3K called **VPS34** to PAS
    - Starting to set a new bed of lipids for a new structure to make a new membrane

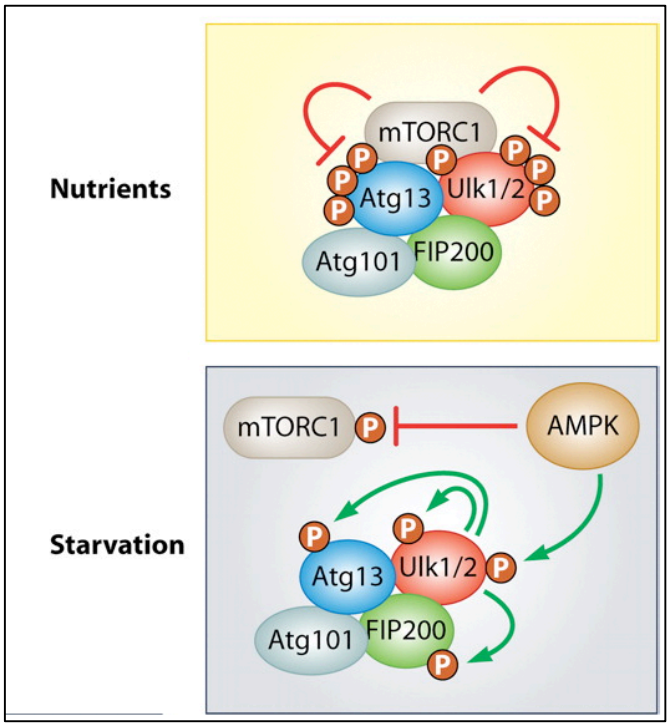


Figure 8: The level of nutrition effects the type of phosphorylation (and therefore function) of the ULK1 Complex

Where does the membrane for the PAS come from? How is it made?

- The PAS is a double membrane structure, very unusual
- Autophagy signal will inactivate TOR, leads to activation of autophagy initiating complex (ULK1, FIP200, ATG13) and bring P13P kinase to create a new lipid patch
  - Vps34 is PI3K
- Actual initiating membrane is still debated, although most agree Endoplasmic Reticulum (ER) is the primary source of the membrane
  - So start with an initial patch on ER and then have a conjugation system from there that allows the growth and elongation around cargo and eventually fusion with lysosome
- Long list of proteins and complexes involved here, >30 genes identified form screens in yeast, called 'Atg' (autophagy)

- A lot of work has been done on flies, on worms, and on the mammalian system
  - There are some differences but there are common key steps and key elements

Assembly of PAS: requires recruitment of ULK and Beclin

- Assume the membrane in Figure 9 is from the membrane as the PAS forms
  - Figure 9 shows that growth factors and amino acids activate TORC1 that will shut down the autophagy initiator complex
  - If AMPK is active, then ULK1 complex is active
- **ULK1 complex** regulates AMBRA/Beclin/VPS34 complex
  - This Beclin-Vps34 complex generates PI3P on ER membrane

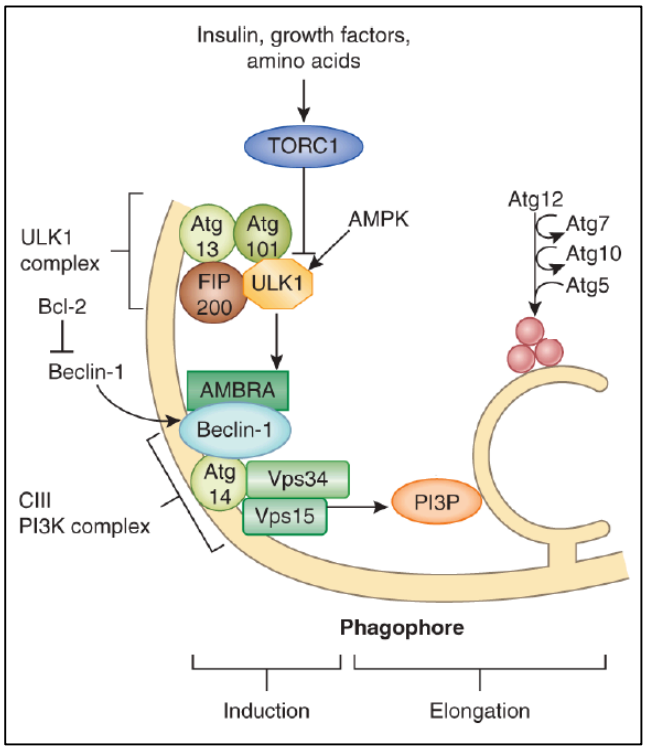


Figure 9: Activation of the ULK1 Complex and Beclin-VPS34 Complex leads to the formation and elongation of the PAS membrane

- Major point here: **chain of phosphorylation** events locally regulates the membrane recruitment of ULK1 and Beclin complexes to PAS, which is generally the ER membrane
- Once complexes are recruited and PI3P is generated, the membrane begins to elongate around cargo to be degraded
- Series of proteins essential to autophagy **(Atg12/7/10/5)** are required for this wrapping event
  - These proteins were the basis of a paper by Ohsumi in Nature in 1998

Autophagy: Elongation

- **Atg12** is small, ubiquitin-like protein
  - There is an E1 and an E2, but there is no E3
  - Atg12 has c-terminal di-glycine motif (like ubiquitin) and is conjugated to Atg5 through an E1 (Atg7) and E2 (Atg10) protein
    - There is no E3 ligase scaffold, as E2 can conjugate directly
- Once conjugated to Atg12, **Atg5** is able to start to oligomerize against another protein called **Atg16.**

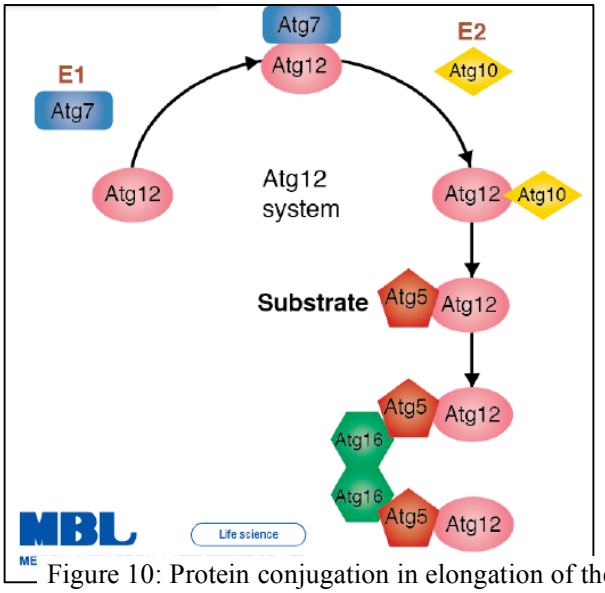


Figure 10: Protein conjugation in elongation of the autophagosomal membrane involves various Atg Proteins (12/7/10/5/16)

- Formation of a strong structure that is like a coat → allow the lining of the membrane
- This oligomer is essential to elongate the autophagosomal membrane around the cargo, but the mechanism is still unclear

- There are two types of conjugation events that make the phagosome during elongation: protein based (Atg12/7/10/5 in Figure 10) and lipid based conjugation (Figure 11)
- To recruit cargo and complete the autophagosome, a specific lipid conjugated protein **LC3** is required
  - LC3 is a small protein (16kDa) that first came out of Ohsumi's first screen as Atg8

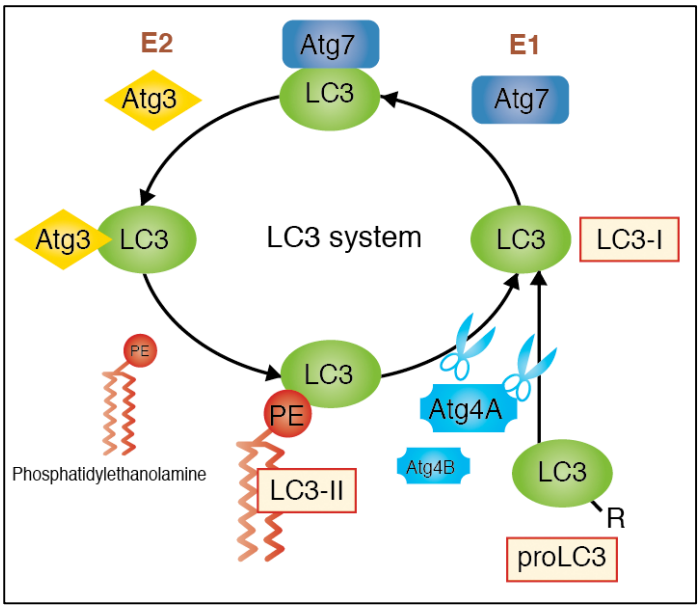


Figure 11: LC3 is a specific lipid-conjugated protein that functions in autophagosomal elongation

- LC3 is first processed at the C-terminus by a cleavage event by protease Atg4
  - There is E1 activity (Atg7) and E2 (Atg3), which will help conjugation of a lipid **PE (phosphatidylethanolamine)**
    - By conjugating to a lipid, LC3 is going to be sitting in the membrane and anchored there (both on the internal and external side)
  - LC3 on internal membrane of autophagosome is degraded
  - LC3 on external membrane is cleaved from the PE lipid and recycled by Atg4

How is the cargo recognized?

- At this point of the expanding autophagosome membrane, there is a coat of protein based oligomers (Atg12/5/16) and LC3 conjugated to PE on the internal and external membrane (Figure 12)
  - The Atg5 oligomeric complex resembles a 'coat' type structure on the inside of the phagophore
  - At this point, the autophagosome can selectively capture cargo or randomly select cargo → how is the cargo recognized?
- Usually, ubiquitinated proteins go to proteasome for degradation → but if there is too much (like a large aggregate in neurodegenerative diseases), it will be captured by the autophagosome instead of going to the proteasome
  - Soluble ubiquitin cargo will go to the proteasome, but the aggregates will go to the autophagosome
- Entire mitochondria can be captured by the autophagosome as well as pathogens like bacteria for degradation
  - They are labelled for degradation by **ubiquitin**
- In starvation conditions, it is considered that the primary cargo is bulk internalization of cytosolic contents
  - Ohsumi didn't believe there could be selectivity so this was his initial belief
- Organelles and protein aggregates are linked to the growing autophagosomal membrane through linker proteins called **adaptors** or **receptors**

- Adaptors all have a common domain called **LIR domain** – LC3 interacting motif
  - These adaptors also had **UBA** domains – ubiquitin binding domain
- Ubiquitin-bound cargo binds to adaptors (like p62), and these adaptors have a LIR domain that links it to LC3
  - These adaptors are critical for incorporation of cargo into growing phagophore
- Most commonly studied adaptor is p62, but others are growing in interest

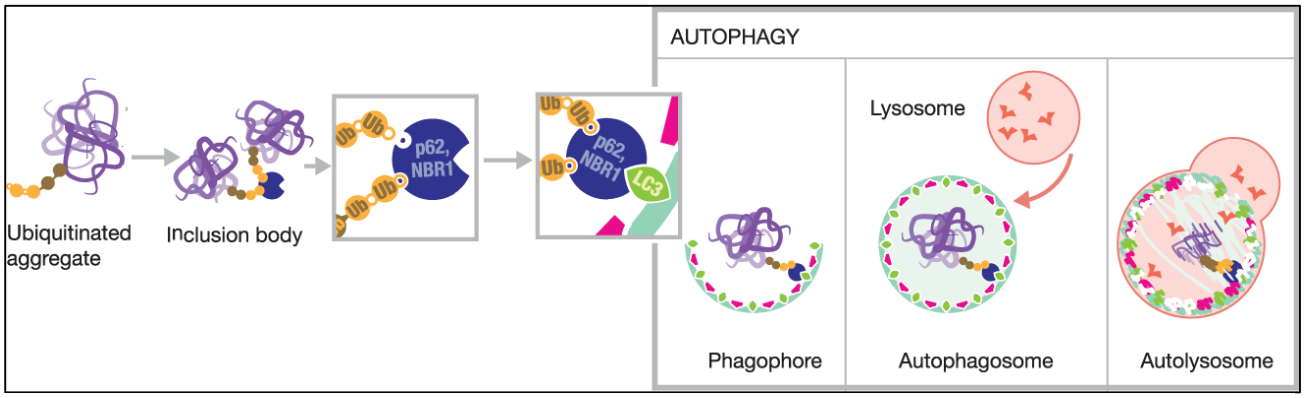


Figure 12: Ubiquitin-bound aggregates bind to adaptor proteins via UBA domains. Adaptor proteins also bind to LC3 on the internal membrane of the expanding autophagosome via LIR domains. This process is how cargo is recognized.

Recognition of Mitochondria as Cargo

- Ubiquitination is also critical for recognition of mitochondria
- Upon loss of electrochemical potential (mitochondrial dysfunction), **PINK1** cannot be imported and accumulates on mitochondrial outer membrane instead
  - If PINK1 is stuck in the channel, it will act as kinase and phosphorylate Parkin
- **Parkin** is E3 ubiquitin ligase that is recruited by PINK1 and ubiquitinates the surface of the mitochondria
  - Mitochondria is then adapted by p62 and other adaptors, and this will target the mitochondria to the phagophore for autophagy
- PINK1 is a **serine/threonine kinase** and it phosphorylates Parkin at a serine in its **ubiquitin-like domain (UBL)**
  - New data suggests that PINK1 also phosphorylates ubiquitin at same conserved serine residue
- PINK1 and Parkin are both mutated in familial forms of Parkinson's Disease

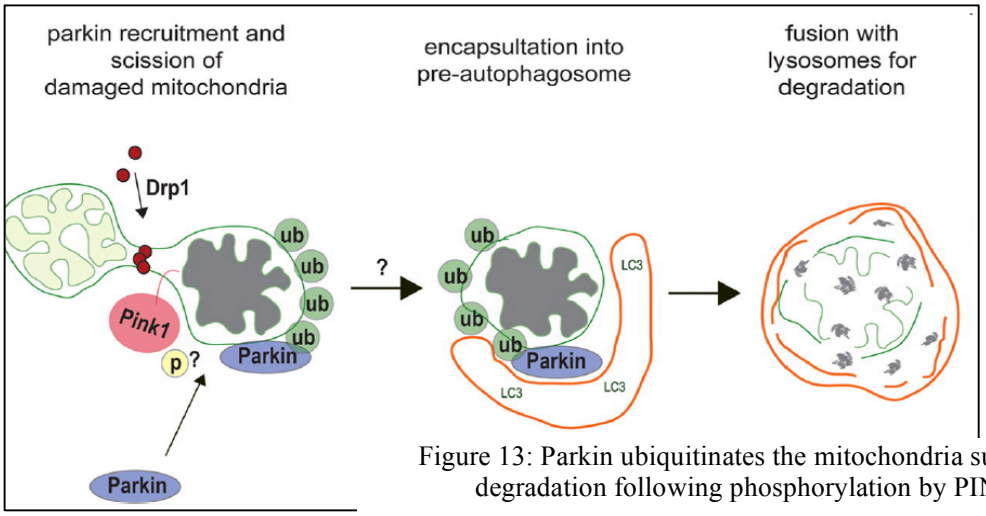


Figure 13: Parkin ubiquitinates the mitochondria surface for degradation following phosphorylation by PINK1

Autophagy: Fusion with Late Endosome/Lysosome

- In Figure 14, it is clear that the LC3 in the lumen of the autophagosome is degraded (and not on the external membrane)
- The autophagosome fuses with late endosome, which requires **Rab7** and some SNARES, but not so clear
    - Evidence: can capture fused autophagosomes and late endosomes that still have multivesicular bodies (MVB) and cargo inside
- Hybrid is called **amphisome**, where MVB and LC3 are visible

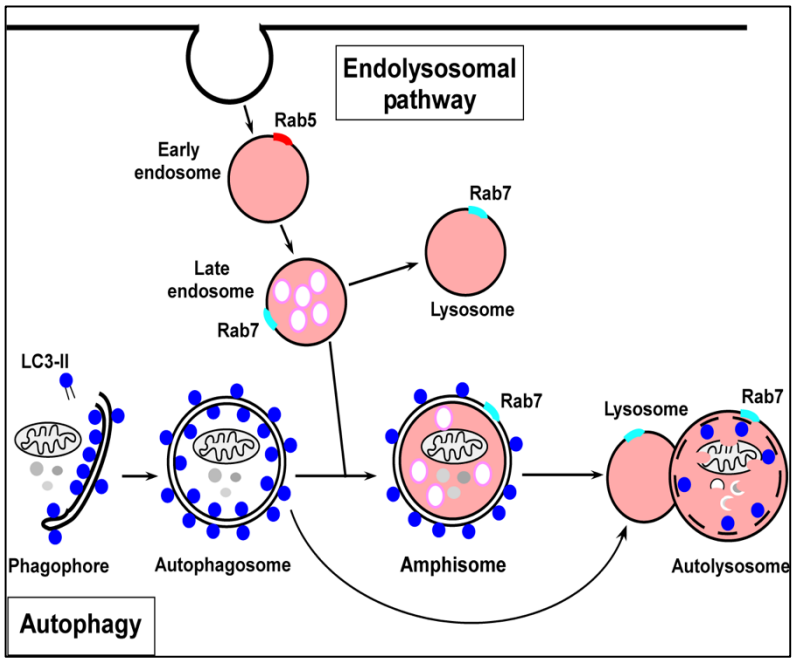


Figure 14: Autophagosome fuses with late endosome to form autolysosome

- Finally, the amphisome fuses again with lysosomes for complete degradation in the **autolysosome**

Summary

- Autophagy is a highly complex and elegant process
    - There is a huge switch about nutritional state of the cell
- We have described the initiating signals that 'sense' the cells metabolic condition in a simple way
- mTOR and AMPK pathways were the focus, but there are many signals that are directed to this machinery
- Once autophagy is initiated, the pre-autophagosomal structure is the seed for engulfment of cellular cargo
    - This can include aggregates, ribosomes, mitochondria, lipid droplets, etc.
- We have not described microautophagy and chaperone-mediated autophagy
- These hint towards additional mechanisms to degrade cellular material that do not require the core 'Atg' machinery
- If this process is not correct, it can lead to diseases

ANAT 365
Lecture #15- Parkinson's Disease-Related Proteins PINK1 and Parkin Repress Mitochondrial Antigen Presentation
Prof. Heidi McBride- Friday, October 7th, 2016

NOTE: This NTC is meant to be used as a study aid to supplement your own class notes. Hence, not all of the text contained in the lecture slides will be reproduced here.

Announcements:

- **Logo Submissions for MACCS close October 8th at 8PM**
  - **Submit them at it.maccs@gmail.com or check the listserv for more information on that**
- **Wine and Cheese: October 13th, 2016 @ 11:00 in Strathcona Anatomy Reading Room**

Please send any comments or questions about NTCs to us through e-mail: macss.academic@gmail.com

Why this Paper was chosen

- Good link between basic cell biology and applied cell biology
- Touches upon vesicle budding from the mitochondria and antigen presentation

Introduction to Mitochondrial Vesicle Trafficking

Defining MDVs

- MDV= Mitochondrial derived vesicles
- Process by which the mitochondria like other organelles, are able to laterally segregate their cargo into vesicular profiles that can then bud off and send its contents to other compartments of the cell
- In order to consider a vesicle an MDV they must be:
  - Cargo selective
  - DRP1 independent
  - 70-150nm in size→ can be imaged through EM
- **TOM20**: outer mitochondrial membrane protein
  - Component of the import receptor
- **PDH**: enzyme found in the center of the mitochondria
- EM colour stained image: see lecture slide 2
  - You would think that the circled portions are simply mitochondrial fragments
  - This is not possible because they are forming without the fission GTPase called **DRP1**→ this is a different mechanism

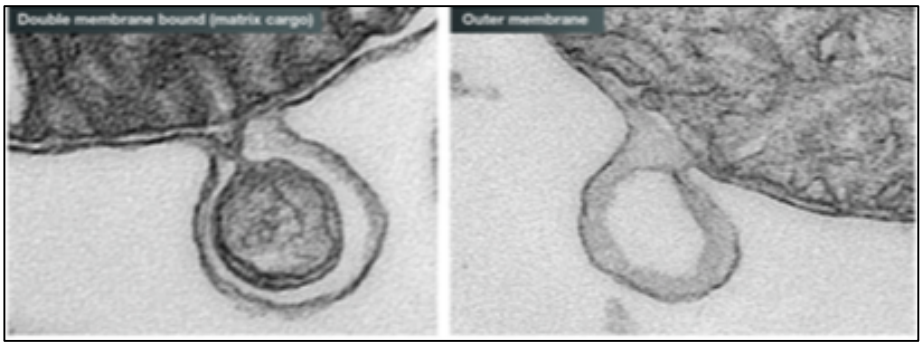
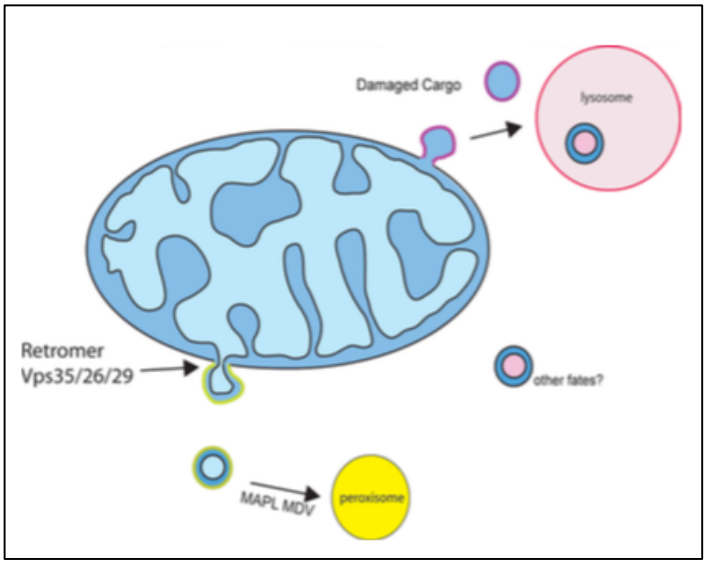


Fig.1 EM of an MDV about the size of a normal vesicle

- You can see the outer and inner layers of the mitochondria budding off

MDV Transport Pathway

- 2 main pathways were identified:
  - MDVs→ Peroxisome
    - **MAPL** (mitochondrial anchored protein ligase) is a mitochondrial membrane protein that was found in the peroxisome
    - Budding off of the mitochondria is dependent of the **retromer complex**
  - MDVs→ lysosome
    - Damaged Cargo is being shuttled to the lysosome for degradation
  - Are their other fates the MDV could undertake?
    - This is what we are going to explore



- The discovery of MDVs:
  - They were studying MAPL-YFP expressing HeLa Cells
    - Noticed that MAPL was budding off the mitochondria in a vesicular looking structure
    - This type of budding looked very distinct from mitochondrial fission events
    - ***See lecture recording at 9:55mins for the video comparison

Hard Fact To Publish

- People were sceptical of the idea that mitochondria could form vesicular bodies
- This shouldn't have been super surprising due to the fact that bacteria bud vesicular structures and mitochondria originate from bacteria
  - Bacterial Vesicles are used to:
    - Fuse with other bacteria
    - Transfer of signalling molecules or DNA
    - Deliver toxins into host to cause sickness

- Interspecies communication

- Researchers needed a function for MDVs in order for people to believe they were real and a useful mechanism
  - Still don't quite understand the peroxisome pathway→ working on it in the lab at the moment

MDV Trafficking to the Lysosome

MDV Generation: Respiration and Stress

- To test whether MDV formation was correlated with oxidative damage, the number of vesicles produced at the surface of the mitochondria were counted under various conditions
- Growth medium for mitochondria
  - Glucose: mitochondria in this state are diabetic and depend on glycolysis for energy production
    - They don't need to go through respiration to survive
  - Galactose: forces mitochondria to use respiration for energy production
    - Increases the # of electrons flowing through the electron transport chain (ETC)

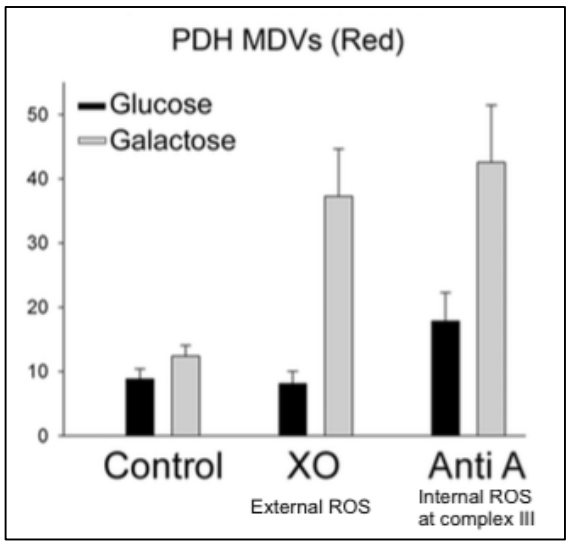


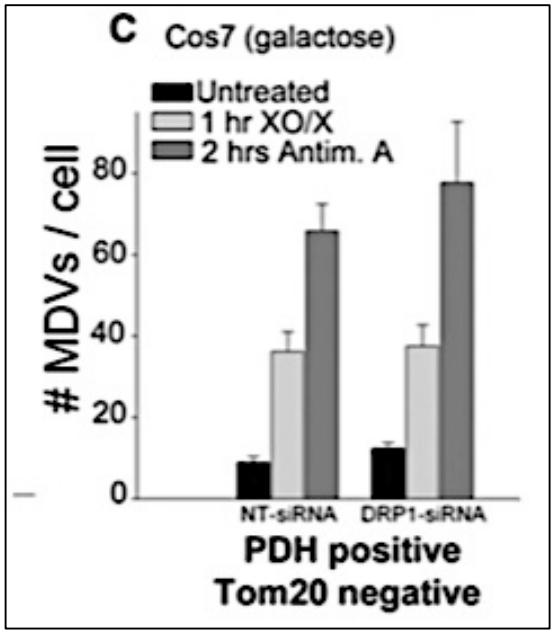
Fig.2

- **ROS**: By product of respiration created by the ETC that can cause oxidative stress on the cell if not eliminated efficiently
  - XO: Stimulates the production of External ROS
  - AntiA: Stimulates the production of Internal ROS
    - Binds to Complex III of the ETC and forces the increased production of ROS inside the mitochondria
- **Experimental Setup**:
  - Control: both conditions yield low number of PDH MDVs
  - XO:
    - Glucose: Mitochondria are not using respiration therefore the level of ROS even if stimulated is low
      - Low # of MDVs
    - Galactose: Mitochondria are undergoing respiration therefore the levels of ROS induced by XO are high
      - High # of MDVs
  - AntiA:
    - Glucose: Mitochondria are not using respiration therefore the level of ROS remains relatively low
      - Low # of MDVs
    - Galactose: Mitochondria are undergoing respiration therefore the levels of ROS induced by AntiA are high
      - High # of MDVs

- **Conclusion**: Oxidative stress increased the production of PDH MDVs in cells that were undergoing respiration (galac.)
    - Same was observed when the experimental conditions were applied to TOM20 MDVs
    - MDV formation is regulated

Testing DRP1 Dependence

- Under the same experimental conditions explained above, the samples were tested with DRP1 variable
- **NT-siRNA**: condition in which DRP-1 is **present**
- **DRP1-siRNA**: condition in which DRP-1 is **not present**
- Removing the DRP1 from the mitochondrial cells didn't prevent them from forming MDV structures under oxidative stress conditions
    - If these structures were mitochondrial fragments, they would need DRP1 to be able to bud of from the mitochondria
    - Remember that MDVs are DRP1 independent



a

Visualizing MDVs within/entering MVB

- Immuno-gold stained the TOM20 proteins:
    - TOM20 +ve vesicular carriers were seen docking onto the late endosomal and MVB membranes
- Gold particles were seen in the internal vesicles of the MVB thus confirming that the vesicular bodies were transferring their content into the MVB itself

Regulating Machinery

- If we can isolate the machinery involved in the regulation of the MVBs then it is easier to convince others that they are vesicular structures
- In the case of dysfunctional mitochondria:
    - Pink1 is arrested in the import channel and phosphorylate Parkin and Ub
    - This chain of events targets the entire mitochondria for degradation
- Q: If this machinery can work on an entire organelle, can it work on a dysfunctional piece of an organelle?
- A: In collaboration with Fon lab we saw that YFP-Parkin was recruited to emerging MDVs stimulated by oxidative stress
    - YFP: Immunostaining molecule
    - Parkin is thus involved in the regulation of MDV formation and the elimination of defective mitochondrial parts
    - It was later shown that Parkin is essential in the formation of MDVs
- Since the beginning of the research it has been discovered that many different types of vesicles can bud off from the mitochondria depending on the stress causing the damage
    - Regulatory machine known so far: Pink1, Parkin, Syntaxin 17, SNAP29 and VAMP7

- Discovery of the SNAREs involved is really important→ continues to reinforce the idea that the structures budding from the mitochondria are really vesicles

Redundancy In Mitochondrial Quality Control

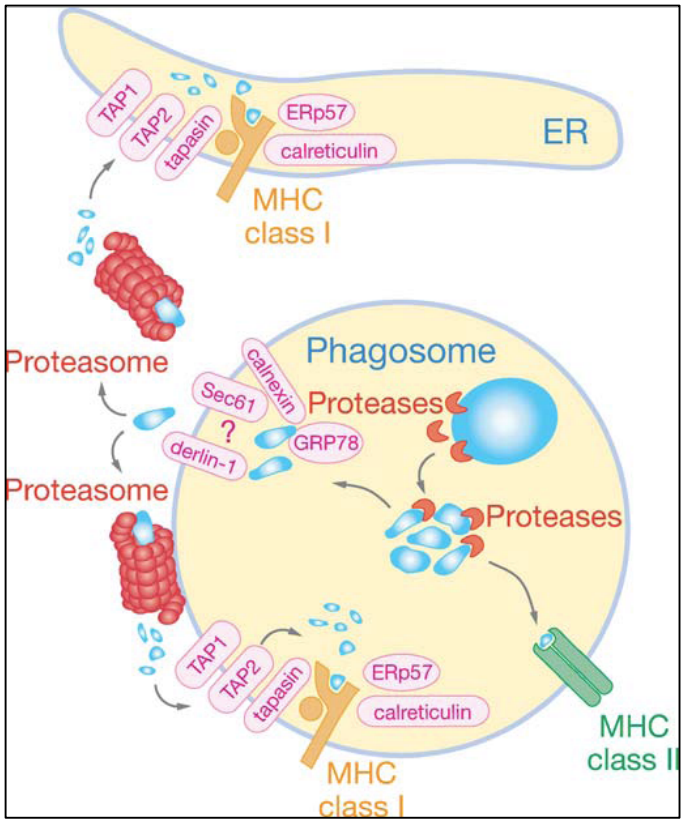
- Mitochondria have many mechanisms that ensure their function and quality:
  - Mitochondrial proteases
    - Used to degrade defective internal molecules
    - A lot of these are conserved in bacteria
  - Ub-Mediated degradation by proteases
    - Defective outer membrane proteins are Ub within the mitochondria and are then targeted to the proteasome
  - Mitochondria bud vesicles (MDVs)→ new mechanism
    - Carry the damaged cargo to the MVB/lysosome for degradation
    - Require the help of Pink1 and Parkin
    - This is thought to be the method employed by the mitochondria when the molecule or complex that requires degradation is too big for the other mechanisms to handle
      - Electron transport chain complexes: if they undergo damage they can release ROS which is dangerous for the mitochondria
      - ETC complex is thus sent out via and MVB to d=be disassembled and the functional proteins and molecules are sent back to the mitochondria for reassembly
  - Fission and depolarization recruits Parkin for mitophagy
    - Defective portion of the mitochondria is pinched off an degraded through the mechanisms of autophagy
- *Q: Is mtDNA packaged into the MDVs that leave the mitochondria?*
- *A: As far as we can tell mtDNA is not packaged into the MDVs however there is a researcher in Texas that has found that neutrophil mitochondria do package mtDNA in their MDVs and process it in the MVB. Once this is done, mtDNA is presented at the surface of the Neutrophil and activated the Toll9 receptor→ launch a complex immune cascade*
  - *This would cause non-infectious sepsis*
- Depending on the target of the damage caused the cargo will change
- ***NOTE: there is an ongoing production of MDVs that is increased in situations of cellular stress

Role of MDVs in Adaptive Immunity

Brief Introduction to Adaptive Immunity

- Adaptive Immunity: Part of the immune system that produces antibodies (Ab) that protect against future infection
  - Professional antigen presenting cells (APCs) will be infected with bacteria/viruses that will be degraded by the proteasome and then presented at the surface MHC complexes
  - MHC expression of antigen fragments→ stimulate degradation by CD$+/CD8+ T-Cells
- **MHC Class 1**:
  - Pathogens are degraded in the proteasome
  - Peptides enter the ER through TAP transporters→ degraded→ loaded onto MHC I at the cell surface

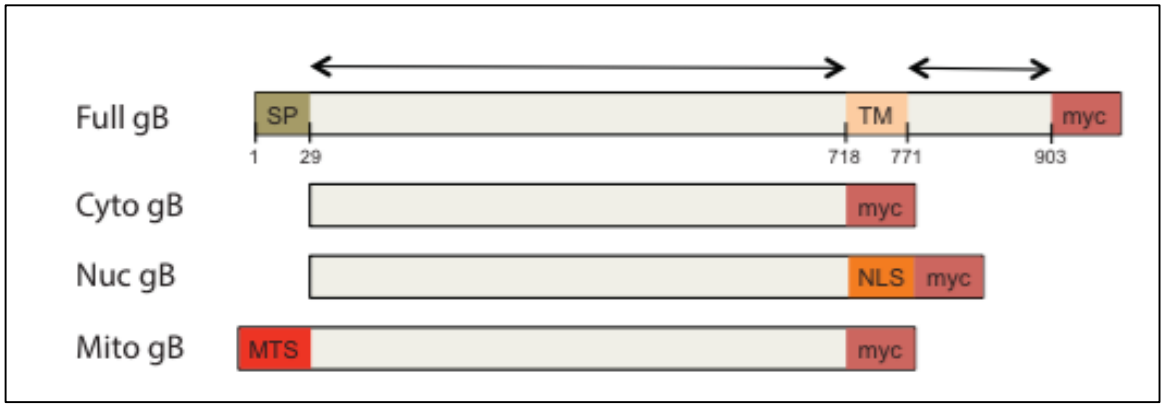
- Antigen fragment loaded into MHC I will recruit and activate cytotoxic T-Cells (CD8+)
- Cross-presentation: MHC I found in the endosome
  - MHC class I is not only limited to the ER



- **MHC Class II**:
  - Usually found on the surface of the endosome/phagosome
  - Pathogens are degraded by proteases
  - Peptides derived from pathogens are loaded onto the MHC II at the cell surface and recruit Helper T-Cells (CD4+)→ triggers antibody production by B-Cells
- In the thymus, we have a lot of antigen presentation occurring with our own cells to build what is called **immuno-tolerance**
  - This suggests that there is a pathway to present normal cell organelles in the thymus
  - Studies were done to see how each organelle is presented except for the mitochondria
    - The reason mitochondria are not as studied is that T-cells seam to be less reactive to mitochondrial content
- A lot of autoimmune diseases that are caused by the reaction of the immune system to mitochondrial fragments
  - Historically it was thought that mitochondrial fragments left the cell because the cell had exploded

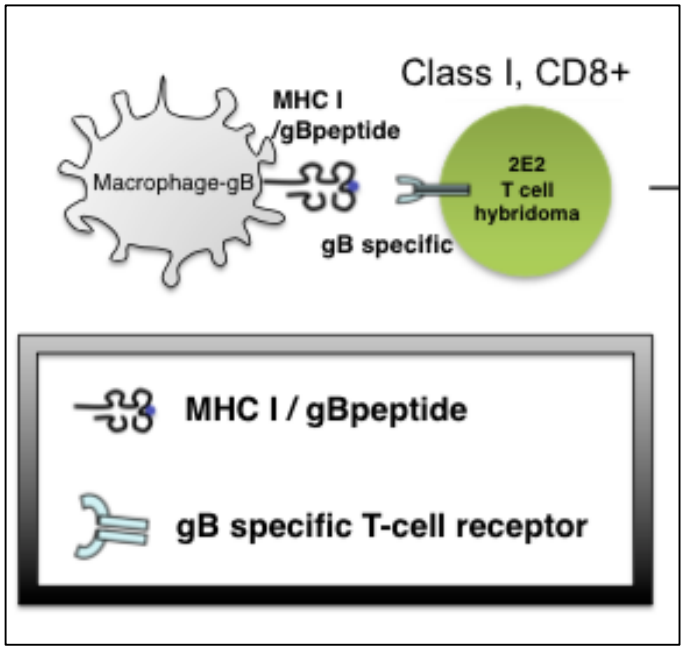
Expression of viral gB

- gB is a protein from the herpes simplex virus (HSV)
  - Used because it is an extremely immunogenic protein because of the way it is folded
- gB protein normally has:
  - SP: Signal peptide that targets gB to the ER
  - TM: Trans membrane domain that anchors it to the surface of the cell
- Changing its domains altered what organelle it was targeted to within the **macrophage**:
  - **Cyto gB**: removing TM and SP resulted in the protein floating throughout the cytosol
  - **Nuc gB**: removing TM and SP and replacing them with NLS targeted the protein to the nucleus
  - **Mito gB**: removing the Tm and SP and replacing them with MTS targeted the protein to the mitochondria



Hybridomas for Quantification of MHC I Presentation

- Remember that we are trying to figure out how the mitochondria is presented to the immune system in the thymus as to allow immuno tolerance to its components
- Experimental setup:
    - Used macrophages expressing gB constructs
    - Peptide fragments from the gB protein are expressed by MHC I at the cell surface
    - gB fragment-MHC I complex is presented to a T-cell Hybridoma
        - Hybridomas are specific in what they are able to recognize as antigens
        - In this case it is recognizing the gB protein fragment bound to MHC I
    - Binding of the gB fragment to the T-cell Hybridoma causes the activation of IL-2
        - IL-2 is a toxic cytokine expressed only when cell are activated
        - By placing B-galactosidase in IL-2 promoter region the activation of IL-2 will produce a high quantity of B-galactosidase
        - Simple assays can measure the enzymatic activity of B-galactosidase and thus acts as a reporter for the T-Cell activation



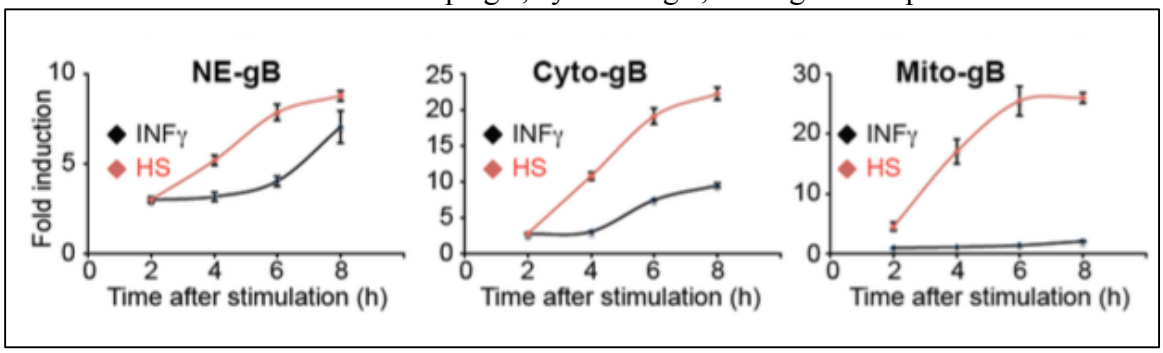
- ***NOTE: In this paper, there is nothing regarding MHC II complexes because they didn't have any Hybridoma CD4+ cells

Heat Shock to Measure MitAP (Mitochondrial Antigen Presentation)

- Setup:

- Expose the macrophage cells to 42 degrees for 10-30 minutes and then return them to 31 degrees
- Incubate the cells with gB and clonal T-Cells
- Calculate the B-gal readout to determine T-Cell activation

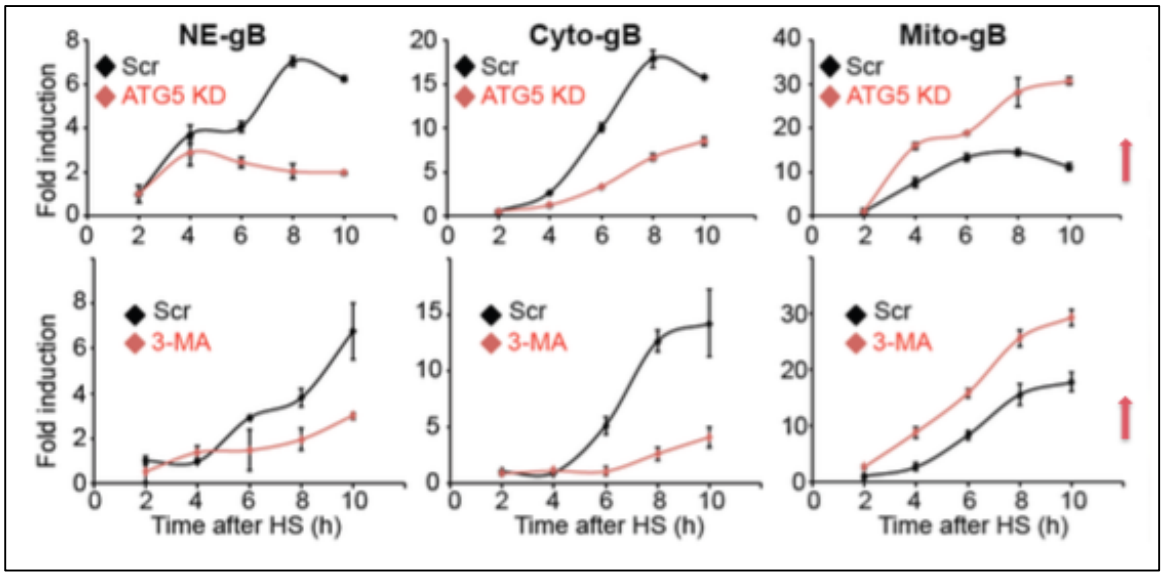
- **IFN-gamma**: mitochondria didn't were not presented to the T-cells when infected with this virus therefore it had to be a different pathway that allowed this presentation to occur
- **HS**: caused Nuclear envelope gB, cytosolic-gB, Mito-gB to be presented to the T-cells



- Notice that the reaction to the gB in the cell occurs long after the HS, that is because we are waiting for the IL-2 transcription to stimulate B-gal production
- *LPS treatement (infection) also induces MitAP*
  - *Skin of bacteria will stimulate the same kind of findings*

Autophagy as the Presentation Model

- Hypothesis: Autophagy is necessary for the presentation of the Nuclear Envelope gB and the Cytosolic gB therefore **mitophagy** must be responsible for the presentation of Mitochondrial gB
- Upper Panel:
  - Atg5: necessary for autophagy→ lines the inside of the autophagosome
    - Knock Down of Atg5→ prevented autophagy from occurring which lead to the lack of presentation of the nuclear envelope and the cytosolic gB. Blocking of the autophagy mechanism didn't hinder the presentation of mitochondrial gB therefore it must be independent of autophagy all together
      - There must be another mechanism used in mitochondria



- Lower Panel: essentially the same experiment as in the upper panel but with the drug version of Atg-5
  - 3-methyladenine blocks PI3kinase VSP34→ inhibits mitophagy
  - The same results are yielded→ base yourself on the first panel

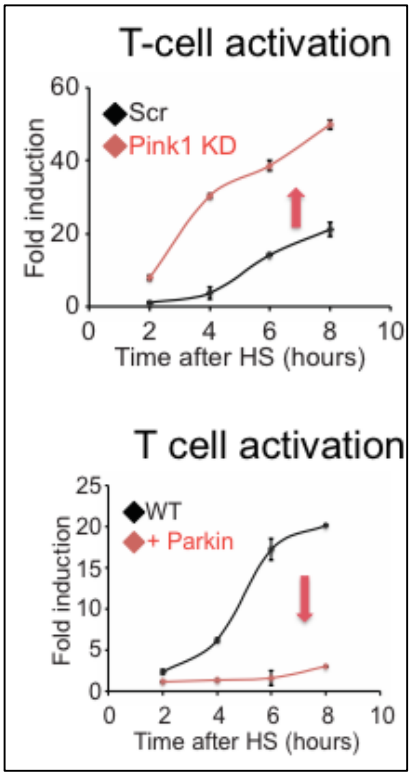
- So hypothesis was wrong, autophagy does not drive the antigen presentation for the mitochondria

Pink1 and Parkin in Antigen Presentation

- Since they are necessary for mitophagy and formation of MDVs, do Pink1 and Parkin regulate antigen presentation for the mitochondria?

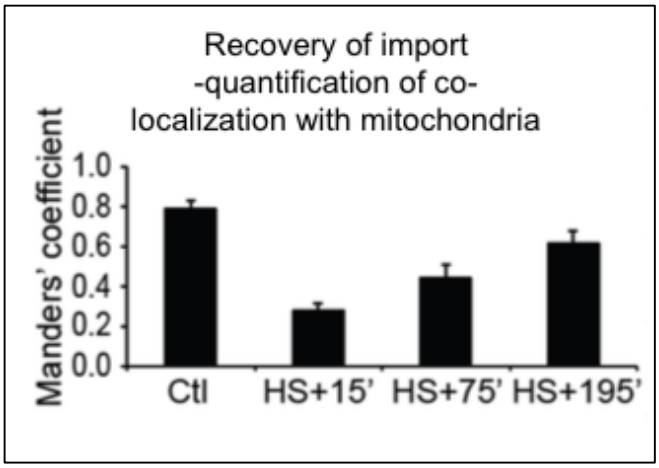
Pink1 and Parkin Represses Antigen Presentation

- 2 main results:
  - Knocking Down Pink1: stimulated antigen-presentation
  - Adding Parkin: blocked the presentation from occurring
    - They added Parkin because they noticed that macrophage cells had really low quantities of the protein
- We conclude from this that Pink and Parkin actually repress/block antigen presentation of mitochondrial content when there is a situation of heat shock or cell damage
- What is happening in the cell after a Heat Shock?
  - GFP-Parkin in recruited to the Mitochondria containing the gB (Mito-gB)
  - Mitophagy is initiated→ the block by parkin is not because it drives mitophagy
  - After the heat stress everything goes back to normal



Mitophagy blocks MitAP

- HS was found to promote the release of gB into vesicular bodies
  - Mander's coefficient measures the degree of colocalization of 2 molecules
  - After 15 minutes of HS, gB exited the mitochondria
    - Biochemical fractioning showed that gB exited through MDVs
  - As cells recovered from the HS pulse (brief exposure), which means that the HS is reversible
    - HS didn't kill or damage the cell



Sorting Nexin 9(SNX9)

- Discovered that it was required for vesicle budding and MitAP
- Snx9:
  - Necessary in actin polymerization at the surface of the cell
    - Serves as a driving force to push vesicles inside the cell
- After the HS the gB comes out of the mitochondria→ you can tell because it isn't colocalized with the TOM20 in the mitochondria
  - Removing Snx9→ Prevented gB from leaving the mitochondria so it remained colocalized with TOM20
- Since Snx9 is an established vesicle trafficking protein, it confirms that gB is leaving the mitochondria in vesicular structures

Recruitment of Snx9

- Snx9 is not normally found on the surface of the mitochondria
  - HS will promote the recruitment of Snx9 to the surface of the mitochondria
- Mitochondria that don't have Snx9→ able to make MDVs however they are unable to bud from the mitochondria
  - Accumulation of unbudded structures at the surface of the mitochondria
  - This is consistent with its role in bringing actin to the neck of a bud for pinching
  - Accumulation is usually really difficult to visualize

Increased Parkin blocks Snx9

- Parkin will block the Snx9 activity as well as gB escape into vesicles
  - Presence of MG-132 which blocks the proteasome functions will allow the recruitment of Snx9 and thus vesicle formation
  - Suggests that when Snx9 is recruited to the mitochondria it is Ub and degraded by the Parkin (E3 ligase)

Rab GTPase

- Wanted to look at the Rab GTPases that work around the late endosome because that is where MDVs are targeted
  - Rab9: required for the return of mannose-6-phosphate receptors to the golgi
  - Rab7: required for vesicle fusion with the late endosome
- Loss of Rab prevents MitAP from occurring
  - Rab7 Knock Down: If you give the mitochondria a HS the gB will leave the mitochondria but will be unable to fuse with the late endosome

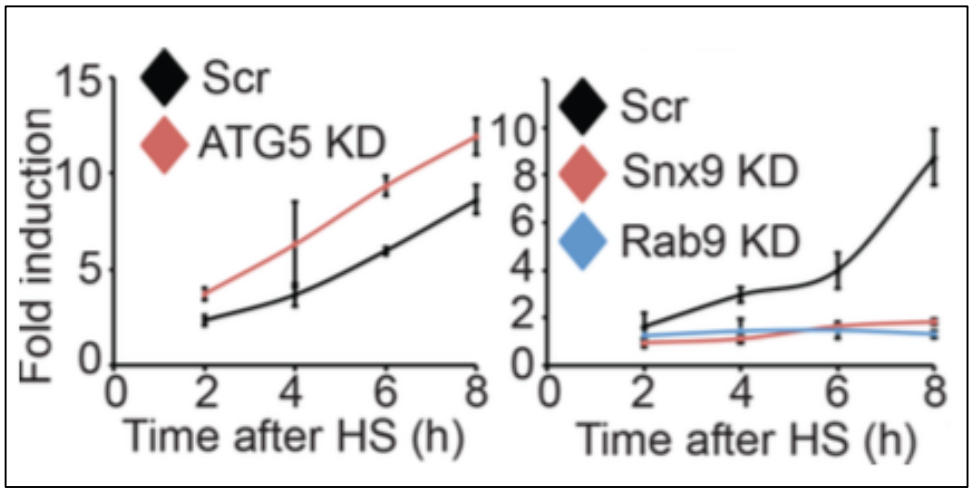
- gB cannot be degraded and therefore you end up with gB not being able to colocalize with the mitochondria
- T-cell activation is blocked

- Rab9 Knock Down: If you give the mitochondria a HS MDVs will form but they will be unable to bud off
  - gB remains colocalized and cannot leave the mitochondria→ see accumulation of MDVs at the mit. surface
  - T-cell activation is blocked

Application of Techniques to Other Systems

Artificial Sytem with gB

- The technique established for demonstrating MitAP has only been showed in vitro up to here, but is this possible in vivo?
- Many autoimmune diseases are caused by antibodies raised against mitochondrial proteins→ indicates that mitochondrial content can activate MHC II
  - **Primary Billiary Cirrhosis**: autoimmune disease in which antibodies attack OGDH (*2-oxoglutarate dehydrogenase or alpha-ketoglutarate dehydrogenase)*
    - OGDH: mitochondrial matrix protein
  - Scientists have been able to create a CD8+ hybridoma specific to OGDH
  - This allows us to look for endogenous mitochondrial presentation in mice
- Endogenous mitochondrial OGDH was presented to T-Cells in 3 different cell types:
  - RAW, primary BMDM (macrophages) and dendritic cells
  - KD of Atg5: promoted the release of OGDH in vesicles
  - Snx9/Rab9 KD: prevented release of OGDH in vesicles

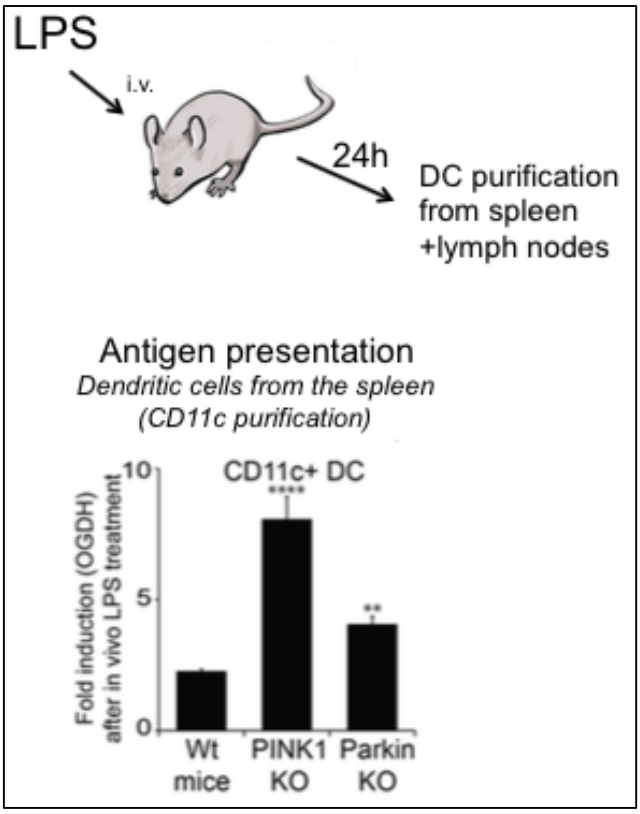


Mouse Model

- Now that it has been shown that this experimental assay with Hybridomas works with endogenous proteins we can move out of the test tube and into a live mouse
- Took 2 month old mice and either KO Pink1, KO Parkin or wild type (wt)
  - Injected LPS (Bacterial "skin") to mimic an infection
  - 24 hours later→ take dendritic cells from the spleen and the lymph nodes
  - Incubate these dendritic cells with T-Cell Hybridomas that are selective for OGDH

- Trying to find out whether or not mitochondrial content is being presented to the T-Cell
- Remember that OGDH is the mitochondrial content being tested for in this case

- Measure activation of T-Cell Hybridoma
  - Wt: low amounts of presentation but these cells wtill have Pink1 and Parkin so it is logical that MitAP be blocked
  - Pink1 KO: great 5 fold increase in MitAP→ no Pink1 to block it
  - Parkin KO: also showed MitAp activation but not as strong as the one seen for Pink1



Summary of MitAP

- Mitochondrial antigens exit in vesicles dependent of Snx9 and Rab9
- Parkin actively inhibits Snx9 by ubiquitination and targeting of Rab9 to the proteasome
- Heat stress and oxidative stress recruit different regulatory machineries for their MDV formation
  - Different types of stress will recruit different MDV machineries
  - A lot to do still
- MDVs are delivered to the endosome dependent of Rab7
- Peptides are translocated into the cytosol:
  - Proteasome will deliver them to the ER for MHC I presentation
  - They can also be presented through the endosome

How do our findings affect our understanding of Parkinson's Disease?

- We now know that Pink1/Parkin are key regulators of immunity through MitAP
- Dopaminergic neurons express MHC I
  - Infection or fever could potentially trigger MitAP in these dopaminergic neurons because these patients have defective Pink1/Parkin
  - Recruitment of killer T-cells to the neurons through MitAP→ destruction of these neurons
- Not sure if dopaminergic neurons expressing MHC I leads to the proposed pathway above, however research is currently being conducted