Tracking autophagic PI3P

For the Medical Student Project Thesis
University of Oslo

Axel Norgren Onsager
Dept. of Biochemistry, the Norwegian Radium Hospital,
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Tutor: Professor Harald Stennmark, PhD, Kay Schink, PhD

1. ACKNOWLEDGEMENTS

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I would also like to thank all the group members for providing a warm, stimulating and friendly atmosphere in the lab. I will also thank my family and friends for their patience and support.

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2. INDEX

3. SUMMARY

Background: The origin and genesis of the autophagosomal membrane is still a matter of debate. There are indications that the phospholipid phosphatidylinositol 3-phosphate (PI3P) may play a pivotal role in early formative stages, by acting as a recruiter of secondary proteins containing PI3P binding FYVE domains (including DFCP1). This project seeks to investigate the role of PI3P in autophagy; where it is located, when it is needed, and how autophagy (as measured by readouts of the autophagosome marker protein LC3) can be regulated by varying levels of PI3P.

Methods: Confocal fluorescencet microscopy, Live Cell Imaging, Fixed cell imaging/ Immunofluorescence(IF), standard biochemical assays, Western blots etc. Cloning methods are used to attach fluorescent markers (GFP, RFP etc) to proteins containing FYVE domains (2XFYVE, DFCP1 etc) and LC3. Their movement and localisation, colocalisation etc. is tracked by live imaging and fixed cell immunofluorescence (IF) using Zeiss Confocal 710 and 780 microscopes, as well as wide field Deltavision microscopy. Autophagy in cells (mainly hTERT-RPE1) is induced by incubation in salt solution (EBSS) for approx. 2h. Site-spec ific, inducible depletion of PI3P at the ER-membrane is achieved by using an ER-targeted phosphatase (MTMR1) in a Rapalogue-system. Quantification of autophagic activity is measured by ImageJ on Western Blots against LC3 (main autophagic marker), and by quantifying LC3 spot formation using manual counting and automated ScanR analysis.

Results:

-Little colocalization is observed between 2XFYVE and LC3
-Starvation induces spot formation of DFCP1 in U2OS and RPE1 cells
-DFCP1 and 2XFYVE label distinct PI3P-pools
-Overexpression of truncated DFCP1 inhibits autophagy

Conclusions and future implications

1) 2XFYVE does not colocalize with autophagy related PI3P-pools
2) DFCP1 and 2XFYVE recognise distinct PI3P pools.
3) Blocking of ER-localised PI3P by overexpression of truncated DFCP1 can reduce autophagy.

By using a targeted Rapalogue system, one future aim is to study the different pools of PI3P, i.e. endosomal pool vs ER pool, and find out which pool(s) is necessary for autophagy, and to what extent. If one could find ways to inhibit autophagy by specifically altering autophagic PI3P, this could have implications ranging from cancer therapy to optimal industrial production of recombinant proteins.

5. INTRODUCTION

5.1. Autophagy

*Autophagy**, or** autophagocytosis**,*** is a catabolic process involving the degradation of a cell's own components through the lysosomal machinery. Cells produce proteins and organelles, and when they are no longer needed, or become dysfunctional, it is important to get rid of them, otherwise the effects on the cell might be deleterious**.**

Different Types of Autophagy. Most significant and interesting of these, is so called "macroautophagy", which is basically what we think of when we speak of autophagy in general terms, often simply referred to as "autophagy".

Macroautophagy involves the enclosing of a portion of cytoplasm, including organelles, by a double membrane to form an autophagosome. The outer membrane of the autophagosome fuses with the lysosome, and the internal material is degraded in the autolysosome. Macroautophagy serves as an important mechanism to remove large protein aggregates, damaged organelles like mitochondria, and also to maintain a general energy balance in the cell, among other things

Microautophagy: Small pieces of the cytoplasm are directly engulfed by inward invagination of the lysosomal or late endosomal membrane.

Chaperone-mediated autophagy: Substrate proteins containing a KFERQ-like pentapeptide sequence are first recognized by cytosolic Hsc70 and cochaperones. Then they are translocated into the lysosomal lumen after binding with lysosomal Lamp-2A.After all three types of autophagy, the resultant degradation products can be used for different purposes, such as new protein synthesis, energy production, and gluconeogenesis.

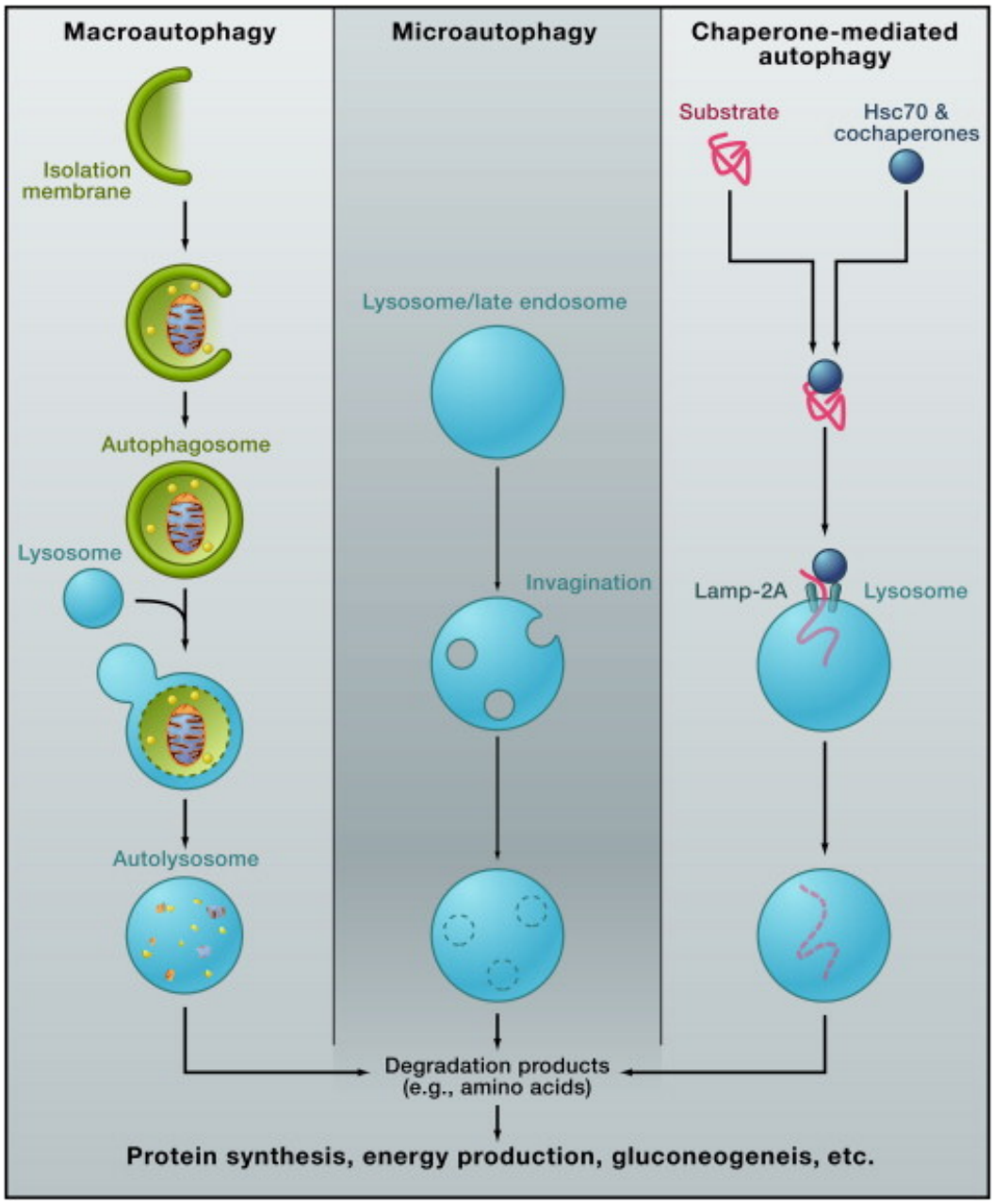

Fig. 1

Mizushima et al.Cell 2011 (Vol. 147, Issue 4, pp. 728-741

5.2 **Why is autophagy interesting from a medical perspective?**

- **Neurodegeneration** (intracellular protein aggregation: pTau/B-amyloid, a-synuclein, Huntingtin etc.)
- **Aging** (aging cells characterized by accumulation of "garbage", aggregates, dysfunctional mitochondria etc.)
- **Cancer** (cancer cells "hijack" autophagic machinery to increase survival)
- **Immunity/infection**, **heart and muscle disorders**

- Autophagy is an essential, basic cell mechanism, important for degradation of various cell components. Autophagy has been shown to be involved in a wide range of disease states, some examples include neurodegenerative disorders (Alzheimers, Parkinsons, Huntington´s etc), cancer, heart and muscle disorders, and overall ageing.

5.3 **Autophagy vs the scientific community**

The interest in and number of publications in the autophagy field is growing exponentially, as ever more implications of defective autophagy are discvered. Autophagy is involved in anyhing from neurodegenerative disease, to cancer to immunity.

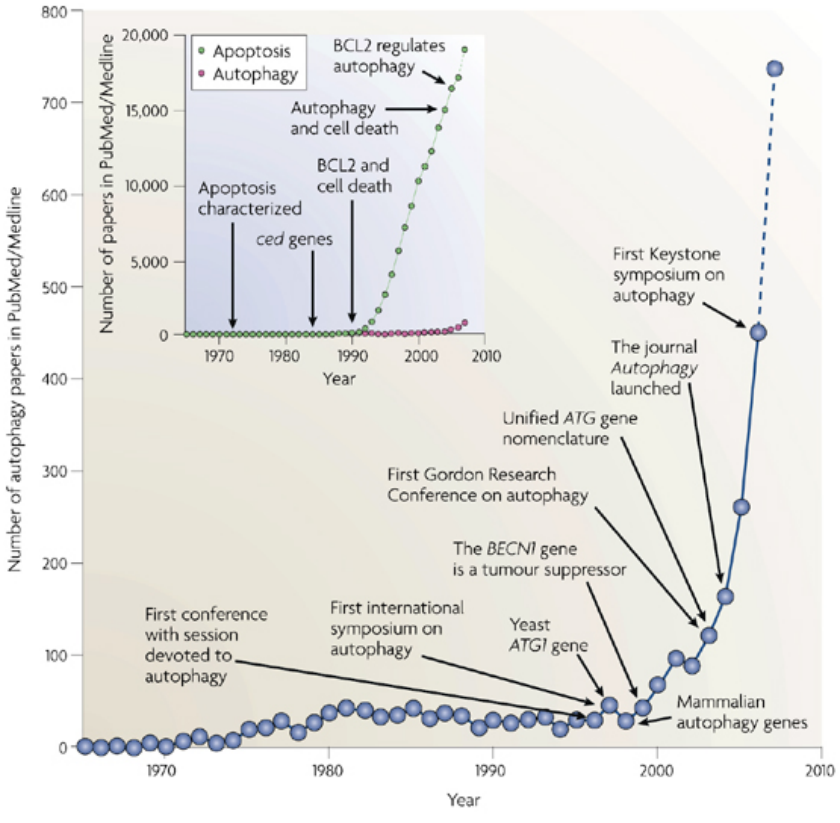

Fig 2. Nature reviews autophagy.

5.4 **Autophagy vs. Aging**

Well- Functioning autophagy seems to be very important for longevity and to avoid aging associated disorders like Alzheimers etc. Autophagy is also essential to clear out

dysfunctional mitochondria, which are believed to play an important role in aging, through the production of ROS.

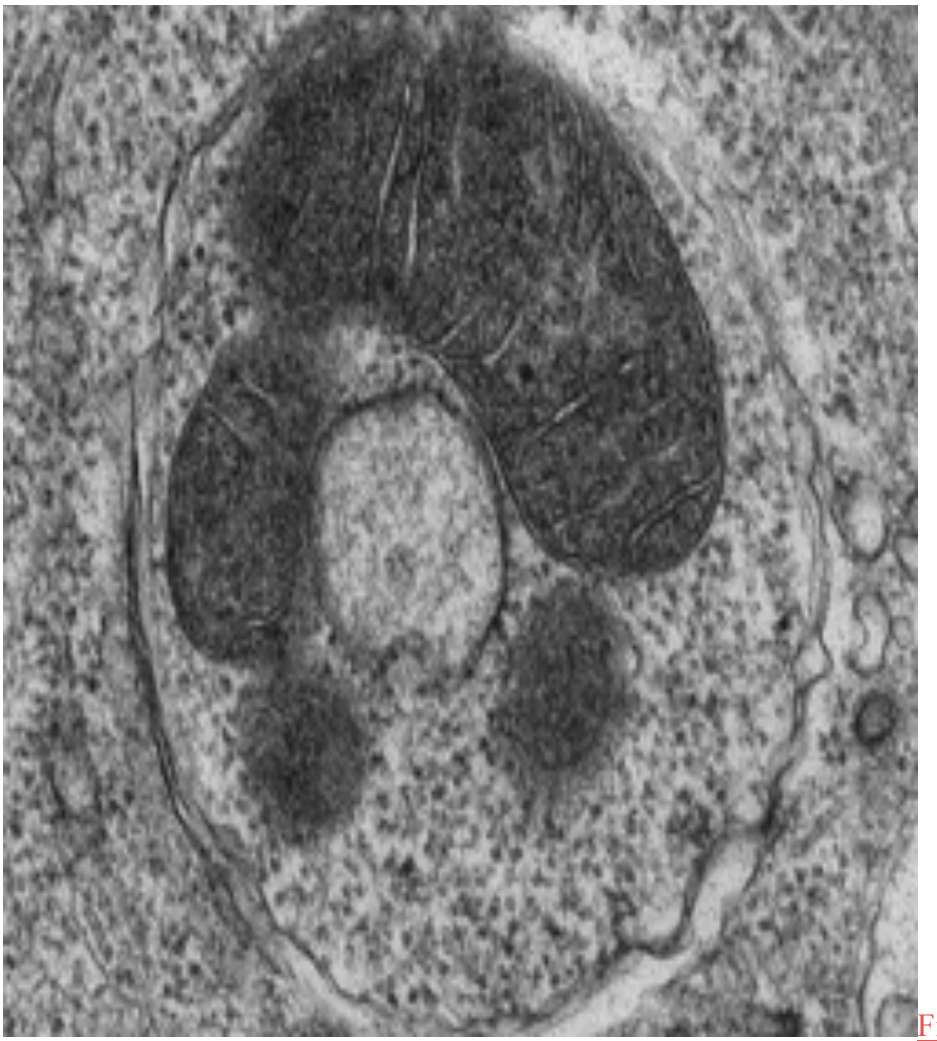

Fig 3. EM-Picture illustrating the engulfment of mitochondria by autophagic membrane. (Cell. 2011 Sep 2;146(5):682-95. **Autophagy and aging.**

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Caloric Restriction (CR) is the only proven method to enhance lifespan in a wide range of species. Basically, you limit the amount of calories, but still eat required vitamins and minerals. CR might be mediated through autophagy. KO of autophagy genes abolishes CR effect on lifespan. (reviewed Cell. 2011 Sep 2;146(5):682-95. **Autophagy and aging.**)

A large experiment involving CR in rhesus monkeys has been conducted in the US. (Mattison, J. A. *et al*. *Nature* http://dx.doi.org/10.1038/nature11432 (2012).). 20 years after the experiment began, the monkeys seemed to be showing many beneficial signs of caloric restriction, including significantly less diabetes, cancer heart and brain disease. When you limit caloric intake, autophagy is upregulated, meaning that some or all of these effects might be due to autophagy. However, no signifcant increase in the final lifespan was discovered in the monkeys in the experiment. Such an effect has been observed in several model organisms before:

- One study found **56 % increase** in average Drosophila lifespan upon enhancing expression of a single autophagy gene (atg8/LC3) (Simonsen et al, 2008).
- **Autophagy inducing drugs** (eg **Rapamycin**) known to increase lifespan in model organisms, including rodents.

5.5 **Origin of autophagosomal membrane**

An open question in the autophagy field is: The autophagosomes: where do they come from?

There are several candidates:

- Endoplasmid reticulum (ER)
- Golgi
- Plasma membrane
- Mitochondria

This is not fully clear at the moment, but ER is likely the most important. It would be reasonable to assume that there exists a flux of membran exchange between the different compartments.

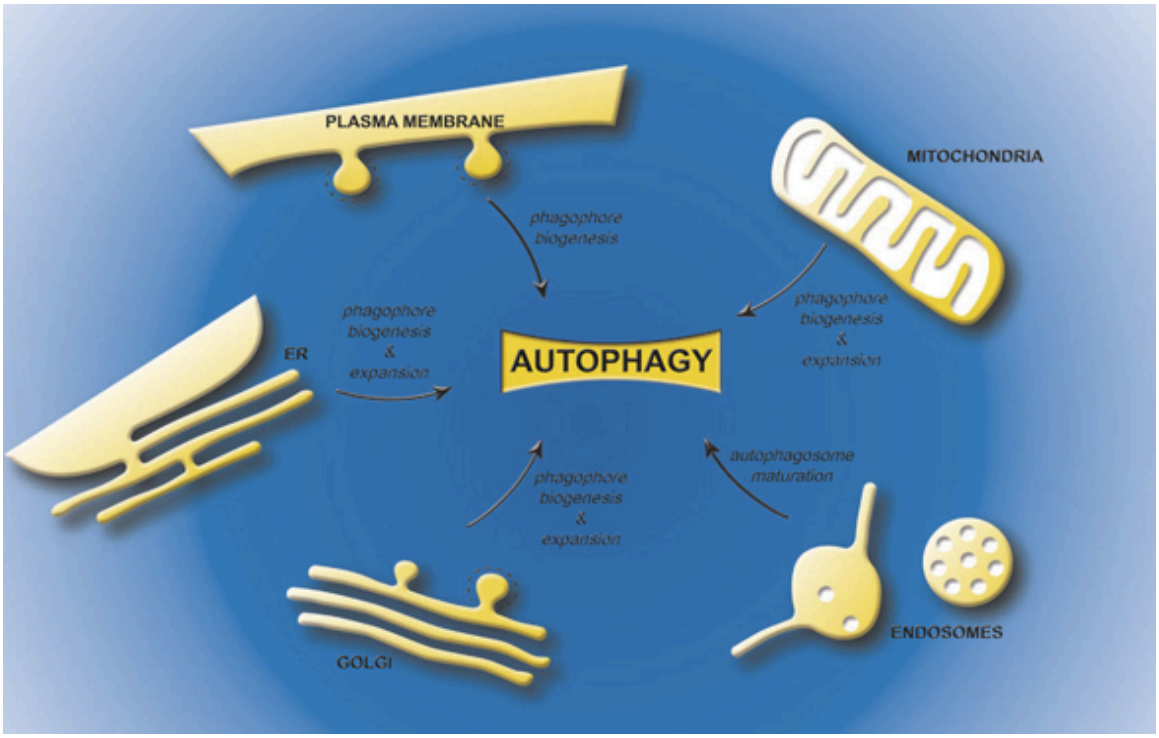

Fig.4 **Mizushima et al.Cell 2011 (Vol. 147, Issue 4, pp. 728-741.**

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5.6 **PI3P = Phosphatidylinositol 3-phosphate**

PI3P is a lipid in the cell membrane. Different hydroxyl groups on the inositol ring structure of the molecule can be phosphorylated, and when the third hydroxyl is the only phosphorylated hydroxyl, we have PI3P.

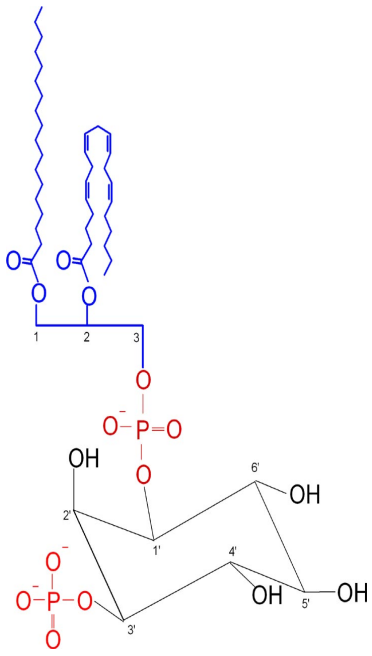


Fig 5. Own drawing

PI3P is:

- A phospholipid normally residing on early endosomes
- Involved in sorting and recycling of endocytic vesicles
- Member of phosphoinositide family, 7 isoforms, varying phosphorylation pattern.
- Proposed involvement in endosomal fusion, motility and sorting, and autophagy. Other functions possible.

Autophagy relevant PI3P is localized/synthesized de novo on ER membrane upon autophagy induction by various stimuli (i.e. aa starvation). It is formed by a special class 3 PI3Kinase-complex, consisting of several subunits including Vps34, Beclin, Vps 15 andAtg14.

Proteins containing FYVE-domains like DFCP1, WIPIs, 2XFYVE and P40PX bind to PI3P, as shown in the figure.

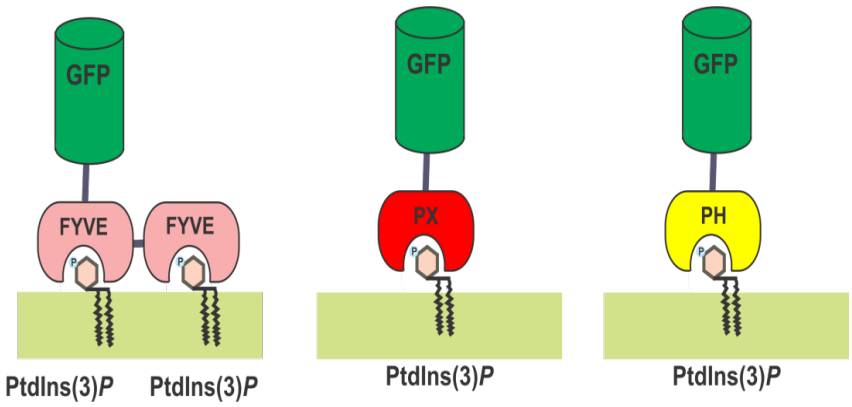


Fig 6. Own drawing.

5.6 **PI3P vs Autophagy- Necessary but not sufficient**

How do we know this? Starvation induced autophagy (as measured by increases in LC3 puncta) is inhibited by:

- Wortmannin (general PI3K-inhibitor)
- 3-MA (more specific PI3K-class III inhibitor),
- Knock-down by siRNA of single components of PI3K-III complex (Vps 34, Beclin 1) (Axe et al 2008)

  As mentioned above, there are several indications that PI3P is necessary for autophagy. The bottom line is: Inhibit the producer of PI3P/the kinase complex → reduced autophagy .

LC3 is a molecule more or less specific to autophagosomes, and hence used as a marker for the level of autophagy, see figure from Mizushima et al below.

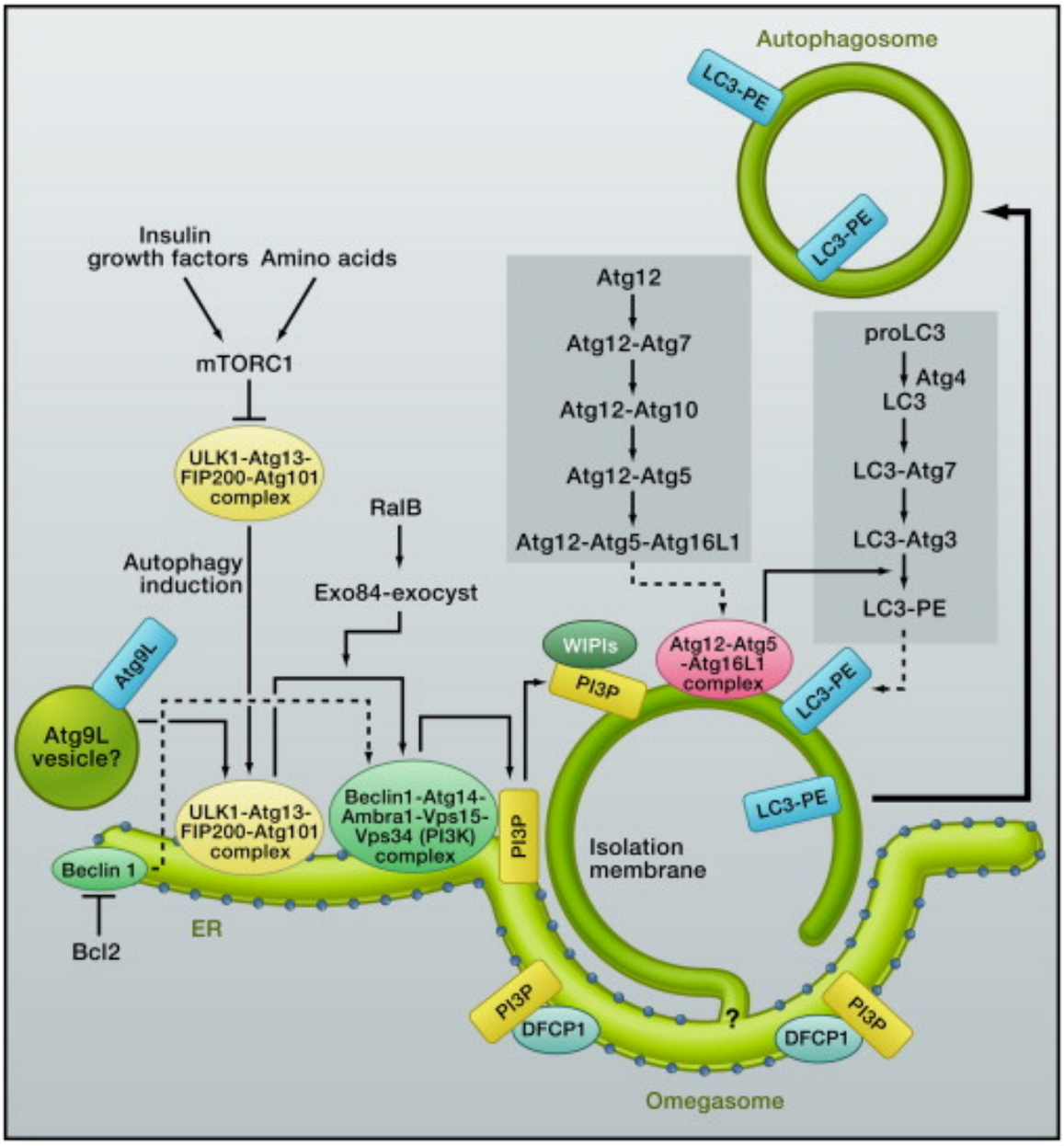


Fig 7. Source: Mizushima et al. Cell 2011

Schematic overview of the formation of the autophagosoe, above. As we can see, the initial formation o f the isolation membrane, the precursor of the autophagosome, is a rather complex event involving many different factor. PI3P is perceivmed to play a central role. PI3P on ER in theory acts as a Platform for Future Growth by Attracting secondary proteins ike DFCP1 and WIPI which again attract other protein complexes necessary for the formation of autophagosomes.

An excerpt from the article in question provides more detail:

“Autophagosome Formation and Atg Proteins in Mammalian Cells.

mTOR complex 1 (mTORC1) suppresses the ULK1 (Atg1 homolog) complex under nutrient-rich conditions. Upon autophagy induction, the ULK1 complex (including ULK1, Atg13, FIP200, and Atg101) is activated and translocates to a certain domain of the endoplasmic reticulum (ER). Once in the ER, the ULK1 complex regulates the class III phosphatidylinositol (PtdIns) 3-kinase complex (including Beclin 1, Atg14(L)/barkor, Vps15, Vps34, and Ambra1), and this regulation is promoted by RalB and an Exo84-containing exocyst complex. Recruitment of Beclin 1 to the PtdIns3-kinase complex is also sensitive to starvation; Beclin 1 forms a complex with ER-associated Bcl-2 under nutrient-rich conditions and is released upon phosphorylation of Bcl-2 by JNK1. Atg9L, a multimembrane-spanning protein, is also involved in an early stage of autophagosome formation (Atg9L is recruited likely on vesicles). Formation of PtdIns(3)P recruits double FYVE-containing protein 1 (DFCP1) and promotes the formation of the omegasome, from which autophagosomes appear to be generated. Other PtdIns(3)P-binding WIPI proteins (WD-repeat protein interacting with phosphoinoside) (Atg18 homologs) are also crucial for the maturation of the omegasome/isolation membrane. The Atg12–Atg5–Atg16L1 complex and the LC3 (Atg8 homolog)–phosphatidylethanolamine (PE) conjugate play important roles in the elongation and closure of the isolation membrane. The Atg12–Atg5–Atg16L1 complex is also required for formation of the covalent bond between LC3 and PE.”

It is hypothesized that autophagosome membrane can come from other sources aswell, including mitochondrial membrane, plasma membrane etc. My own theory is that all membranes in the cell are in more or less constant flux.

If we zoom in on the actual formation stage of the autophagosome, we see how the PI3Kinase complex influences the level of PI3P, and how different proteins like WIPIs and DFCP1 bind to PI3P, and again influence the formation of the isolation membrane, or autophagosome precursor.

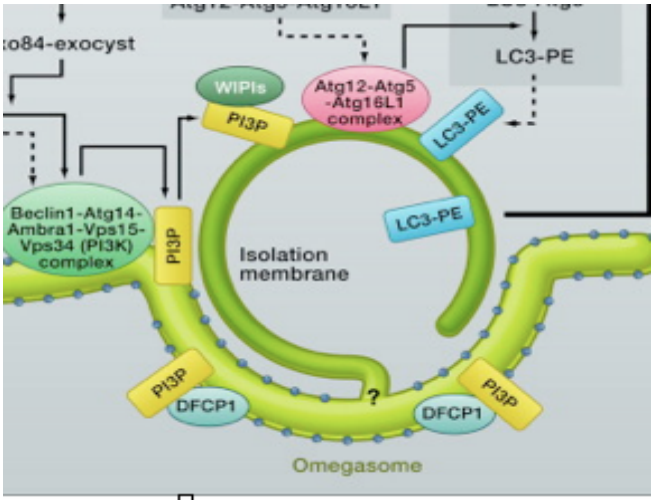


Fig 8. Source: Mizushima et al. Cell 2011

6. Objective: This project seeks to investigate the role of PI3P in autophagy; where it is located, when it is needed, and how autophagy (as measured by readouts of the protein LC3) can be regulated by varying levels of PI3P.

7. Methods: Summary: Confocal fluorescence microscopy, Live Cell Imaging, Fixed cell imaging/ Immunofluorescence(IF), standard biochemical assays, Western blots etc. Cloning methods are used to attach fluorescent markers (GFP, RFP etc) to proteins containing FYVE domains (2XFYVE, DFCP1 etc) and LC3. Their movement and localisation, colocalisation etc. is tracked by live imaging and fixed cell immunofluorescence (IF) using Zeiss Confocal 710 and 780 microscopes, as well as wide field Deltavision microscopy. Autophagy in cells (mainly hTERT-RPE1) is induced by incubation in salt solution (EBSS) for approx. 2h. Site-specific, inducible depletion of PI3P at the ER-membrane is achieved by using an ER-targeted phosphatase (MTMR1) in a Rapalogue-system. Quantification of autophagic activity is measured by ImageJ on Western Blots against LC3 (main autophagic marker), and by quantifying LC3 spot formation using manual counting and automated ScanR analysis.

7.1 **Cells**

Cells used were mainly hTERT-RPE1 cells. Autophagy in cells (mainly hTERT-RPE1) is induced by incubation in salt solution (EBSS) for approx. 2h

7.2 **Western Blotting**

Western blotting is an analytical technique used to quantify and detect the amount of a specific protein in a sample. Quantification of autophagic activity is measured by ImageJ on

Western Blots against LC3 (main autophagic marker), and by quantifying LC3 spot formation using manual counting and automated ScanR analysis.

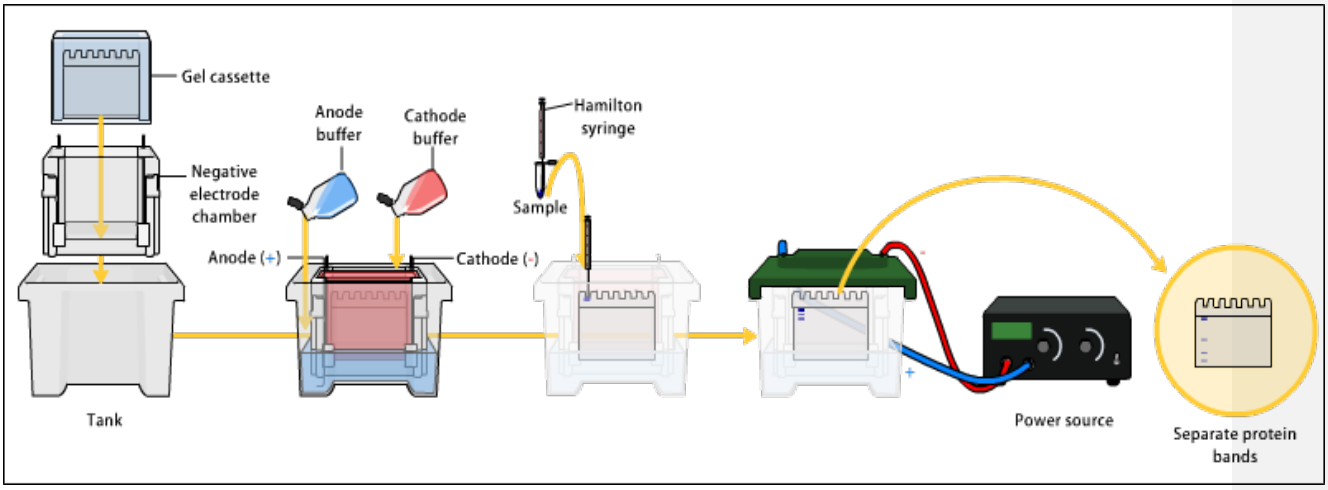


Fig 9: An illustration, showing schematically the process of Western blotting. Source: http://en.wikipedia.org/wiki/Western_blot

7.3 **Methods of Tracking PI3P / Microscopy**

How can we track the actions of PI3P? We need special probes binding selectively to PI3P. Proteins containing FYVE domains bind more or less specifically to PI3P.

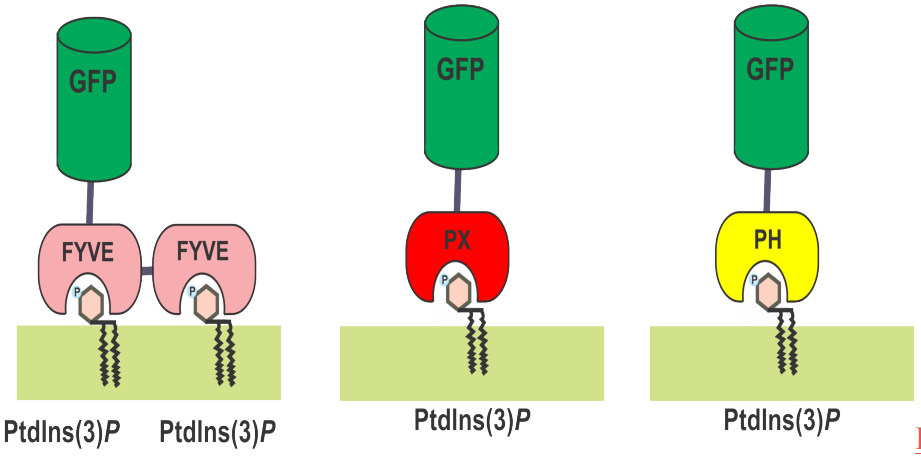


Fig 10. Own drawing

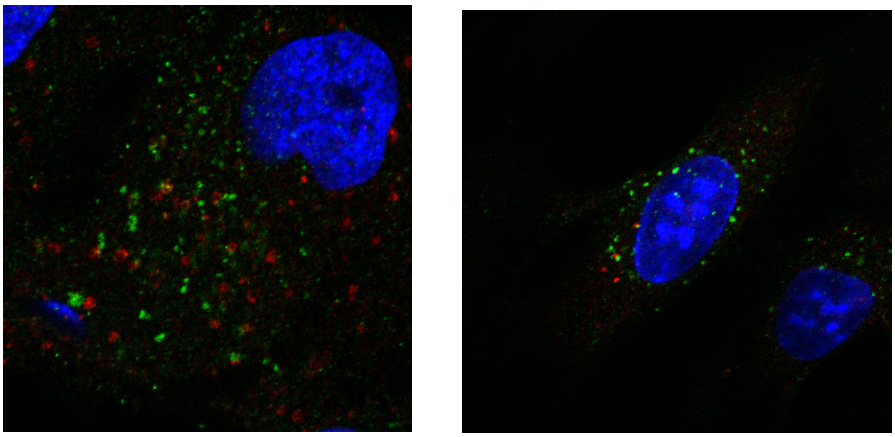
- Attach fluorescent markers to proteins, GFP family: GFP, RFP, YFP etc. using cloning methods. to proteins containing FYVE domains (2XFYVE, DFCP1 etc) and LC3. Their movement and localisation, colocalisation etc. is tracked by live imaging

and fixed cell immunofluorescence (IF) using Zeiss Confocal 710 and 780 microscopes, as well as wide field Deltavision microscopy.

- Fluorescence microscopy in live and fixed cells.
- Zeiss LSM710/780 confocal microscope, (+ Deltavision/wide field microscopy)
- Simultaneous staining for specific autophagosome markers, (LC3, Atg5), ER markers like VapA. Stain for autophagosomal markers, like LC3, look for colocalization and stain structural markers in the ER and other cell compartments to get a sense of where we are…

8**. Results**

- **1) Little colocalisation between LC3 and 2XFYVE**



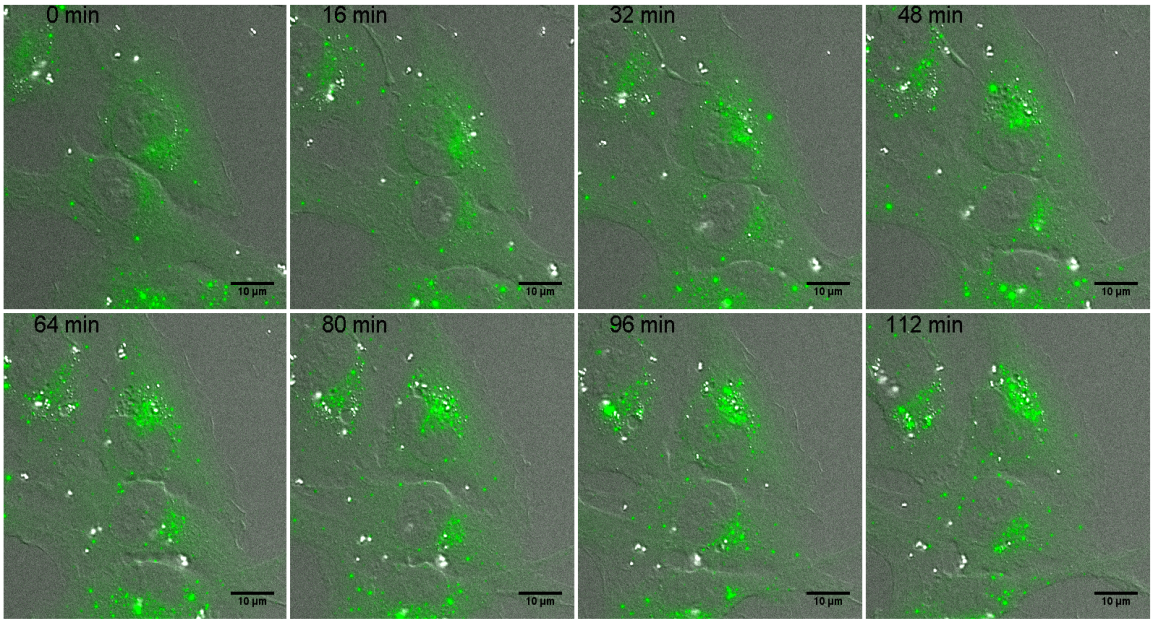
(Starved RPE1-2XFYVE cells, LC3, 2XFYVE-GFP). Non-starved/Fed control sample

IF pictures of induced autophagy by starvation for 2h50min (in saline solution (EBSS)).. Autophagosomes are visualized by using antibodies against the protein LC3 in red, the most common marker for autophagosomes. Strong autophagy induction as seen in increase of red LC3 dots

GFP labelled 2XFYVE proteins that are supposed to bind PI3P show no colocalisation with the LC3. Other group (Axe et al) showed colocalisation between LC3 and a couple of other FYVE containing proteins, DFCP1and ER-FYVE. 2XFyve, unlike these other two proteins, lacks an ER targeting domain, which might explain the results as the relevant PI3P is supposed to be on the ER. Cell lines used were mammalian RPE1 cells (meOH fixation

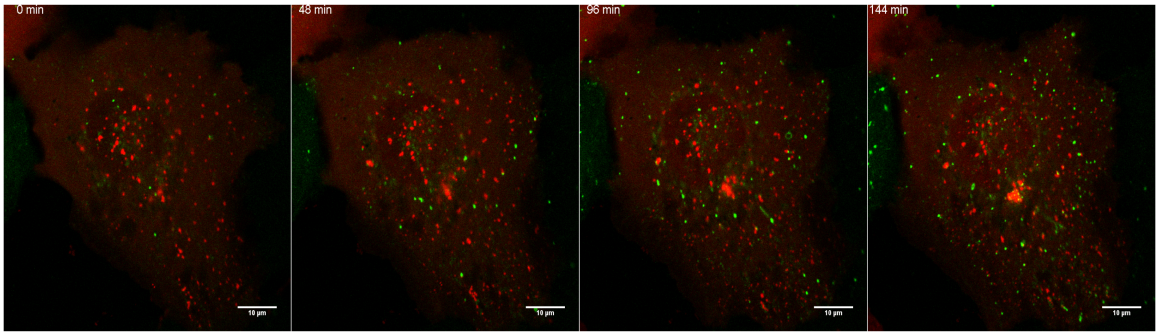
(primary anti-lc3 rabbit, secondary antibody dy555(red), anti-gfp, cy2(green)). A control of fed cells were used for comparison.

2) Starvation induces spot formation of DFCP1 in U2OS and RPE1 cells

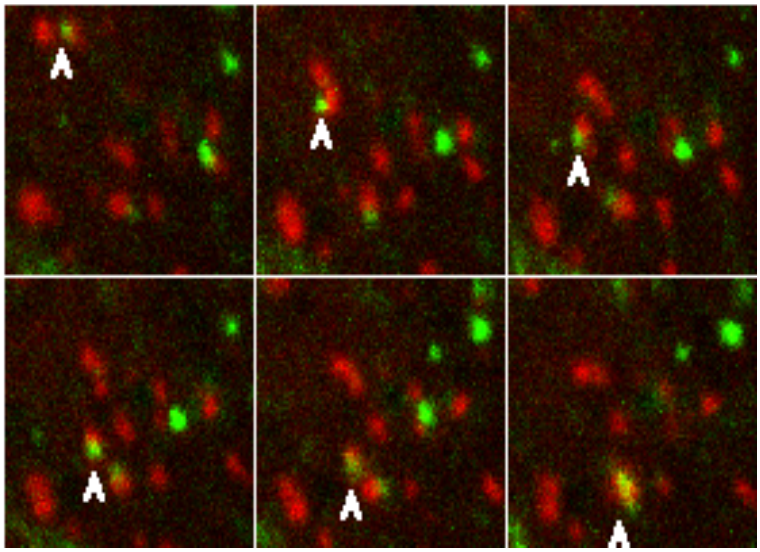


U2OS and RPE1 cells were transduced with lentiviruses expressing DFCP1 under control of the weak PGK promoter. These stable cells expressing GFP-DFCP1 were starved live 2h, using EBSS.

3) DFCP1 and 2XFYVE label distinct PI3P-pools



Live microscopy of U2OS cells expressing GFP-DFCP1 and mCherry-2xFYVE under starvation conditions. Little colocalisation between the two putative PI3P binding proteins could be observed.



DFCP1 and 2XFYVE recognize two separate but related pools of PI3P, as associating structures are seen.

4) Overexpression of truncated DFCP1 inhibits autophagy

To further elucidate the role of DFCP1, we created a C-terminal construct by molecular cloning, containing only the 2xFYVE and ER-binding domain. We analyzed the effect on autophagy by overexpressing this truncated version in RPE1 and U2OS cells, and running Western Blots Immunofluorescence microscopy runs. The idea was that the truncated version containing only ER-targeting domain plus2XFYVE would bind to PI3P on the ER and "block" downstream autophagy.

WT DFCP1

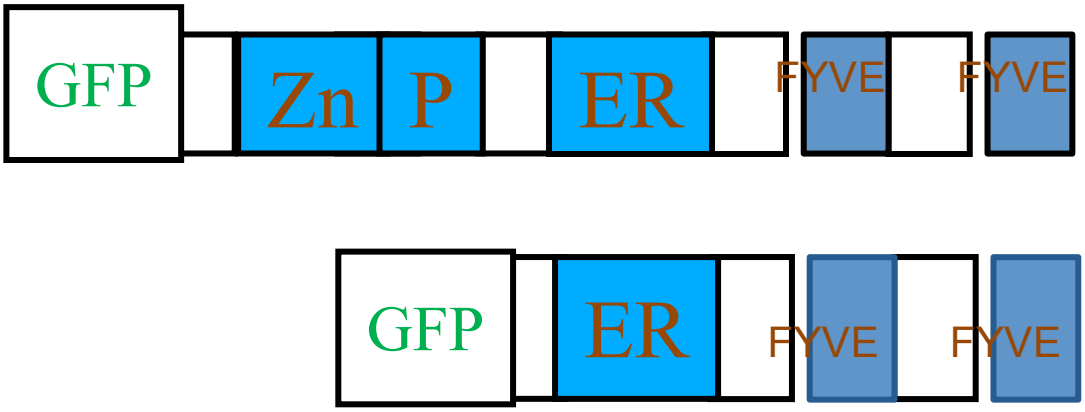
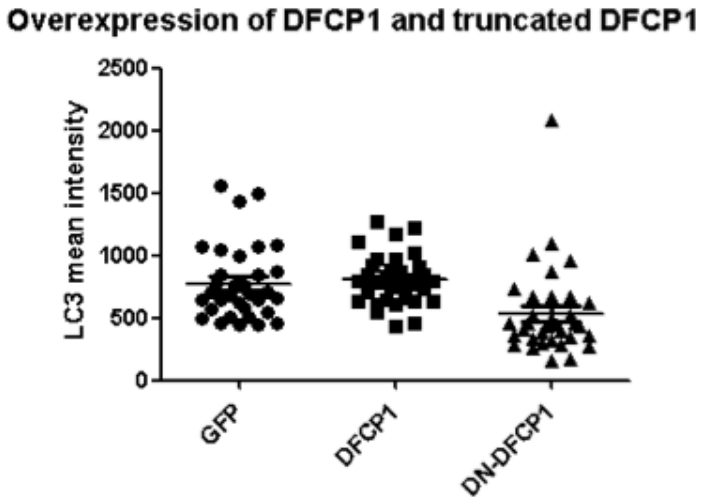


Fig 11 own drawing



I did a small-scale quantification to verify this: Using ImageJ on 34 transfected sample cells from each type from ScanR pictures, I measured the LC3 mean intensity, and using statistical software, we came up with the following data. We see statistically lower LC3 values in cells transfected (48h) w/ truncated DFCP1. No effect seen on cells transfected with full length protein. It is claimed in the literature that overexpression of WT DFCP1 inhibits autophagy. (Axe et al, 2008)

Initial Western Blots seemed to confirm that less autophagy was going on in cells overexpressing truncated DFCP1. Western Blots were supportive of the Immunofluorescence/ScanR, showing an inhibitive effect on autophagy from overexpression of the truncated DFCP

9. **Conclusions:**

1) 2XFYVE does not colocalize with autophagy related PI3P-pools

2) DFCP1 and 2XFYVE recognise distinct PI3P pools

3) Blocking of ER-localised PI3P by overexpression of truncated DFCP1 can reduce autophagy

10. Possible future work to further elucidate role of PI3P in autophagy:

PI3P level manipulation:

I) Reduce amount of PI3P

II) Increase amount of PI3P

I) Targeted reduction of PI3P: ER Targeted Phosphatase

Analyse the role of ER-localized PI3P for autophagy ➔ construct finished, functional test follows.

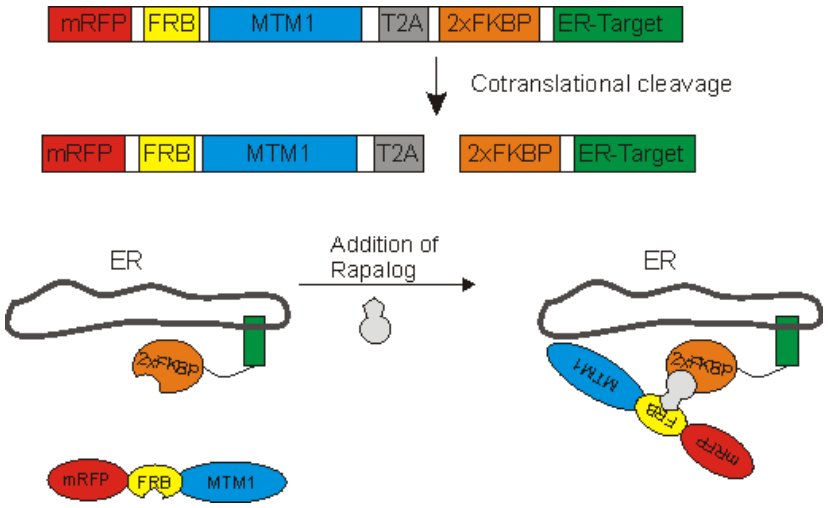


Fig 12 own drawing

The construct we made contains a phosphatase that, in theory, specifically targets ER localised PI3P and converts it into PIP by removing a phosphate group.

II) Targeted increase of PI3P levels (planned)

The question is: how can we artificially but nondisruptively increase the concentration of a specific phospholipid, like PI3P. In the past, membrane-

permeant phospho- inositide derivatives using bioactivatable protecting groups have been successfully developed.

These compounds have the negative charges masked with acetoxymethyl ester groups that permit passive cell entry. Once inside cells, endogenous esterases and lipases efficiently remove these groups, thereby regenerating negative charges and biological activity.

'Caged' derivatives are even more sophisticated— they are similar derivatives carrying a photoactivatable protecting group that prevents biological activity until its removal by a flash of light.» ie Membrane-permeant caged PI3P (Schultz et al, 2010)

PI3P containing photoactivatable protecting group, cleaved by photoactivation, would possibly enable region-specific upregulation of PI3P.

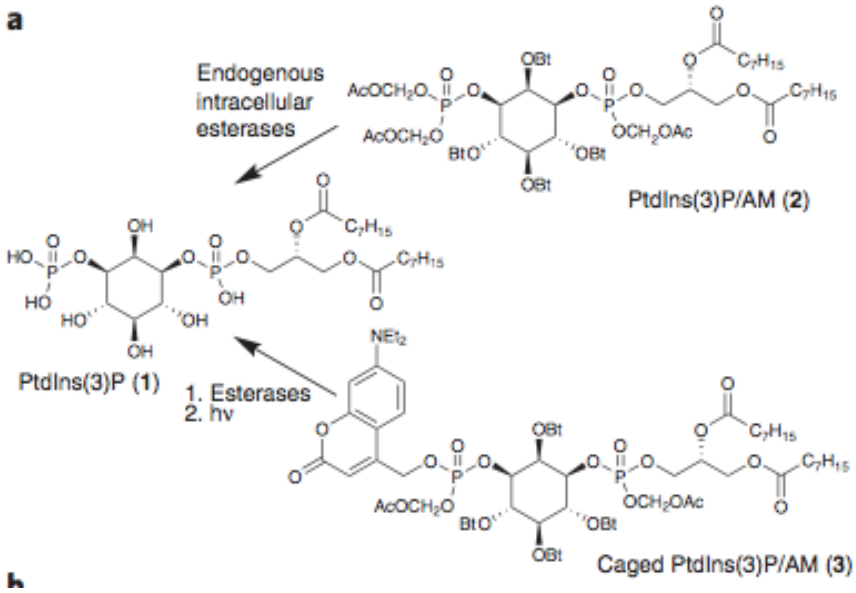


Fig 13. (Schultz et al, 2010)

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