Ego3, a regulator of TORC1 signaling, is degraded by the intralumenal fragment pathway

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ABSTRACT

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Target of rapamycin complex 1 (TORC1) is a conserved protein kinase complex whose activity controls metabolism in eukaryotic cells. To function, TORC1 and its regulators are localized to lysosome membranes. The mechanism(s) that control TOR complex protein lifetimes remain enigmatic. The IntraLumenal Fragment (ILF) pathway is a selective protein degradation pathway that involves lysosomes. Thus, I hypothesized that components of TORC1, EGO and SEA complexes may be degraded by this ILF pathway.

Using the yeast *S. cerevisiae* and its vacuolar lysosome (or vacuole) as models, I tested this hypothesis by first imaging live cells by fluorescence microscopy. I found that several subunits of TORC1, EGO and SEA complexes tagged with GFP are sorted into boundaries between docked vacuoles and accumulate within the vacuole lumen. However, when I isolated vacuoles from cells and repeated this experiment in vitro, I found that nearly all components re-localized to endosomes. The only exception was Ego3, a subunit that tethers the EGO complex to vacuole membranes. I then applied rapamycin, an inhibitor of TOR, to induce TOR protein downregulation and degradation. In vivo, I found that rapamycin stimulated sorting of TOR complex . Whereas, in vitro, rapamycin only enhanced sorting, internalization and degradation of Ego3.

In conclusion, many components of TOR signaling complexes seem to be degraded by the ILF pathway including Ego3. Thus, I speculate that the ILF pathway may play an important role in downregulating TOR activity.

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LIST OF ABBREVIATIONS

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AMPK	AMP-activated protein kinase
ATP	adenosine triphosphate
CHX	cycloheximide
DAG	diacylglycerol
DDM	*n*-Dodecyl β-D-maltoside
DMSO	Dimethyl Sulfoxide
EGO	Exit from G_0
F.I.	fusion inhibitors
FI	fluorescence intensity
FM4-64	*N*-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide (lipophilic dye for fluorescence microscopy)
FoxO	Forkhead box class O
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescence protein
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOPS	homotypic fusion and vacuole protein sorting
ILF	intralumenal fragment

PI3K	phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl Fluoride
RAP	rapamycin
Raptor	regulatory-associated protein of TOR
SC	synthetic complete
SEA	Seh1-associated
SEM	standard error mean
TIRF	total internal reflection fluorescence
(m)TOR	(mechanistic) target or rapamycin
mTORC1/2	mechanistic target of rapamycin complex ½
MVB	Multivesicular body
Ub	ubiquitin
UBD	ubiquitin binding domain
UPP	Ubiquitin Proteasome Pathway
YPD	yeast extract peptone dextrose

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INTRODUCTION

In all organisms, from yeast to humans, aging represents the accumulation of physical and physiological changes of an individual over time (Bowen et al., 2004). In humans, aging is among the largest risk factors for disease (Holroyd et al., 2008; Christensen et al, 2009; Dilling et al., 2014). Researchers have been investigating the molecular basis of aging for decades, and the underlying mechanisms are beginning to be revealed, although its cause and valid strategies to prevent this process are still largely unknown. At the cellular level, aging is promoted by an accumulation of damage overtime within the cell, ultimately leading to dysfunction and cell death. Currently, one of the most widely accepted aging theory involves the target of rapamycin (TOR) pathway, mainly target of rapamycin complex one (TORC1), a key regulator of cellular metabolism (Evans et al., 2011; McCormick et al., 2011). This theory involves the regulation of autophagy by TORC1. When TORC1 is active, its inhibition of autophagy leads an accumulation of toxic materials such as protein aggregates and damaged organelles therefore causes cell death (Budanov et al., 2010). Since TORC1 is an important player in aging, the study of its degradation, and proteins that regulate its activity, may give us an insight into the mechanism by which the cell downregulates TORC1 signaling therefore impacting the cellular aging program.

Target of Rapamycin Complex 1(TORC1) and Aging

TORC1 is a highly conserved signaling complex found in all eukaryotic species from yeast to humans. It functions as a mediator between external stimuli such as caloric input and cell growth and proliferation (Kim et al., 2002; Hay and Sonenberg, 2004; Gonzalez and Rallis, 2017). To be activated, all components of TORC1 must assemble into a functional complex on lysosome (or vacuole in yeast) membranes. In term of acidic digestive compartments, the yeast vacuole and the lysosome are analogous. They also both recycle degradation products to replenish cellular storage. However, unlike lysosome, the vacuole serves as a storage of nutrients such as amino acids, metal ions, and metabolic biproducts (Li and Kane, 2009).

It has been shown that when TORC1 is inhibited, the lifespan of several eukaryotic organisms is extended (Fabrizio et al., 2001; Harrison et al., 2009; Robida-Stubbs et al., 2012). For example, blocking TOR signaling extends lifespan in yeast, worms, flies and mice. Importantly, rapamycin, a TORC1 inhibitor, prevents age-related diseases and extends lifespan even in old mice. Rapamycin also delays decline in cognitive function if administered starting at a young age in mice. Caloric restriction is another way that can extend lifespan. Caloric restriction prolongs lifespan in yeast, worms, flies, rodents, and possibly primates. The life-prolonging effects of caloric restriction has been shown to be largely due to inhibition of TOR, as suggested by the findings that TORC1 inhibition mimics starvation and caloric restriction does not extend lifespan in yeast and flies if TORC1 signaling is defective (Hansen et al., 2007; Power et al., 2006) Moreover, knocking out S6K (ribosomal S6 kinase), a downstream substrate of TORC1, in mice correlates with extended life span and gives a similar phenotype to mice on calorie restricted diets (Selman et al., 2009). Whereas S6K overexpression renders *D. melanogaster* resistant to lifespan extension by rapamycin (Kapahi et al., 2009)

4E-BP (eukaryotic translation initiation factor 4E-binfding protein) is the other important downstream target of TORC1, that is also thought to play a role in lifespan extension (Zid et al., 2009). When phosphorylated by TORC1, 4E-BP dissociates from the translation machinery halting protein translation. Reduced protein synthesis has also been shown to extend lifespan in many species (Arnsburg et al., 2014). When not phosphorylated by TORC1, 4E-BP activates stress responsive genes, such as the tumor suppressor FoxO. These stress genes can exert a positive effect on lifespan by protecting cells and tissues from age-related damage, through transcriptional programs (Giannakou et al., 2008). For example, FoxO activates expression of glutamine synthetase (GS) and increased GS expression then inhibits TORC1 activity, representing a negative feedback loop. Furthermore, FoxO inhibits TORC1 signaling by inducing sestrins express which leads to activation of AMPK, a negative regulator of TORC1 signaling (Tettweiler et al., 2005). In all, it is clear that TORC1 inhibition leads to lifespan extension. Thus, it is of interest to identify the cellular processes that downregulate (or degrade) TOR signaling protein levels as a potential target in strategies that promote longevity.

Components of TORC1 singling in S. cerevisiae

In *S.cerevisiae*, TORC1 is composed of the serine/threonine protein kinase Tor1 or Tor2 bound to Kog1, Tco89, and Lst8. All of these components have mammalian orthologs except Tco89 (Loewith et al., 2002). Lst8 is a 36 kDa protein that has WD40 repeats and binds to the kinase domain of Tor1. Although its precise function is not fully understood, knockdown studies in mammals and yeast suggest that Lst8 also stimulates TORC1 signaling (Loewith et al., 2002; Chen and Kaiser, 2003; Kim et al., 2003). Kog1 (or Raptor in mammals), a seven WD-40 repeat and quadruple HEAT domain containing protein, is the largest component of TORC1 at 150 kDa. When Kog1/Raptor is knocked down in mammalian, worm, or fly cells, the phenotype mimics treatment with the TORC1 inhibitor rapamycin or depletion of Tor1 suggesting that Raptor stimulates mTOR signaling (Hara et al., 2002; Kim et al., 2002; Sarbassov et al., 2004). Kog1/Raptor interacts with Tor1 and its substrates S6K1 (ribosomal protein S6 kinase 1) and 4EBP1 (eukaryotic initiation factor 4E binding protein 1). In turn, TORC1-mediated phosphorylation of 4EBP1, for example, stimulates downstream protein synthesis by inducing the release of 4EBP1 from eIF4E, allowing it to associate with other factors that drive cap-dependent translation to support cell growth (Kim et al., 2002; Foster et al., 2010). Tco89, although has no mammalian orthologs, is an important regulatory subunit of TORC1 in yeast. It mediates the control of the Exit from G0 complex (EGOC) of TORC1 (Loewith and Hall, 2011).

Upstream of TORC1, there are four major stimuli thought to be integrated by this signaling complex: growth factors, energy status, stress and nutrient availability. The mechanisms of involving the former three stimuli implicate the phosphatidylinositol 3-kinase (PI3K), Akt, TSC1/TSC2 and Rheb, a guanosine triphosphate (GTP)-binding protein in upstream signaling (Sarbassov et al., 2005). Nutrient availability, especially amino acids levels, on the other hand, involves stimulation of Rag GTPases (e.g. RagA – D in mammals) that activate TORC1 on lysosome membranes (Kim et al., 2008).

Specifically, amino acids are sensed upstream of the EGO complex (EGOC, orthologus Ragulator in mammals) which consists of Gtr1 (orthologous to RagA and RagB), Gtr2

(orthologous to RagC and RagD), Ego1 and Ego3 in S. cerevisiae, whereby Ego1 and Ego3 are membrane associated proteins that serve as important components of EGOC for its localization to the vacuole membranes for TORC1 stimulation (Binda et al., 2009). For example, the presence of amino acids promotes GTP-binding to Gtr1, mediated by the guanine exchange factor (GEF) Vps39, and GTP hydrolysis of Gtr2, rendering Gtr2::GDP, which together activate TORC1 (Binda et al., 2009). Conversely, activation of Gtr1 GTPase activity rendering Gtr1::GDP terminates TORC1 signaling. This is mediated by the two SEA complexes: SEACIT, containing Iml1, Npr2 and Npr3 subunits, acts the GTPase activating protein (GAP) for Gtr1 to inhibit TORC1 signaling, whereas SEACAT (containing Sea2, Sea3, Sea4, Sec13 and Seh1 subunits) inhibits SEACIT GAP activity, thus positively regulating TORC1 (Algret et al., 2014). Thus, I reasoned that altering the protein lifetimes of subunits with any of these four protein complexes, SEACAT, SEACIT, EGOC and TORC1, should influence TOR signaling. But currently it is unclear how TOR signaling proteins are degraded in all eukaryotic cells.

The ILF pathway and selective degradation of lysosome membrane proteins

Although the function of TORC1 and its regulatory protein complexes has been well studied, how components are degraded or downregulated is unknown. Ubiquitin Proteasome Pathway (UPP) is a well-conserved process in eukaryotic organisms responsible for selective degradation of soluble, cytoplasmic proteins (Hasselgren et al., 1997). Upon stimulation (e.g. phosphorylation or misfolding), a client protein is labeled with ubiquitin, a highly conserved and ubiquitously expressed small protein composed of 76 amino acids. Protein labeling involves three important enyzmes: the ubiquitin activating enzyme or E1 enzyme, the ubiquitin conjugating enzyme or E2 enzyme, and the ubiquitin ligase or E3 ligase. Ubiquitin-tagged client proteins are sorted into and degraded by the 26S proteasome, a large cylindrical multi-enzyme complex within the cytoplasm (Tu et al., 2012). However, the proteasome is primarily responsible for degradation of soluble, cytosolic proteins, not membrane-associated proteins, like many of the subunits of TORC1 and its regulatory complexes (Tu et al., 2012). Thus, it possible that another process is responsible for their degradation, like ones known to degrade lysosomal polytopic (i.e. membrane spanning) proteins.

Recently, a new cellular pathway responsible for selective lysosome membrane protein degradation was described in the model organism *Saccharomyces cerevisiae* called the ILF (intralumenal fragment) pathway: Immediately prior to homotypic fusion between vacuoles (yeast lysosomes), resident polytopic proteins are sorted into an area of membrane encircled by fusogenic lipids and proteins assembled in a ring at the vertex that forms the interface (or "boundary") between docked organelles (Wang et al., 2002; McNally et al., 2017). Upon lipid bilayer merger at the vertex ring, boundary membranes and the protein embedded in them are internalized as an intralumenal fragment that is then degraded by lumenal hydrolases. Although no membrane-associated proteins have been shown to be degraded by this pathway, it is feasible that they too can be sorted and entrapped in boundary membranes (Mattie et al., 2017), as the intermembrane distance between sandwiched membranes (8 nm) is wide enough to include proteins bound to the outer leaflets of the bilayers. Thus, I hypothesized that perhaps membrane-associated proteins within complexes responsible for TOR signaling may be sorted and degraded by the ILF pathway.

MATERIALS AND METHODS

Yeast strains and reagents

Strains listed in **Table 1** are*Saccharomyces cerevisiae*used in this study. All yeast cultures were grown at 30ºC in YPD media (1% yeast extract, 2% peptone, 2% dextrose), or synthetic complete minimal media (SC, 0.67% yeast nitrogen base, 2% dextrose, amino acids and vitamins as needed), unless otherwise indicated. Liquid cultures were grown with rotary agitation at 200 rpm for aeration, and 1% agar was added to the medium for colony growth on plates. Biochemical and yeast growth reagents used are acquired from either Sigma-Aldrich, Invitrogen or BioShop Canada Inc. Fusion reactions reagents used were prepared in 20 mM Pipes-KOH, pH 6.8, and 200 mM sorbitol (Pipes Sorbitol buffer, PS).

Fluorescence microscopy

Images presented in this study were captured using a Nikon Eclipse TiE inverted microscope equipped with a motorized laser TIRF illumination unit, Photometrics Evolve 512 EM-CCD camera, CFI ApoTIRF 1.49 NA 100x objective lens, and 488 nm or 561 nm (50 mW) solid-state lasers operated with Nikon Elements software. The brightness and contrast, inversion and sharpness of images shown were adjusted using ImageJ Software. ImageJ was also used to generate Fluorescence intensity profiles of GFP fluorescence.

FM4-64 was used to stain live yeast cells to label vacuole membranes and prepared for imaging using a pulse-chase method as previously described (Brett et al., 2008). 0.4 ml of a seed culture grown overnight in SC medium at 30ºC was added to 4ml of YPD medium containing 0.5 µl of a 5 mM FM4-64 stock in DMSO (50 µM final), then grown for 1 hour at 30ºC to allow cellular dye uptake. Afterwards, cells were pelleted at 4500 rpm for 1 minute, washed with 1 ml SC medium, resuspended in 3 ml SC medium, and grown for another 1-2 hours at 30ºC before imaging, allowing the accumulation of dye in vacuole compartments. For isolated vacuole imaging, 0.5 µl of FM4-64 stock was added to freshly isolated vacuoles, which were then

incubated at 27ºC for 10 min., and placed on ice prior to imaging, as in Wang et al. (2002). Preparing samples for the rapamycin treatment, live cells were treated with 7 μM rapamycin for 120 minutes at 30 °C after first hour of FM4-64 staining. Time-lapse videos were captured using a Chamlide TC-N incubator (Live Cell Instruments) at 30 °C with cells plated on coverslips coated with concavalin-A (1 mg/ml in 50 mM HEPES, pH 7.5, 20 mM calcium acetate, 1 mM $MnSO_4$).

Vacuole isolation and homotypic vacuole fusion

Vacuoles were isolated from yeast cells used the protocol previously described (Haas, 1995). Yeast cells were spheroplasted with Lyticase and centrifuged for 90 min. at 125,000 g on a 15/8/4/0% Ficoll® (GE Healthcare) gradient dissolved in PS buffer (20 mM Pipes-KOH pH 6.8, 200 mM Sorbitol). Isolated vacuoles were obtained from the 4%/0% interface, and quantified using the Bradford assay (Thermo Scientific), based on Bradford (1976). 6 μg of vacuoles isolated from GFP derivative strains were used in fusion reactions in standard fusion reaction buffer with 0.125 M KCl, 5 mM $MgCl_2$, 1 mM ATP and 10 μM CoA. FM4-64 was used to stain membrane of isolated vacuoles by treating vacuoles with 3 μM FM4-64 for 10 minutes at 27 °C. Reactions were incubated at 27 °C for 60 minutes, unless otherwise noted, and placed on ice prior to visualization by HILO microscopy. For rapamycin treatment, isolated vacuoles were pretreated for 15 minutes at 27 °C with 7 μM Rapamycin, then incubated for another 30 minutes.

Western blot analysis

Isolated vacuoles from yeast strains expressing a GFP-tagged vacuolar membrane protein was used for sample preparation. Fusion reactions were prepared as previously described. Samples were incubated for 0 (ice), or 60 minutes at 27 °C. After incubation, protease inhibitors (6.7 μM leupeptin, 33 μM pepstatin, 1 mM PMSF and 10.7 mM AEBSF), 1% DDM and 5X laemmli sample buffer were added. Samples were probed for α-GFP (Abcam, Cambridge, UK). All samples were repeated at least two times. GE Amersham Imager 600 was to acquire the images of blots by chemiluminescence. The acquired images were then edited and prepared using Adobe Photoshop and Illustrator CC software.

Data analysis and presentation

To ensure the reliability of data, both biological replicate and technical replicate were taken into account. Each experiment was repeated at least twice on different days (biological replicate) and a great number of micrographs were taken (n>20) for each GFP strain to ensure unbiasedness (technical replicate). For cumulative probability calculations, 50 vacuoles were used for each strain (n=50). For the purpose of quantitating isolated vacuole micrographs, I calculated the relative boundary membrane or lumenal GFP fluorescence using only docked vacuoles by measuring their surface area, the length of the boundary. Using ImageJ software, measurements of GFP fluorescence intensities were measured using a ROI 4x4 pixels in diameter and acquiring a fluorescence value on the outer vacuole membrane, within the lumen and on the boundary membrane. Data are reported as mean ± SEM. Student T-test is used the test the significance the data compared to control Vph1-GFP. Quantitative data was acquired using ImageJ software. Figures were prepared using Adobe Illustrator CC software.

Table 1. List of yeast strains used in this study

Strain	Genotype	Source
BY4741	*MATα his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*	Huh et al., 2003
Ego1-GFP	BY4741, *Ego1-GFP::HIS3MX*	Huh et al., 2003
Ego3-GFP	BY4741, *Ego3-GFP::HIS3MX*	Huh et al., 2003
Fet5-GFP	BY4741, *Fet5-GFP::HIS3MX*	Huh et al., 2003
Iml1-GFP	BY4741, *Iml1-GFP::HIS3MX*	Huh et al., 2003
Kog1-GFP	BY4741, *Kog1-GFP::HIS3MX*	Huh et al., 2003
Sea3-GFP	BY4741, *Sea3-GFP::HIS3MX*	Huh et al., 2003
Tco89-GFP	BY4741, *Tco89-GFP::HIS3MX*	Huh et al., 2003
Vph1-GFP	BY4741, *Vph1-GFP::HIS3MX*	Huh et al., 2003

RESULTS

EGO complex proteins Ego1 and Ego3 are sorted into the ILF pathway for degradation in live yeast cells

To test this hypothesis, I studied baker's yeast (*Saccharomyces cerevisiae)* and its vacuolar lysosome as models by examining the sorting, internalization and degradation of six different GFP-tagged components of TORC1 (Tco89, Kog1), EGO (Ego1, Ego3), SEACIT (Iml1) or SEACAT (Sea3) complexes during homotypic fusion events (see Figure 1). First, I studied live yeast cells grown to mid-log phase in rich medium (when TOR signaling is expected to be activated) using HiLo microscopy and examined the distribution of the GFP-tagged proteins on vacuole membranes stained with FM4-64 (Figure 2A). I found that all GFP-tagged proteins examined were sorted into boundary membranes with the exception of Kog1-GFP, a component of TORC1, which primarily localized to puncta adjacent to vacuole membranes, likely representing endosomal compartments.

ILF client proteins are recognized and actively sorted in to boundary membranes, and as such, must be enriched with boundary membranes. It is apparent when I first looked the FI plots (Figure 2A). Then I conducted line scan analysis (Figure 2B) to determine if any of the protein of my interest is an ILF client (i.e. enriched in the boundary). If proteins were enriched in the boundary, the intensity of GFP signal (represented by the peak of the blue curve in the graph) would be more than that of the FM4-64 signal (represented by the red curve). The line scan analysis suggests Ego1-GFP is enriched as the other proteins are uniformly distributed. Ego3-GFP also seems to be enriched although its not very clear. To further determine which TOR signaling proteins were ILF clients, I compared their distributions to those observed for Vph1-GFP, the stalk domain of the V-type H^+-ATPase protein complex that is known to be uniformly distributed on vacuole membranes, and Fet5-GFP, a membrane-bound iron/copper oxidase that is known to be excluded from boundary membranes (Wang et al., 2002; McNally et al., 2017). I expected that GFP-tagged proteins with significantly higher boundary fluorescence values than these proteins to be ILF clients. I found that, as compared to GFP-tagged Vph1 or Fth1, only boundary fluorescence of GFP-tagged EGO complex subunits Ego1 and Ego3 were higher (Figure 2C), confirming enrichment in boundary membranes suggesting that they are

constitutively degraded by the ILF pathway under unstimulated conditions in yeast cells collected from a culture growth to mid-log phase. Of the remaining proteins studied (besides Kog1-GFP), only Tco89 showed enough membrane fluorescence for subsequent reliable micrographic analysis. Although a small number of Tco89 shows enrichment in the boundary membrane (Figure 2A), its average distribution on two fusing vacuoles is more like that of Fet5-GFP (Figure 2B). This observation suggests Tco89 is not as actively sorted into the boundary membrane as Ego1 and Ego3.

Upon fusion, polytopic proteins embedded in boundary membranes should be internalized into the lumen. To confirm, I calculated Ego1-GFP, Ego3-GFP and Tco89-GFP lumenal fluorescence values and found that they were significantly higher than Vph1-GFP and Fet5-GFP (Figure 2D), again supporting that these proteins were internalized into the vacuole lumen by the ILF pathway. To confirm, I recorded vacuole fusion events with live yeast cells and found that Ego1-GFP and Ego3-GFP decorated ILFs formed during vacuole fusion (Figure 2E). I exclusively studied Ego1-GFP and Ego3-GFP as the FI of Tco89-GFP was too low for live cell video. I found that both protein is indeed internalized into the vacuolar lumen by forming intralumenal fragments during membrane fusion events over time (Figure 2E). This result is even more apparent when comparison is made with Vph1-GFP, a protein known to be degraded by the ILF pathway. Thus, I conclude that GFP-tagged Ego1 and Ego3, subunits of the EGO complex, seem to be clients of the ILF pathway that are constitutively turned over in unstimulated, actively growing cells. Furthermore, these results are the first to suggest that the ILF pathway may also degraded membrane-associated proteins as well as polytopic proteins.

EGO complex protein Ego3 is sorted into the ILF pathway in vitro

All of the molecular machinery underling the ILF pathway should copurify with isolated vacuoles, allowing further biochemical characterization in vitro (McNally et al., 2017). Thus, if Ego1 and Ego3 are degraded by the ILF pathway, then I reasoned that this process should be recapitulated in vitro. After isolating vacuoles, initiating fusion in vitro and examining GPF localization, I confirmed that Ego3-GFP is sorted into boundary membranes during homotypic

vacuoles fusion in vitro (Figure 3A). However, all other proteins studied, including Ego1-GFP, localized to puncta adjacent to vacuole membranes, resembling endosomal compartments (Figure 3A). These results were not entirely unexpected, as TOR signaling complex localization is known to be responsive to cytoplasmic amino acid or nutrient levels (González and Hall, 2017). Removing these organelles from the cytoplasm (and nutrients) likely mimics nutrient depravation, which has been shown to shunt TOR components from vacuole/lysosome to endosome membranes, to supposedly shut off TOR signaling (Hailey et al., 2010).

Because it continued to localize to boundary membranes, I further investigated Ego3-GFP internalization by recording its vacuole distribution over the course of the fusion reaction in vitro. I observed Ego3-GFP is indeed internalized into the lumen of fusing vacuoles over time (Figure 3B). I then confirmed that Ego3-GFP was found in boundary membranes and accumulated in the lumen of vacuoles over the course of the fusion reaction by calculating the GFP fluorescence of Ego3-GFP over time within the lumen relative to the total fluorescence (Figure 3C). Maximum lumenal fluorescence was observed at 30 minutes, as it seemed to decrease afterwards (at 60 minutes). This loss of signal is likely a product of GFP protein degradation in the vacuole, accounting for the observed loss of signal. Regardless, based on these results, I conclude that, unlike other TOR signaling proteins, Ego3 downregulation may not involve relocation to endosome membranes (possibly in response to changing cytoplasmic nutrient levels), but rather is mediated by sorting into the ILF pathway for degradation.

Ego3 degradation by the ILF pathway is stimulated by the TOR inhibitor rapamycin

Having established that the ILF pathways can contribute to TOR signal protein downregulation under unstimulated conditions, I next determined whether it also mediates protein degradation when TOR signaling is inactivated by the inhibitor rapamycin. First, using HiLo microscopy, I examined the distribution of the GFP-tagged TOR proteins on vacuole membranes after 7 μM rapamycin treatment in living cells and in vitro (Figure 4A). The concentration of rapamycin I used in this study was used in another study of our lab (McNally and Brett, 2017) where no growth abnormality was observed. In live cells, I found that all GFP-tagged proteins were found

in boundary membranes, including Kog1-GFP, which was found on puncta – presumably representing endosomal compartments – in untreated cells (see Figure 2A), suggesting that perhaps upon activation, all TOR proteins could be degraded by this pathway. However, when I examined isolated vacuoles, I found that only Ego1-GFP and Ego3-GFP are enriched in boundary membranes after rapamycin treatment (Figure 4A and B). Other TOR proteins continue to localize to endosomal compartments, as observed in the absence of rapamycin (Figure 2A), suggesting that rapamycin does not drive the relocation of these proteins to vacuole membranes for degradation by the ILF pathway. An alternatively explanation is that rapamycin perhaps cannot override TOR relocation to endosomes in response to nutrient withdrawal, which is consistent with the interpretation that TOR is inactive on endosome membranes.

In the case of GFP-tagged Ego3, which seems to be constitutively degraded by the ILF pathway, I found that rapamycin treatment significantly increased boundary enrichment (Figure 4B) and lumenal fluorescence (Figure 4C), suggesting that TOR inhibition does indeed drive its degradation. Moreover, this response was selective to Ego3-GFP, as Vph1-GPF and Fet5-GPF degradation by the ILF pathway was unaffected by rapamycin treatment (Figure 4A – C). Finally, to demonstrate that Ego3-GFP protein degradation was stimulated by rapamycin treatment, I assessed GFP cleavage from Ego3-GFP by western blot analysis (Figure 4D). I found that more GFP was cleaved from Ego3 after TOR inactivation by rapamycin, supporting the conclusion that TOR inactivation by rapamycin stimulates Ego3 degradation by the ILF pathway. All things considered, these results show that the ILF pathway contributes to TOR signaling protein degradation, and this is stimulated by rapamycin.

DISCUSSION

In this study, I demonstrate with confidence that GFP-tagged Ego3, and possibly other components of TOR signaling protein complexes, is sorted into ILFs for degradation upon fusion of two vacuoles both in vivo and vitro. This seems to mediate constitutive turnover of Ego3 and is stimulated when TOR signaling was inhibited by rapamycin. Whereas other TOR signaling proteins studies are downregulated through relocalization to endosome membranes as previously reported. Why Ego3 downregulation differs from other proteins in this pathway, how this study improves our understanding of the ILF pathway, and possible physiological relevance in context tot longevity is discussed.

New mechanism for TORC1 inhibition

Since TORC1 is an important protein complex for regulating cell growth and metabolism, it is crucial to understand what regulate TORC1 itself. So far, there are three major ways that TORC1 can be regulated: through dephosphorylation of Sch9 (substrate of TORC1), EGO complex and feedback loop/ribosome biogenesis homeostasis (Loewith and Hall, 2011). Although the detail mechanism is different, these elements all regulate TORC1 in the same way, by activation or inhibition. Inhibition of TORC1 has major consequences on cell growth and metabolism. When TORC1 is inhibited, protein synthesis and ribosome biogenesis are greatly reduced. Stress responses, which are normally inhibited by TORC1, are active when TORC1 is turned off. Furthermore, nutrient uptake is also reduced when TORC1 is inhibited (Loewith and Hall, 2011). Knowing that TORC1 inhibition is responsible for regulating many cellular processes, what are some ways that TORC1 can be inhibited? Nitrogen starvation is a way that can inhibit TORC1 activity by reduction of Sch9 phosphorylation. Another way is using Rapamycin, a known TOR inhibition that mimics nitrogen starvation. Moreover, TORC1 regulator EGO complex can trigger its inhibition. In this study, I demonstrate the degradation of components of EGO, TORC1, and SEA complex directly by the ILF pathway might be another way the cell regulates TORC1 activity through inhibition. In my experiments, I observed Ego3 is degraded at a higher level compared to control when the cell is under rapamycin treatment, which mimics cellular

starvation. This result suggests that in response to starvation, the cell uses the ILF to degrade the components of the EGO, TORC1, and SEA, therefore downregulating TORC1 activity.

Although most components of EGO, TORC1 and SEA complex I studied is shown to be degraded in vivo, I find all of these components except Ego3-GFP is concentrated in endosomal compartments when studied in vitro. Previous studies suggest when components of TORC1 is inactive, they are sorted into endosomal compartments (Chiang et al., 2010). By isolating the vacuoles, these TORC1 components might be rendered inactive therefore sorted into endosomal compartments. There must be an important reason why Ego3-GFP is spared from this sorting process. So what is Ego3 and why is it important? Ego3 is a subunit of the EGO complex. This complex is located on the endosomal and vacuolar membranes and composed of Gtr1, Gtr2, Ego1, and Ego3 subunits. It has a crucial role in cell growth and autophagy regulation through relaying amino acid signals to activate TORC1 (De Virgilio et al., 2009). It was shown that Ego3 assumes a homodimeric structure similar to that of the mammalian MP1-p14 heterodimer and the C-terminal domains of the yeast Gtr1-Gtr2 heterodimer, both of which function in TORC1 signaling. This unique homodimer conformation of Ego3 is required for its binding to Ego1 and its recruitment to the vacuolar membrane. Furthermore, the Ego3 also has a binding site for Gtr1-Gtr2, suggesting it is an important mediator for docking of the Gtr GTPases to the vacuolar anchor Ego1 (Zhang et al., 2012). These finds suggest Ego has a scaffolding role within the EGO complex that is important for its regulatory function. However, it's worthy to note Ego3 also participate in other cellular processes. For example, it was shown that Ego3, also known as Slm4, also participate in endosomal-vacuolar trafficking of plasma membrane proteins (Wuttke et al., 2012). Although there are redundant proteins that perform endosomal-vacuolar trafficking such as Rcr2 and Vps20 (Wuttke et al., 2012), it is worthy in the future to study how Ego3 degradation by the ILF pathway affects endosomal-vacuolar trafficking. For purpose of further investigation of the regulation of TORC1 signaling by the ILF pathway, it is worthy to delete Ego3 to observe the effect of this deletion on TORC1 signaling. Moreover, cycloheximide, a known activator of TOR signalling (Hall et al., 2011), could be applied to isolated vacuoles to confirm the localization of components of TORC1 other than Ego3 is really caused by their inactivation.

ILF pathway also degrades soluble cytosolic proteins

Prior to this study, only polytopic proteins are shown to be degraded by the ILF pathway. For example, Fth1, an iron transporter that is embedded in the vacuolar membrane, was shown to be degraded by the ILF pathway (McNally and Brett, 2017). Here I show that the ILF pathway might also degrade cytoplasmic proteins if they are membrane soluble. Ego3 is a membrane soluble protein that has no transmembrane domain. It is has a very small size of 18 kDa (Zhang et al., 2012). In this study, I show that ILF pathway is able to degrade such protein. When two vacuoles are fusing, the distance between membranes at the contact zone is only around eight nm. Thus, a bulky protein is unlike to enter this confined space. Therefore, I speculate in order for a membrane soluble protein to be degraded by the ILF pathway, it has to be satisfy certain size requirement.

Another interesting conclusion can be made from the fact that ILF degrades membrane soluble proteins such as Ego3. Since the protein is membrane soluble, meaning it is the on the cytosolic side of the vacuolar membrane, the sorting machinery for ILF pathway must also be on the cytosolic side of the membrane. Then what is the mechanism behind the sorting of components of EGO, TORC1 and SEA for degradation by the ILF pathway? Although I did not investigate the mechanism in this study, a previous study done by our laboratory suggests a molecular sieving mechanism is involved (McNally et al., 2017). More precisely, this study suggests a ubiquitin-based mechanism is involved in the sorting for proteins into the ILF pathway (Figure 5). Ubiquitin is a highly conserved and ubiquitously expressed small protein composed of 76 amino acids. The first step of labeling involves the attachment of ubiquitin to a target protein. This process involves three important enyzmes: the ubiquitin activating enzyme or E1 enzyme, the ubiquitin conjugating enzyme or E2 enzyme, and the ubiquitin ligase or E3 ligase. In the case of ILF pathway, evident suggests Sna4 is an important mediator protein for the target protein and ubiquitin ligase.If ubiquitylation mediates degradation, labeled proteins must be recognized at the vertex ring for sorting into the ILF pathway and these three important proteins containing partial potential ubiquitin binding domains (UBDs) are involved: Vps11, Vps18, and Vps39 (Nickerson et al., 2009). These proteins along with three other subunits form

HOPS, the lysosomal multisubunit tethering complex important for MVB-lysosome and homotypic lysosome fusion (Balderhaar and Ungermann, 2013). HOPS is an important mediator for multiple fusion events at the yeast vacuole, including homotypic vacuole fusion, which is important for the ILF pathway. For future studies, in order to understand better the degradation of Ego3, it is worthy to investigate the exact mechanism behind its labeling.

Physiological relevance

It is known TORC1 is a nutrient sensor and growth controller that plays an essential role in aging. One of mechanism that Tor controls nutrient level in the cell is changing nutrient transporter expression on the lysosomal membrane (McNally and Brett, 2017). Since the ILF pathway both degrades lysosomal nutrient transports and components of TORC1 and its regulators, the degradation pathway might play a mediator role between nutrient availability and aging. The pathway could control cell growth by changing the availability of nutrient transporters on the vacuolar membrane following TORC1 signalling. Although speculative at this point, it is worthy to further investigate the involvement of the ILF pathway in aging in the future.

Cancers are very common aging disorders. Many types of cancers increase significantly with age since aging has many negative effects on cellular processes including metabolism and waste disposal. For example, prostate cancers are the most frequent cancer in older males compared to younger males (Hsing et Chokkalingam, 2006). As for older females, breast cancers are the most frequent compared to younger females (Gaffield et al., 2009). Since ILF could participate in regulation of cellular aging, it is possible to speculate it could be a potential target for cancer treatments.

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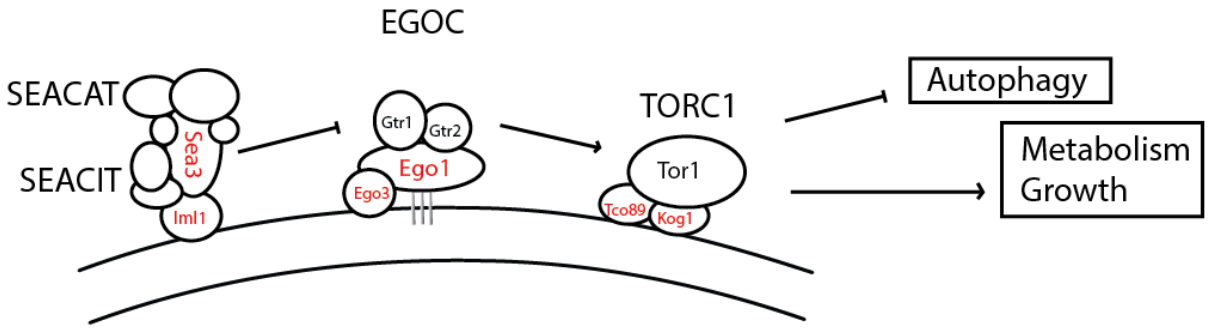
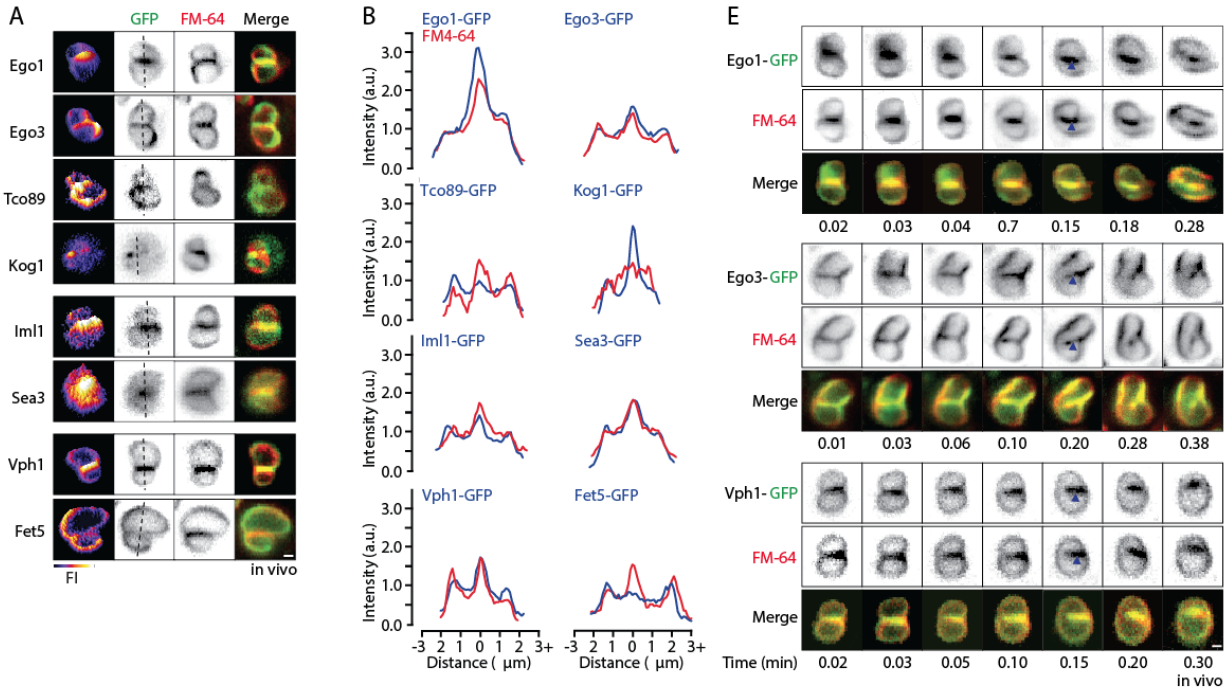
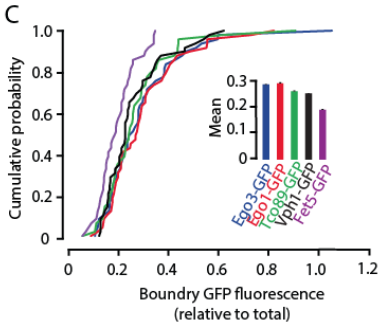


Figure 1. Overview of TORC1 signaling in *S.cerevisiae*. The proteins of interest of this study are highlighted in red.


A
GFP
FM-64
Merge
Ego1
Ego3
Tco89
Kog1
Iml1
Sea3
Vph1
Fet5
FI
in vivo
B
Ego1-GFP
FM4-64
Ego3-GFP
Tco89-GFP
Kog1-GFP
Iml1-GFP
Sea3-GFP
Vph1-GFP
Fet5-GFP
Intensity (a.u.)
Distance (µm)
E
Ego1-GFP
FM-64
Merge
0.02 0.03 0.04 0.7 0.15 0.18 0.28
Ego3-GFP
FM-64
Merge
0.01 0.03 0.06 0.10 0.20 0.28 0.38
Vph1-GFP
FM-64
Merge
Time (min) 0.02 0.03 0.05 0.10 0.15 0.20 0.30
in vivo


C
Cumulative probability
Boundry GFP fluorescence
(relative to total)
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Ego3-GFP
Ego1-GFP
Tco89-GFP
Vph1-GFP
Fet5-GFP

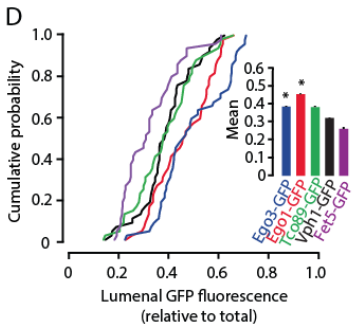

D
Cumulative probability
Lumenal GFP fluorescence
(relative to total)
Mean
Ego3-GFP
Ego1-GFP
Tco89-GFP
Vph1-GFP
Fet5-GFP

Figure 2. Components of EGO, TORC1 and SEA complex are constitutively degraded by ILF pathway *in vivo*

(A) Micrographs of *in vivo* yeast cells expressing six different GFP-labeled components of EGO, TORC1 and SEA complex illustrating their presence at the boundary of two fusing vacuoles except Kog1-GFP. GFP fluorescence intensity profile plots (left panel). Scale bars, 1 μm. **(B)** Line-scan analysis of GFP-labeled proteins shown in A along dotted line illustrating GFP (blue) or FM4-64 (red) fluorescence distribution. **(C)** Cumulative probability curves of GFP fluorescence intensity within the boundary membrane (relative to outside) for Ego1-GFP, Ego3-GFP and Tco89-GFP *in vivo* **(D)** Cumulative probability curves of lumenal GFP fluorescence intensity of of Ego1-GFP, Ego3-GFP and Tco89-GFP *in vivo*. When compared to Vph1-GFP using student t-test ($t < 0.05$), the lumenal fluorescence of Ego1-GFP ($t = 6.28 \times 10^{-8}$) and Ego3-GFP ($t = 0.004$) are significant higher. **(E)** Images captured from *in vivo* time-lapse videos of live yeast cells undergoing vacuole fusion, expressing Ego1-GFP, Ego3-GFP or Vph1-GFP stained with FM4-64 to label the vacuolar membrane.

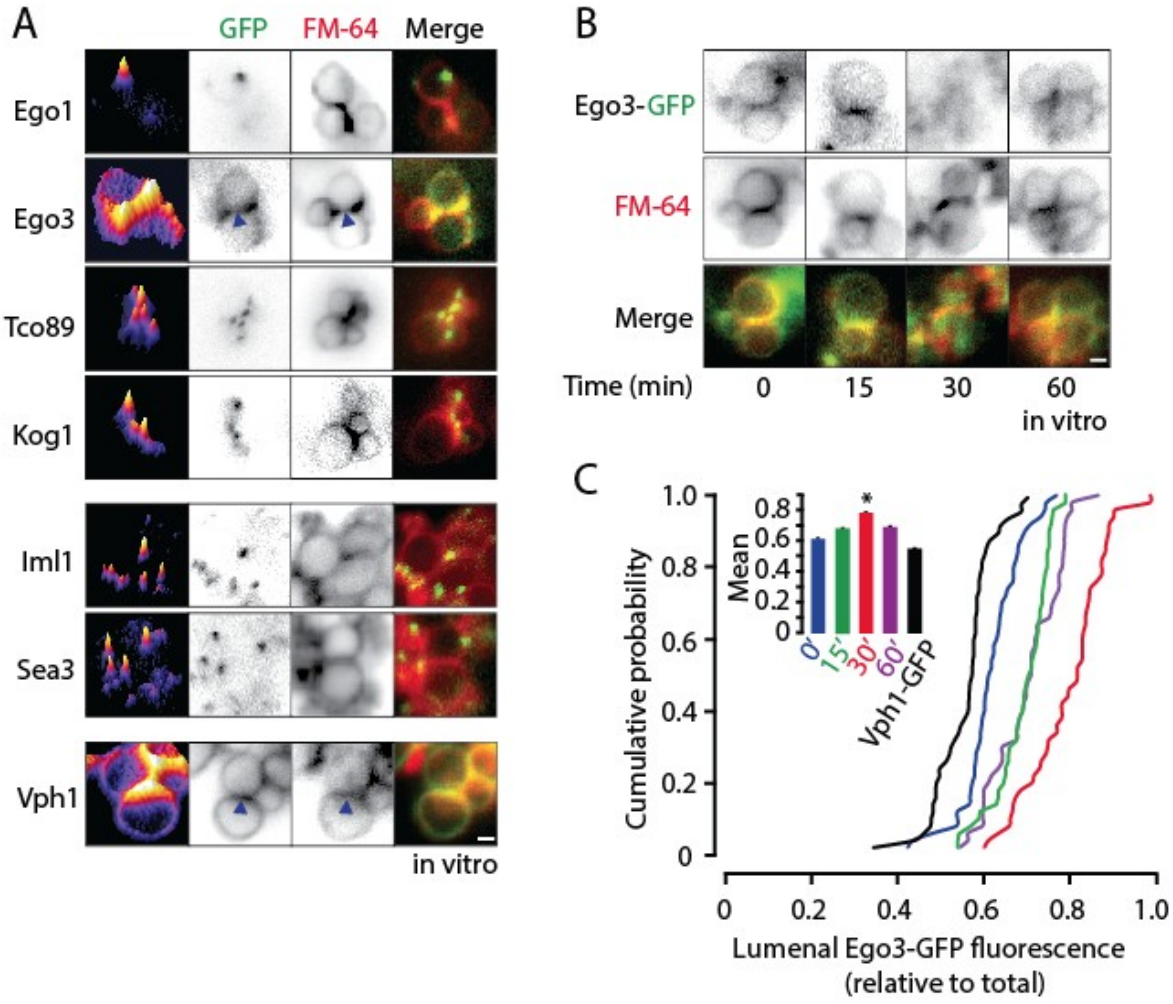

A
GFP
FM-64
Merge
Ego1
Ego3
Tco89
Kog1
Iml1
Sea3
Vph1
in vitro
B
Ego3-GFP
FM-64
Merge
Time (min)
0
15
30
60
in vitro
C
Cumulative probability
1.0
0.8
0.6
0.4
0.2
0
Mean
0.8
0.6
0.4
0.2
0
*
0'
15'
30'
60'
Vph1-GFP
0
0.2
0.4
0.6
0.8
1.0
Lumenal Ego3-GFP fluorescence
(relative to total)

Figure 3. Ego3-GFP is constitutively degraded by ILF pathway *in vitro*

(A) Micrographs of *in vitro* yeast cells expressing six different GFP-labeled components of EGO complex, TOR1 complex and SEA complex. Only Ego3-GFP is present at the boundary of two fusing vacuoles. The other GFP labeled proteins are concentrated in puncta. GFP fluorescence intensity profile plots (left panel). Scale bars, 1 μm. **(B)** Images captured from *in vitro* time-lapse videos of isolated vacuoles undergoing vacuole fusion, showing internalization of Ego3-GFP into the lumen of two fusing vacuoles. **(C)** Cumulative probability curves of lumenal GFP fluorescence intensity of isolated vacuoles containing Ego3-GFP measured after 0, 15, 30, 60 minutes. When compared to Vph1-GFP using student t-test ($t < 0.05$), the lumenal fluorescence of Ego3-GFP (3.42×10^{-6}) at 30mins is significant higher.

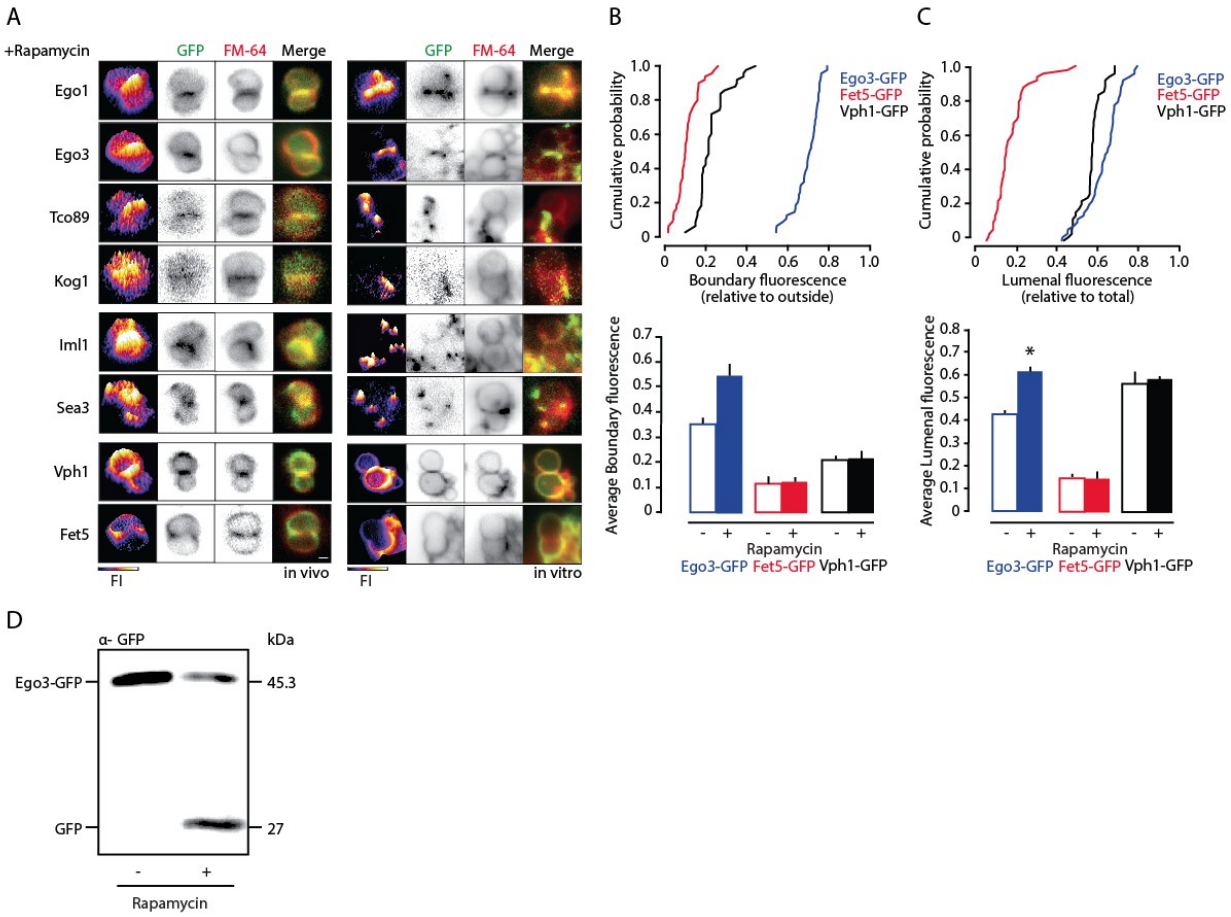
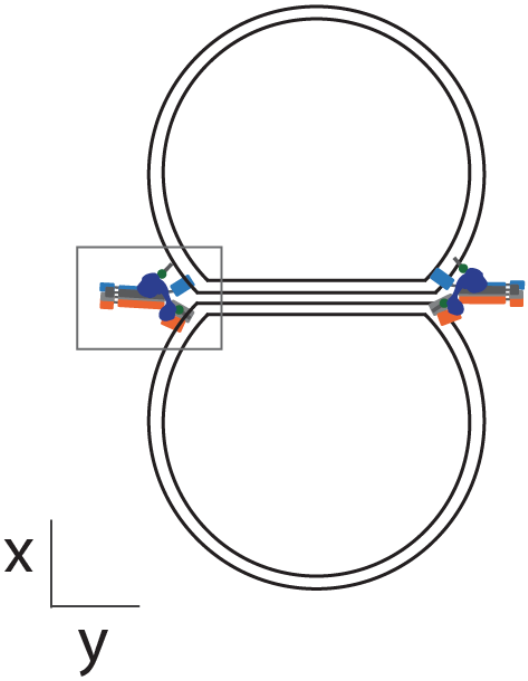

A
+Rapamycin
GFP
FM-64
Merge
Ego1
Ego3
Tco89
Kog1
Iml1
Sea3
Vph1
Fet5
FI
in vivo
in vitro
B
Cumulative probability
Boundary fluorescence
(relative to outside)
Ego3-GFP
Fet5-GFP
Vph1-GFP
Average Boundary fluorescence
Rapamycin
C
Lumenal fluorescence
(relative to total)
Average Lumenal fluorescence
*
D
α- GFP
kDa
Ego3-GFP
45.3
GFP
27
Rapamycin

Figure 4. The effect of Rapamycin on degradation of components of TORC1, EGO and SEA complex *in vivo and in vitro*

(A) Micrographs of *in vivo and in vitro* yeast cells expressing six different GFP-labeled components of EGO, SEA and TORC1 complex in the presence of Rapamycin. GFP fluorescence intensity profile plots (left panel). Scale bars, 1 μm. **(B)** Cumulative probability curves of GFP fluorescence intensity within the boundary membrane (relative to outside) for Ego3-GFP in the presence of Rapamycin *in vitro*. Bar graph showing average boundary fluoresce intensity of Ego3-GFP in the absence or presence of Rapamycin. **(C)** Cumulative probability curves of lumenal GFP fluorescence intensity of Ego3-GFP *in vitro*. Bar graph showing average lumenal fluoresce intensity of Ego3-GFP in the absence or presence of Rapamycin. When compared to Ego3-GFP under no rapamycin treatment using student t-test ($t < 0.05$), the lumenal fluorescence of Ego3-GFP under rapamycin treatment ($t = 1.19 \times 10^{-4}$) is significant higher. **(D)** Western blot analysis of Ego3-GFP degradation in the absence or presence of Rapamycin.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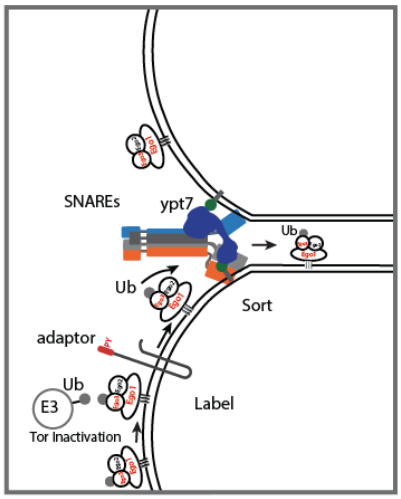
SNAREs
ypt7
Ub
Ub
Sort
adaptor
Ub
E3
Tor inactivation
Label

Figure 5. Proposed labeling and sorting of EGO complex by the ILF pathway. Following Tor inactivation, E3 ligase, with the help of a possible adaptor protein Sna4, labels Ego3 with ubiquitin. Once ubiquitinylated, Ego complex is recognized and sorted into the boundary of two fusing vacuoles by UBDs (not shown here) for degradation by the ILF pathway.