# Correlative Roles of Exocyst Complex and Septins in Autophagy

A thesis submitted for partial fulfillment of degree of

MASTER OF SCIENCE

As part of Integrated PhD program

(Biological Sciences)

By

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April 2017

## DECLARATION

I hereby declare that the work described in this thesis entitled **'Correlative roles of Exocyst complex and Septins in Autophagy'** is the result of investigations carried out by myself under the guidance of **Dr. Ravi Manjithaya** at Autophagy Laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore-560064, India. This work has not been submitted elsewhere for the award of any other degree.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described has been based on findings of other investigators. Any omission, which might have occurred by oversight or misjudgement, is regretted.

24th April, 2017 Sarika Chinchwadkar

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## CERTIFICATE

This is to certify that the work described in this thesis entitled **'Correlative roles of Exocyst complex and Septins in Autophagy'** is the result of investigations carried out by Ms. Sarika Chinchwadkar at Autophagy laboratory, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, under my supervision.

24th March, 2017

Dr. Ravi Manjithaya

# Acknowledgments

I take this opportunity to express my gratitude to all the people who have motivated, inspired and supported me throughout my MS thesis.

First and foremost I extend my gratitude to my supervisor Dr. Ravi Manjithaya for giving me an opportunity to work on this project. I am especially grateful to Sunaina for mentoring me throughout the course of my thesis. This work wouldn't have been possible without her support.

I express my warm thanks to Ramesh sir (Dr. GR Ramesh) for teaching me the basic and important aspects of research.

I take this opportunity to thank MBGU faculty members Prof. Uday Kumar Ranga, chairman, MBGU, Prof. M.R.S. Rao, Prof. Anuranjan Anand, Prof. Tapas Kumar Kundu, Prof. Hemlatha Balaram, Prof. Maneesha Inamdar, Prof. Namita Surolia, Dr. Kaustuv Sanyal and Dr. James Chelliah for their invaluable support.

I was lucky to have wonderful lab mates in Piyush, Suresh, Gaurav, Somya, Veena, Vidya, Greg, Vishwa Dr. Sreedevi, Dr. Lakshmi, Shashank, Anindita, Swati and our lab assistant Ramji. I am thankful to them for making my stay in the lab comfortable and for their indispensable assistance.

I thank all my batch mates and other friends in the department for making my stay at JNC joyful.

Last but not the least I thank my parents and my elder brother for their love and encouragement. All this would not have been possible without the support of my elder brother who has always motivated me and made me realize my potential.

**List of Abbreviations**

| Abbreviations | Full forms |
|---|---|
| kDa | Kilo Dalton |
| $^{O}C$ | Degree Celsius |
| µL | Microliter |
| M | Molar |
| Atg | Autophagy Related |
| CMA | Chaperone mediated autophagy |
| PT | Permissive temperature |
| NPT | Non permissive temperature |
| AP | Autophagosomes |
| GFP | Green Fluorescent protein |
| h | Hours |
| TOR | Target of Rapamycin |
| WT | Wild Type |
| Ts | Temperature sensitive |
| Cvt | Cytoplasm-to-Vacuole Transport |
| PE | Phosphatidylethanolamine |

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# SYNOPSIS

Autophagy is an evolutionarily conserved intracellular degradation process which maintains cellular homeostasis. This involves the capture and delivery of cytoplasmic contents by the hallmark organelles called autophagosomes to the vacuole/lysosome for degradation. Despite more than 50 years of investigations in the field of autophagy, the process of de novo autophagosome biogenesis is still enigmatic. The key questions regarding the origins and dynamic contribution of autophagosomal membranes for its biogenesis are poorly understood. A genetic screen performed in our lab revealed the involvement of two multisubunit protein complexes, Exocyst complex and Septins in early stages of autophagy. In depth studies on these complexes suggest their roles in autophagosome biogenesis, particularly in the trafficking of vesicles that contribute membrane to the growing autophagosome. Past studies have demonstrated a role for these complexes in exocytosis, membrane tethering and cytokinesis where they also interact with each other. As our lab has identified autophagy related roles for these complexes (exocyst and septin), my studies have explored the possibilities of these complexes interacting during autophagosome biogenesis.

I started by genomically tagging exocyst and septin subunits to be used for live cell imaging. Next, I determined the colocalization between exocyst components and septin under autophagic conditions. We found that these complexes interacted during autophagic conditions also. To further understand the correlative role of exocyst-septin interaction in autophagy, I studied association of complexes individually with autophagy protein Atg9 in the absence of the other functional complex. Using live cell microscopy, colocalization experiments between these complexes and autophagy proteins have shed light on the possible rearrangement of these complexes during autophagy that may be distinct from their canonical compositions.

# Chapter 1

# Introduction to Autophagy and Autophagosome Biogenesis

## 1.1 Autophagy

The term autophagy was coined by the Nobel laureate Christian De Duve in 1963 that means 'self-eating'[1].Autophagy is a bulk intracellular degradation process which involves sequestration of cytoplasmic contents into double membranous structures called autophagosomes (AP). Autophagosomes later fuse with the vacuole/lysosome wherein the cargo is degraded by acidic hydrolases and the resultant nutrients are then recycled into the cytoplasm. The process of autophagy is accomplished by interplay of a wide array of AuTophaGy-related (Atg) proteins. It is highly conserved across eukaryotes from yeast to mammals. Genetic studies in yeast have so far identified 40 different autophagy related genes (*ATG*)[2] and these studies have provided mechanistic understanding of the process of autophagy at the molecular level. There are three defined types of autophagy. They are microautophagy, macroautophagy and chaperone mediated autophagy (CMA). Common to all three types of autophagy is the proteolytic degradation of the cytoplasmic contents in the vacuole/lysosome. In microautophagy, cytoplasmic contents are directly gulped by the lysosomes through membrane invaginations/protrusions. Whereas, macroautophagy is mediated by the double membrane organelles called autophagosomes that sequester the cytosolic cargoes and subsequently fuses with the vacuole/lysosome to form autolysosomes/autophagolysosomes. The cargoes are degraded in the autolysosomes and nutrients are recycled into cytoplasm. Macroautophagy can be both general and selective in degrading specific organelles, proteins and pathogens. CMA is carried out only by mammalian cells. In CMA, the cargoes containing the specific KFERQ motif are recognized and unfolded by chaperones (such as Hsc-70) and later transported to the lysosome for degradation[3]. Macroautophagy is the most extensively studied of all the three types of autophagy and hereafter the term autophagy refers to macroautophagy.

## 1.2 Process of autophagy

The process of autophagy is divided into five sequential steps that are summarized below (Figure 1).

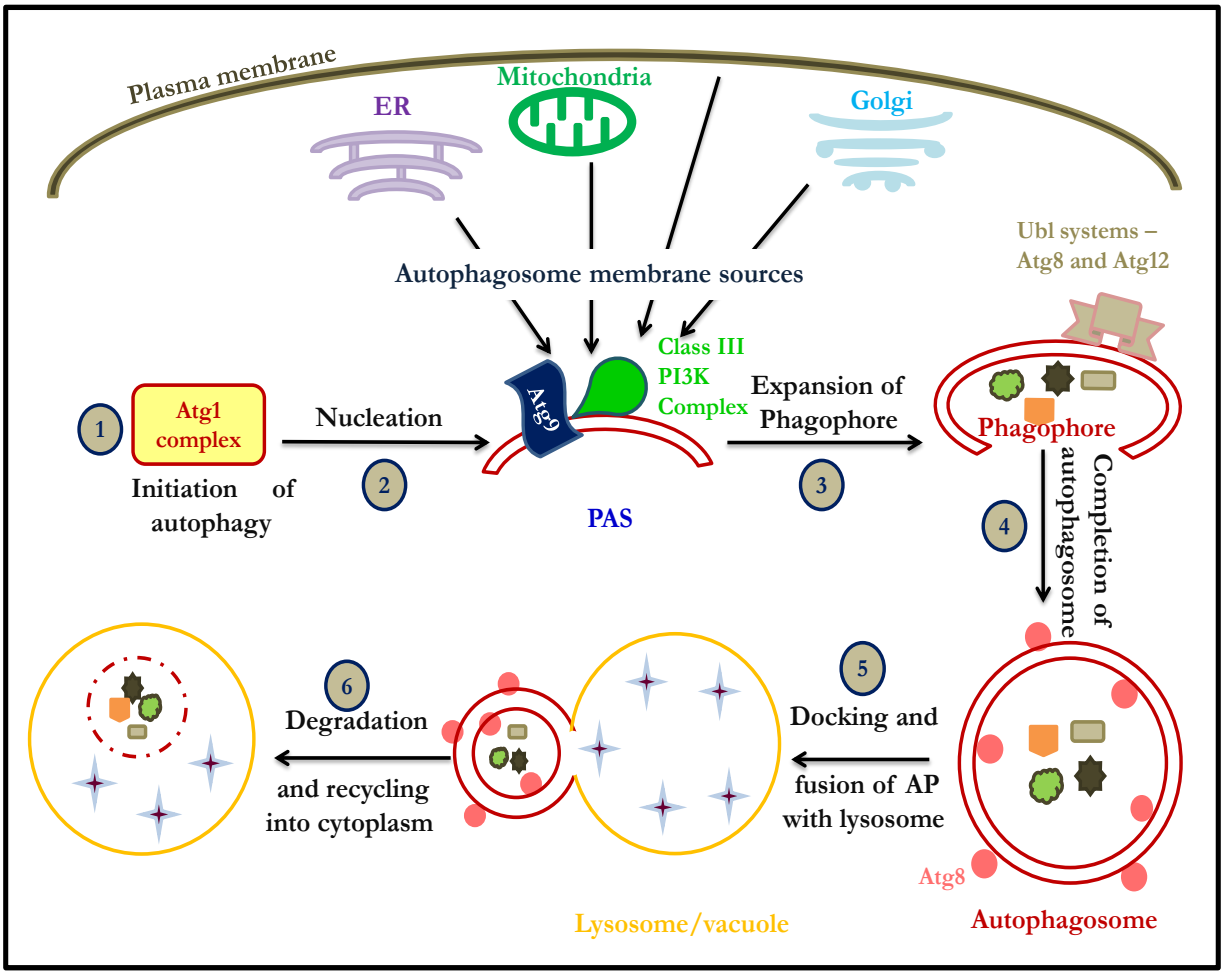


**Figure 1: Schematic representing various steps in the process of autophagy.** The steps include autophagy initiation, nucleation of the phagophore, expansion of the phagophore into complete autophagosome, docking and fusion of autophagosomes with vacuole and finally degradation of the cargo and recycling of the nutrients into cytoplasm. These steps are comprehensively described in the text.

### 1.2.1 Induction of autophagy

Basal autophagy occurs constitutively inside all eukaryotic cells and helps in maintaining cellular homeostasis. It is essential for turnover of the cytosolic components. However, autophagy can also be induced by nutrient (amino acids and/or Growth Factor) starvation [4]. Nitrogen starvation in yeast acts as a powerful trigger for stimulating autophagy. Autophagy can also be induced by deficiency of other essential factors such as carbon, auxotrophic amino acids, nucleic acids and sulphate; although less efficiently as compared to nitrogen starvation[5]. Inhibitors of TOR signalling such as rapamycin and Torin 1 induce autophagy even under nutrient rich conditions. Autophagy induction by both nutrient starvation and rapamycin is regulated by TOR (Target of Rapamycin) signalling[6]. TOR is a Ser/Thr kinase that is a master regulator of nutrient, growth and stress signalling[7] .TOR forms two different complexes, TOR Complex 1 (TORC1) and TOR Complex 2 (TORC2) of which TORC1 negatively regulates autophagy in yeast. In other words, nutrient starvation and rapamycin induce autophagy by impeding TOR activity[6].

### 1.2.2 Nucleation of phagophore/isolation membrane

Autophagy induction leads to the formation of phagophore/isolation membrane. Initiation of phagophore begins at the phagophore assembly site/pre-autophagosomal structure (PAS). PAS is a dynamic assembly site of Atg proteins in the periphery of yeast vacuole where Atg-proteins assemble in hierarchical manner [8]. Atg17/Atg11 is the first Autophagy related protein to be recruited to the PAS. In general autophagy, Atg17-Atg31-Atg29 complex act as a scaffold for Atg1 complex [9]. In a selective form of autophagy called as Cytoplasm to Vacuole Transport (Cvt) pathway, Atg17 is substituted by Atg11. Upon nutrient starvation, TORC1 activity is reduced that leads to dephosphorylating Atg13. This in turn facilitates the incorporation of Atg1-Atg13 into Atg17-Atg29-Atg31 complex[10-13] . Atg1 further acts as a scaffold for the recruitment of downstream autophagy related proteins. Once the nucleation of phagophore is initiated, it is elongated by the continuous addition of the membranes by the interplay of core Atg proteins.

Class III PI3K is a complex involved in initial stages of autophagosome formation. It is composed of four proteins in yeast-Vps34 (catalytic unit), Vps15, Atg6/Vps30, and

Atg14. It phosphorylates third hydroxyl group of phosphatidylinositol (PI) to produce phosphatidylinositol 3-phosphate (PI3P). PI3P is required for recruitment of PI3P-binding proteins such as Atg18[14, 15].Concerted roles of these autophagy related complex proteins lead to the elongation of phagophore into a complete double membrane autophagosome.

### 1.2.3 Autophagosome biogenesis and maturation

The growing end of PAS and phagophore requires the continuous supply of membranes for the formation of autophagosome. Although autophagosomes are the hallmark organelle of autophagy that separate the cytoplasmic content and eventually transport them to the vacuole for degradation, their biogenesis is very complex. Despite more than 50 years of investigations in the field of autophagy, the process of autophagosome biogenesis is still enigmatic. The key questions pertaining to the origins and dynamic contributions of autophagosomal membrane for its biogenesis still remains elusive.

Various studies have reported that peripheral membrane sources such ER[16], plasma membrane[17], mitochondria[18]and Golgi Apparatus[19-21]contribute to the autophagosome biogenesis by donating their membranes. This indicates that the formation of autophagosome is a *de novo* process. Unlike many organelles, it doesn't generate from the pre-existing autophagosome. Membranes from these sources are delivered to the PAS and to the early autophagic structures by Atg9 vesicles. Atg9 is the transmembrane autophagy-related protein associated with the vesicles of 30-60 nm diameter called as Atg9 vesicles. The shuttling of the Atg9 vesicles between the peripheral membrane sources (such as ER, Golgi, etc.) and the PAS is defined as Atg9 trafficking. Because of the perpetual association of Atg9 with these membranes, it is believed that Atg9 vesicles deliver membranes that contribute to the developing autophagosome. Atg9 vesicles collect membranes from various peripheral sources and deliver them to the PAS[22-25]. The anterograde movement of Atg9 vesicles from various membrane donors to PAS involves Atg23 and Atg27[26] whereas the retrograde transport from PAS to the cytoplasmic reservoirs is aided by Atg1, Atg2 and Atg18[23]. Homotypic fusion between the Atg9 vesicles are thought to provide a membranous platform for initiation and growth of the isolation membrane that eventually forms the autophagosome. Evidence for such coalescence of Atg9 vesicles comes from the

observation that Atg1 kinase complex anchors Atg9 vesicles at the PAS to initiate autophagy hence suggesting the major contribution of Atg9 trafficking in autophagosome biogenesis[13]. Autophagosome biogenesis can also be followed by using Atg8, a PAS and autophagosome marker[27]. It associates with the early autophagic compartments and autophagosomes by conjugating with the lipid molecule phosphatidylethanolamine (PE). Atg8 is synthesised as a precursor protein which is truncated at its C-terminal by Cysteine protease, Atg4. This exposes C-terminal Glycine of Atg8 that is required for conjugation of Atg8 to the amino group of PE[28]. Atg4 also recycles Atg8 embedded on the outer membrane of autophagosome by cleaving the bond between PE and Atg8[29]. Two ubiquitin-like conjugation systems Atg7-Atg3-Atg10 and Atg5-Atg12-Atg16 support the conjugation of truncated Atg8 to PE. Atg7 and Atg3, the E1-like and E2-like enzymes respectively facilitate the bonding of C-terminal glycine of Atg8 to the amino group of PE[30]. Atg12 conjugation system (Atg12-Atg5-Atg16 complex) assembles on isolation membrane and acts as E3-like enzyme[31].Thus Atg9, Atg8 conjugation system, ubiquitin-like conjugation complexes and Atg4 together orchestrate the biogenesis and maturation of autophagosome biogenesis. Completely formed autophagosomes enclosing the cargo, then migrate towards the vacuole for fusion.

### 1.2.4 Fusion of Autophagosome with vacuole

Just like any other vesicle fusion event, autophagosome fusion also involves Rab GTPases, tethering complexes and the SNARE machinery. Ypt7 is Rab GTPase that in coordination with Sec17 and Sec18 mediates the homotypic fusion of the vacuole[32-34]. Absence of these proteins blocks the fusion between autophagosomes and vacuole leading to the accumulation of autophagosomes. Class C VPS complex also known as HOPS complex (Homotypic fusion and Vacuolar Protein Sorting complex) is a multisubunit tethering complex (MTC) that docks the vesicles at their accurate target. Vps18, Vps11, Vps16, Vps33, Vps39, and Vps41 are the six subunits that make up the HOPS complex[35, 36].Vam3[37], Vam7[38] and Vti1[37] are some of the SNARES reported to mediate the autophagosome fusion with vacuole.Ccz1 and Mon1 are two additional proteins that form a stable complex to regulate the homotypic vacuolar fusion in coordination with SNAREs[34].

### 1.2.5 Degradation of cytoplasmic cargo

Fusion of the outer membrane of the autophagosome with vacuole forms a single membrane vesicles in the autolysosomes/autophagolysosomes called as autophagic bodies. They constitute cytoplasmic cargo entrapped in the inner membrane of autophagosomes that are destined for degradation by vacuolar hydrolases. Aut15/Aut5/Cvt17 (now Atg15) is a putative lipase reported to degenerate the single membrane of autophagic bodies inside the vacuole[39, 40]. Pep4 and Prb1 are vacuolar proteases required for degradation of autophagic bodies. The absence of these proteases lead to the accumulation of autophagic bodies inside the vacuole post starvation[41]. Autophagic bodies were also found to accumulate in the yeast vacuole in the absence of Vacuolar Membrane H(+)-ATPase (Vma) indicating that acidification is important for degradation of autophagic bodies[42].

### 1.2.6 Reuse of the breakdown products

Degradation of macromolecules inside the vacuole produces monomeric units which are transported to the cytosol for reuse. In yeast, Aut4 (now Atg22) is a membrane protein that is important for disintegration of autophagic bodies[43]. Atg22 along with other vacuolar proteases such as Avt3 and Avt4 expel the amino acids out of the vacuole[44].

Autophagy regulates wide range of physiological functions which we have summarized in our review titled as ‘Multifaceted Housekeeping Functions of Autophagy’ (Chinchwadkar, S. *et al*. 2016).

Most of the steps in the process of autophagy involve either membrane trafficking or vesicle trafficking. Trafficking is the movement of vesicles loaded with proteins and biochemical signals from the synthesis and packaging locations to specific release locations. It is tightly regulated process to ensure the correct cargo delivery to the correct destination organelle. Trafficking machinery involves tethering complexes for accurate docking of the vesicles, fusion machinery SNAREs, cytoskeleton, Rab GTPases and many other accessory proteins. Vesicles are ferried on the roads of cytoskeleton by drivers called motor proteins to the target location. Multisubunit tethering complexes prevent the vesicle escape until SNARE mediated fusion takes place (Figure 2). This universal eukaryotic phenomenon has a key role in

synaptic neurotransmission, endocrine secretion, mucus secretion, granular product secretion by neutrophils etc.

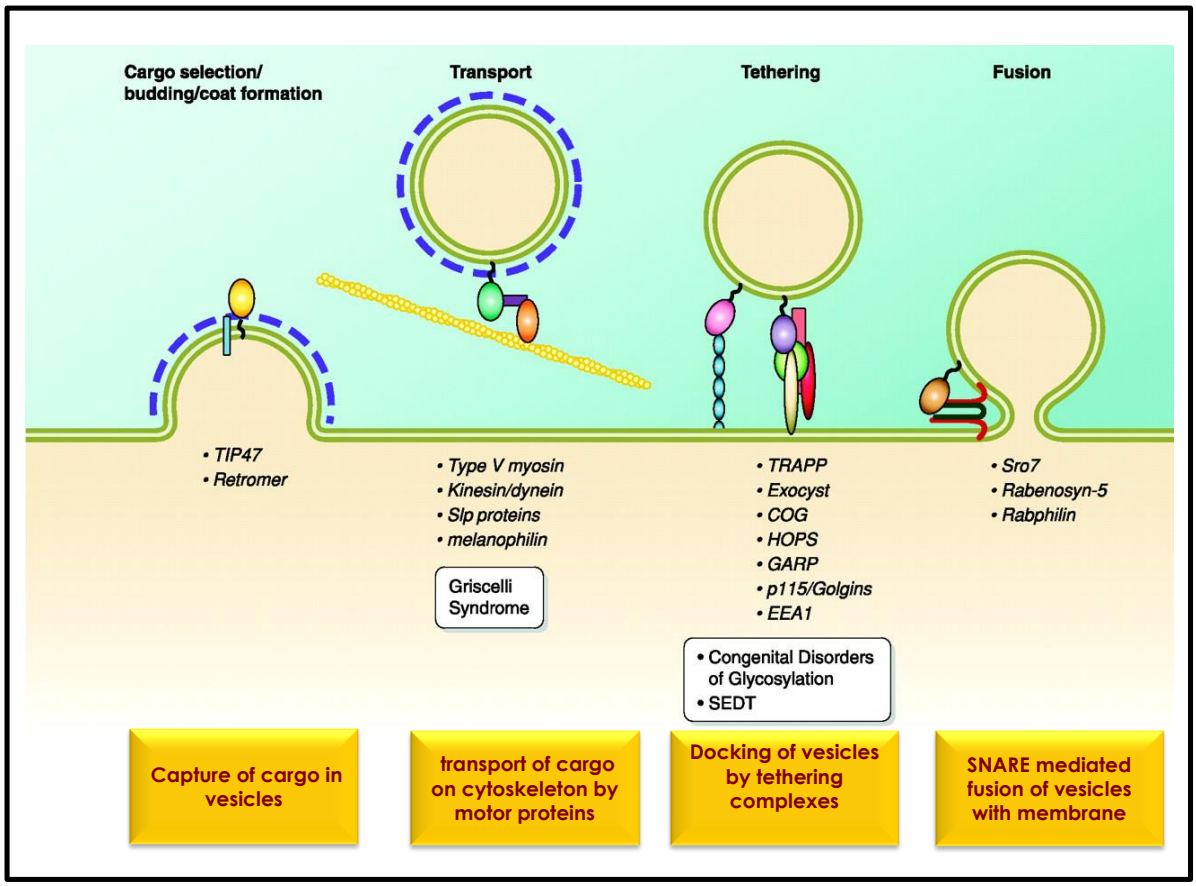


**Figure 2: Schematic representing the components of vesicle trafficking. Modified with permission from Alex H. Hutagalung and Peter J. Novick, 2011**

The components of trafficking such as tethering factors and cytoskeleton are involved in the process of autophagy.

## 1.3 Components of vesicle trafficking and autophagy

### 1.3.1 Conserved Oligomeric (COG) Golgi complex

COG complex is an evolutionarily conserved MTC comprising of 8 subunits - Cog1, Cog2, Cog3, Cog4, Cog5, Cog5, Cog6, Cog7 and Cog8. In *S. cerevisiae*, it is divided into two lobes – lobe A (Cog2–4) and lobe B (Cog5–8) coupled to each other by Cog1 [45-51]. Cog complex interacts with Rab proteins Ypt1 and Ypt6, intra Golgi SNARE molecules[51, 52] and COP1 coat complex [50, 51] at *cis*/medial Golgi membranes where it is situated [48, 53, 54]. Cog complex primarily mediates the retrograde trafficking (*trans*-to-*cis*) of vesicles loaded with Golgi resident proteins and glycosylation enzymes by tethering COPI vesicles to the *cis*-Golgi membranes [51]. It is also known to act as a tether in anterograde trafficking from endoplasmic reticulum (ER) to Golgi [45, 46, 51].

It serves many purposes in yeast like localizing enzymes in *trans*-Golgi network [55] and sorting of cargo during ER exit [56].Apart from its canonical tethering role, COG complex is also involved in autophagy. Yen *et al* have shown that COG complex participate in the formation of double membrane autophagosomes and Cvt vesicles. Immunoblot analysis reveals that COG mutants possess defects in Cvt pathway, autophagy and pexophagy (selective autophagy of peroxisomes). Moreover Atg8 and Atg9 are mislocalized in these mutants. Subunits of COG were also found to localize at PAS [19]. However the exact mechanistic roles of COG complex as tethering factor in autophagy related trafficking is still unclear.

### 1.3.2 Transport Protein Particle (TRAPP) complex

TRAPP complex is highly conserved multisubunit tethering complex that mediate vesicle tethering, Golgi trafficking and autophagy. Four TRAPP complexes have been identified so far acting in discreet trafficking events such as secretion and autophagy. Eleven subunits of the TRAPP complex have been identified thus far which are conserved in yeast and humans. Seven out of these are small subunits and four are large. In yeast, these subunits are designated either as TrsN (N represents molecular weight in kDa) or Bet3/5. TRAPP complexes act like GEFs that catalyse the

GDP/GTP exchange on small Rab GTPases. Four small subunits self-assemble to form stable core TRAPP complex. Trs20/Sedlin is a small subunit that bridges the core TRAPP complex to the larger subunits to form TRAPP II and TRAPP III complexes. This core complex possesses GEF activity for Rab GTPase Ypt1.

Six out of seven small subunits constitute TRAPP I whereas other TRAPP complexes are formed when larger subunits associate with the smaller ones. Core subunits of the TRAPP complex include Bet5, Bet3, Trs23 and Trs31. Subunits specific to different complexes are mentioned in the table below (Table 1).

**Table 1: Specific subunits of different TRAPP complexes**

| Sr.No. | Subunit group | Specific subunit |
|---|---|---|
| 1 | TRAPP I-associated | Trs33 |
| 2 | TRAPP II-specific | Trs65, Trs120, Trs130 |
| 3 | TRAPP III-specific | Trs85 |
| 4 | TRAPP IV-specific | Trs33 |

Mutation analyses have revealed that TRAPP I, TRAPP II and TRAPP III complexes act at different levels – at early Golgi, at late Golgi and in autophagy respectively. TRAPP I acts on Rab GTPase Ypt1 and mediates ER to Golgi transport. TRAPP II acts on Ypt31/32 to facilitate Golgi to PM transport. Ypt1 activation by TRAPP III guides the assembly of the PAS during autophagy[57].

Of all the tethering factors known, role of TRAPP complex in autophagy is very well studied. Trs85 containing TRAPP III complex is important for Cvt pathway, general autophagy and selective autophagy of peroxisomes; as the cells deficient in Trs85 are unable to organise the PAS[58, 59]. Trs85 is required for targeting Ypt1 to the PAS [60]**.** Biomolecular fluorescence complementation (BiFC) assay disclosed that Ypt1 and Trs85 interact at PAS and also with Atg9 membranes[61, 62]. Additionally, Ypt1 helps in recruiting Atg1 and Atg11 on the PAS [61, 63].

Although Trs85 containing TRAPP III complex is specific to autophagy and is extensively studied, there are few reports that suggest the role of other TRAPP complexes in autophagy as well. Trs130, a subunit specific to TRAPP II complex regulate Cvt pathway and autophagy. Transport of autophagy proteins Atg8 and Atg9

to the PAS is abrogated in *trs130* temperature sensitive (Ts) mutants. Moreover autophagy defects in strains temperature sensitive for Trs130 and Trs65 (another TRAPP II specific subunit) are rescued by overexpressing Ypt31/Ypt32 (TRAPP II specific Rab GTPases) and not by Ypt1 (TRAPP I specific Rab GTPase)[64]. Recently TRAPP IV complex has been identified (with Trs33 as a specific subunit) to regulate autophagy in Ypt1 dependent manner. Trs33 is required for recruiting core TRAPP and Ypt1 to the PAS in the cells that lack Trs85[65].

### 1.3.3 Golgi associated retrograde protein (GARP) complex

It is a multisubunit complex that is composed of four subunits-Vps51, Vps52, Vps53, Vps54[66, 67]. As the name suggests, it is involved in the retrograde trafficking of vesicles from endosomes to the trans-Golgi network. Mutations in three of its subunits (Vps52, Vps53, Vps54) lead to aberrant sorting of the vacuolar carboxypeptidase Y. These mutations also incorrectly localizes Golgi membrane proteins to the vacuole[66]. GARP complex associates with two trans-Golgi resident monomeric GTP binding proteins called as Arl1-GTP[68] (the Arf/Arl/Sar protein family) and Ypt6-GTP[69](the Rab family) and this association is recently found to be significance for autophagy. Arl1 and Ypt6 control the anterograde trafficking of Atg9 by transporting the GARP complex to the PAS[70, 71].

Our lab is interested in studying the role of one such tether called Exocyst complex in autophagy. A genetic screen of Ts mutants was performed in our laboratory. We identified that all the exocyst Ts mutants except one (exo70.29/37) showed complete block in general autophagy and pexophagy (unpublished work, Sunaina Singh MS thesis). Exocyst complex and its role in autophagy is described in detail in chapter 2.

### 1.3.4 Cytoskeleton in autophagy

Actin has several roles in autophagy like supporting the phagophore and transporting the autophagosomes to the lysosome for proper fusion. Drugs that depolymerize F-actin (cytochalasin D and Latrunculin B) inhibit the formation of autophagosomes[71, 72]. Hence F-Actin polymerisation is required for autophagosome formation. Just like actin filaments, microtubules also regulate autophagosome formation. However their role is confined to starvation induced autophagy; they do not regulate autophagosome formation at basal level[71, 73].When microtubules are treated with high doses of nocodazole (inhibits microtubule polymerisation), autophagosome formation is

prevented under starvation conditions[73, 74].The roles of cytoskeletal elements actin[75]and microtubules[76]in autophagy have been comprehensively reviewed by Kruppa *et al*., 2016 and Mackeh *et al*.,2013. Our lab is interested in studying the role of one such cytoskeletal element named septins in autophagy. Septins have been recently considered as the fourth component of cytoskeleton. In a genetic screen that was previously done in our, we found that autophagy (both general and selective autophagy of peroxisomes) is affected in the septin Ts mutants. Septins are explained in detail in chapter 3.

# Chapter 2
# Role of Exocyst Complex in Autophagy

## 2.1 Introduction

### 2.1.1 Exocyst complex

Exocyst is a hetero-octameric tethering complex which anchors the post Golgi secretory vesicles in close proximity to the plasma membrane prior to the SNARE-mediated fusion. It comprises of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 in *Saccharomyces cerevisiae*. These subunits are named as *EXOC1*, *EXOC2*, *EXOC3*, *EXOC4*, *EXOC5*, *EXOC6*, *EXOC7* and *EXOC8* in mammalian cells respectively (Figure 3). It is an evolutionarily conserved complex in eukaryotes from yeast to humans. Exocyst complex is also called as Sec6/8 complex in higher eukaryotes. This complex was first identified in budding yeast *Saccharomyces cerevisiae* in a genetic screen of temperature sensitive (Ts) mutants for secretion[77]. Further studies on these mutants revealed that at least 14 of them are involved specifically in vesicular transport from the Golgi apparatus to the plasma membrane. Yeast cells that are deficient in the individual subunits show the defects in secretion. A study suggested the role of 3 (Sec6-Sec8-Sec15 complex) out of these 14 subunits in exocytosis [78]. A year later the number of proteins involved in exocytosis was increased to 7 from 3 with the new additions of Sec3, Sec10, Sec5 and Exo70. These seven proteins existed as a complex and together performed exocytosis[79]. A few years later Exo84 was identified and characterized in *Saccharomyces cerevisiae* as the eighth component of exocyst complex making the complex an octamer. Exo84 is an essential protein required for polarized secretion[80].The molecular weights of individual subunits ranges from 84kDa-155kDa with Exo70 (71 kDa) being the smallest and Sec3 (155kDa) the largest subunit[81]. The mammalian exocyst complex first purified from rat brain extracts is also composed of 8 proteins with a combined molecular weight of 743 kDa. Mammalian exocyst complex is homologous to the yeast 834 kDa exocyst complex[82].

Sec3 and Exo70 are localized on the plasma membrane and Sec15 is present on the vesicles that are targeted to the plasma membrane for secretion. Sec8 forms the core subunit of the exocyst complex[83].

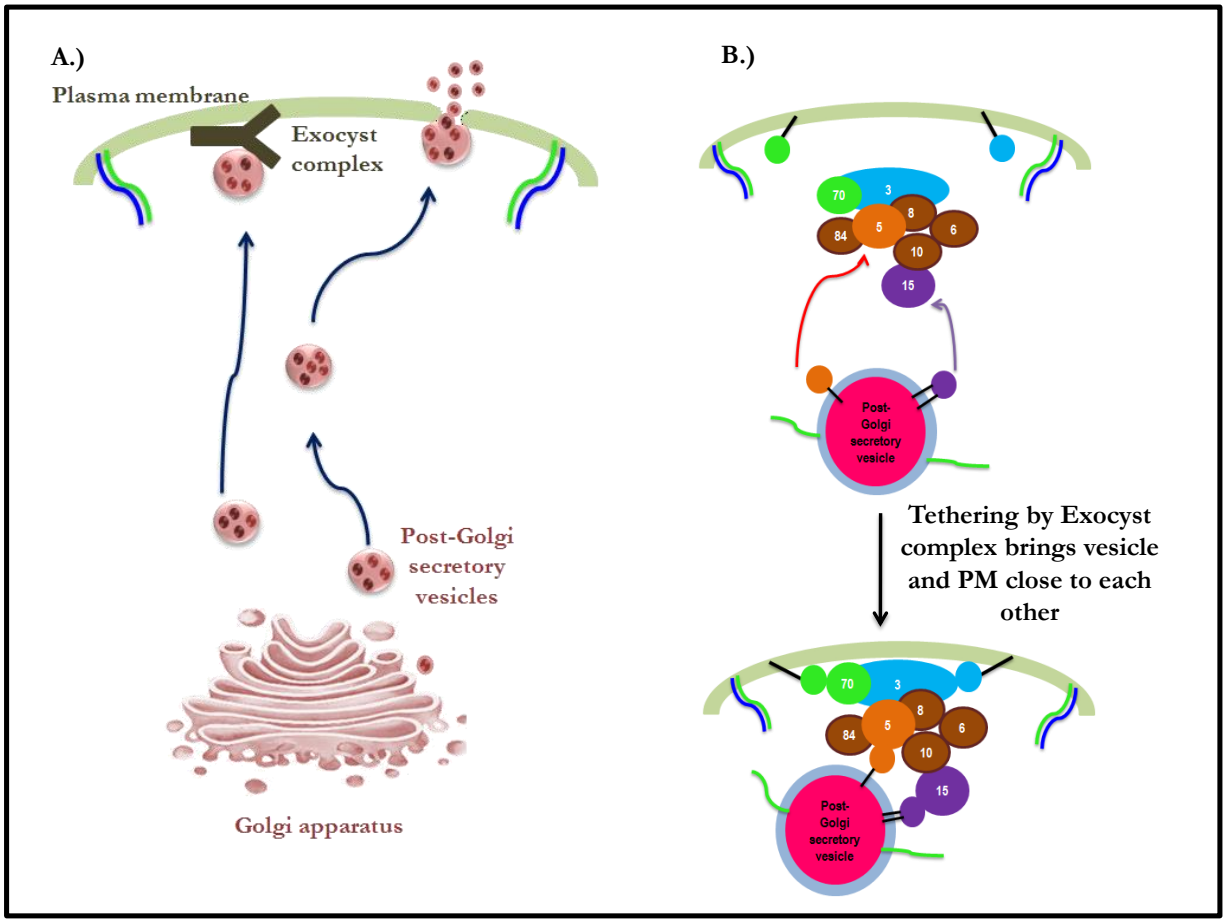


**Figure 3: Role of exocyst complex. A)** Exocyst complex tethers the post Golgi secretory vesicles to the Plasma membrane before their fusion **B)** Zoomed in representation of the tethering role of exocyst complex prior to SNARE mediated fusion

The exocyst complex mostly accumulates at the active cell surface expansion regions such as bud tip and mother bud neck region. Studies have shown that exocyst is regulated by small GTPases such as Sec4, Cdc42 and also by the members of the Rho, Ral and Rab families. Exocyst acts as effectors to these molecules[84]. Exocyst is involved in multitude of physiological processes such as vesicle tethering, cell migration and tumor invasion, primary ciliogenesis, cytokinesis, epithelial cell membrane trafficking, polarized secretion in budding yeast, autophagy and tumorigenesis[83].

### 2.1.2 Autophagy and Exocyst at the crossroads

Recent reports have suggested a molecular link between the two important processes, Exocytosis and Autophagy. Both the processes are found to be dysfunctional in large number of diseases like cancer, neurodegeneration and chronic inflammatory diseases. Variety of biological processes such as immune response, cell growth, cell proliferation, apoptosis and multicellular organelle development are essential for exocytosis as well as autophagy again suggesting a commonality between them. Exocytosis and autophagy are mediated by exocyst complex and set of Atg proteins respectively. Depending upon the extracellular and/or intracellular milieu, exocytosis and autophagy may be competitively, cooperatively or independently regulated (Brooks et al, doi 10.5772/39111).

Autophagy and exocytosis involve membrane trafficking and fusion events and therefore it is speculated that both the processes utilize similar groups of molecules (such as GTPases, MTC exocyst, SNAREs etc.) that facilitate the membrane trafficking and fusion events.

The following reports exemplify this notion of intersection of autophagy and exocyst components.

1) Exocyst and its effector RalB are important in both exocytosis and autophagy. RalB when interacts with Exo84, initiates autophagy whereas autophagy is deactivated when RalB interacts with Sec5 subunit. It is postulated that exocyst forms a scaffold for initiation of autophagy complexes in mammalian cells[85].

2) In mammalian cells, Rab11 which interacts with the sec15 subunit of the exocyst complex and mediates the tethering of the vesicles to the plasma membrane is also known to facilitate the fusion of the autophagosome with the endocytic compartments[86].

3) The cells with impaired Exocytic Rab-GTPase Sec4 and Sec2 (Sec4 GEF) activity are known to effect the anterograde movement of Atg9 which in turn influences the recruitment of Atg8 to the PAS in yeast[20].

4) VAMP7 which is SNARE protein involved in exocytosis also mediates the fusion of autophagosome with the lysosome[87].

Despite all these information, the physical localization of exocyst complex and its association with autophagy machinery in yeast are not known. Therefore our objective was to determine these associations for which we undertook live cell fluorescence imaging based approach. The possible steps in autophagy where exocyst perhaps have a tethering role could be homotypic fusion of Atg9 vesicles, fusion of Atg9 vesicles with early autophagosomal structures and heterotypic fusion of autophagosomes with vacuole. These scenarios are diagrammatically illustrated below (Figure4).

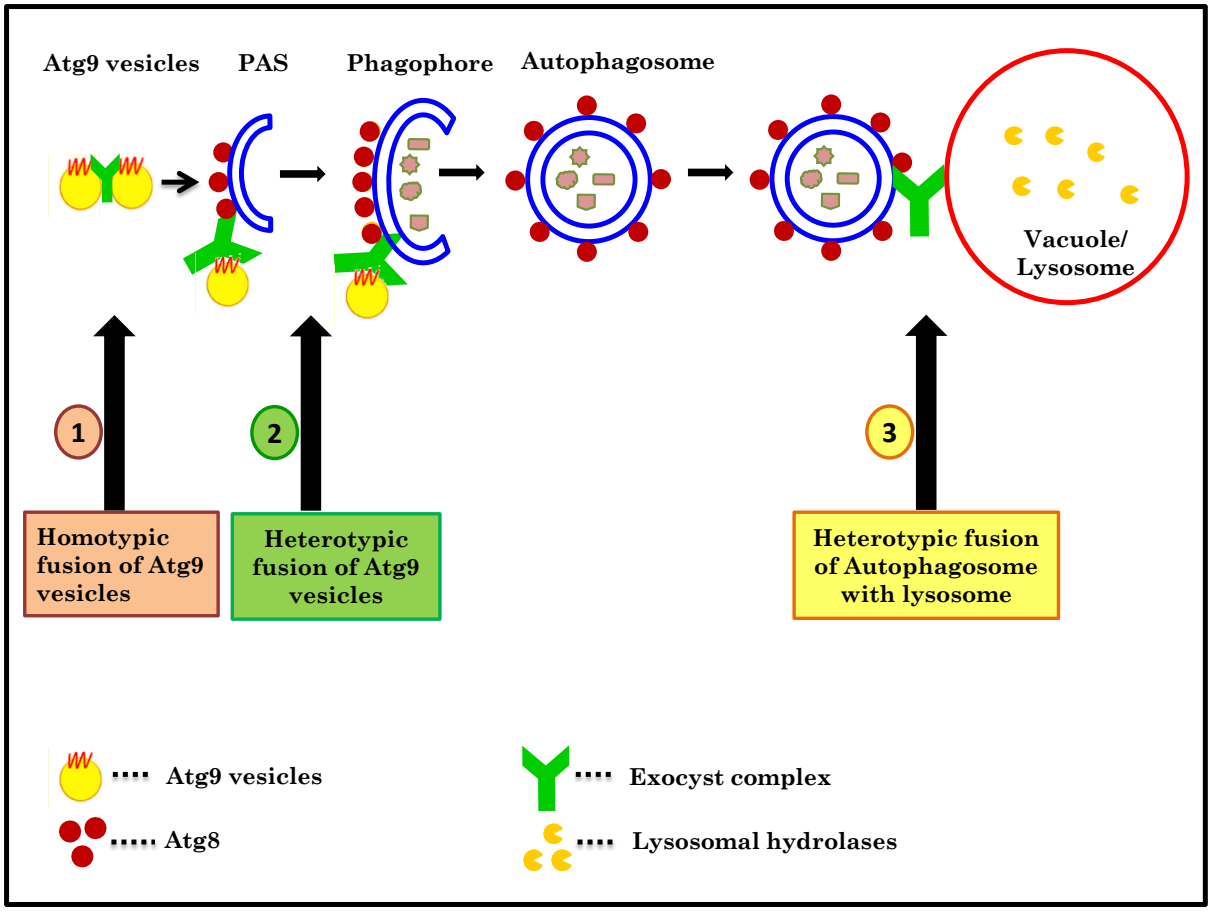


**Figure 4: Possible scenarios of the tethering role of exocyst complex in the process of autophagy.** Exocyst complex might act as a tether for 1) homotypic fusion of Atg9 vesicles, 2) heterotypic fusion of Atg9 vesicles with early autophagosomal structures and 3) in the heterotypic fusion of autophagosomes with vacuole.

## 2.2 Materials and methods

### 2.2.1 Live cell microscopy

Single colony was inoculated in SD-URA (2% dextrose, 0.5% ammonium sulphate, 0.17% yeast nitrogen base, 0.002% histidine, 0.002% methionine, 0.003% lysine, 0.006% leucine) and incubated overnight at 30 $^{O}$C at 200 rpm. Secondary inoculation was performed at$A_{600}$ equivalent to 0.2 and allowed to grow till the culture reached log phase (an $A_{600}$ between 0.6 and 0.8). Cells were transferred to starvation medium (autophagy induction) and time lapse videos were acquired within an hour of inducing autophagy.

## 2.3 Role of Exocyst complex in Autophagy

### 2.3.1 Results and discussion

Previous studies from our lab have shown that in the temperature sensitive mutants of exocyst complex, both general and selective autophagy of peroxisomes (pexophagy) are affected. PAS is a site where autophagy specific dynamic membrane trafficking of vesicles takes place. All the membrane donors converge at PAS and contribute their membranes for autophagosome biogenesis. Therefore we thought if exocyst as a tether has any role at PAS. The exocyst complex might act as a tether for homotypic fusion of Atg9 vesicles or for mediating the fusion between Atg9 vesicles and developing membrane. Hence, in order to know whether exocyst complex associates with PAS, colocalization experiments were performed where we looked for colocalization between PAS marker Atg8 and exocyst subunits. The strains used for colocalisation experiments are depicted in table 4 in Appendix-AI. In these strains, different exocyst subunits are tagged with GFP at their C-termini. Also they harbour pRS316 centromeric low copy number plasmid containing Atg8 tagged with 2x-mCherry. pRS316 plasmid possesses uracil selection marker.

Colocalisation events between different exocyst subunits and 2x-mCherry Atg8 were observed. Number of colocalisation events per 1000 viable cells are plotted (Figure 6B). These experiments suggested that the exocyst associated with either PAS or autophagosome, as Atg8 is a marker for both the autophagic structures. Therefore to address whether exocyst associates with PAS, autophagosomes or both, we performed similar experiments in Δ*ypt7* background. This would also explain whether the events are dynamic or just the chance events. Ypt7 is a Rab GTPase which is essential for the fusion of autophagosomes (AP) with the vacuole. Absence of Ypt7 blocks the fusion of autophagosomes with vacuole resulting in accumulation of AP in the cytoplasm which appears as red puncta (Figure 5). Interestingly

number of colocalisation events between exocyst subunits and the Atg8 increased in Δ*ypt7* background (Figure 6). Also there were cells in which two colocalization events were observed simultaneously suggesting that exocyst associates with both PAS and autophagosomes and that the colocalisation events are actual dynamic events. These are initial observations based on one experiment and need to be further validated by repeating experiments.

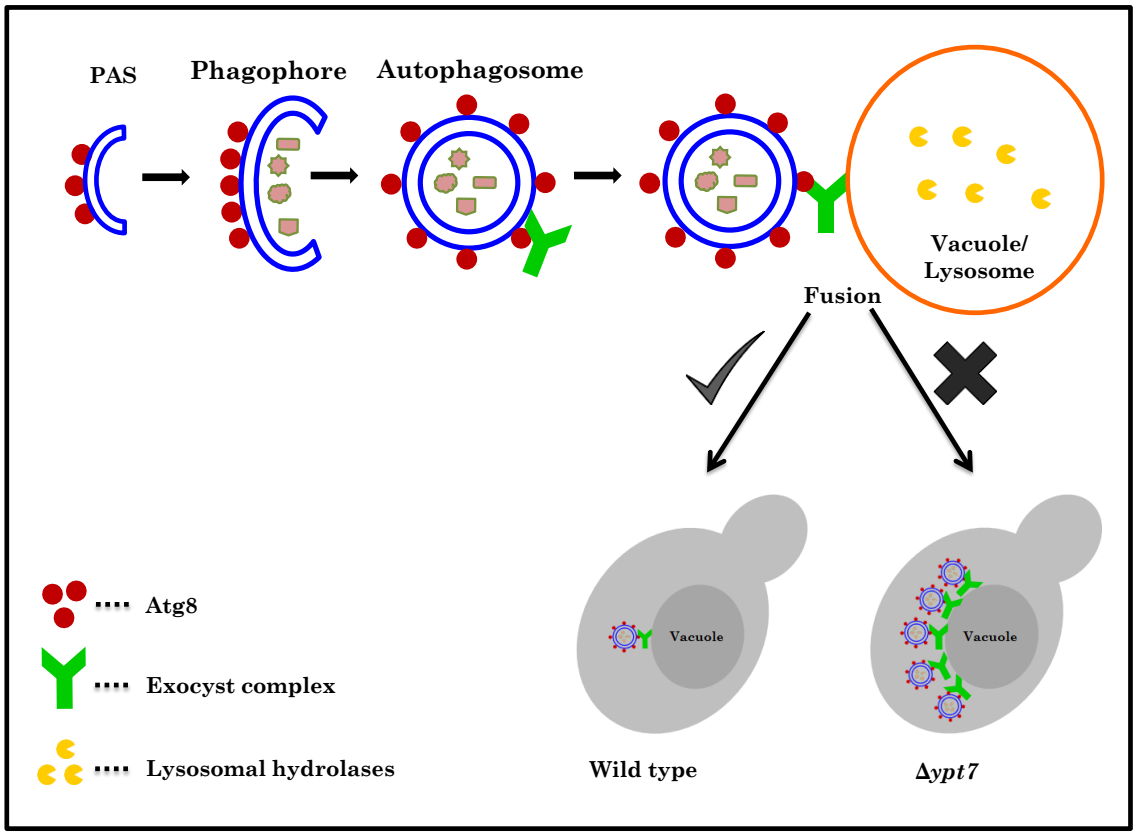


**Figure 5: Accumulation of autophagosomes in Ypt7 deficient yeast cell.**

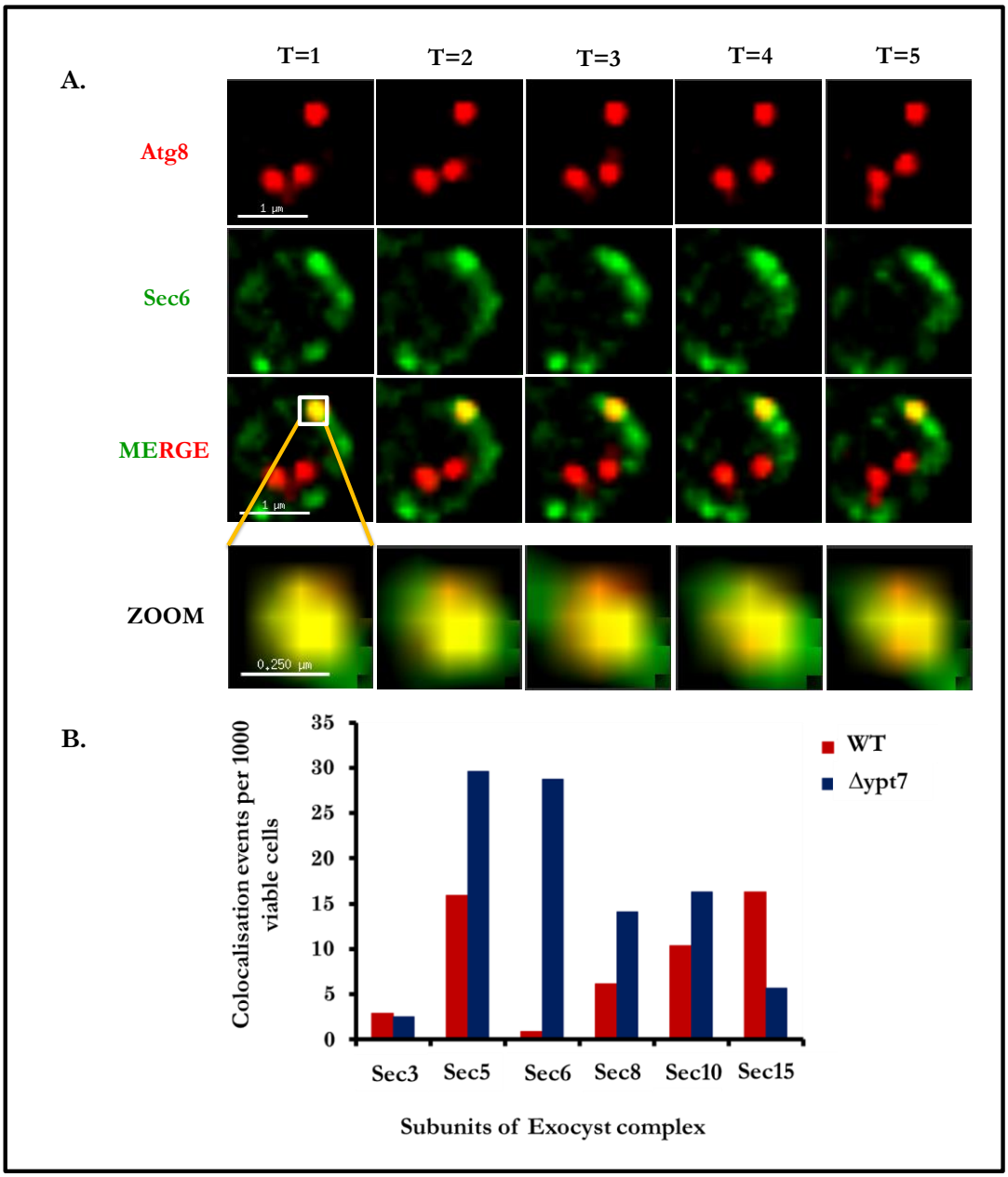


**Figure 6: A) Micrographs representing the colocalization between Sec6-GFP and 2x-mCherry-Atg8 in Δypt7 across different time points. B) Quantification of colocalization between exocyst subunits and Atg8 in WT and Δypt7 background.**

**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.08 sec) and FITC (transmittance 32%, exposure 0.08 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm and projected to maximum intensity. Images were acquired for 5-7 minutes till the signal completely bleached. Colocalization events were manually counted using cell counter plugin of Fiji software**

# Chapter 3

# Correlative Roles of Exocyst complex and Septins in Autophagy

## 4.1 Introduction

### 4.1.1 Septins

Septins are GTP binding proteins that form heterooligomeric complexes and higher order structures. Septins were discovered in budding yeast *Saccharomyces cerevisiae* about 4 decades ago in a pioneering cell division cycle screens performed by Lee Hartwell and group**.** Four cell division cycle (*CDC*) genes (*CDC3, CDC10, CDC11* and *CDC12*) were characterized in this study that have functions in cytokinesis[88].

They are present in many eukaryotes but are absent from plants and prokaryotes. Protistans lack true septins. The numbers of genes that encode septins vary from organisms to organisms ranging from one in *Chlamydomonas reinhardtii*, two in *Caenorhabditis elegans* to 13 in *Homo sapiens*[89]. There are 7 septins in *Saccharomyces cerevisiae*, 5 of which (Cdc3, Cdc10, Cdc11, Cdc12 and Shs1) form a ring at the mother-bud neck region that are important for cytokinesis; and the other two (Spr3p and Spr28p)[90] are required for sporulation. Humans have 13 isoforms of septins SEPT1-SEPT12 and SEPT14. SEPT13 is now considered as one of the many SEPT7 related pseudogenes called as *SEPT7P2***.**In humans, septins play a diverse role from cilliary movements to cage formation around bacteria during infections

### 4.1.2 Molecular architecture of Septins

Molecular weights of individual septins range from 30-65 kDa. Most of the septins contain a GTPase domain which belongs to the GTPase superclass of P-loop NTPase (**P**hosphate-binding loop **N**uceotide **T**ri **P**hosphatases). The other renowned members of P-loop NTPase include kinesin, myosin and Ras proteins[91]**.** Nevertheless, GTPase domain of septins contain a stretch of 53 highly conserved amino acids called as Septin Unique Element (SUE), making this domain a unique among the family. The functions of this distinctive stretch are still unknown[92]. Amino (N-terminal) and carboxy (C-terminal) termini regions flanking the GTPase domain of septins are also conserved. Amino terminal region contains a proline rich polybasic region that binds to phospholipids (phosphoinositides). Whereas carboxy terminal

region possesses coiled coil domains that are thought to be important for certain septin-septin and septin-substrate interactions[93]. Apart from this, there are two conserved interfaces in septins that are involved in the formation of septin-septin interactions and their subsequent complex assembly. They are G-interface (Guanine nucleotide binding domains) and NC-interface (N- and C- terminal extensions)[94]. Recently septins have been recognised as the fourth component the cytoskeleton because of their resemblance to filaments and their association with distinct regions of cell membrane[95, 96], actin filaments[97] and microtubules[98, 99]. The interactions of septin with actin, microtubule and phospholipid membrane can influence the assembly of septins into higher-order structures (filaments and rings). EM studies in budding yeast suggest that septin filaments are nearly 10 nm thick and surround the mother-bud neck region. It was corroborated through fluorescence microscopy studies that septins localize in a ring form at the bud neck that is septating. Hence septins are considered as the major components of bud-neck filaments during cell septation[100-102].

### 4.1.3 Septin Filament Assembly

All septins control cellular processes by forming hetrooligomeric protein complexes (rods) that further assemble into higher-order structures. They form heterooligomeric complexes of rod shape which assemble end-to-end into biologically active long paired linear filaments. These filaments in turn assemble end to end (Figure 7) to form higher-order structures such as rings, hourglass or gauzes[94, 103-105]. EM studies have shown that septin filaments are non-polar that makes them distinct from actin filaments and microtubules. In this term, they are non-polar like intermediate filaments. Septins are reckoned as more stable than other cytoskeletal elements for they do not undergo rapid turnover like that of actin filaments or microtubules. The different number and kinds of septins involved in hetero-oligomer formation in different species are listed in the table 2[94, 103-105].

**Table 2: Number and kinds of septins involved in hetero-oligomer formation in different species**

| Sr.No. | Organism | No of Subunits | Oligomer formed |
|---|---|---|---|
| 1. | *Caenorhabditis elegans* | 2 | Tetramer |
| 2. | *Saccharomyces cerevisiae* | 4 | Octamer |
| 3. | *Homo sapiens* | 3 | Hexamer |

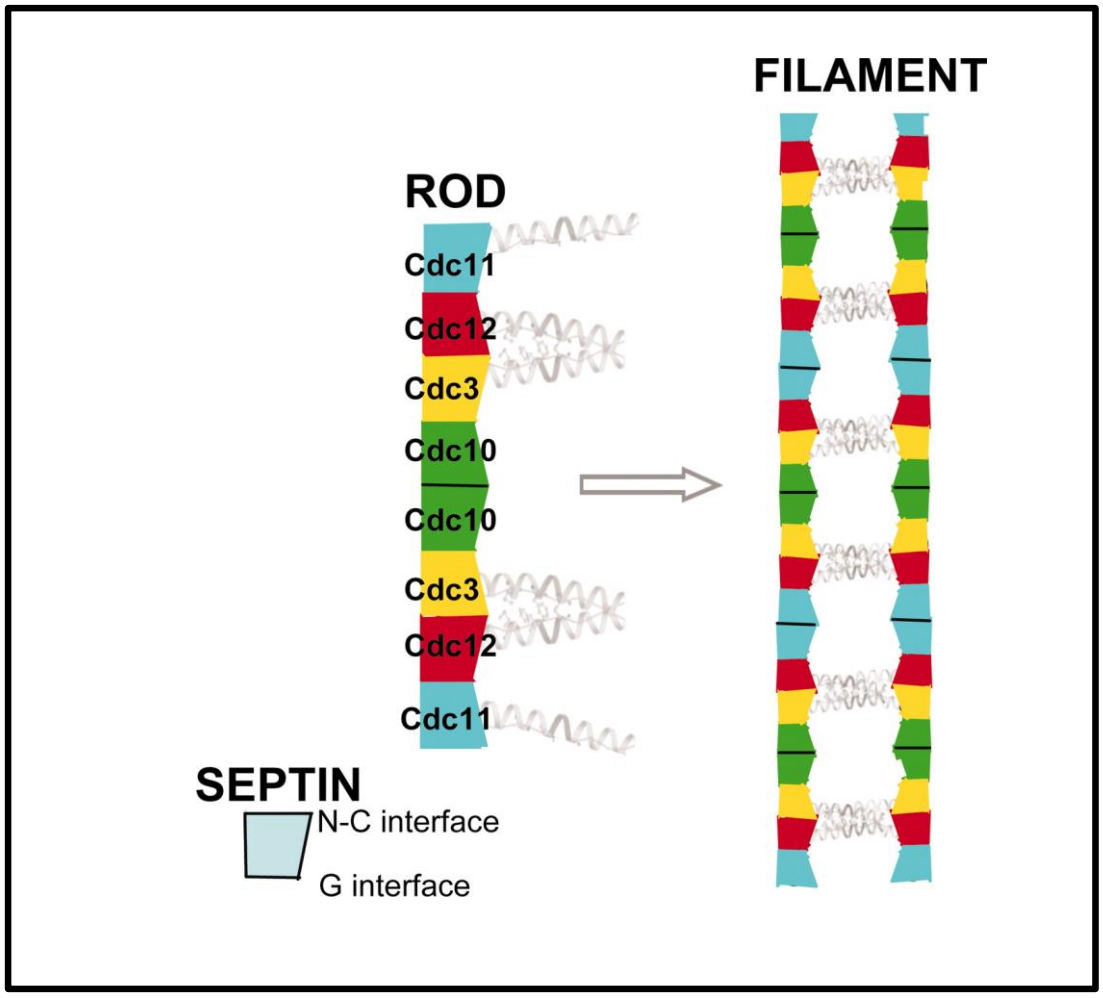


**Figure 8: Schematic diagram of the yeast septin rod, filament, and paired filament assemblies. With permission from Aurelie Bertin *et al*., 2008**

The mechanism of septin filament assembly and septin complex formation is similar in different organisms in spite of involvement of different number of septins in the stable complex. Filament assembly in *C. elegans* and mammals require the G-interface interactions between the monomer units of septins whereas in budding yeast NC-interface interactions are involved.

Structural studies have elucidated that septins can bind and hydrolyse GTP in both yeast and metazoans. GTP-bound state might have a structural role for most of the septins suggesting that its biochemical properties are similar to alpha-tubulin[106-108]. Nucleotide binding and hydrolysis might also have regulatory roles in septin-septin interactions and in polymerisation of septin filaments[94, 104, 109]. However, this is still notional[110, 111].

### 4.1.4 Septins and autophagy

Mostowy *et al* for the first time reported the connection between septin assembly and selective form of autophagy called xenophagy. Cytosolic *Shigella flexeneri* that are entrapped into septin cages are targeted to autophagy as is indicated by the colocalisation of key autophagic proteins p62 and Atg8 with *Shigella*-septin cages. Depletion of proteins involved in the early stages of autophagy such as p62, Atg5, Atg6 (Beclin1) or Atg7 by RNA interference reduces septin caging around Shigella. Similarly, depletion of SEPT2 or SEPT9 significantly reduced the levels of autophagic components p62 and Atg8-II again suggesting the interdependence of septin assembly and autophagy[112].

Recent findings unveiled that the assembly of septin cages around cytosolic *Shigella flexeneri* is mediated by mitochondria and that it inhibits the proliferation of *S. flexeneri*[113].

The role of septins in general autophagy for the first time was reported from our laboratory[114]. In a screen of subset of septin Ts strains, it was observed that both general autophagy and pexophagy are affected in budding yeast *S. cerevisiae*. Septins colocalize with autophagic proteins Atg8 and Atg9 under starvation condition (autophagy induction) and also have a tendency to relocate from mother-bud neck region to cytoplasm upon autophagy induction. Experiments reveal the role of septins in the initial stages of autophagy particularly in autophagosome biogenesis. However the mechanisms behind this are not completely understood and needs to be discerned.

### 4.1.5 Interplay between Exocyst and Septin

There are examples of the processes that are jointly regulated by exocyst and septins. The canonical roles of exocyst and septins are in exocytosis and cytokinesis respectively.

However, exocyst complex plays an important role in cytokinesis. During cytokinesis, split septin rings act as diffusion barriers by limiting the membrane-remodelling factors such as exocyst complex[115] and polarisome[116] to the abscission site. These factors mediate the trafficking of vesicles at the cell-cell junction and hence are required for proper furrowing and abscission during cytokinesis. Just like exocyst has role in cytokinesis, septins also affect the exocytosis. Dynamic assembly and disassembly of septins is required for exocytosis of secreted and transmembrane proteins in various cell types[117]. Moreover, in mammalian brain tissue, septins immunoprecipitate with exocyst complex[118].Thus both exocyst and septins regulate exocytosis and cytokinesis. Studies from our lab have shown that exocyst and septins regulate autophagy. Our unpublished results suggest that exocyst and septins associate with Atg8 and Atg9 under starvation conditions (autophagy induction). In addition to this, exocyst and septins are also found to affect anterograde and retrograde movement of Atg9 vesicles respectively (Figure 9). Given the interdependent roles of exocyst and septins in exocytosis and cytokinesis and the observations from our lab, led us to speculate if both these complexes cooperate with each other to regulate autophagy. We tested this hypothesis by performing various colocalization experiments which are described below. We started by genomically tagging WT and Ts strains. We then analysed the colocalization between exocyst components (Sec3, Sec8, and Exo84) and septin cdc10 under starvation conditions. Ts strains are versatile, convenient and powerful tools to study the functions of essential genes. These are conditional mutants which generally mimic the WT phenotype at permissive temperatures (PT). While at NPT, because the concerned protein is ablated, mutant phenotype is observed. This is a very specific approach, as the protein that harbours the mutation is only targeted at the NPT[119]. Thus we exploited this inherent property of Ts strains to study the correlative roles of exocyst and septins in autophagy. One of each of the components of exocyst and septins were tagged in the same strain. This created genomically tagged fluorescence protein in WT and Ts mutants for both exocyst and septins. For example, in WT strain exocyst subunits were GFP tagged and septin Cdc10 with mCherry. Ts Strains were subsequently transformed with pRS316 centromeric low copy number plasmid containing Atg9-2x-mCherry. In exocyst Ts strains septin subunit is tagged with GFP and transformed with Atg9-2x-mCherry. Similarly septin Ts strain possess GFP tagged exocyst subunits and Atg9-2x-mCherry. This allowed us to study the Atg9-septin and Atg9-exocyst associations in the absence of functional exocyst components and/or septins respectively.

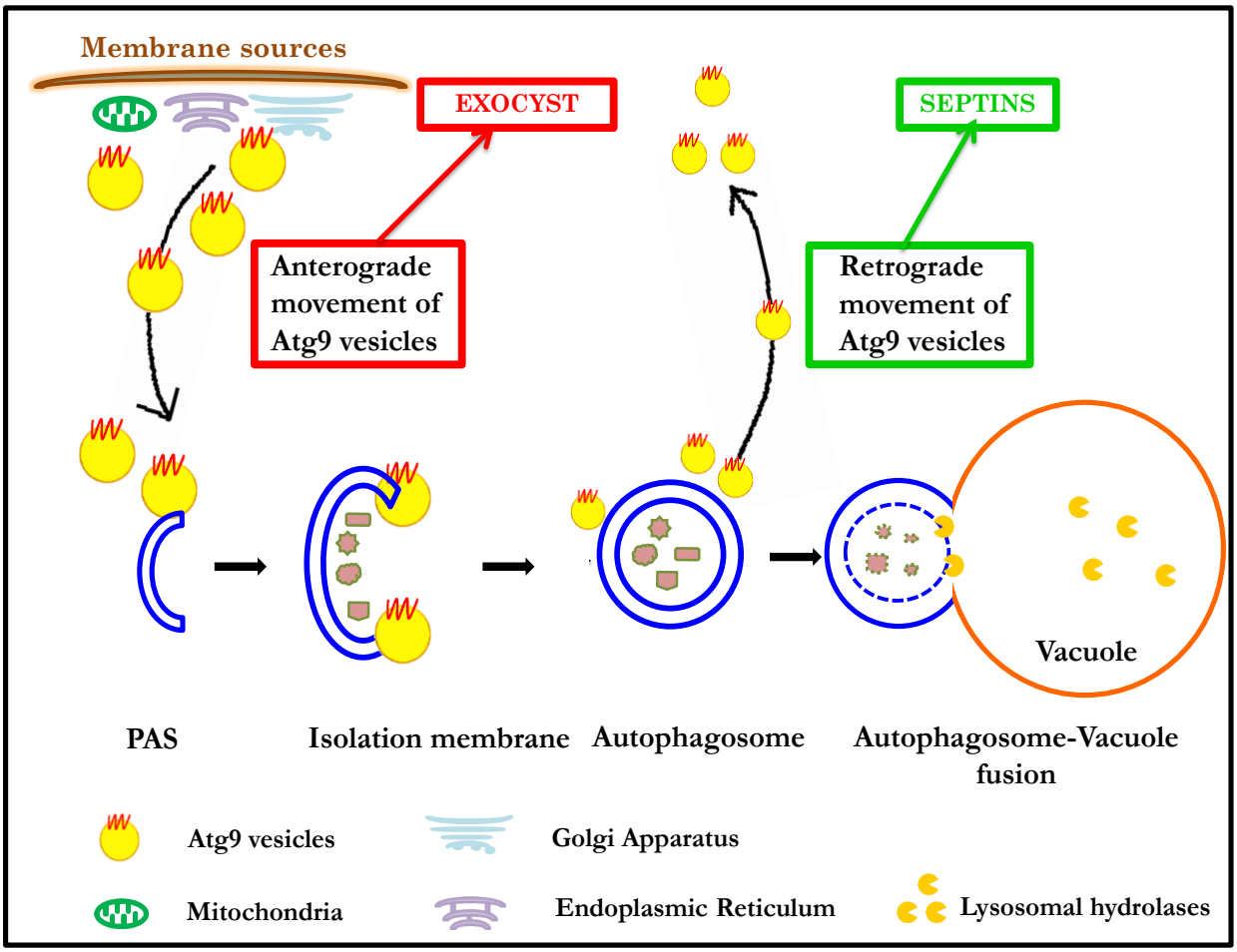


**Figure 9: Role of exocyst and septins in Atg9 trafficking.** Exocyst complex affects the anterograde movement of Atg9 vesicles whereas septins affect the retrograde movement of Atg9 vesicles.

## 4.2 Genetic manipulation of yeast strains

### 4.2.1 Strains used

The strains used in this study are listed in table 5 in Appendix A-II**.** GFP tagged strains and Ts strains were kind gifts from Dr.Kausik Chakraborty's laboratory (IGIB, Delhi) and Prof. Charlie Boone's laboratory (University of Toronto, Canada).

### 4.2.2 Genetic Manipulation of yeast strains

Genomic DNA was isolated from the strains in which the gene of interest was already tagged with GFP at its C-terminus. Using isolated genomic DNA as template and the short primers (listed in the table 6 in appendix A-III), C-terminal region of the gene of interest (GOI) (GFP tagged) was PCR amplified. Forward primer anneals approximately 200bp upstream of the stop codon whereas reverse primer is complementary to the region approximately 200bp downstream of stop codon in the 3' UTR. Flanking regions of the amplicon are homologous to the 3' UTR of the GOI as depicted in the schematic (Figure 10). These homologous regions mediate the recombination consequently integrating the GFP at the 3'UTR of the GOI.

Amplicons contain histidine selection marker downstream of the GFP sequence. These PCR amplicons were verified by agarose gel electrophoresis (Figure 11).

WT and Ts strains were then transformed with these amplicons to C-terminally tag desired genes with GFP. High efficiency standard transformation protocol (Lithium acetate based) was used for transforming yeast cells[120]. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 22$^{O}$C/25$^{O}$C/30$^{O}$C at 200 rpm and were harvested at an $A_{600}$ equivalent to 0.6-0.8. Log phase cells were then incubated at 22$^{O}$C/25$^{O}$C with transformation mix (1M Lithium Acetate, 2 mg/mL Salmon sperm carrier DNA, 50% w/v PEG-3350) containing desired amplicons (1-1.5 µg). After 10-12 hours of incubation, cells were pelleted and spread onto a selection plate (SD-His).

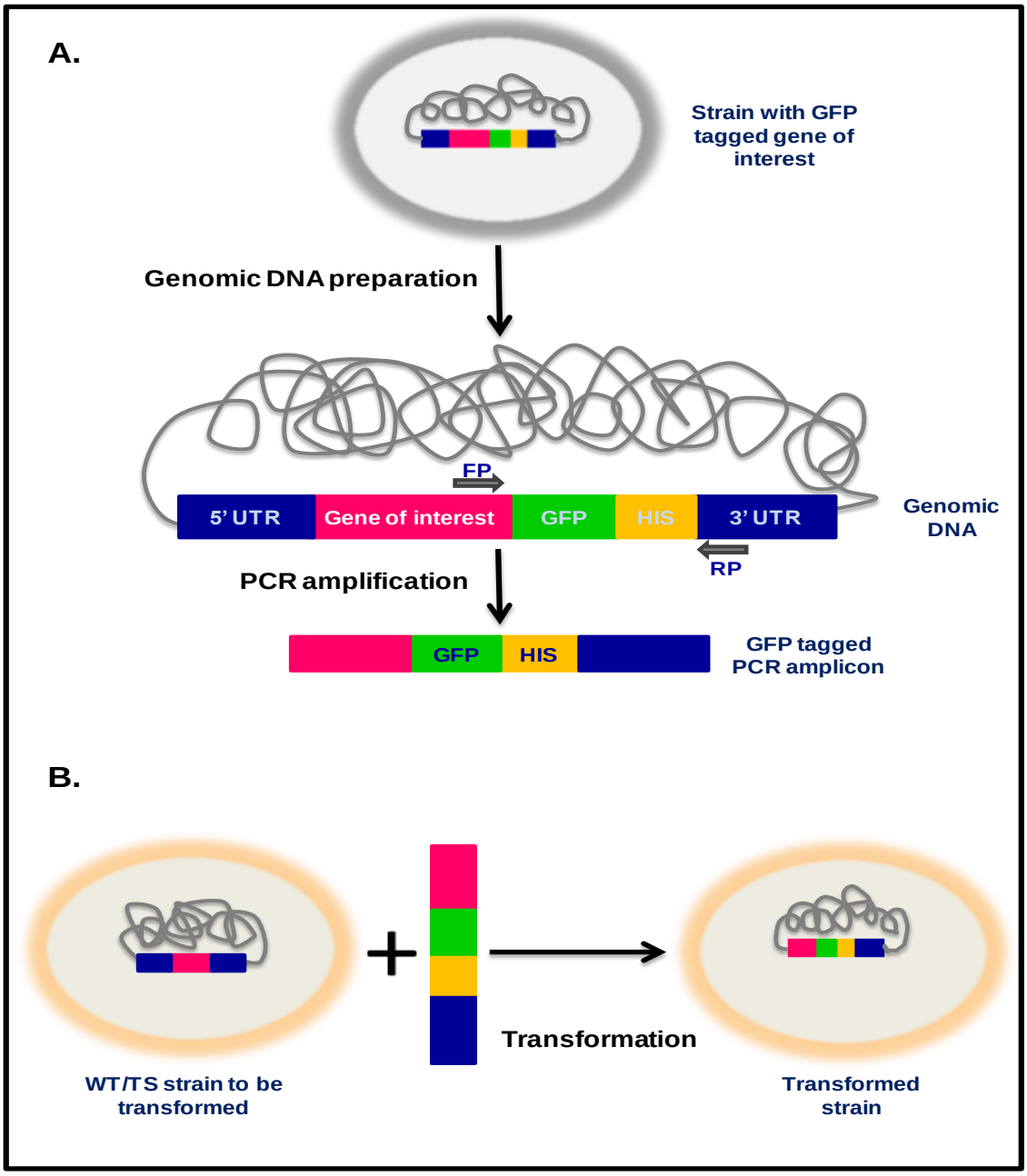


**Figure 10: Genetic manipulation of the yeast *Saccharomyces cerevisiae*. C-terminal GFP tagging of genes in different strains.**

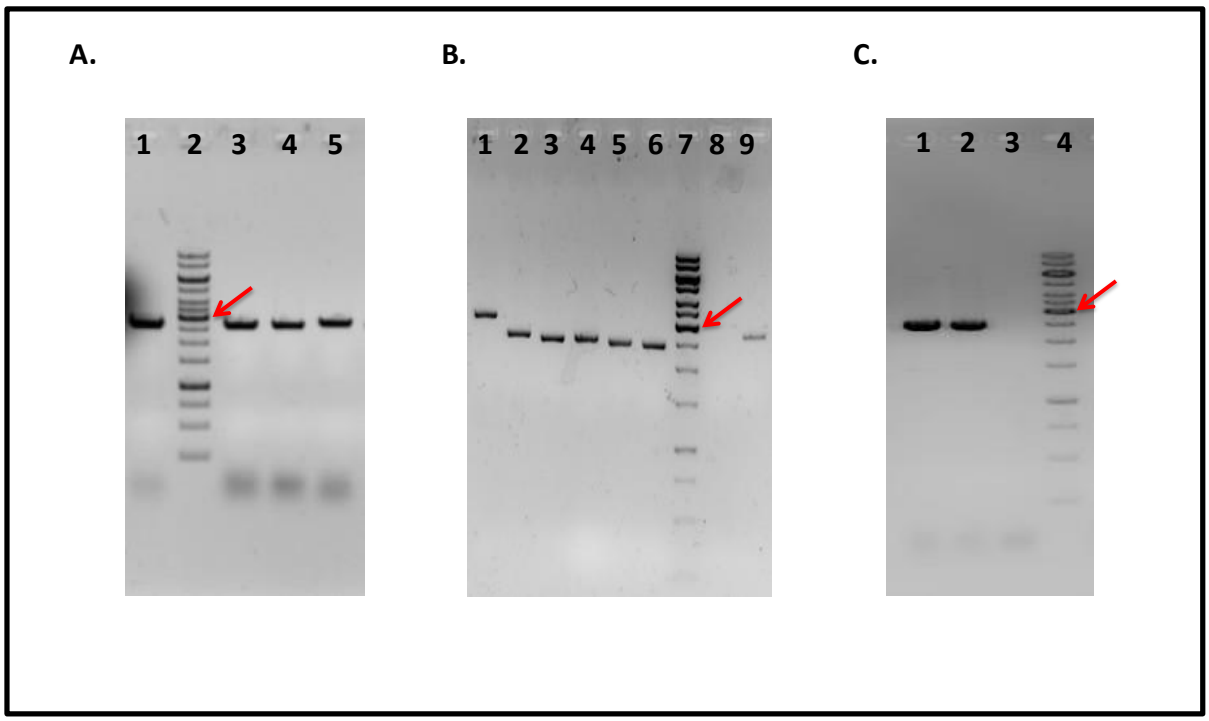


**Figure 11: Verification of GFP-tagged PCR amplicons used for genomically tagging yeast strains**. **(A)** PCR verification of septin-GFP amplicons, lane 1, positive control representing C-terminal Cdc10-GFP amplicon; lane 2, ladder (GeneRuler 1 kb DNA ladder); lanes 3-5, PCR amplicons of C-terminal Cdc10-GFP, Cdc11-GFP and Shs-GFP. **(B)** PCR verification of exocyst-GFP amplicons. Lanes 1-6, PCR amplicons of C-terminal sec3-GFP, sec5-GFP, sec6-GFP, sec8-GFP, sec10-GFP, sec15-GFP; lane 7,ladder (GeneRuler 1 kb DNA ladder); lane 8, No DNA control; lane 10,positive control representing C-terminal Cdc10-GFP amplicons.**(C)** verification of exocyst-GFP amplicons Lane 1, positive control representing C-terminal Sec6-GFP amplicons; lane 2,PCR ampliconof C-terminal Exo84-GFP; lane 3,No DNA control; lane 4,ladder (GeneRuler 1 kb DNA ladder). Red arrows indicate 3 kb band in the ladder. All the PCR amplicons are approximately 2.5 kb in size.

Plates were incubated either at 22$^{O}$C, 25$^{O}$C or 30$^{O}$C for 48-60 h depending on the background of strain (Note). Single isolated colonies were obtained which were patched on secondary selection plate (SD-His) and were then screened for GFP signal using fluorescence microscope. Correct integration was verified by looking into functional colocalization.

**NOTE:** WT strains were throughout grown at 30$^{O}$C. Permissive temperature (PT) for exocyst Ts and septin Ts strains is different. Septin Ts strains were grown at 22$^{O}$C whereas exocyst Ts strains were grown at 25$^{O}$C.

Above mentioned GFP tagged strains were transformed with centromeric low copy number plasmids pRS316 containing Atg9-2xmCherry[120]. This plasmid contain uracil selection marker. Single colonies obtained on the selection plate (SD-URA) were patched on secondary plate and screened for mCherry signal using fluorescence microscope. Functional Atg9 was visualized as multiple discrete red punctate structures.

The various combination of strains constructed using this strategy are tabulated in Appendix A-IV (Tables 7-10)**.** All the tags are integrated at the genomic loci and expressed under the endogenous promoter**.** The strains constructed here were then used for performing colocalization experiments which are explained below.

## 4.3 Study of Colocalisation between Different Exocyst Subunits and Septin Subunit Cdc10 under Starvation Condition

### 4.3.1 Strains used in the study

Following strains were used to study the colocalisation between exocyst subunits (Sec3, Sec8, and Exo84) and septin Cdc10 under starvation condition. In these strains, exocyst subunits are tagged with GFP whereas Cdc10 is mCherry tagged.

**Table 3: Strains used to study colocalization between exocyst subunits and septins under starvation conditions**

| Sr.No. | Strain Name | Background | Selection Markers |
|---|---|---|---|
| 1 | Sec3-GFP + Cdc10mCherry | WT | GFP-His and mCherry-KanMx |
| 2 | Sec8-GFP + Cdc10mCherry | WT | GFP-His and mCherry-KanMx |
| 3 | Exo84-GFP + Cdc10mCherry | WT | GFP-His and mCherry-KanMx |

### 4.3.2 Study of colocalisation between Exocyst subunits and Septin Cdc10 through fluorescence microscopy (live cell imaging)

Cells were grown in YPD and incubated at 30 $^{O}$C overnight. Actively growing log phase cells ($A_{600}$ 0.6-0.8) were washed twice with sterile water to remove traces of media and then transferred into SD-N medium (1 $A_{600}$) (Nitrogen starvation medium) to induce autophagy. Images were acquired 0, 2, 8 h post starvation to analyse potential colocalisation events between different exocyst subunits (Sec3, Sec8 and Exo84) and septin Cdc10.

### 4.3.3 Results and discussions

Although exocyst and septins interact with each other under normal physiological conditions[118], we wanted to identify the same under starvation conditions. Colocalization experiments were performed to identify the association between GFP tagged exocyst components (Sec3, Sec8 and Exo84) and mCherry tagged septin cdc10. We observed colocalization between exocyst components (Sec3, Sec8 and Exo84) and Cdc10 at 0, 2, 8 h after inducing autophagy in agreement with previously known interactions under growth conditions (Figures 12, 13 and 14). Interestingly exocyst and septins also colocalize under starvation conditions particularly at non bud neck region.

As discussed earlier, exocyst and septins interact at mother-bud neck region and regulate exocytosis and cytokinesis. However, during starvation when autophagy is prevalent, these processes (exocytosis and cytokinesis) cease to occur. Eventually there is also a gradual loss of colocalization of these complexes at the bud-neck region. Despite this, both exocyst and septins were found to colocalize under starvation conditions that to at non bud neck regions. This suggests that these proteins might interact to carry out autophagy related functions.

Although this conjecture is based on circumstantial evidence that exocyst and septins are involved in autophagy and that they might colocalize with each other under starvation conditions for autophagy purposes; it cannot be ruled out at this juncture that they might colocalize for carrying out other cellular trafficking related functions other than in autophagy (as exocytosis and cytokinesis are already ruled out).

Studies from lab have revealed that not all the components of exocyst and septins are required for autophagy. The exocyst subunits Exo70 and Sec15 are not involved in autophagy. Similarly septins Cdc3 and Cdc12 also does not affect autophagy. Therefore to determine the exocyst subunits that colocalize with septin Cdc10 under starvation conditions, we observed colocalization with three different subunits (Sec3, Sec8 and Exo84) of exocyst

complex. Percentage of colocalisation events between exocyst subunit Sec3 and Cdc10 did not change under starvation conditions. While the percentage colocalization between other exocyst subunits (Sec8 and Cdc10; Exo84 and Cdc10) increased. This suggests that interaction of all the exocyst components with Septin Cdc10 is variable under autophagic conditions. However subunit participation has to be further looked for remaining subunits of exocyst and septin. Also these associations have to be validated by biochemical approaches such as immunoprecipitation under starvation conditions.

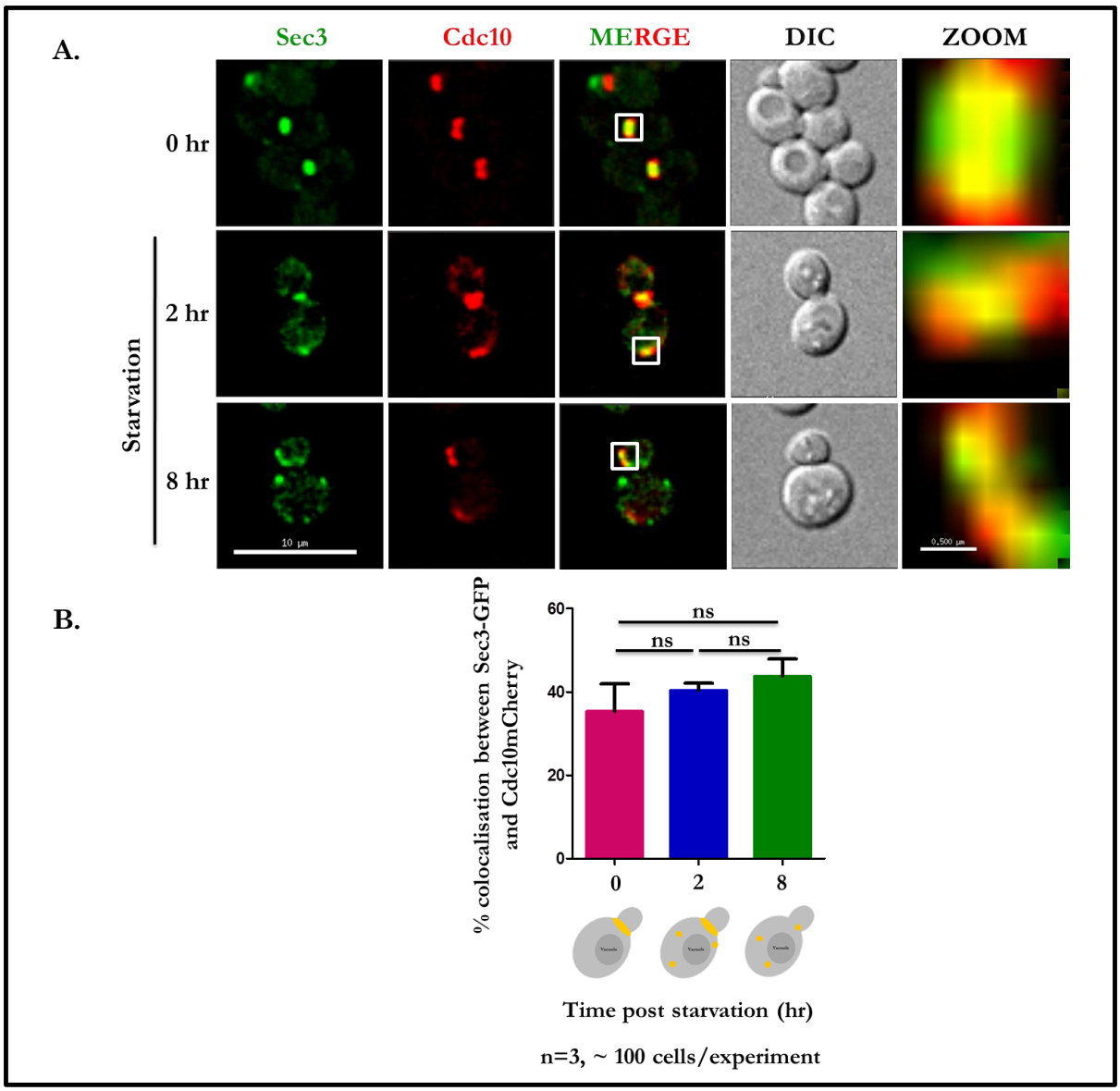


**Figure 12: Colocalisation between Sec3-GFP and Cdc10-mCherry under starvation conditions; A) Micrographs representing colocalisation between Sec3-GFP and Cdc10-mCherry post 2 hr and 8 hr of starvation. Scale bar represents 10 µm. For inset view scale bar represents 0.5 µm. B) Quantification of colocalisation between Sec3 and Cdc10 under starvation.**
**Image acquisition: images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 32%, exposure 0.08 sec) and FITC (transmittance 32%, exposure 0.1 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks were taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

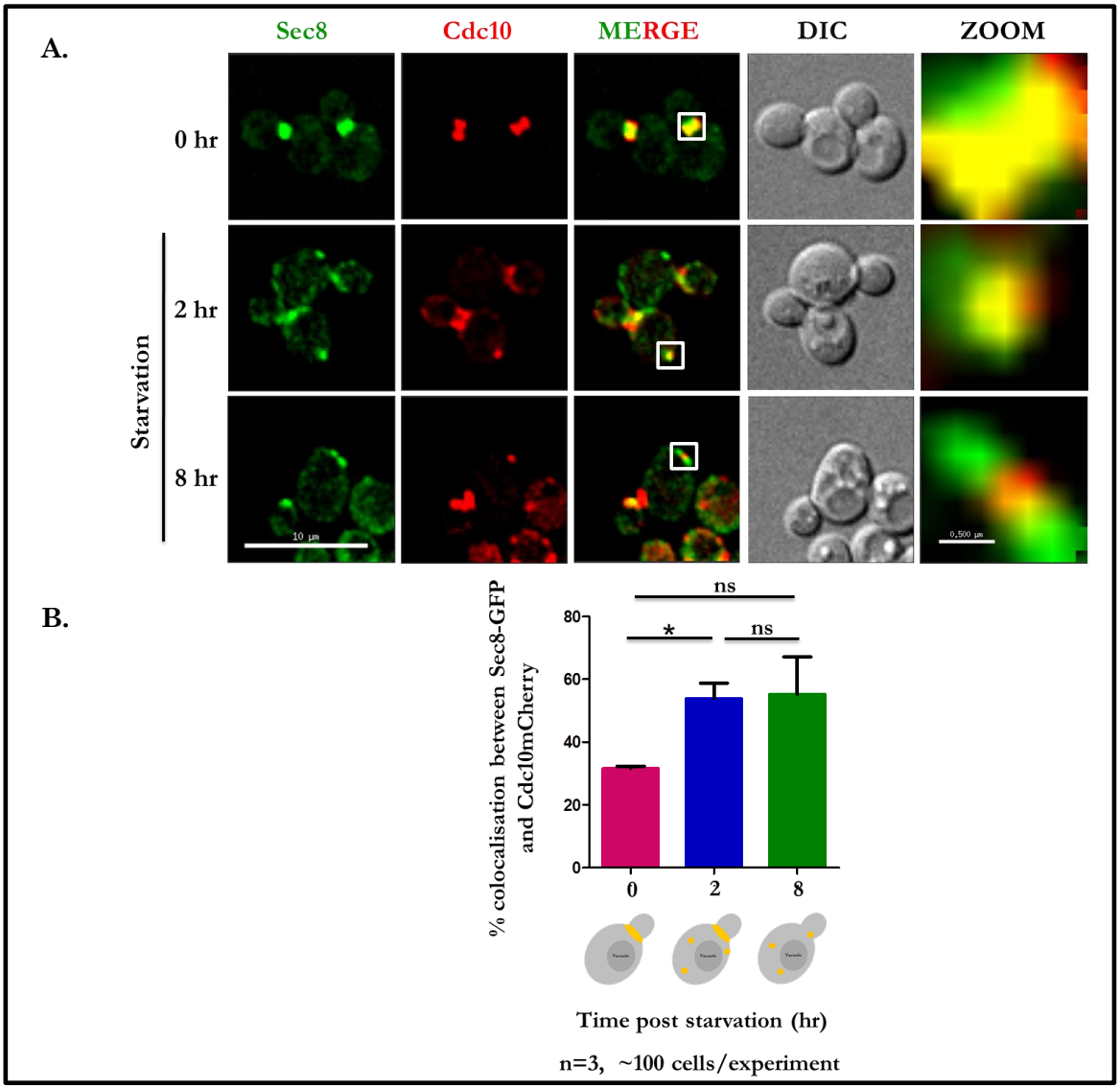


**Figure 13: Colocalisation between Sec8-GFP and Cdc10-mCherry under starvation conditions; A) Micrographs representing colocalisation between Sec8-GFP and Cdc10-mCherry post 2 hr and 8 hr of starvation. Scale bar represents 10 µm. For inset view scale bar represents 0.5 µm. B) Quantification of colocalisation between Sec8 and Cdc10 under starvation.**
**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.08 sec) and FITC (transmittance 50%, exposure 0.1 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

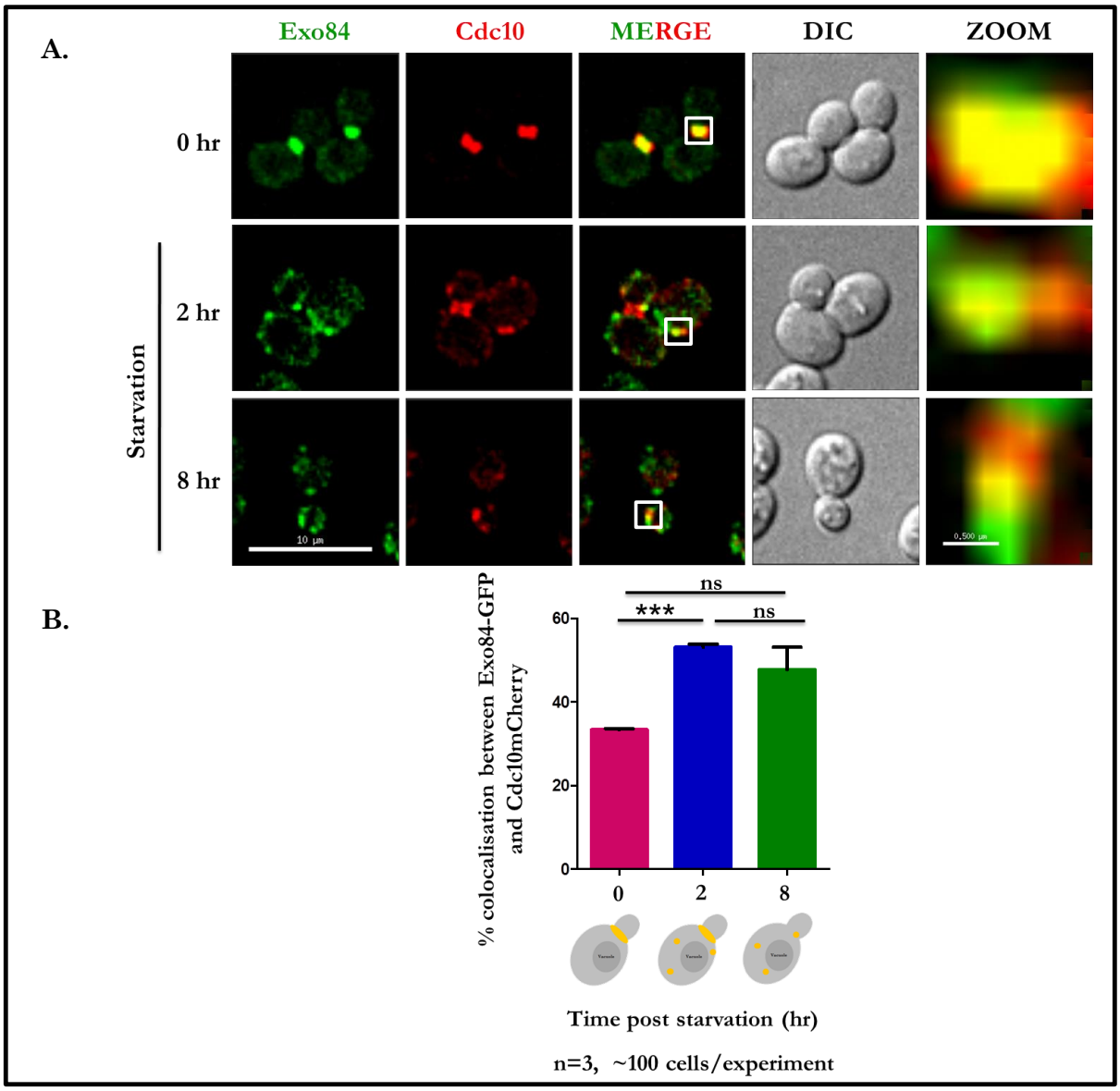


**Figure 14: Colocalisation between Exo84-GFP and Cdc10-mCherry under starvation condition; A) Micrographs representing colocalisation between Exo84-GFP and Cdc10-mCherry post 2 hr and 8 hr of starvation. Scale bar represents 10 µm. For inset view scale bar represents 0.5 µm. B) Quantification of colocalisation between Exo84 and Cdc10 under starvation.**
**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.08 sec) and FITC (transmittance 50%, exposure 0.1 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

## 4.4 Study of Dynamic Association between Septins and Atg9 in the absence of functional Exocyst Subunits

### 4.4.1 Study of dynamic association through live cell imaging

Strains with temperature sensitive alleles for exocyst subunits (*sec3-2* and *sec8-6*) were used in this study. Exocyst Ts strains provide a platform to study dynamic association between septins and Atg9 in the absence of functional exocyst subunit. Cells were grown at permissive temperature of 25$^{O}$C in SD-URA medium at 200 rpm. Secondary inoculation was done starting from 0.2 $A_{600}$ and grown till log phase (0.6-0.8 $A_{600}$).

Log phase cells were split into two parts such that each part has nearly 3 $A_{600}$ equivalents of cells. Cells were washed twice with sterile water to remove residual media and transferred into SD-N medium (1 $A_{600}$) to induce autophagy. Cells were then incubated at two different temperatures (PT and NPT). The part of cells to be incubated at 37 $^{O}$C were transferred in SD-N medium which was pre-maintained at 37$^{O}$C. 700 µL of cells were collected from both the pools (permissive and restrictive) after 0, 1, 2 h of inducing autophagy and images were acquired.

Experiments were performed with the following strains.

1) Cdc10-GFP and Atg9-2x-mCherry in sec3-2 background
2) Shs1-GFP and Atg9-2x-mCherry in sec8-6 background

### 4.4.2 Results and Discussions

In previous experiments we observed that exocyst and septins colocalize during starvation conditions at non bud neck region. Moreover studies from lab suggest that exocyst and septins affect Atg9 trafficking. But how exocyst and septin regulate autophagy is still a question. Therefore, it would be of prime importance to determine as to how this exocyst-septin interaction regulates autophagy. One of the ways is to look for combined interaction of exocyst-septin with autophagy related proteins. It can be achieved by two ways – first through Biomolecular Fluorescence complementation (BiFC) assay. In this approach, fluorescent protein (say GFP) is fragmented into two parts (N- and C- terminal) and each part is associated with two proteins (say Septin and Atg9). The fluorescence (green) is observed only when these to proteins come in close proximity to each other. The third protein (Exocyst) under study can be tagged with other fluorophore (RFP). The association of these three proteins can be observed by visualising colocalisation (Yellow) between them. Second

method could be to determine triple colocalization. This involves tagging of all the three components (Exocyst, septins and Atg) with different tags in the same strain.

Another approach to find the cooperative role of exocyst and septin in autophagy is based on the observation that exocyst and septins affect Atg9 trafficking. It can be achieved by determining the association of one complex with Atg9 in the absence of the other functional complex. To test this hypothesis, we made use of Ts strains available for exocyst subunits and septins. Exocyst Ts strains used in this study possessed GFP tagged septins and Atg9-2x-mCherry. Similarly in septin Ts, exocyst components were GFP tagged and contained Atg9-2x-mCherry. This allowed us to determine if either complex regulates the Atg9 association of the other.

We observed the colocalisation between Septin subunits (Cdc10-GFP and Shs1-GFP) and Atg9-2x-mCherry in different exocyst Ts strains (*sec3-2* and *sec8-6*) under starvation conditions. Exocyst Ts strains provide an opportunity to study septin-Atg9 association in the absence of functional exocyst subunits. Work from lab has shown that septins associate with Atg9 and exocyst has mild effect on Atg9 trafficking. Because no change in Cdc10-Atg9 and Shs1-Atg9 associations was observed at NPT in *sec3-2* and *sec8-6* respectively, it suggests that septin involvement in Atg9 trafficking is not influenced by exocyst. At this stage the question that remains is whether exocyst-septin interaction during starvation still has any role in other stages of autophagy other than Atg9 trafficking (Figures 15 and 16).

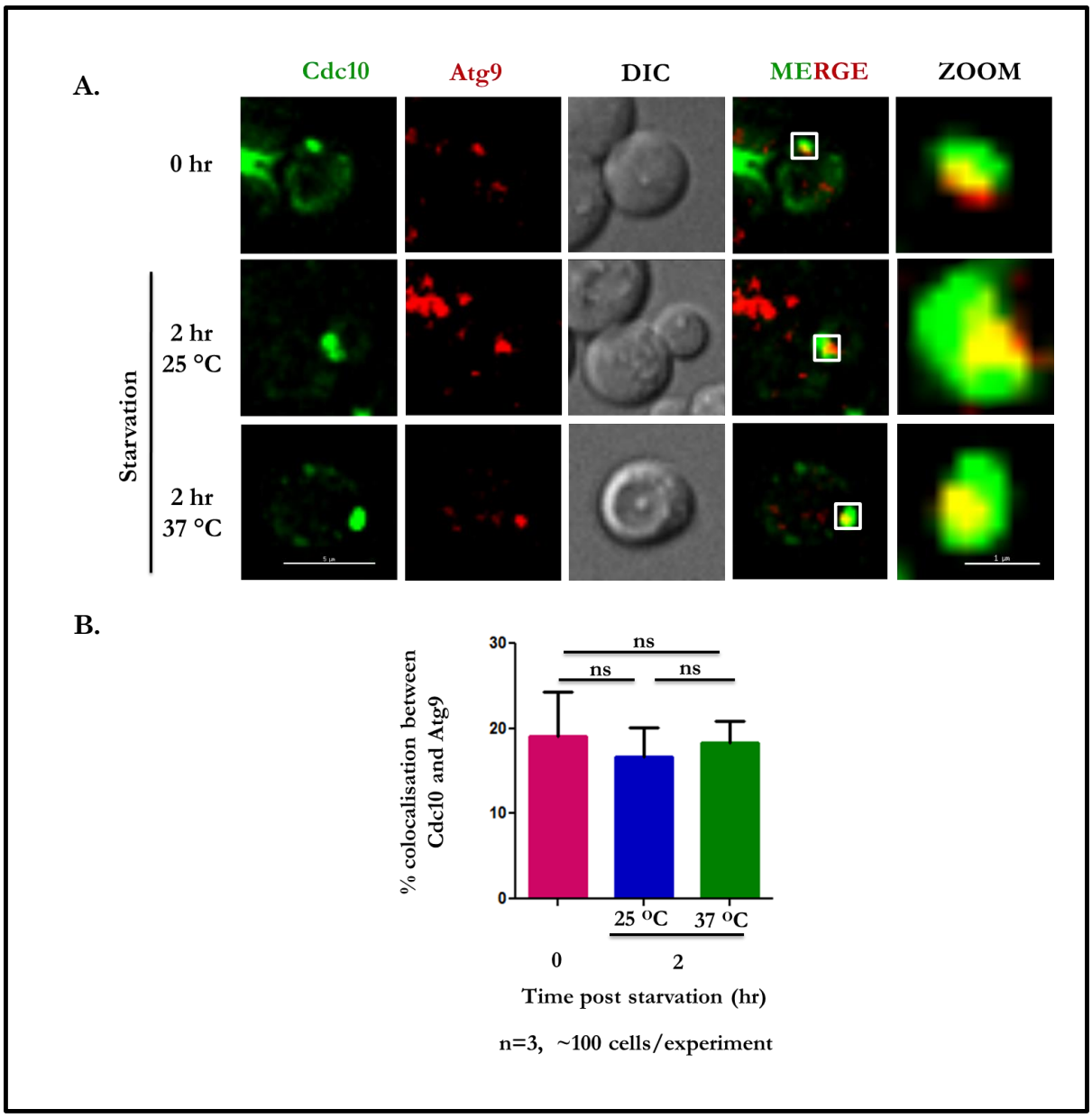


**Figure 15: Colocalisation between Cdc10-GFP and Atg9-2xmCherry in sec3-2 (in the absence of functional Sec3 exocyst subunit); A) Micrographs representing colocalisation between Cdc10-GFP and Atg9-2xmCherry at PT and NPT for 0 hr and 2 hr. Scale bar represents 5 µm. For inset view scale bar represents 1 µm. B) Quantification of colocalisation between Cdc10 and Atg9 in *sec3-2* post starvation at both PT and NPT.**
**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.8 sec) and FITC (transmittance 32%, exposure 0.025 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

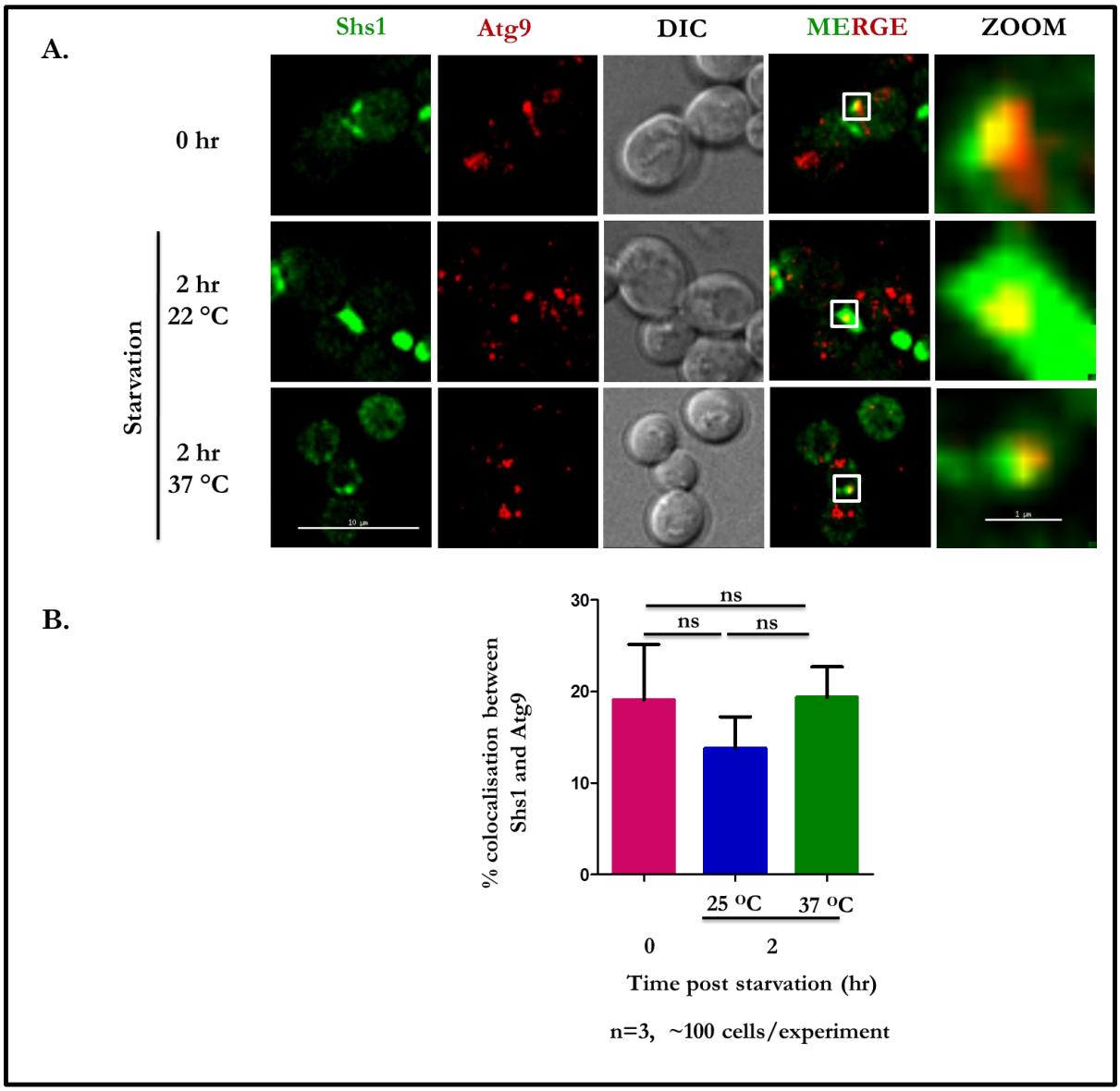


**Figure 16: Colocalisation between Shs1-GFP and Atg9-2xmCherry in *sec8-6* (in the absence of functional Sec3 exocyst subunit); A) Micrographs representing colocalisation between Shs1-GFP and Atg9-2xmCherry at PT and NPT for 0 hr and 2 hr. Scale bar represents 10 µm. For inset view scale bar represents 1 µm. B) Quantification of colocalisation between Cdc10 and Atg9 in *sec8-6* post starvation at both PT and NPT.**
**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.8 sec) and FITC (transmittance 32%, exposure 0.05 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

Our lab has shown that septins translocate from the mother-bud neck region to the cytoplasm under autophagic conditions in budding yeast *Saccharomyces cerevisiae*[114]. Next we wanted to analyse if this disassembly of septins from mother-bud neck region is exocyst dependent. For this we studied the dissociation of septins in the absence of functional exocyst subunits (exocyst Ts background) by measuring the intensity of septin-GFP at the mother-bud neck region. For all the WT and Ts strains, we observed a decrease in the intensity of septin-GFP at the mother-bud neck region under starvation conditions at both PT and NPT. This dissociation of septins was only marginally (6-12%) accelerated in Ts strains at NPT (that is in the absence of functional exocyst subunits) as compared to that of WT strains. These results indicate that disassembly of septins from mother-bud neck region under starvation is not influenced by exocyst components (Figure 17).

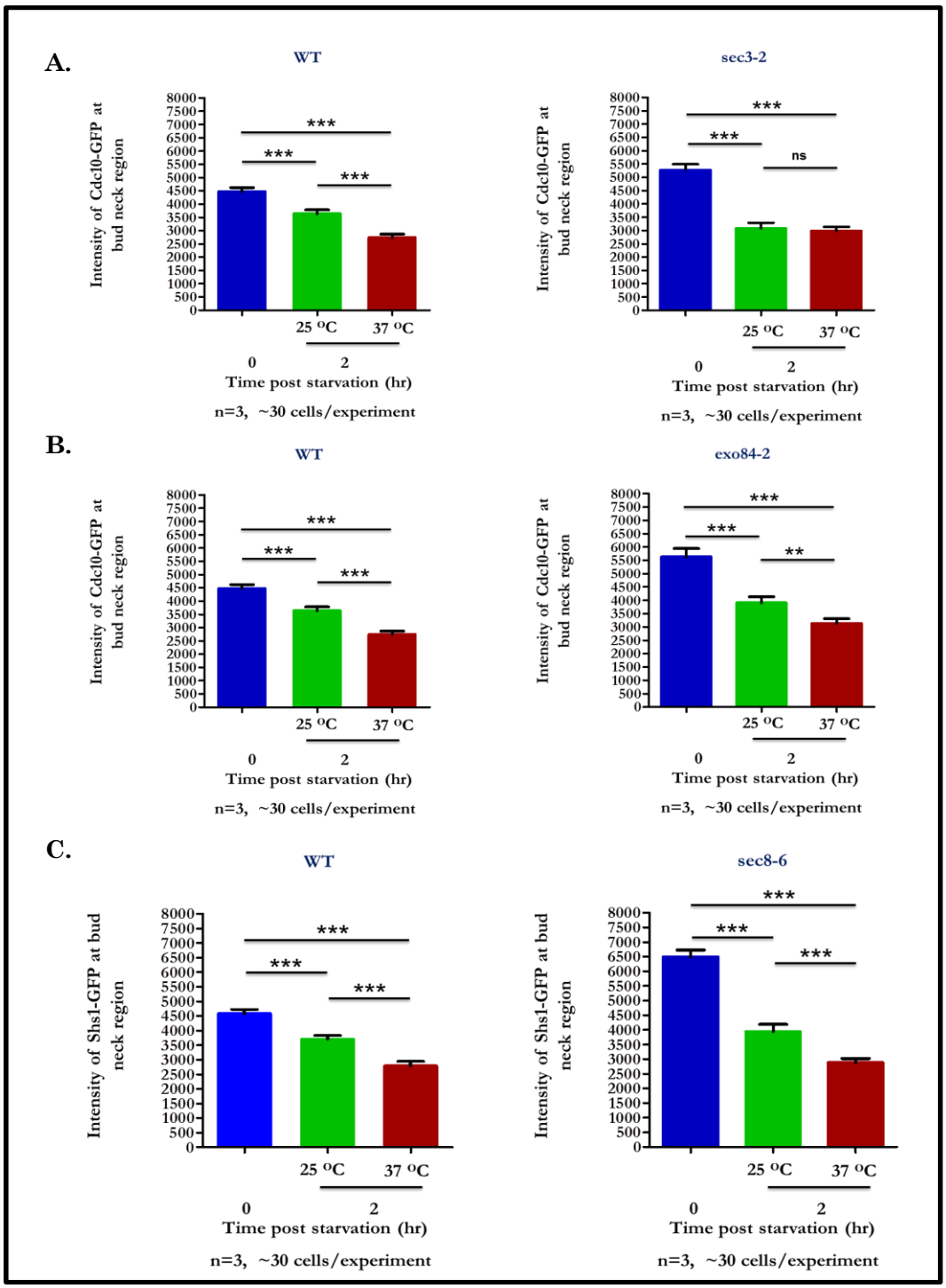


**Figure 17: Measurement of intensity of septin-GFP at the bud neck region to analyse its dissociation upon starvation in WT and Ts backgrounds. A) Cdc10-GFP intensity measurement in WT and *sec3-2*. B) Cdc10-GFP intensity measurement in WT and *exo84-2*. C) Cdc10-GFP intensity measurement in WT and *sec8-6*.**
**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter (% transmittance and exposure were kept constant for tests and control)], quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm and projected by maximum intensity. Intensity of Septin-GFP was measured using measure plugin of Fiji software. Statistics – unpaired t-test.**

## 4.5 Study Dynamic Association between Exocyst and Atg9 in the Absence of a Functional Septin Subunit

### 4.5.1 Study of dynamic association through live cell imaging

For this study, strain with temperature sensitive allele of septin Cdc10 (*cdc10-5*) was used. Cells were grown in SD-URA medium at 22 $^{O}$C (permissive temperature) at 200 rpm. Log phase cells (0.6-0.8 $A_{600}$ were split into two parts and washed twice with sterile water to remove traces of media. Cells were then transferred in SD-N medium (1 $A_{600}$) to induce autophagy and the two parts were then incubated at 2 different temperatures, one at 22 $^{O}$C (permissive) and the other at 37 $^{O}$C (restrictive).

Cells were withdrawn from both the pools at different time intervals (post 1 and 2 h of starvation) and images were acquired using fluorescence microscope. Imaging was also done before transferring the cells into SD-N (0 h).

Strain contain genomically tagged Sec3-GFP and Atg9-2x-mCherry and it harbours a temperature sensitive allele of Cdc10 (*cdc10-5*).

### 4.5.2 Results and discussions

It is identified in our lab that exocyst associates with Atg9 under starvation conditions. In addition to this, studies also reveal that septins have role in retrograde trafficking of Atg9 vesicles and hence we set out to determine whether exocyst-Atg9 association is dependent on septins. For this we studied the association between exocyst subunit Sec3 and Atg9 in *cdc10-5* (that is in the absence of functional Cdc10). Just like in septins no change in Sec3-Atg9 association was observed at NPT in *cdc10-5* suggesting that role of exocyst in Atg9 trafficking is not influenced by septins. (Figure18). Although too early to speculate, these studies along with other experiments might shed light on the possible epistatic influence of these complexes in various stages of autophagic flux.

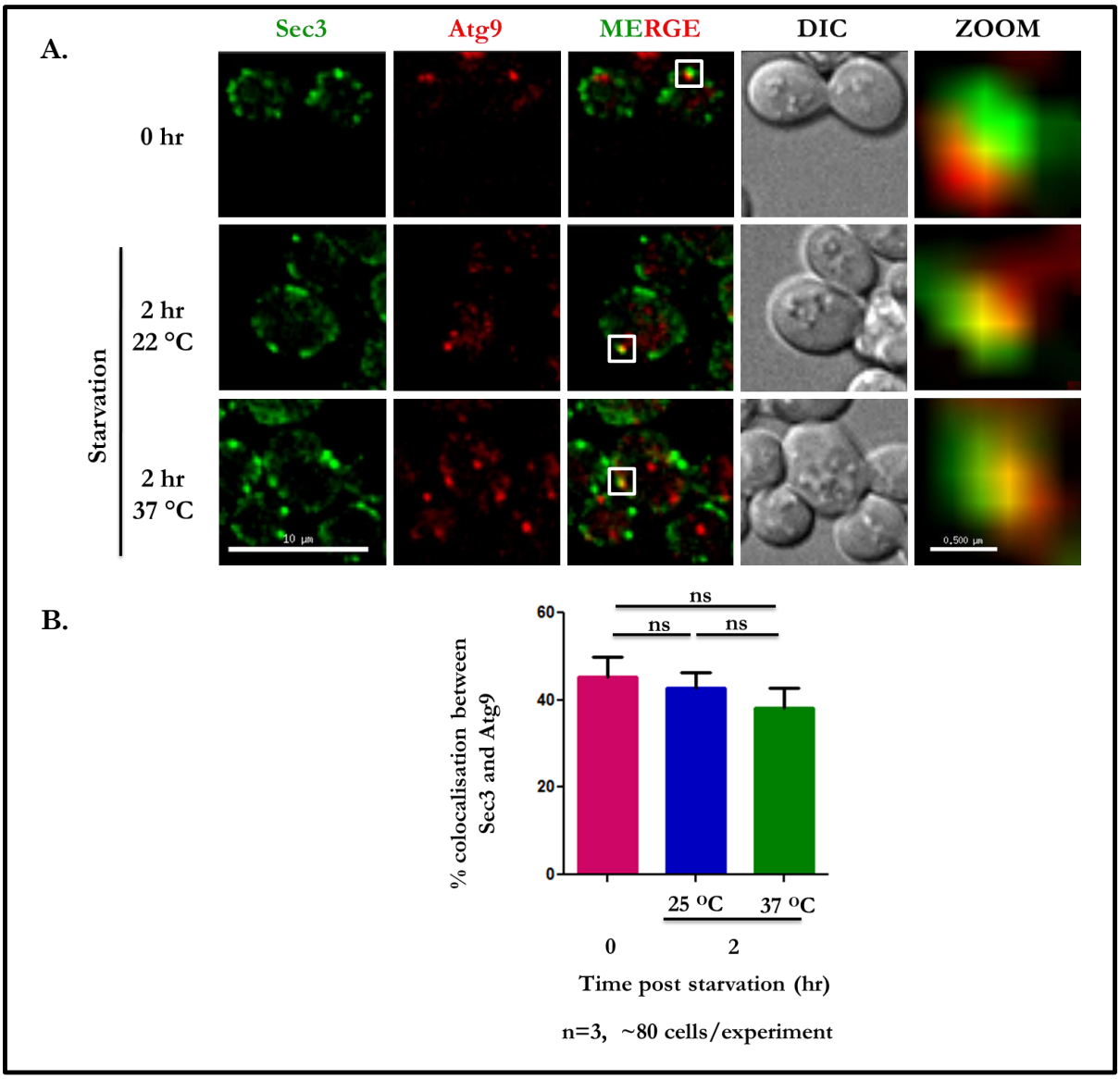


**Figure 18: Colocalisation between Sec3-GFP and Atg9-2xmCherry in *cdc10-5* (in the absence of functional Cdc10 septin); A) Micrographs representing colocalisation betweenSec3-GFP and Atg9-2xmCherry at PT and NPT for 0 hr and 2 hr. Scale bar represents 10 µm. For inset view scale bar represents 0.5 µm. B) Quantification of colocalisation betweenSec3and Atg9 in *cdc10-5* post starvation at both PT and NPT.**

**Image acquisition: Images were acquired using Delta vision microscope [Olympus 60X/1.42, Plan ApoN objective; excitation and emission filter A594 (transmittance 50%, exposure 0.8 sec) and FITC (transmittance 32%, exposure 0.15 sec), quad-mCh polychroic and 2 X 2 binning]. Approximately 25 stacks was taken with distance between each one of them 0.5 µm. Images were deconvolved using enhanced ratio algorithm. Colocalization events were manually counted using cell counter plugin of Fiji software. Statistics – unpaired t-test.**

# Chapter 4

# Conclusions and Future Perspectives

Through our studies we have tried to establish the mutual roles of exocyst and septins in autophagy. Our findings indicate that exocyst and septins associate with each other during the stages of autophagy. Based on colocalization experiments by tagging various members of exocyst and septins, our studies indicate that the number and kinds of subunits of both the multisubunit protein complexes that participate may be different in autophagic conditions as opposed to what is known under growth conditions. Defined composition of exocyst and septin that are involved in exocytosis and cytokinesis may be different in size and composition during autophagy. To pursue these experiments and strengthen our observations, we want to perform colocalization experiments with other subunits of exocyst and septins. These colocalization studies have to be corroborated by biochemical approaches such as size-exclusion chromatography and/or immunoprecipitation under starvation conditions (autophagy induction).

Septin dissociation from mother-bud neck region is only marginally increased under starvation condition in the absence of functional exocyst subunits. This indicated that septin dissociation under starvation is independent of exocyst components. We would further like to check this dissociation with other combination of exocyst Ts strain.

Exocyst and septins both are known to independently affect Atg9 trafficking. However, observation reveals that when one subunit of one of the complexes was disabled, Atg9 trafficking by the other was not affected. We intend to determine the mutual role of exocyst and septin in Atg9 trafficking through Biomolecular fluorescence complementation (BiFC). This assay provides a platform to study colocalization of all the three components (Atg9, exocyst and septins) simultaneously.

These observations raise many questions regarding the functional consequences of the interactions between septins and exocyst under autophagic conditions.

## APPENDIX A-I

**Table 4: List of strains used to study colocalisation between exocyst components and Atg8**

| Sr.No. | Strain | Strain background |
|---|---|---|
| 1 | Sec3-GFP + 2x-mCherry-Atg8 | WT |
| 2 | Sec5-GFP + 2x-mCherry-Atg8 | WT |
| 3 | Sec6-GFP + 2x-mCherry-Atg8 | WT |
| 4 | Sec8-GFP + 2x-mCherry-Atg8 | WT |
| 5 | Sec10-GFP + 2x-mCherry-Atg8 | WT |
| 6 | Sec15-GFP + 2x-mCherry-Atg8 | WT |
| 7 | Sec3-GFP + 2x-mCherry-Atg8 | Δypt7 |
| 8 | Sec5-GFP + 2x-mCherry-Atg8 | Δypt7 |
| 9 | Sec6-GFP + 2x-mCherry-Atg8 | Δypt7 |
| 10 | Sec8-GFP + 2x-mCherry-Atg8 | Δypt7 |
| 11 | Sec10-GFP + 2x-mCherry-Atg8 | Δypt7 |
| 12 | Sec15-GFP + 2x-mCherry-Atg8 | Δypt7 |

## APPENDIX A-II

**Table 5: List of strains used for genomic tagging**

| Sr.No. | Strain | Strain background |
|---|---|---|
| 1 | Sec3-GFP | WT |
| 2 | Sec5-GFP | WT |
| 3 | Sec6-GFP | WT |
| 4 | Sec8-GFP | WT |
| 5 | Sec10-GFP | WT |
| 6 | Sec15-GFP | WT |
| 7 | Exo84-GFP | WT |
| 8 | Cdc10-GFP | WT |
| 9 | Cdc11-GFP | WT |
| 10 | Shs1-GFP | WT |
| 11 | *sec3-2* | Ts |
| 12 | *sec5-24* | Ts |
| 13 | *sec6-4* | Ts |
| 14 | *sec8-6* | Ts |
| 15 | *sec8-9* | Ts |
| 16 | *sec10-2* | Ts |
| 17 | *sec15-1* | Ts |
| 18 | *exo70.29/37* | Ts |
| 19 | *exo70.38* | Ts |
| 20 | *exo84-2* | Ts |
| 21 | *cdc10-1* | Ts |
| 22 | *cdc10-2* | Ts |
| 23 | *cdc10-5* | Ts |
| 24 | *cdc11-3* | Ts |
| 25 | *cdc11-4* | Ts |
| 26 | *cdc11-5* | Ts |

## APPENDIX A-III

### Table 6: List of primers used in the study

| Sr.No. | PRIMER NAME | TAG | PRIMER SEQUENCE |
|---|---|---|---|
| 1. | CDC10GFPFP | C-terminal GFP tag | TGAAGGGTTCAGAGCAAGAC |
| 2. | CDC10GFPTRP | C-terminal GFP tag | CCGAATCATAAGGATAAATCTTGAA |
| 3. | CDC11GFPFP | C-terminal GFP tag | ACACGTGAGGAGCAAATACG |
| 4. | CDC11GFPTRP | C-terminal GFP tag | CCGGCCCGGGTCGATAATGACGATCCACACA |
| 5. | SHS1GFPFP | C-terminal GFP tag | GCACACGAATTGAAATTGAGG |
| 6. | SHS1GFPTRP | C-terminal GFP tag | CCTTCCCTCAACAACAAAGG |
| 7. | SEC3I6 | C-terminal GFP tag | CCAGTAGGCTTGAAGAATTCAG |
| 8. | SEC3R | C-terminal GFP tag | TGGACAATGAAGCTAACTAATATTCTGTTC |
| 9. | SEC5GFPF | C-terminal GFP tag | GGGCGTGCTTACAAAACTGTTTC |
| 10. | SEC5GFPR | C-terminal GFP tag | CGAATTTGACAGTGCCATCATAC |
| 11. | SEC6GFPF | C-terminal GFP tag | AATACCGCCGTAACATGGAGGC |
| 12. | SEC6GFPR | C-terminal GFP tag | CATTCCTACTGAAGGCACAGC |

| | | | |
|---|---|---|---|
| 13. | SEC8GFPF | C-terminal GFP tag | GTAACCTCGAAAAAGAGCAGTCG |
| 14. | SEC8GFPR | C-terminal GFP tag | TGATGTTGTAATGTTCCTTCAGGTATC |
| 15. | SEC10GFPF | C-terminal GFP tag | GGGTCATTTAGCAGACATTGGAAG |
| 16. | SEC10GFPR | C-terminal GFP tag | CGTGGGCCTAAGAAAAAGGT |
| 17. | SEC15GFPF | C-terminal GFP tag | CTGAATCAGCGAGTGTCATTG |
| 18. | SEC15GFPR | C-terminal GFP tag | TTGAAGTGAACTTTTTCGATGC |
| 19. | EXO84GFPF | C-terminal GFP tag | AGATTGGTGTAGCGACGAAG |
| 20. | EXO84GFPR | C-terminal GFP tag | CTGACTGACGATGAGTGCTC |

# APPENDIX A-IV

## List of strains constructed in this study

**Table 7: C-terminal tagging of septin subunits in exocyst TS strains**

| Sr.No. | Background strain | Amplicon | Strain constructed |
|---|---|---|---|
| 1 | *sec3-2* | Cdc10-GFP-His | Cdc10-GFP-His in *sec3-2* |
| 2 | *sec5-24* | Cdc10-GFP-His | Cdc10-GFP-His in *sec5-24* |
| 3 | *sec6-4* | Cdc10-GFP-His | Cdc10-GFP-His in *sec6-4* |
| 4 | *sec8-6* | Cdc10-GFP-His | Cdc10-GFP-His in *sec8-6* |
| 5 | *sec8-9* | Cdc10-GFP-His | Cdc10-GFP-His in *sec8-9* |
| 6 | *sec10-2* | Cdc10-GFP-His | Cdc10-GFP-His in *sec10-2* |
| 7 | *sec15-1* | Cdc10-GFP-His | Cdc10-GFP-His in *sec15-1* |
| 8 | *exo70.29/37* | Cdc10-GFP-His | Cdc10-GFP-His in *exo70.29/37* |
| 9 | *exo70.38* | Cdc10-GFP-His | Cdc10-GFP-His in *exo70.38* |
| 10 | *exo84-2* | Cdc10-GFP-His | Cdc10-GFP-His in *exo84-2* |
| 11 | *sec3-2* | Cdc11-GFP-His | Cdc11-GFP-His in *sec3-2* |
| 12 | *sec5-24* | Cdc11-GFP-His | Cdc11-GFP-His in *sec5-24* |
| 13 | *sec6-4* | Cdc11-GFP-His | Cdc11-GFP-His in *sec6-4* |
| 14 | *sec8-6* | Cdc11-GFP-His | Cdc11-GFP-His in *sec8-6* |
| 15 | *sec8-9* | Cdc11-GFP-His | Cdc11-GFP-His in *sec8-9* |
| 16 | *sec10-2* | Cdc11-GFP-His | Cdc11-GFP-His in *sec10-2* |
| 17 | *sec15-1* | Cdc11-GFP-His | Cdc11-GFP-His in *sec15-1* |
| 18 | *exo70.29/37* | Cdc11-GFP-His | Cdc11-GFP-His in *exo70.29/37* |
| 19 | *exo70.38* | Cdc11-GFP-His | Cdc11-GFP-His in *exo70.38* |
| 20 | *exo84-2* | Cdc11-GFP-His | Cdc11-GFP-His in *exo84-2* |
| 21 | *sec3-2* | Shs1-GFP-His | Shs1-GFP-His in *sec3-2* |
| 22 | *sec5-24* | Shs1-GFP-His | Shs1-GFP-His in *sec5-24* |

| | | | |
|---|---|---|---|
| 23 | *sec6-4* | Shs1-GFP-His | Shs1-GFP-His in *sec6-4* |
| 24 | *sec8-6* | Shs1-GFP-His | Shs1-GFP-His in *sec8-6* |
| 25 | *sec8-9* | Shs1-GFP-His | Shs1-GFP-His in *sec8-9* |
| 26 | *sec10-2* | Shs1-GFP-His | Shs1-GFP-His in *sec10-2* |
| 27 | *sec15-1* | Shs1-GFP-His | Shs1-GFP-His in *sec15-1* |
| 28 | *exo70.29/37* | Shs1-GFP-His | Shs1-GFP-His in *exo70.29/37* |
| 29 | *exo70.38* | Shs1-GFP-His | Shs1-GFP-His in *exo70.38* |
| 30 | *exo84-2* | Shs1-GFP-His | Shs1-GFP-His in *exo84-2* |

**Table 8: C-terminal GFP tagging of exocyst subunits in Septin TS strains**

| Sr.No. | Background strain | Amplicon | Strain constructed |
|---|---|---|---|
| 1 | *cdc10-1* | Sec3-GFP-His | Sec3-GFP-His in *cdc10-1* |
| 2 | *cdc10-2* | Sec3-GFP-His | Sec3-GFP-His in *cdc10-2* |
| 3 | *cdc10-5* | Sec3-GFP-His | Sec3-GFP-His in *cdc10-5* |
| 4 | *cdc11-3* | Sec3-GFP-His | Sec3-GFP-His in *cdc11-3* |
| 5 | *cdc11-4* | Sec3-GFP-His | Sec3-GFP-His in *cdc11-4* |
| 6 | *cdc11-5* | Sec3-GFP-His | Sec3-GFP-His in *cdc11-5* |
| 7 | *cdc10-1* | Sec5-GFP-His | Sec5-GFP-His in *cdc10-1* |
| 8 | *cdc10-2* | Sec5-GFP-His | Sec5-GFP-His in *cdc10-2* |
| 9 | *cdc10-5* | Sec5-GFP-His | Sec5-GFP-His in *cdc10-5* |
| 10 | *cdc11-3* | Sec5-GFP-His | Sec5-GFP-His in *cdc11-3* |
| 11 | *cdc11-4* | Sec5-GFP-His | Sec5-GFP-His in *cdc11-4* |
| 12 | *cdc11-5* | Sec5-GFP-His | Sec5-GFP-His in *cdc11-5* |
| 13 | *cdc10-1* | Sec6-GFP-His | Sec6-GFP-His in *cdc10-1* |
| 14 | *cdc10-2* | Sec6-GFP-His | Sec6-GFP-His in *cdc10-2* |
| 15 | *cdc10-5* | Sec6-GFP-His | Sec6-GFP-His in *cdc10-5* |
| 16 | *cdc11-3* | Sec6-GFP-His | Sec6-GFP-His in *cdc11-3* |
| 17 | *cdc11-4* | Sec6-GFP-His | Sec6-GFP-His in *cdc11-4* |
| 18 | *cdc11-5* | Sec6-GFP-His | Sec6-GFP-His in *cdc11-5* |
| 19 | *cdc10-1* | Sec8-GFP-His | Sec8-GFP-His in *cdc10-1* |
| 20 | *cdc10-2* | Sec8-GFP-His | Sec8-GFP-His in *cdc10-2* |

| | | | |
|---|---|---|---|
| 21 | *cdc10-5* | Sec8-GFP-His | Sec8-GFP-His in *cdc10-5* |
| 22 | *cdc11-3* | Sec8-GFP-His | Sec8-GFP-His in *cdc11-3* |
| 23 | *cdc11-4* | Sec8-GFP-His | Sec8-GFP-His in *cdc11-4* |
| 24 | *cdc11-5* | Sec8-GFP-His | Sec8-GFP-His in *cdc11-5* |
| 25 | *cdc10-1* | Sec10-GFP-His | Sec10-GFP-His in *cdc10-1* |
| 26 | *cdc10-2* | Sec10-GFP-His | Sec10-GFP-His in *cdc10-2* |
| 27 | *cdc10-5* | Sec10-GFP-His | Sec10-GFP-His in *cdc10-5* |
| 28 | *cdc11-3* | Sec10-GFP-His | Sec10-GFP-His in *cdc11-3* |
| 29 | *cdc11-4* | Sec10-GFP-His | Sec10-GFP-His in *cdc11-4* |
| 30 | *cdc11-5* | Sec10-GFP-His | Sec10-GFP-His in *cdc11-5* |
| 31 | *cdc10-1* | Sec15-GFP-His | Sec15-GFP-His in *cdc10-1* |
| 32 | *cdc10-2* | Sec15-GFP-His | Sec15-GFP-His in *cdc10-2* |
| 33 | *cdc10-5* | Sec15-GFP-His | Sec15-GFP-His in *cdc10-5* |
| 34 | *cdc11-3* | Sec15-GFP-His | Sec15-GFP-His in *cdc11-3* |
| 35 | *cdc11-4* | Sec15-GFP-His | Sec15-GFP-His in *cdc11-4* |
| 36 | *cdc11-5* | Sec15-GFP-His | Sec15-GFP-His in *cdc11-5* |
| 37 | *cdc10-1* | Exo84-GFP-His | Exo84-GFP-His in *cdc10-1* |
| 38 | *cdc10-2* | Exo84-GFP-His | Exo84-GFP-His in *cdc10-2* |
| 39 | *cdc10-5* | Exo84-GFP-His | Exo84-GFP-His in *cdc10-5* |
| 40 | *cdc11-3* | Exo84-GFP-His | Exo84-GFP-His in *cdc11-3* |
| 41 | *cdc11-4* | Exo84-GFP-His | Exo84-GFP-His in *cdc11-4* |
| 42 | *cdc11-5* | Exo84-GFP-His | Exo84-GFP-His in *cdc11-5* |

**Table 9: Transformation of GFP tagged strains with plasmids**

| Sr.No. | Background Strain | Plasmid pRS316 | Strain Constructed |
|---|---|---|---|
| 1 | Cdc10-GFP-His in *sec3-2* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec3-2* |
| 2 | Cdc10-GFP-His in *sec5-24* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec5-24* |
| 3 | Cdc10-GFP-His in *sec6-4* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec6-4* |
| 4 | Cdc10-GFP-His in *sec8-6* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec8-6* |
| 5 | Cdc10-GFP-His in *sec8-9* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec8-9* |
| 6 | Cdc10-GFP-His in *sec10-2* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec10-2* |
| 7 | Cdc10-GFP-His in *sec15-1* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *sec15-1* |
| 8 | Cdc10-GFP-His in *exo70.29/37* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *exo70.29/37* |
| 9 | Cdc10-GFP-His in *exo70.38* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *exo70.38* |
| 10 | Cdc10-GFP-His in *exo84-2* | pRS316-Atg9-2x-mCherry | Cdc10-GFP and Atg9-2x-mCherry in *exo84-2* |
| 11 | Cdc11-GFP-His in *sec3-2* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec3-2* |
| 12 | Cdc11-GFP-His in *sec5-24* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec5-24* |
| 13 | Cdc11-GFP-His in *sec6-4* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec6-4* |

| | | | |
|---|---|---|---|
| 14 | Cdc11-GFP-His in *sec8-6* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec8-6* |
| 15 | Cdc11-GFP-His in *sec8-9* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec8-9* |
| 16 | Cdc11-GFP-His in *sec10-2* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec10-2* |
| 17 | Cdc11-GFP-His in *sec15-1* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *sec15-1* |
| 18 | Cdc11-GFP-His in *exo70.29/37* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *exo70.29/37* |
| 19 | Cdc11-GFP-His in *exo70.38* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *exo70.38* |
| 20 | Cdc11-GFP-His in *exo84-2* | pRS316-Atg9-2x-mCherry | Cdc11-GFP and Atg9-2x-mCherry in *exo84-2* |
| 21 | Shs1-GFP-His in *sec3-2* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec3-2* |
| 22 | Shs1-GFP-His in *sec5-24* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec5-24* |
| 23 | Shs1-GFP-His in *sec6-4* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec6-4* |
| 24 | Shs1-GFP-His in *sec8-6* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec8-6* |
| 25 | Shs1-GFP-His in *sec8-9* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec8-9* |
| 26 | Shs1-GFP-His in *sec10-2* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec10-2* |
| 27 | Shs1-GFP-His in *sec15-1* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *sec15-1* |
| 28 | Shs1-GFP-His in *exo70.29/37* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *exo70.29/37* |
| 29 | Shs1-GFP-His in *exo70.38* | pRS316-Atg9-2x-mCherry | Shs1-GFP and Atg9-2x-mCherry in *exo70.38* |
| 30 | Shs1-GFP-His in *exo84-2* | pRS316-Atg9-2x- | Shs1-GFP and Atg9-2x-mCherry |

| | | | |
|---|---|---|---|
| | | mCherry | in *exo84-2* |
| 31 | Sec3-GFP-His in *cdc10-5* | pRS316-Atg9-2x-mCherry | Sec3-GFP and Atg9-2x-mCherry in *cdc10-5* |

**Table 10: C-terminal tagging of exocyst subunits in Cdc10-mcherry strain**

| Sr.No. | Background Strain | Amplicon | Strain constructed |
|---|---|---|---|
| 1 | Cdc10-mCherry-KanMx | Sec3-GFP-His | Sec3-GFP-His and Cdc10-mCherry in WT |
| 2 | Cdc10-mCherry-KanMx | Sec8-GFP-His | Sec8-GFP-His and Cdc10-mCherry in WT |
| 3 | Cdc10-mCherry-KanMx | Exo84-GFP-His | Exo84-GFP-His and Cdc10-mCherry in WT |

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