Unconventional role of an autophagy protein, Atg11 in maintaining genome stability in *Saccharomyces cerevisiae*.

A thesis submitted in partial fulfillment for the degree

Master of Science

(Biological Sciences)

As part of the Integrated Ph.D Program.

By

Jigyasa Verma

Molecular Biology and Genetics Unit,

Jawaharlal Nehru Centre for Advanced Scientific Research,

Jakkur, Bangalore- 560064, India

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Declaration

I hereby declare that this thesis entitled **"Unconventional role of an autophagy protein, Atg11 in maintaining genome stability in *Saccharomyces cerevisiae*"** is an authentic record of research work carried out by me towards my Master of Science under the supervision of **Dr. Kaustuv Sanyal** at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India and that this work has not been submitted elsewhere for the award of any other degree.

In keeping with the norm of reporting scientific observations, due acknowledgements have been made whenever the work described was based on the findings of other investigators. Any omission, which might have occurred by oversight or misjudgment, is regretted.

Jigyasa Verma
Place: Bangalore
Date:

Certificate

This is to certify that the work described in this thesis entitled **"Unconventional role of an autophagy protein, Atg11 in maintaining genome stability in *Saccharomyces cerevisiae*"** is the result of the investigations carried out by **Miss. Jigyasa Verma** towards her Master of Science as part of the Integrated PhD program at the Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my guidance, and that the results presented in this thesis have not previously formed the basis for the award of any other diploma, degree or fellowship.

Kaustuv Sanyal
Associate Professor
Molecular Biology and Genetics Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
Bangalore 560064

Place: Bangalore
Date:

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Most importantly I thank my parents for the constant encouragement and emotional fall back that they have offered me all through these years. I am extremely grateful to them.

Nothing great was ever achieved without enthusiasm

-Ralph Waldo Emerson

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Abbreviations

° C: degree Celsius

ml: milli litre

µl: micro litre

µg: micro gram

ng: nano gram

bp: base pair

kb: kilo base pairs

mb: mega base pairs

mM: milli molar

OD_{600}: Optical density at wavelength of 600nm

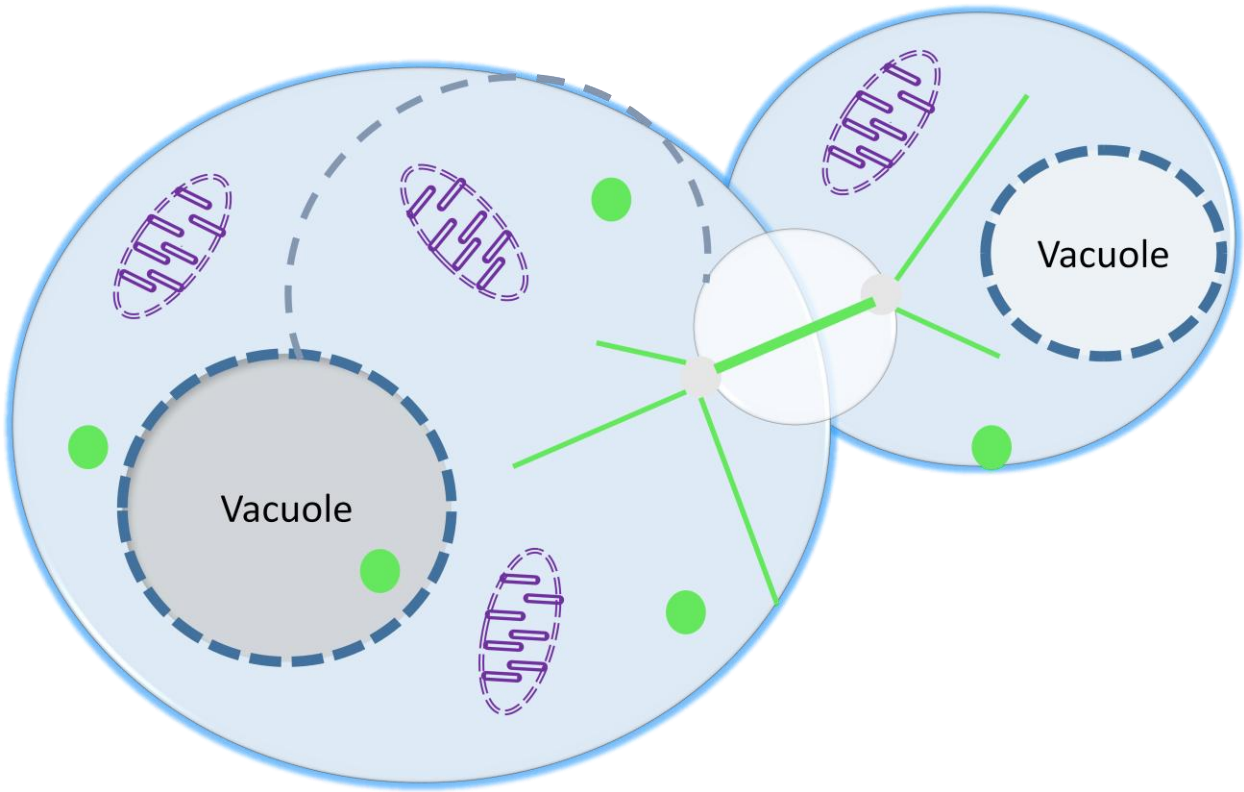
mins: minutes

rpm: revolutions per minute

sec: second

PBS: phosphate buffer saline

Connecting Dots: Genome Stability and Autophagy



Introduction

1.1 Genome instability

Any compromise in genome integrity, whether it is due to point mutations or gross chromosomal rearrangements, is known as genome instability. The integrity of the genome is crucial for the propagation of correct information to subsequent generations. Genome instability has been implicated in neurodegenerative diseases, aging, immunodeficiency and tumor formation. Intrinsic as well as extrinsic factors can cause these changes. DNA replication infidelity and unfaithful chromosome segregation are the intrinsic factors causing genome alterations. Exogenous insults include exposure to genotoxic agents such as ultraviolet light, oxidative stress, chemical mutagens, and radiation.

1.2 Mitotic cell cycle of the budding yeast

The mitotic cell cycle is a series of events leading to the generation of two daughter cells identical to the parent cell (Mitchison and Salmon 2001). The mitotic cell cycle can be broadly divided into two stages a) interphase which involves genome duplication and growth; b) mitosis phase in which the duplicated genome is divided equally between the daughters.

Interphase can be further sub-divided into G1, S and G2 stages. G1 and G2 phases (gap phases) involve the growth of cells in preparation for DNA synthesis (S) phase and mitotic (M) phase, respectively. However, it should be noted that S, G2 and M phases are not exclusive and can be overlapping in yeast.

Mitosis can be sub-divided into: a) prophase in which chromatin condenses to form sister chromatids and spindle pole body (SPB) duplication initiates, b) metaphase in which

bi-orientation of the chromosomes is established c) anaphase during which spindle fiber shortening and the poleward movement of the SPBs occur and d) telophase in which chromatin decondenses and spindle breakdown takes place. Completion of the cell division is brought about by cytokinesis where the cell divides into two daughter cells.

In the budding yeast, *Saccharomyces cerevisiae* the discernible events of the various phases of the cell cycle are as follows (Figure 1). In G1, polarized growth commences after bud site selection. In S phase, small buds grow by the accumulation of organelles and other components made in the mother cell during G1. In G2, the actin cytoskeleton depolarizes, leading to a switch from apical to isotropic growth. Finally, in the M phase buds acquire a copy of the genome and participate in cytokinesis (Loewen et al. 2007).

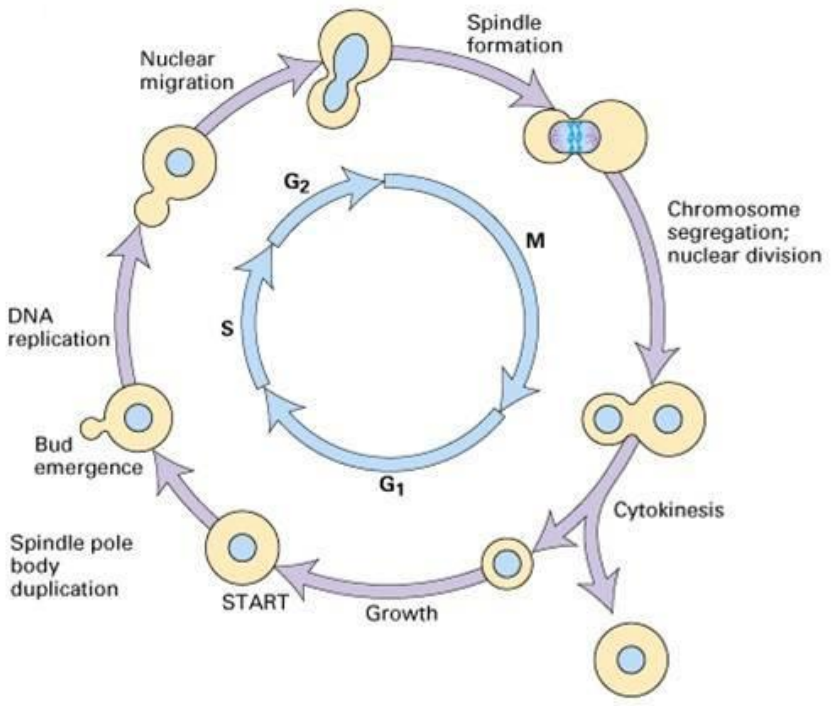


Figure 1: Budding yeast cell cycle. Schematic representing major events during the budding yeast cell cycle (https://voer.edu.vn/m/cell-cycle-regulation-in-the-budding-yeast/1ce212d4).

Eukaryotic chromosomes are surrounded by a double membrane called the nuclear envelope (NE). Based on the integrity of the NE during cell division, mitosis can be either closed or open. If the NE remains intact during division, the cell is known to be undergoing closed mitosis. For example, the budding yeast *S. cerevisiae* undergoes closed mitosis. On the other hand, if the NE breaks down during prophase, then the cells undergo open mitosis, for example somatic cells of higher eukaryotes undergo open mitosis. A prevalent intermediate between open and closed mitosis is the partial disassembly of the NE, known as semi-closed mitosis. The NE only partially opens up near the centrosomes/SPBs to allow cytoplasmic microtubules to reach the nuclear interior without the need for major rearrangements of NE components. The NE finally breaks down during anaphase. Early embryos of *Caenorhabditis elegans* and lower eukaryotes such as the filamentous fungus *Aspergillus nidulans* undergo semi-closed mitosis (Guttinger et al. 2009; Boettcher and Barral 2013).

1.3 Components of the chromosome segregation machinery

Components of the chromosome segregation in eukaryotes consist of the centromere-kinetochore complex, microtubule and its associated proteins, checkpoint proteins, molecular motors, and microtubule organizing centre.

1.3.1 Centromere

The centromere is defined as a specialised locus on the chromosome onto which a multi-protein complex called the kinetochore assembles in order to facilitate accurate chromosome segregation.

The centromere (*CEN*) is a rapidly evolving region (Figure 2). Based on the length, presence of repetitive DNA elements and whether the kinetochore assembly is centromere sequence dependent or not, centromeres can be classified into point, small regional and large regional (Roy and Sanyal 2011). Point centromere span a short length (< 400 bp) of DNA and are genetically defined. *S. cerevisiae* and other yeasts like *Candida glabrata, Kluyveromyces lactis* harbor a point *CEN*. On the contrary, large regional centromeres span longer stretches of DNA. Centromeres of humans and certain fungi like *Schizosaccharomyces pombe* and *Neurospora crassa* fall under this category. Placed in between these two classes are the small regional centromeres (4-6 kb in length) in *Candida* species. The regional centromeres are often epigenetically defined (Drinnenberg et al. 2014). The centromeric protein-A (CENP-A) in humans and Cse4 in yeast, is a histone H3 variant found exclusively in the centromeric nucleosome. Cse4/CENP-A mediates the binding of other kinetochore proteins onto the centromere.

The physical association of the kinetochore onto the centromere leads to a notion that these regions would be transcription poor. However, reports suggest that centromeric transcription is important for the localization of the histone H3 variant CENP-A as well as the inner kinetochore protein CENP-C (Gent and Dawe 2012; Grenfell et al. 2017).

Monocentric chromosomes are the ones in which the *CEN* is localised to a single region on a chromosome. The presence of more than one centromere on a monocentric chromosome may lead to the formation of what is known as a dicentric condition. Microtubules originating from the opposite spindle poles may get attached to the two centromeres of the same chromatid. Consequently the chromatid experiences two opposing poleward forces during its movement in anaphase. This causes the DNA to break.

However, a kinetochore mutant may lead to stable inheritance of a dicentric chromosome, in contrast to the wild-type which causes extensive structural alterations in the chromosome. Also, on deleting the centromere of a monocentric chromosome, a neocentromere is formed at a non-native locus which then acts as a functional *CEN* (Fukagawa and Earnshaw 2014).

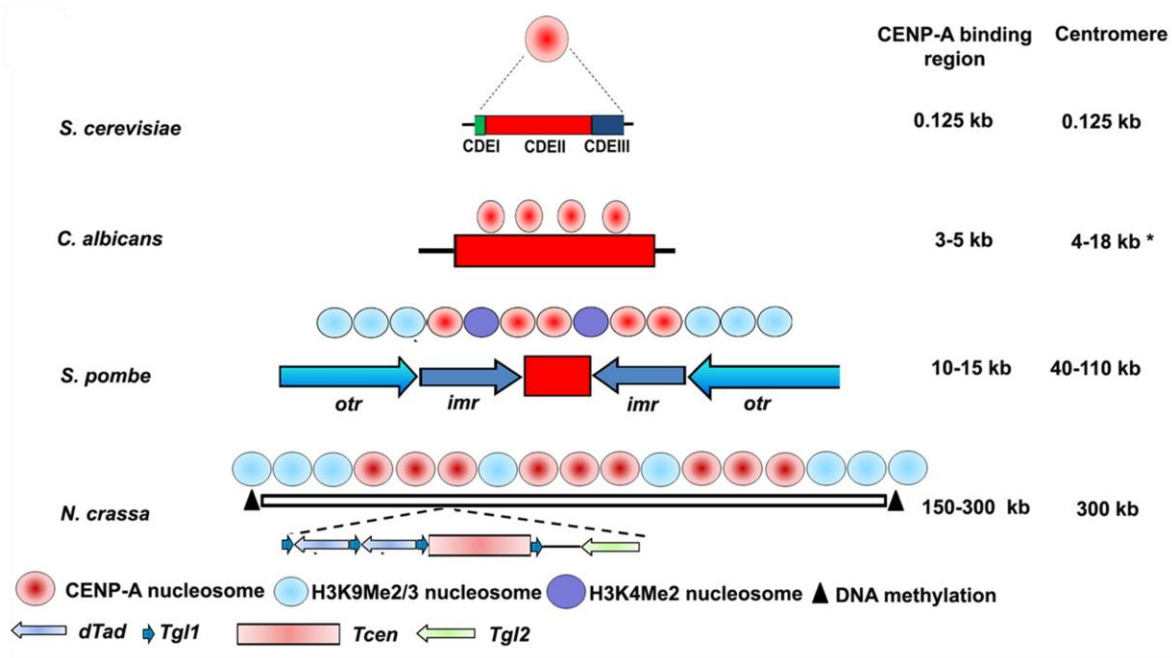


Figure 2: Centromere diversity in fungi. Fungal kingdom exhibits huge diversity in the centromeres harbored by them; point *CEN*s (*S. cerevisiae*), small regional *CEN*s (*C. albicans*) and large regional *CEN*s (*S. pombe and N. crassa*) (Roy and Sanyal 2011).

1.3.2 Kinetochore

An important aspect of chromosome segregation involves the movement of chromosomes. For accomplishing this, chromosomes have to be linked to a molecular force that can perform this function. The linking of the centromeric chromatin to the spindle microtubules is performed by a multi-protein complex called the kinetochore (KT). This

complex is composed of more than 100 proteins in *S. cerevisiae*. With the exception of few species specific complexes, the proteins of various layers of the KT are conserved to a great extent from yeast to humans (Figure 3).

On the basis of their co-isolation, the KT proteins are believed to be made up of various sub-complexes. The KT proteins close to the centromere are known as inner KT proteins, and the ones mediating the attachment to the microtubule are known as outer KT proteins.

Despite the overall conserved structure, there are differences in KT assembly and its regulation in different species. Proteins of the two layers of the KT are constitutively present across the cell cycle in *S. cerevisiae* and *C. albicans* (Roy et al. 2013), except for a short duration during S phase when the centromere is duplicated. However, in metazoans only the inner KT is constitutively present at the centromere throughout the cell-cycle. The subsequent assembly takes place during mitosis. It has been proposed that when KT proteins fail to find their appropriate binding partners, they undergo ubiquitin mediated degradation and do not become incorporated into the kinetochore complex (Rodrigo-Brenni et al. 2004).

Most kinetochores bind multiple microtubules, except in budding yeast, where they attach to only a single microtubule (Peterson and Ris 1976; Winey et al. 1995). Lack of proper kinetochore-microtubule attachments leads to activation of the spindle assembly checkpoint (discussed in Section 1.3.4) and cell-cycle progression is halted till improper attachments are corrected.

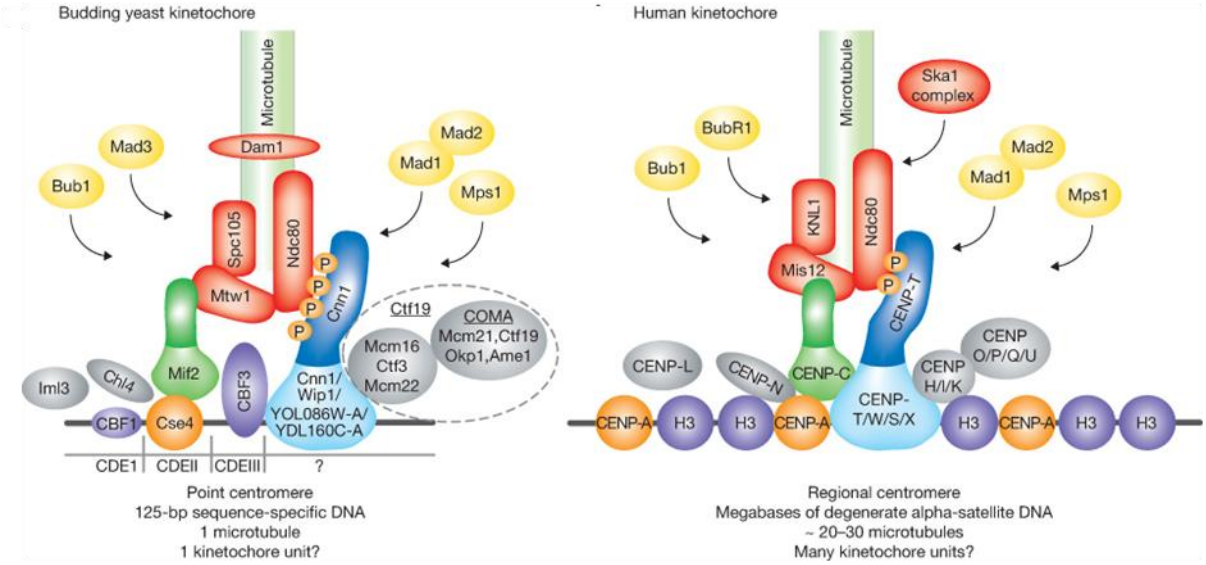


Figure 3: Evolutionary conservation of the kinetochore structure from yeast to humans. (Gascoigne and Cheeseman 2012).

1.3.3 Microtubules

Microtubules constitute the fibers of the mitotic spindle and are made up of α and β heterodimers. α tubulin is coded by *TUB1* and *TUB3* (Schatz et al. 1986) and β tubulin by *TUB2* (Neff et al. 1983). Most of the α tubulin mutants are sensitive to microtubule de-polymerising drugs and exhibit defective microtubule structures at restrictive temperature (Schatz et al. 1988). In contrast to this, *TUB2* mutants are benomyl-resistant (Thomas et al. 1985).

Microtubules are polar in nature and oscillate between phases of growth and shrinkage, a property known as dynamic instability. This dynamic instability of microtubules results from GTP hydrolysis of the β subunit (Kline-Smith and Walczak 2004). A cargo is transported along these tracts with the help of motor proteins. Defects in motor protein function result in abnormal spindle phenotypes like short spindle, long spindle or crooked spindle (Hildebrandt and Hoyt 2000).

Microtubules are of three types, based on the direction in which they emanate and the interactions they perform (Figure 4). The microtubules which make contacts with the kinetochores of sister chromatids are known as kinetochore microtubules (kMTs). The second type known as the interpolar microtubules (ipMTs), form an overlapping array of spindle. The third type known as the cytoplasmic or astral microtubules (cMTs), originate from poles and emanate towards the cell cortex. cMTs are required for the nuclear and spindle movement into the daughter cell (Civelekoglu-Scholey and Scholey 2010).

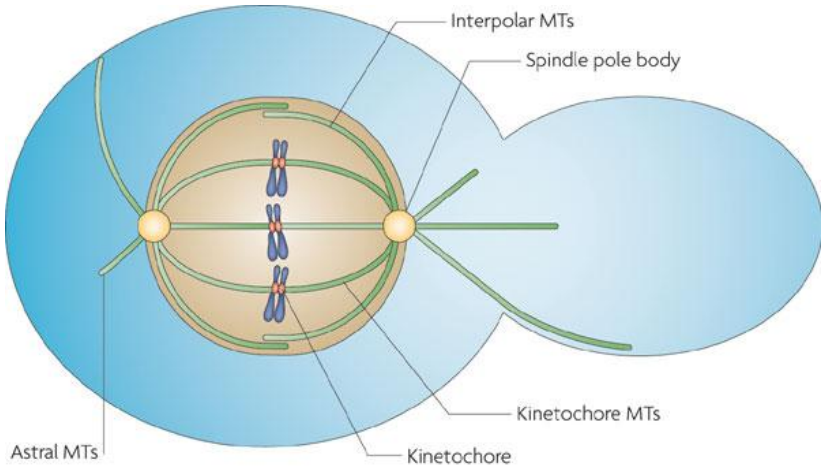


Figure 4: Three types of microtubules in *S. cerevisiae.* kMTs are required for sister chromatid segregation, ipMTs are required for nuclear pole separation and cMTs interact with the cell cortex and the septin ring at the bud neck to orient the mitotic spindle across the mother daughter cell junction (Gladfelter and Berman 2009).

1.3.4 Kinetochore-microtubule attachment and checkpoint proteins

The bipolar attachment of a chromosome, also known as amphitelic attachment is essential to avoid mis-segregation during mitosis. Bi-orientation occurs when each kinetochore on a sister chromatid binds to only one spindle pole. Mono-oriented

attachments like monotelic, when one of the kinetochores on a sister chromatid binds to one of the spindle poles or syntelic, when both the sister kinetochores bind to microtubules emanating from the same pole are corrected by the cell. Merotelic attachments take place when additional attachments at the kinetochore apart from the normal bi-orientation are formed (Figure 5).

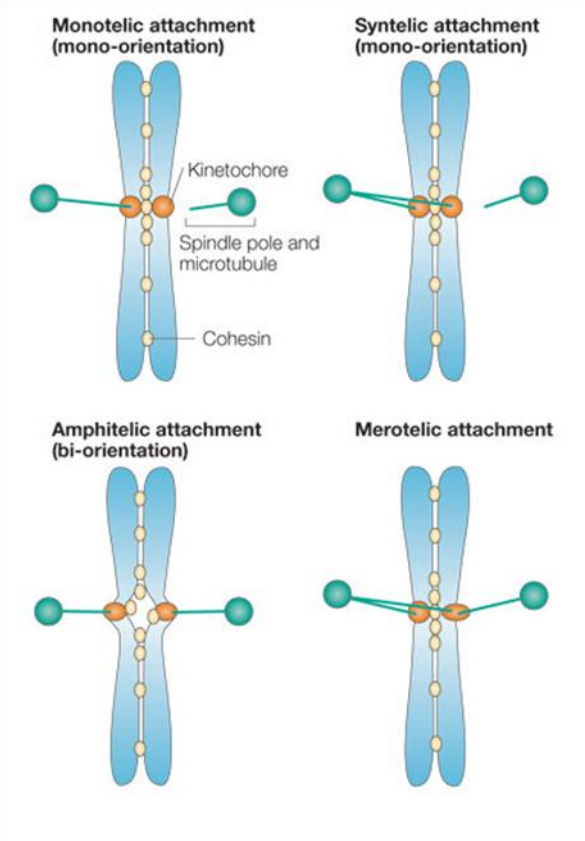


Figure 5: Attachments between kinetochore and spindle. Errors in attachment exist, including syntelic attachments (in which both sister kinetochores are attached to microtubules from the same spindle pole) and merotelic attachments (in which a single kinetochore is attached to microtubules from both spindle poles). As cells progress through mitosis, the erroneous attachments are corrected, leading to end-on, bi-oriented attachments, in which sister kinetochores are attached to microtubules from opposite spindle poles to support faithful chromosome segregation (Tanaka 2010).

Checkpoint proteins comprise of the cellular surveillance system which monitors different steps during cell division and delays cell cycle until those defects are corrected. Attachment of kinetochores to microtubules during metaphase is monitored by the spindle assembly checkpoint (SAC). The SAC proteins sense the lack of tension at the microtubule-kinetochore interface (Musacchio 2015) and delay progression to anaphase. Defects in spindle function, kinetochore assembly or spindle pole duplication all activate the checkpoint, as the checkpoint senses the tension at the KT-MT attachment.

A complete lack of KT-MT attachments, partial or weak KT-MT attachments, or a defect in achieving bi-orientation, all of them lead to chromosome mis-segregation. Depending on the kind of defect, yeast KT mutants exhibit different phenotypes.

When KT-MT attachment is completely absent, the entire nuclear mass remains in the mother cell and no segregation of the genetic material takes place (Figure 6A). Mutants of the CBF3 complex (inner kinetochore) exhibit this phenotype. The CBF3 complex is required for the assembly of all kinetochore proteins but is not sufficient to mediate MT attachments in vitro (Sorger et al. 1994). In contrast to the CBF3 mutants, mutants in the Ndc80 complex (outer kinetochore) do not completely abolish the kinetochore structure despite a lack of MT attachment (Janke et al. 2001), but exhibit a similar phenotype. Meta-stable MT-KT attachments lead to a metaphase arrest with a short spindle and the nuclear mass stuck at the bud neck (Figure 6B). Nuf2 is a subunit of the Ndc80 complex. A mutation in the *NUF2* gene, encoding the outer kinetochore protein Nuf2, leads to metastable KT-MT attachments (He et al. 2001). Mutants in this class do not completely abolish kinetochore assembly.

Defects in achieving bi-orientation result in unequal chromosome segregation between the mother and the bud (Figure 6C). Some mutants of the Ipl1 and Dam1 complexes (outer kinetochore) exhibit this phenotype (Chan and Botstein 1993; Cheeseman et al. 2001). As these mutants are able to segregate chromosomes, these proteins regulate bi-orientation but do not mediate attachment.

Absence of non-essential kinetochore proteins in the Ctf19 complex (inner kinetochore), motor proteins, microtubule associated proteins such as Bim1 and Bik1, and the mitotic checkpoint proteins exhibit subtle chromosome loss phenotypes, probably due to functional redundancy.

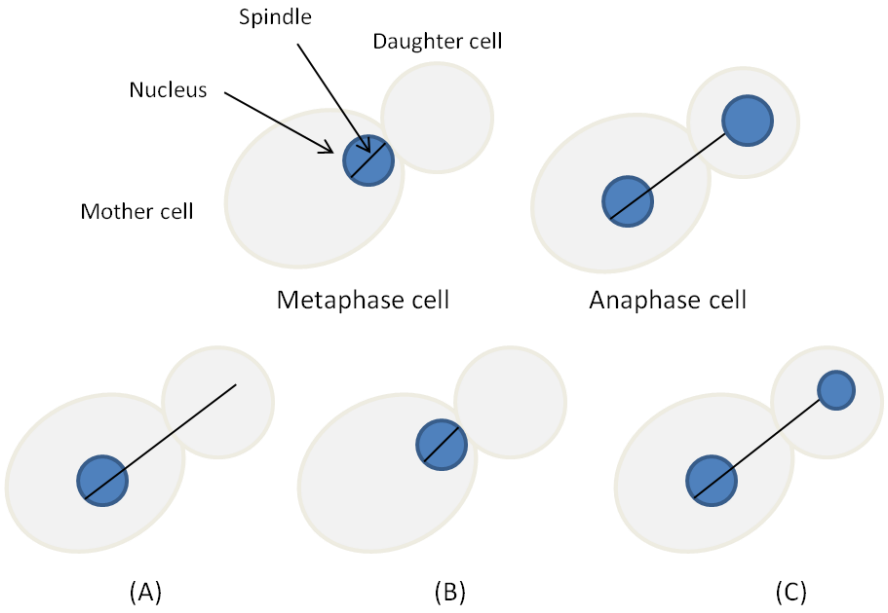


Figure 6: Phenotypes of budding yeast kinetochore mutants. (A) Complete absence of MT-KT attachments leads to non-segregation of the nuclear mass. **(B)** Unstable KT-MT interactions result in short spindle, metaphase arrest and nuclear mass present at the bud neck. **(C)** Defects in achieving bi-orientation leads to unequal chromosome segregation. Adapted from (Biggins and Walczak 2003)

1.3.5 Molecular motors

Motor proteins are molecular machines that utilize the energy of ATP hydrolysis to move along microtubules to deliver various cargos in the cell. Depending on the direction of movement of its cargo, a motor can be plus or minus end directed. Kinesin-related motors generate forces either plus end directed or minus end directed, whereas dyenin generate minus end directed forces. Neither of the two classes of proteins are essential (Hildebrandt and Hoyt 2000). *S. cerevisiae* has six kinesins and a single dynein heavy chain (Hildebrandt and Hoyt, 2000). Kinesins are encoded by *CIN8, KIP1, KIP2, KIP3, KAR3* and *SMY1,* and dynein is encoded by *DYN1*. Premature movement of motor proteins across the cortical cytoskeleton, in the absence of endoplasmic reticulum-septin tether, leads to early movement of the spindle into the daughter leading to segregation defects (Loewen et al. 2007). The offloading model proposes that dynein is delivered by microtubule plus ends to the bud cortex, where dynein becomes anchored to generate forces for microtubule sliding (Lee et al. 2005). Hence, motor proteins play a very important role in mediating the movement of the nucleus and the spindle into the daughter cell during mitosis (Figure 7).

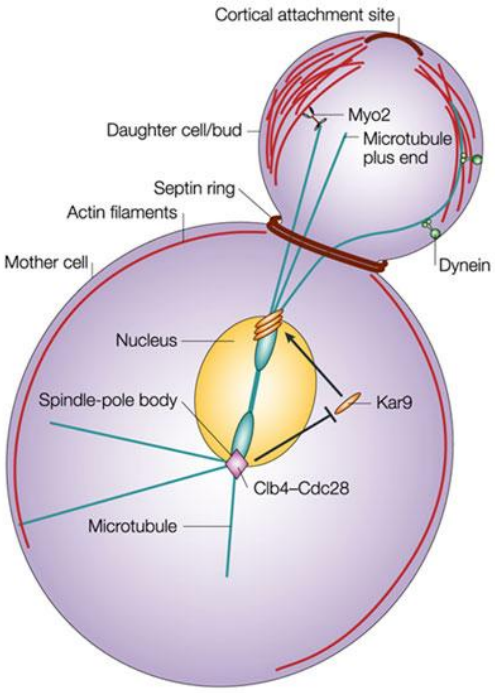


Figure 7: Motor proteins mediate attachment of the spindle to cortical cytoskeleton. Microtubule attachment to the bud-tip can maintain attachment to a microtubule that is growing and shortening. Bud attachment and proper spindle positioning is necessary for equal segregation of the genome to mother and daughter cells (Pearson and Bloom 2004).

1.3.6 Spindle pole body (SPB)

Microtubules emanate from bodies known as microtubule organizing centres (MTOCs), also known as centrosomes in animals and spindle pole bodies (SPBs) in fungi (Jaspersen and Winey 2004; Kilmartin 2014). Duplication of a SPB takes place at the G1/S boundary, after which the duplicated SPBs move away from each other towards opposite ends and microtubules begin to emanate from them. This duplication takes place only once during the cell cycle and is essential for the formation of a bipolar spindle and chromosome segregation. Supernumerary centrosomes lead to defects in segregation.

1.4 Turnover of the proteins of the segregation machinery.

Protein turnover maintains the balance between protein synthesis and protein degradation in a cell. This process has two important implications. First, it ensures that misfolded and aggregated proteins are degraded. Second, signaling inside the cell is regulated by spatial and temporal protein turnover (for example degradation of cyclins during cell division) (Tin Su 2001; Chang et al. 2003). Components of the chromosome segregation machinery that are known to undergo rapid turnover include kinetochore proteins, motor proteins and spindle assembly checkpoint proteins (King et al. 2000; Hoffman et al. 2001; Howell et al. 2004; Shah et al. 2004). This turnover is partially mediated by microtubule binding.

Major intracellular degradation systems include ubiquitin proteasome system (UPS) and autophagy (Ciechanover 2005). UPS clears most soluble proteins in the cytoplasm and nucleus and plays a key role in degrading short-lived and misfolded proteins. On the other hand, autophagy degrades cytoplasmic protein aggregates. Crosstalk between UPS and autophagy has been reported (Kraft et al. 2010). Inhibition of proteasome stimulates autophagic activity, probably as a compensatory strategy (Iwata et al. 2005; Pandey et al. 2007).

1.5 Cellular homeostasis, autophagy and its role in genome stability.

Autophagy is predominantly a cytoplasmic process involving the capture of cargo from the cytoplasm and delivery to the vacuole for degradation and recycling (Mizushima

et al. 2011). However, degradation of nuclear components has also been reported in *Tetrahymena thermophila* and *Aspergillus oryzae* (Lu and Wolfe 2001; Shoji et al. 2010).

1.5.1 The emerging role of autophagy in maintenance of genome stability.

It is becoming increasingly clear that autophagy plays a key role in maintaining genomic stability (Matsui et al. 2013; Vessoni et al. 2013). Inactivation of autophagy reduces DNA damage repair in the cell (Bae and Guan 2011; Lin et al. 2015; Liu et al. 2015; Hewitt et al. 2016). Autophagy regulates the number of centrosomes in the cell by degrading Cep63 (Watanabe et al. 2016). Cep63 plays a role in centrosome duplication along with Cep152 (Brown et al. 2013). Absence of Atg11 in *S. cerevisiae* makes it sensitive to a microtubule de-polymerising drug, benomyl (Strome et al. 2008). Beclin1, the human homolog of ScAtg6 plays a role in chromosome congression and proper outer kinetochore assembly (Fremont et al. 2013). Taken together these data suggest that autophagy proteins play a protective role in the context of chromosome mis-segregation.

1.5.2 Significance of autophagy for cellular homeostasis.

The serendipitous discovery of acid phosphatase by Christian De Duve in 1949 while studying insulin metabolism in rat liver cells led to the discovery of lysosomes, which laid the stage for the discovery of the process of autophagy years later. In the subsequent years, electron microscope studies of different cell types revealed the presence of vesicles containing engulfed cytoplasmic material. As these vesicles contained the cell's own contents, the process came to be known as 'autophagy' meaning 'self eating' by De Duve in

1963 and the knowledge about the process has considerably increased since then (Ohsumi 2014).

The cargo for degradation could be cytoplasmic long lived proteins, aggregated or misfolded proteins, damaged or superfluous organelles or intracellular pathogens. Their breakdown in lysosomes feeds macromolecules back into the cytoplasm which fuels anabolic pathways and helps the cell survive during conditions of stress (Xie and Klionsky 2007). Autophagy occurs at a basal level in normal conditions but is enhanced under conditions of nutrient starvation, hypoxia, infection, low ATP/AMP ratio, high intracellular ROS levels or drugs. This process of cellular homeostasis is conserved from yeast to mammals (Reggiori and Klionsky 2013).

1.5.3 Mechanism of degradation by autophagy.

The molecular machinery of autophagy was largely identified in *S. cerevisiae* by independent groups (Tsukada and Ohsumi 1993; Thumm et al. 1994; Harding et al. 1995). Thus far, 37 autophagy related (ATG) genes are known in yeast. Homologs of many of them have been identified in mammals.

The process of autophagy can be divided into 3 broad steps:

1) Autophagosome nucleation and formation: In this step, a double-membrane vesicle, known as the autophagosome is formed at the phagophore assembly site (PAS) in yeast (Suzuki et al. 2007). This site is found close to the vacuolar membrane. Proteins involved at this step are Atg1 through Atg10, Atg12, Atg13, Atg14, Atg16, Atg17, Atg18, Atg29, Atg31, Vps15 and Vps34. The isolation membrane grows by sequential addition of membrane and

is completed when the vesicle is completely sealed separating the cargo from rest of the cytoplasm.

2) Autophagosome docking and fusion: These autophagosomes are transported in a dynein dependent manner along microtubules and eventually get fused with the lysosomes (Kimura et al. 2008; Monastyrska et al. 2009; Yang et al. 2011). The proteins mediating the fusion of the autophagosome to the lysosome are Vam3, Vam7, Ykt6, Vit1, SNAP, NSF, Sec17, Sec18, Sec19, Ypt7 and the HOPS complex.

3) Autophagosome degradation in the vacuole: Lysis of autophagic bodies in the vacuoles is mediated by Atg15 (vacuolar lipase). The contents of the autophagosome are degraded by vacuolar proteases.

The process of autophagy can be selective or non-selective depending on the specificity of the cargo sequestered for degradation (Figure 8). It is non-selective when bulk cytoplasmic contents are captured and selective when cargoes are specifically targeted using adaptor proteins that bridge the cargo with the core autophagy machinery.

Although a part of a degradative process, autophagy proteins also mediate the biosynthesis of vacuolar proteins in the cell. The Cvt (cytoplasm-to-vacuole targeting) pathway is one such example whose membrane dynamics and mechanism are almost the same as that of selective autophagy.

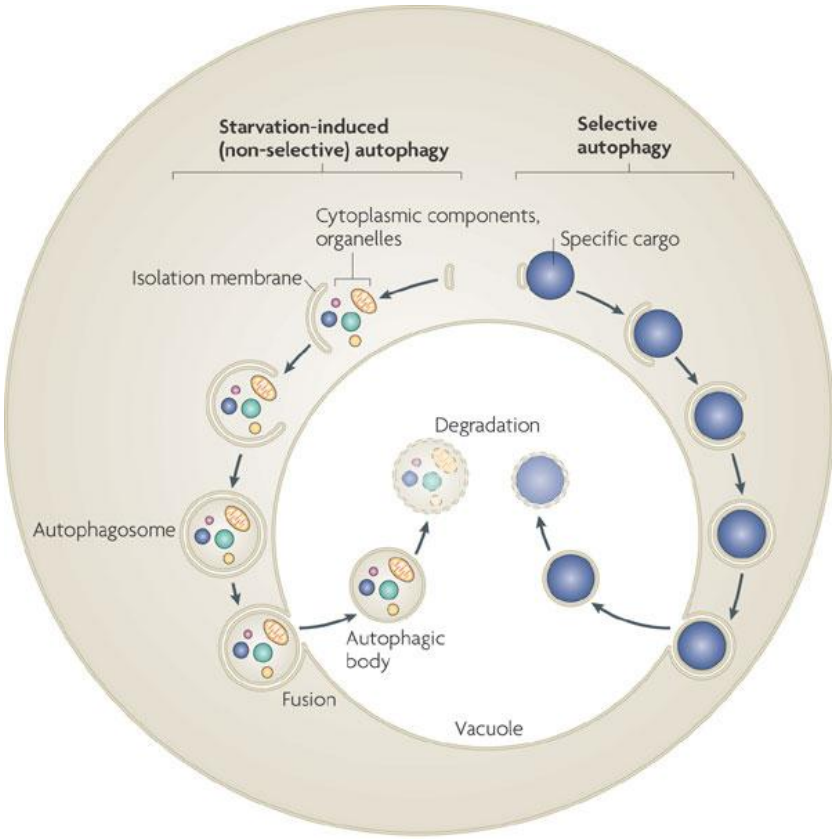


Figure 8: Selective and non-selective autophagy in yeast. In starvation-induced (non-selective) autophagy, the isolation membrane mainly non-selectively engulfs cytosolic constituents and organelles to form the autophagosome. In selective autophagy, specific cargoes (protein complexes or organelles) are enwrapped by membrane vesicles that are similar to autophagosomes, and are delivered to the vacuole for degradation (Nakatogawa et al. 2009).

1.6 Rationale of the current study.

Observations which led us to investigate the role of autophagy proteins in the functioning of the segregation machinery in *S. cerevisiae* are as follows. First, a report by Fremont et al. (2013) showed the role of Beclin1 (human homolog of ScAtg6) in mediating chromosome congression and proper outer kinetochore assembly. On silencing Beclin1, the proportion of misaligned chromosomes increases significantly as compared to the control. Second, with a detailed analysis of the known interactions between autophagy proteins and segregation machinery in *S. cerevisiae,* we were able to identify multiple physical interactions. The significance of these physical interactions is not defined as yet in *S. cerevisiae.* As this organism undergoes closed mitosis, physical interaction between these two sets of proteins is intriguing. Functioning in isolated compartments of the cell, could autophagy proteins be playing any role in regulating chromosome segregation in *S. cerevisiae*? Hence, we laid our aim to investigate if chromosome segregation gets affected in the absence of autophagy proteins in *S. cerevisiae.*

Results

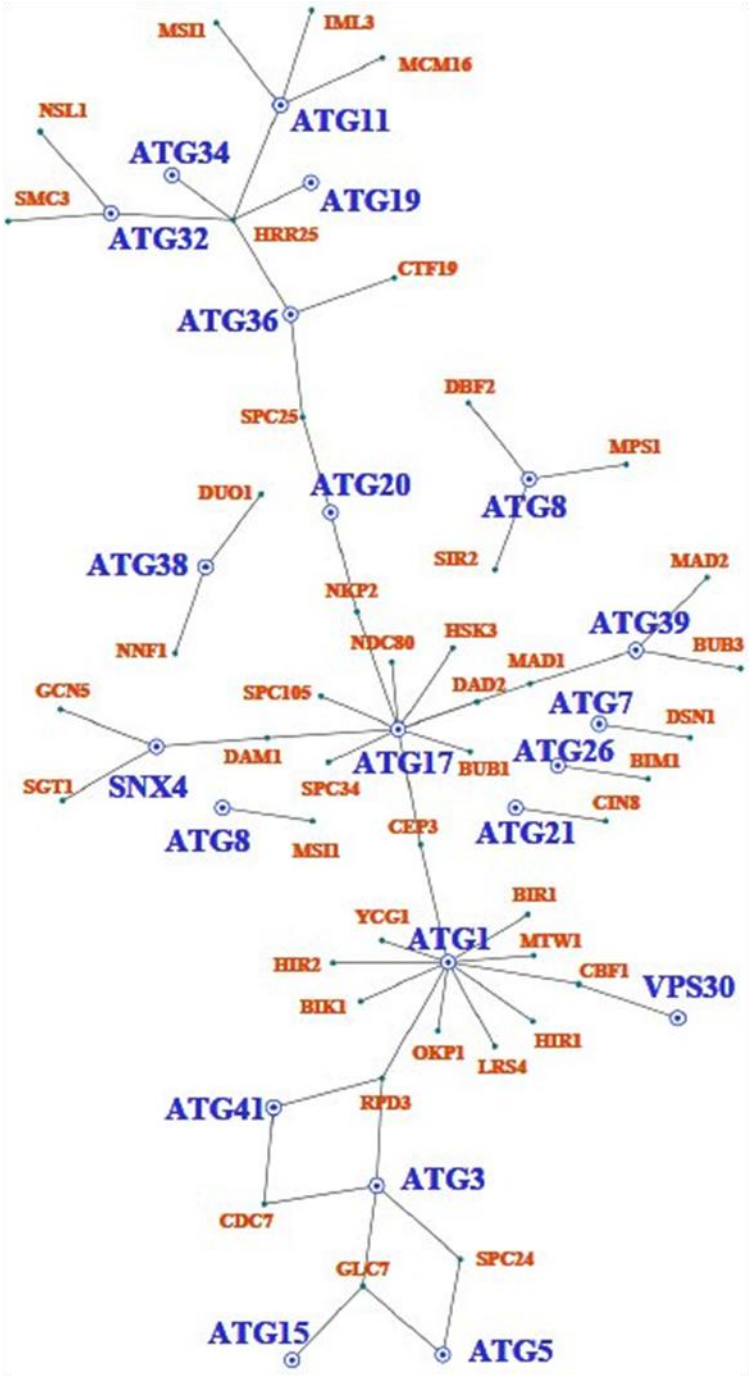
2.1 Analysis of interactions between autophagy proteins and cell cycle proteins in *S. cerevisiae.*

All reported interactions between autophagy proteins and proteins of the replication and segregation machinery were identified from the *Saccharomyces* Genome Database (SGD) using HTML and Python scripts. A complete database of all the reported interactions was created using JSON scripts and plotted.

2.1.1 Autophagy proteins interact physically and genetically with proteins of the replication and segregation machinery in *S. cerevisiae.*

On a detailed analysis of all the reported interactions, we found that autophagy proteins interact physically or/and genetically with the components of the replication and segregation machinery (Figure 9A). Among the proteins of the replication and segregation machinery, we found a significantly higher number of interactions of the autophagy proteins with the proteins of the segregation machinery. Interacting partners of the segregation machinery included inner kinetochore proteins (Cep3, Ctf19 and Okp1) as well as outer kinetochore proteins (Dad2, Dam1, Dsn1, Duo1, Hsk3, Mtw1, Ndc80, Spc24, Spc25, Spc34, Okp1, Nsl1 and Nnf1); microtubule associated proteins Bim1 and Bik1; checkpoint proteins Bub1, Bub3, Mad1 and Mad2 and the molecular motor Cin8. Among all the autophagy proteins, Atg1 and Atg17 were found to be interacting with the maximum number of proteins of the segregation machinery (Figure 9B) (this part of the study was done in collaboration with Ratul Chowdhury, University of Pennsylvania).

(A)


MSI1
IML3
MCM16
NSL1
ATG11
ATG34
ATG19
SMC3
ATG32
HRR25
CTF19
ATG36
DBF2
SPC25
MPS1
ATG20
ATG8
DUO1
ATG38
SIR2
MAD2
NKP2
ATG39
HSK3
NDC80
NNF1
BUB3
MAD1
GCN5
SPC105
DAD2
ATG7
DSN1
DAM1
ATG17
BUB1
ATG26
BIM1
SGT1
SNX4
SPC34
CIN8
ATG8
CEP3
ATG21
MSI1
BIR1
YCG1
ATG1
MTW1
HIR2
CBF1
VPS30
BIK1
HIR1
OKP1
LRS4
ATG41
RPD3
ATG3
CDC7
SPC24
GLC7
ATG15
ATG5

(B)

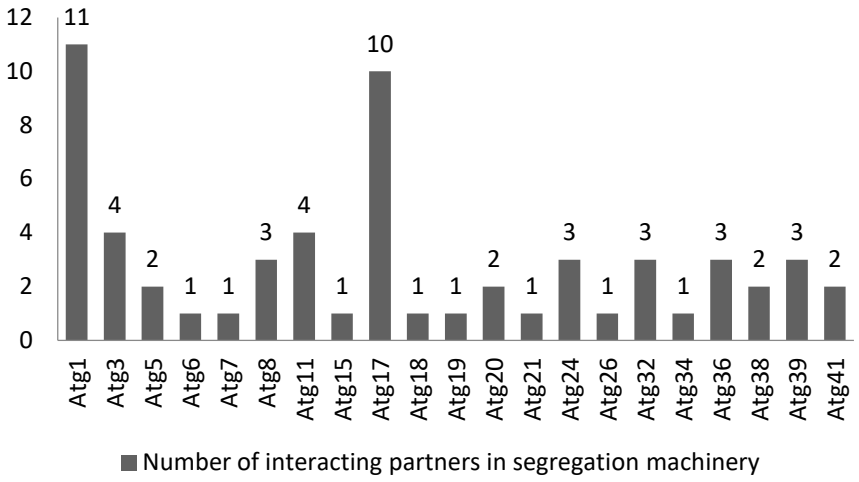


Figure 9: Reported interactions of autophagy proteins with proteins of the segregation machinery in *S. cerevisiae*. (A) Interacting partners of autophagy proteins with proteins of the replication and segregation machinery. The length of the interacting lines does not indicate the strength of the interaction. **(B)** Number of proteins of the segregation machinery that each autophagy protein interacts with. Interaction with a single protein, physical or/and genetic has been represented once.

Hence, proteins of the autophagy machinery were found to be interacting with many proteins of the segregation machinery. Since the most number of interactions have been reported with Atg1 and Atg17, hence as a starting point we laid our aim to investigate if the absence of any of these two autophagy proteins, Atg1 and Atg17, would result in segregation defects.

2.2 Investigating the role of Atg1 and Atg17 in chromosome segregation

We employed the deletion mutants of *ATG1* and *ATG17* and probed for defects in chromosome segregation. Two commonly used techniques discussed below were used to assay for defects.

a) **Sensitivity to microtubule de-polymerising drugs:** To generate unattached kinetochores, cells are exposed to microtubule depolymerizing drugs which deprive kinetochores of their attachment partners. A defect in kinetochore-microtubule interaction sensitizes the cells to microtubule de-polymerising drugs as compared to the wild-type (Stearns et al. 1990). Benomyl and thiabendazole inhibit the microtubules from polymerizing. Kinetochore mutants, as well as mitotic checkpoint mutants, are sensitive to compounds such as benomyl (Spencer et al. 1990; Hoyt et al. 1991; Li and Murray 1991).

b) **Mitotic stability of monocentric plasmid:** The mitotic stability of a monocentric plasmid is lesser in mutants having defects in chromosome segregation (Maine et al. 1984). The fraction of cells carrying the monocentric plasmid will be substantially lower in a segregation mutant than in wild-type cells. However, it should be noted that this loss of monocentric plasmid can also be attributed to a defect in the replication of the plasmid (Fig 10). A defect in segregation would cause the replicated copies to accumulate in mother cells. On the other hand, a defect in replication would not cause an increase in the copy number of the monocentric plasmid (Sinha et al. 1986; Roy et al. 1997). Hence, by determining the copy number of the plasmid, one can differentiate a replication defect from a segregation defect.

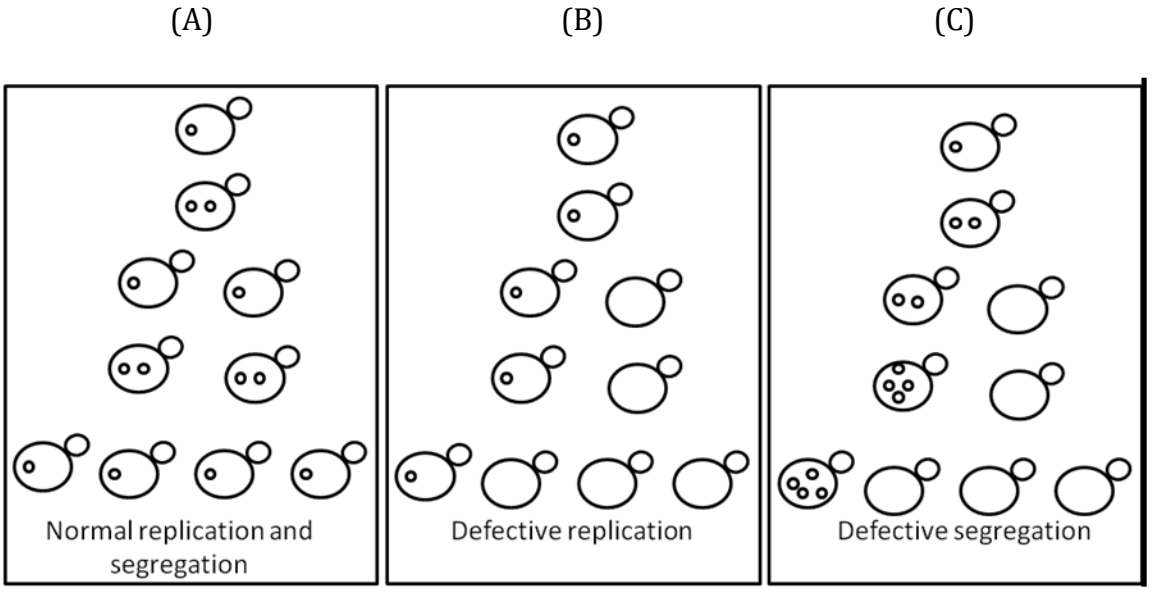


Figure 10: Schematic showing mitotic stability of monocentric plasmid. (A) A monocentric plasmid is stably inherited if the replication/segregation machinery of the cell is intact. **(B)** If there is a replication defect, there will not be enough copies of the plasmid for all the dividing cells to inherit, resulting in loss of the plasmid in some cells. **(C)** In case of a defect in segregation, the monocentric plasmid post duplication will not be equally segregated to the two daughter cells, resulting in some cells not acquiring the plasmid.

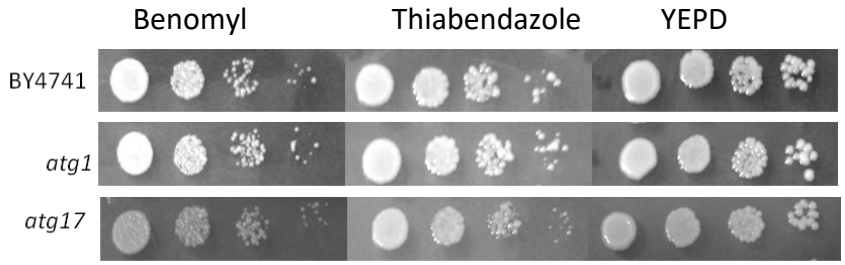
2.2.1 *atg1* and *atg17* are not sensitive to microtubule de-polymerising drugs.

atg1, atg17 and the isogenic wild-type strain (BY4741) were tested for their sensitivities to benomyl and thiabendazole. We observed that both *atg1* and *atg17* were as sensitive as the wild-type at a concentration of 15µg/ml benomyl and 50µg/ml thiabendazole (Fig. 11A). Thus, both *ATG1* and *ATG17* deletions did not alter the tolerance of cells to defects in microtubule assembly caused by the presence of microtubule de-polymerising drugs.

2.2.2 Monocentric plasmid is mitotically stable in *atg1* and *atg17*.

We compared the mitotic stability of a monocentric plasmid in *atg1* and *atg17* and the wild-type strain (described in Section 4.3). The average mitotic stability of the monocentric plasmid in *atg1* and *atg17* was found to be 85±0.6% and 84±1.6% respectively, whereas the isogenic wild-type strain showed a mitotic stability of 84% (Fig. 11B). Hence, there was no significant increase or decrease in the stability of the monocentric plasmid in *atg1* and *atg17* as compared to the wild type. These results indicate that Atg1 and Atg17 do not play any essential role in the process of chromosome replication or segregation.

(A)



(B)

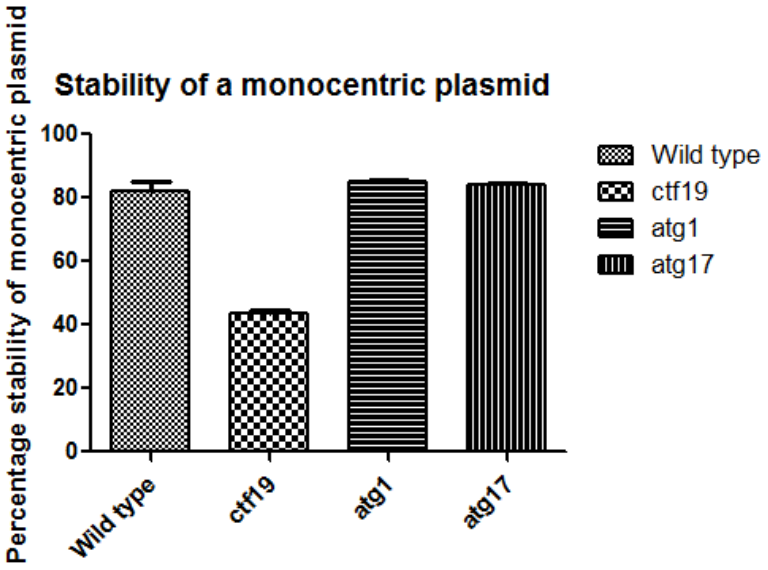


Figure 11: *atg1* and *atg17* do not show chromosome segregation defects. (A) Sensitivity of BY4741 (wild type), *atg1* and *atg1* to benomyl (15µg/ml) and thiabendazole (50µg/ml). *atg1* and *atg17* are not sensitive to either of the microtubule de-polymerising drugs as compared to the wild type. **(B)** Mitotic stability of a monocentric plasmid in *atg1, atg17* and the isogenic wild-type strain (n=3). Student's t-test was applied and the difference between the mitotic stabilities of *atg1, atg17* and wild-type was found to be non-significant.

The result of the assays performed with *atg1* and *atg17* indicate that Atg1 and Atg17 do not play any essential role in mediating genome stability in *S. cerevisiae.* Hence, the basis of the physical interactions between Atg1 and Atg17 with components of the segregation machinery could not be established.

2.3 Screening autophagy mutants for sensitivity towards genotoxic agents.

Since other autophagy proteins, apart from Atg1 and Atg17, have also been reported to interact with components of the segregation machinery (Figure 9B), we determined the sensitivity of a collection of 34 other autophagy deletion mutants (a kind gift from Dr. Ravi Manjithaya's Lab at JNCASR) against microtubule de-polymerising drugs, benomyl and thiabendazole. This was our attempt to determine the significance of the reported physical interactions between the other autophagy proteins and components of the segregation machinery. Sensitivity to microtubule de-polymerising drugs would indicate a defect in kinetochore-microtubule attachment in the absence of the respective autophagy protein.

2.3.1 *atg6, atg11* and *atg15* are sensitive to microtubule de-polymerising drugs.

On screening 36 autophagy deletion mutants for their sensitivities towards microtubule de-polymerising drugs, we observed that most of the *ATG* deletion mutants were not significantly more sensitive as compared to the wild-type (Fig. 12). *atg6, atg11* and *atg15* were found to be sensitive, *atg11* mutant being the most sensitive of the three. This indicates that absence of Atg11 renders the cell sensitive to drugs inhibiting microtubule-kinetochore interaction. Hence, *atg11* has an inherent defect in microtubule-kinetochore interactions, which is getting exacerbated in the presence of the drug.

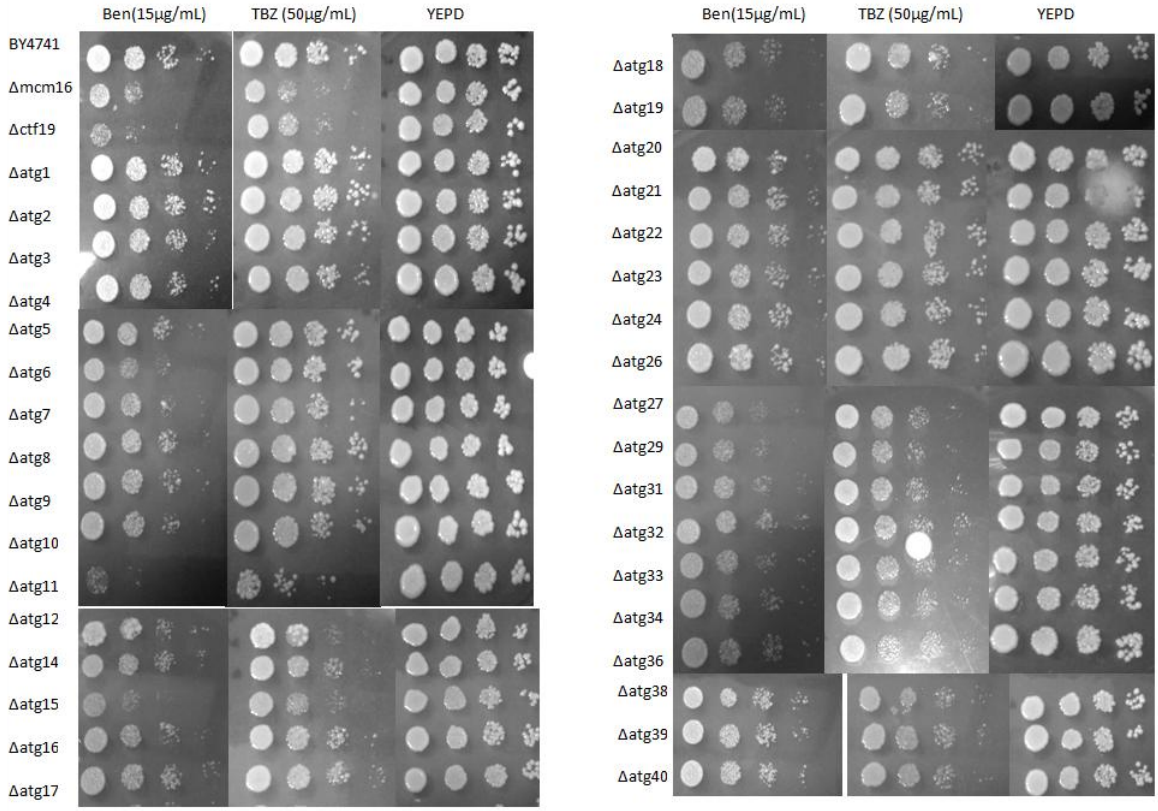


Figure 12: Sensitivity of autophagy deletion mutants to microtubule de-polymerising drugs. Sensitivity of the indicated autophagy deletion mutant to benomyl (15µg/ml) and thiabendazole (50µg/ml). Most of the *atg* strains were not significantly sensitive to the two drugs. *atg6, atg11* and *atg15* were found to be sensitive.

2.3.2 *atg11* is also sensitive to a DNA damaging agent hydroxyurea.

To determine if the sensitivity of *atg11* to microtubule de-polymerising drugs could be by virtue of a defect occurring upstream (replication or DNA damage repair), we determined the sensitivity of *atg11* to a DNA damaging drug hydroxyurea (200mM). Any defect occurring upstream to the process of segregation will lead to loss of viability subsequently. We found that *atg11* is hypersensitive to the DNA damaging drug hydroxyurea (Fig 13). *ctf19* which has defects in kinetochore assembly and hence KT-MT interactions, but no reported defect in replication or repair, was as sensitive as the wild-type to hydroxyurea. This implies that Atg11 plays a role in the basic DNA metabolic processes of replication, repair or/and segregation.

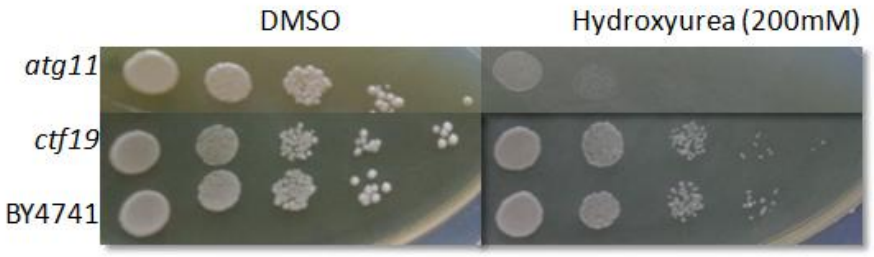


Figure 13: *atg11* is sensitive to hydroxyurea. Sensitivity of *atg11* and isogenic wild-type strain to hydroxyurea (200mM).

Hartwell and Smith (1985) have shown that mutants that affect DNA replication cause chromosome loss with a high genetic recombination frequency, while those mutants that affect the segregation of chromosomes cause a high chromosome loss without elevating recombination frequencies significantly. Hence, the frequency of recombination in *atg11* can be used to determine the kind of defect in this mutant.

2.4 Investigating the role of Atg11 in chromosome segregation.

atg11 was the most sensitive to two microtubule de-polymerising drugs among all the autophagy deletion mutants tested. We further went onto determine the mitotic stability of a monocentric plasmid and nuclear segregation in absence of Atg11, in order to ascertain if Atg11 plays any role in maintaining genome stability.

2.4.1 Monocentric plasmid is mitotically unstable in *atg11*.

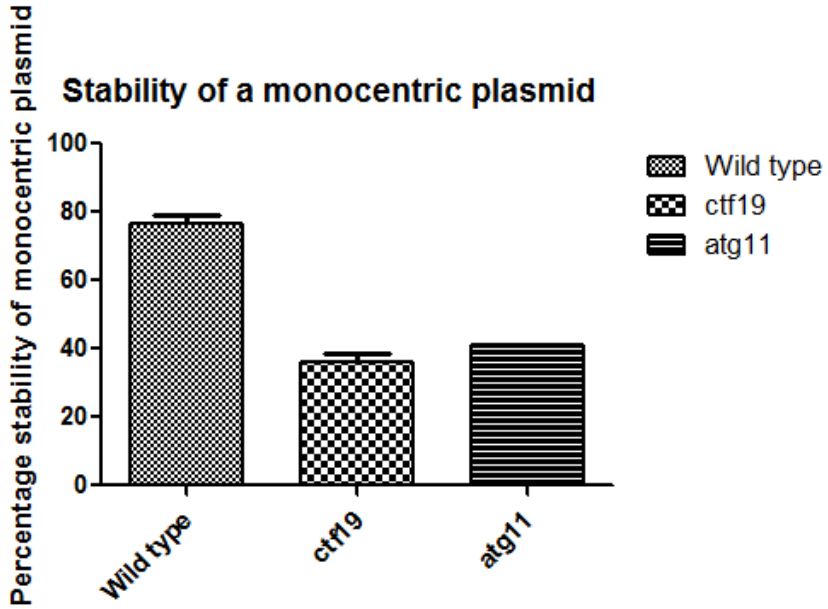
We compared the stability of a monocentric plasmid in *atg11* and wild-type strain (described in Section 4.3). We found that the average mitotic stability of the monocentric plasmid in *atg11* was 41±0.1%, whereas the isogenic wild-type strain showed a mitotic stability of 76±5% (Fig. 14A). This implies that in the absence of Atg11, the cell is not able to stably maintain/propagate the monocentric plasmid. Either insufficient number of copies of the monocentric plasmid is leading to some cells not inheriting the plasmid, or else, improper segregation is leading to the duplicated plasmid being retained within the mother cell. In this scenario, determining the copy number of the plasmid will help determine the type of defect.

2.4.2 *atg11* has defective nuclear segregation.

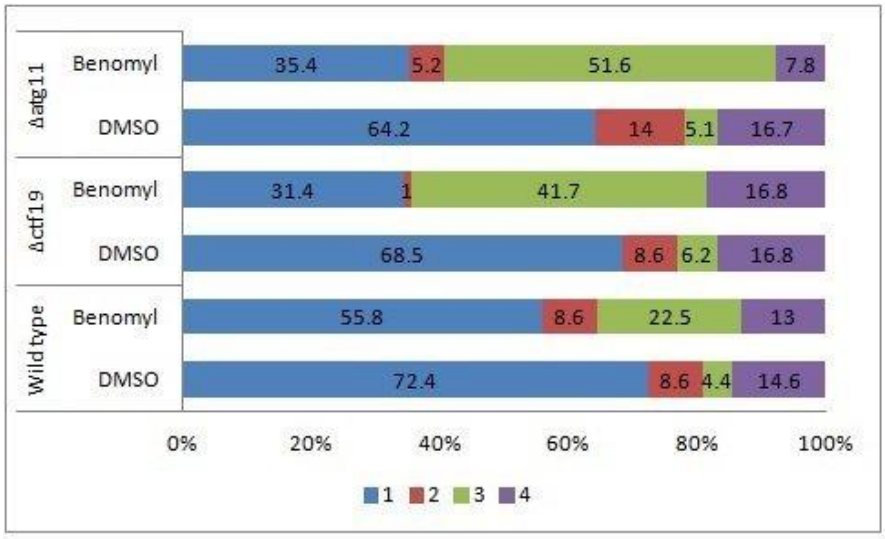
Examination of possible cell cycle defects revealed that *atg11* shows a G2/M accumulation with 2N DNA content in a logarithmically growing culture (Figure 14C). Cytological analysis of *atg11* and the isogenic wild-type on treating with 50 μg/ml

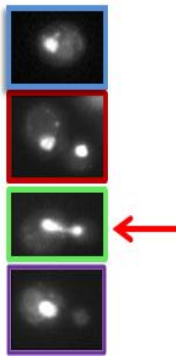
thiabendazole (described in Section 4.4) revealed an accumulation of large budded cells with the nucleus at or near the neck (51.6% in *atg11* and 22.5% in wild-type) (Figure 14B). A similar G2/M delay is seen in *ctf13-30, ndc10-42* (Doheny et al. 1993), *cep3-1/-2* (Strunnikov et al. 1995), and *skp1-4* (Connelly and Hieter 1996) kinetochore structural mutants at their non-permissive temperatures. The increased percentage of large-budded cells with nucleus at the bud neck and the relative DNA content of growing cells in *atg11* all point towards a defect that causes the *atg11* cells to pause in late S, G2 or in an early phase of mitosis. Despite this pause, *atg11* cells do not grow significantly slower than the wild-type cells at 30°C (Figure 14D). A similar observation has been made for the *chl1*, which also accumulates large-budded cells in growing cultures (Gerring et al. 1990). As suggested for *chl1*, it is possible that the large-sized buds of the *atg11* spend less time in G1 and make up for the time lost as a result of pausing in G2. This is also consistent with the lowered fraction of G1 cells in *atg11* (Figure 13B).

(A)

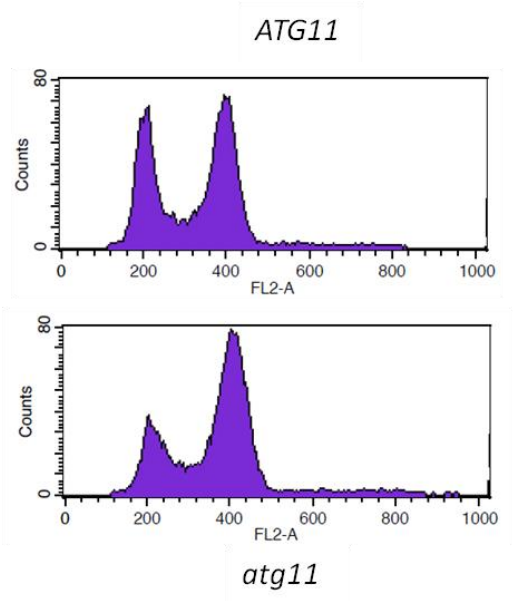


(B)


Δatg11
Benomyl
35.4
5.2
51.6
7.8
DMSO
64.2
14
5.1
16.7
Δctf19
Benomyl
31.4
1
41.7
16.8
DMSO
68.5
8.6
6.2
16.8
Wild type
Benomyl
55.8
8.6
22.5
13
DMSO
72.4
8.6
4.4
14.6
0%
20%
40%
60%
80%
100%
1
2
3
4



(C)


ATG11
Counts
FL2-A
atg11

(D)

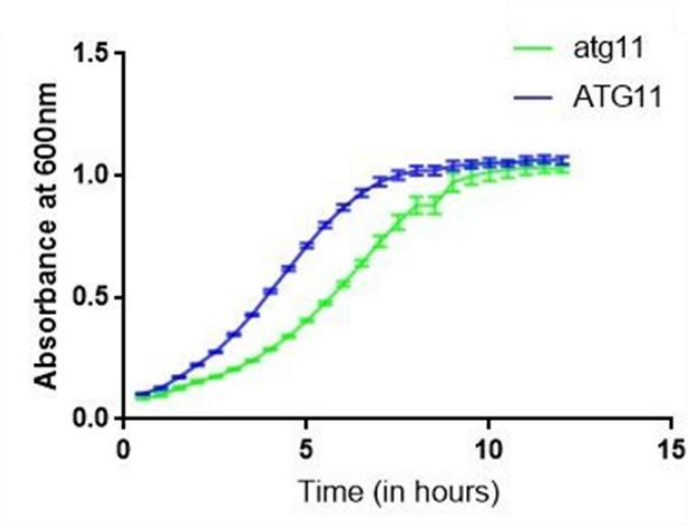

atg11
ATG11
Absorbance at 600nm
Time (in hours)

Figure 14: *atg11* has defects in chromosome segregation (A) *atg11* has decreased mitotic stability of a monocentric plasmid (n=3). Student's t-test was applied and the difference between the stabilities of *atg11* and wild-type was found to be significant with p=0.0002. **(B)** Nuclear segregation with or without drug (DMSO control) treatment in *atg11* and isogenic wild-type strain. Population of large budded cells with un-segregated nuclear mass was increased two-fold in *atg11* as compared to wild-type upon drug treatment. **(C)** FACS profile of *ATG11* and *atg11.* As compared to wild-type, a higher percentage of cells of *atg11* were observed to be in G2/M. In *ATG11,* there was 0.5 fold greater proportion of cells in G2 than G1, whereas in *atg11* the increase was 2 fold. **(D)** Growth curve of *ATG11* and *atg11*. Doubling time for wild type is 107 mins, whereas for *atg11* is 125 mins. Growth curve was performed at standard conditions of growth (30°C, 300rpm) in 96 well plate varioskan in YEPD.

2.5 Understanding the role of Atg11 in mediating faithful chromosome segregation.

Defects in nuclear segregation can be by virtue of improper assembly of the kinetochore on the centromere or defective microtubule dynamics in the cell. We assayed for these in *atg11* by performing the following:

a) **Transcription read-through assay**: A defect in kinetochore assembly may lead to defective kinetochore functioning. An assay has been described that helps determine if the kinetochore assembly in a cell is improper (Doheny et al. 1993) (described in Section 4.5). In this assay, the transcript originates at a strong promoter (*GAL1*), passes through the *CEN* sequence and proceeds to the reporter gene *lacZ* (Figure 14). When the transcription machinery reaches *CEN,* most of the transcripts are expected to terminate owing to the presence of the kinetochore complex at the centromere. If a strain harbours a mutation in a gene whose product

is required for kinetochore assembly, the transcription is expected to be more relaxed resulting in production of β galactosidase. The decrease in activity is not likely due to a defect in splicing caused by introduction of nucleotides into the actin intron. In independent studies looking at splicing, upto 200 bp have been inserted at this site in actin intron without affecting splicing or levels of mRNA produced (Klinz and Gallwitz 1985). The reporter construct is maintained in a single copy by integrating the construct into a genomic locus. This is to minimize the appearance of false positives (due to multiple copies of the reporter), or false negatives (due to the rapid loss of the reporter).

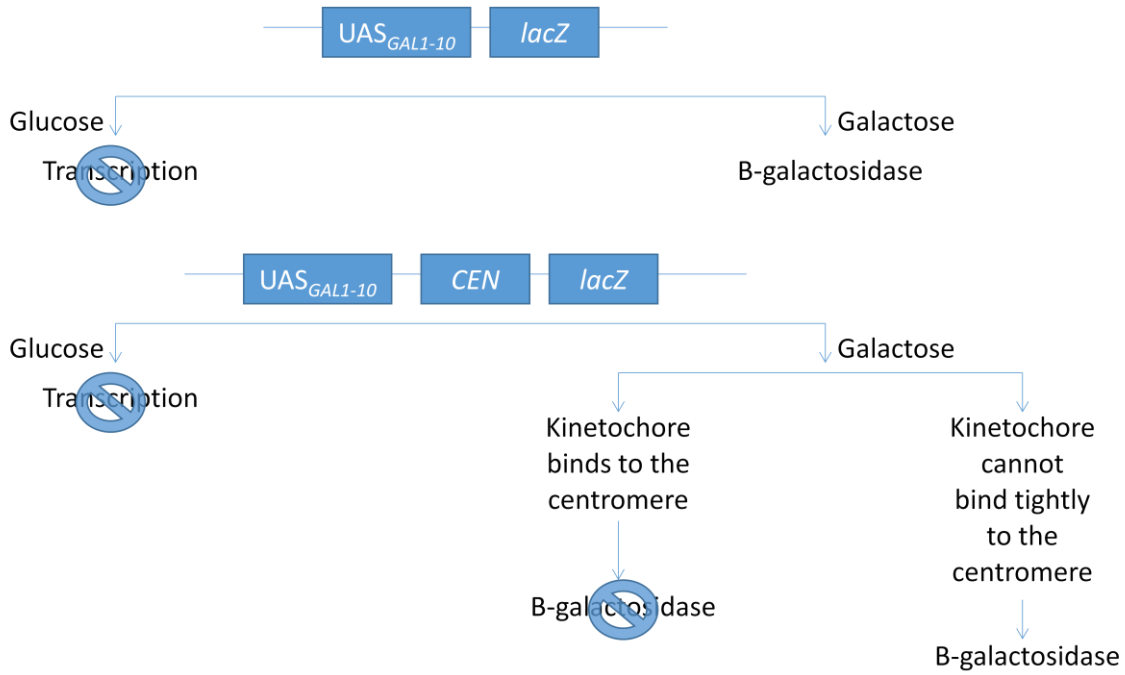


Figure 15: Transcription read-through reporter construct and assay. A *CEN* sequence is placed in between a strong promoter (*GAL1*) and a reporter gene *lacZ α*. The amount of β galactosidase produced is used as readout for kinetochore assembly onto the centromere (Reynolds and Lundblad 1995). A transcript originating at the *GAL1* promoter has to pass

the *CEN* sequence to reach the *lacZ* gene. The kinetochore assembled over *CEN* will act as a physical hindrance resulting in termination of transcription. If a mutation affects kinetochore assembly, transcription machinery can move through the centromere, thus giving higher levels of β-galactosidase.

b) **Spindle morphology**: Defects in the assembly of the various components of the kinetochore (explained in Section 1.3.4) can lead to defects in spindle morphology. Common defects observed are short spindle, elongated spindle, crooked spindle or altered angle of the spindle with respect to the mother-bud axis. Spindle morphology can be examined microscopically (by immunofluorescence/tagging a tubulin protein, in this case *TUB1*) and compared with that of the wild-type. By examining the spindle morphology in *atg11,* we would be able to determine the type of kinetochore defect in the mutant.

2.5.1 The transcription block offered by a kinetochore is not overcome in *atg11*.

We found the β-galactosidase units synthesised in *atg11* and wild-type to be 9.5±0.2 and 7.5±0.7 Miller units, respectively (Fig. 13). This implies that the inner kinetochore components are assembling properly over the centromere. Hence, the defect in *atg11* lies in the components mediating the attachment to the MTs or the motor proteins, microtubule associated proteins such as Bim1 and Bik1, or the mitotic checkpoint proteins.

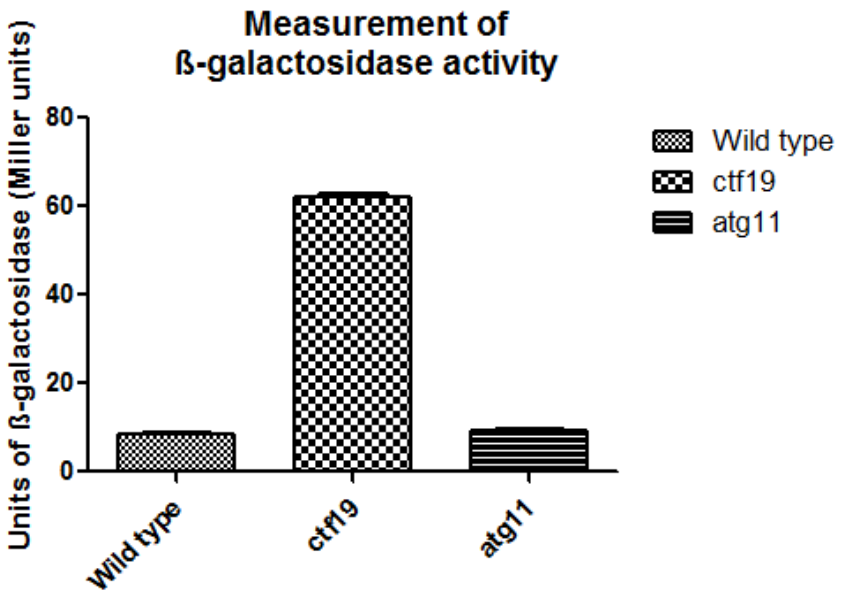
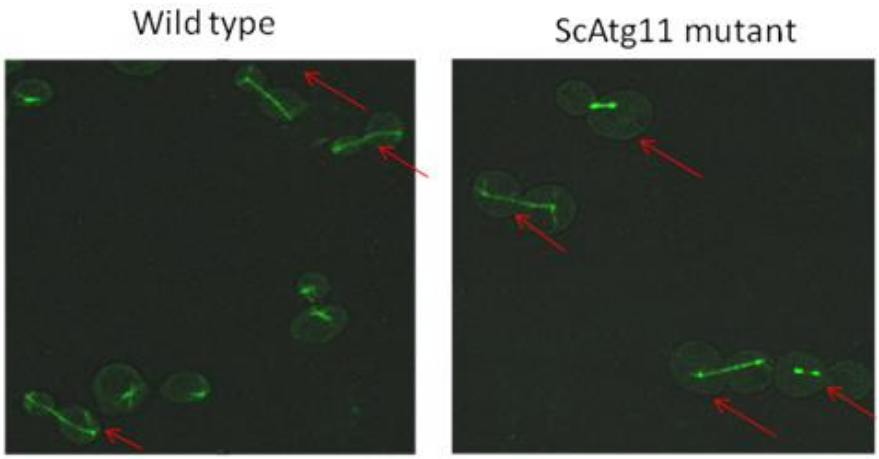


Figure 16: Inner kinetochore assembly is unperturbed in *atg11*. Units of β-galactosidase were measured in *atg11* and the isogenic wild-type strain (n=3). Student's t-test was applied and the difference between the Miller units in *atg11* and wild-type was found to be non-significant.

2.5.2 *atg11* has a short anaphase spindle.

Tubulin staining revealed a difference in the size of the anaphase spindle in *atg11* as compared to the wild-type (Fig. 14A). The average spindle size in wild-type and *atg11* was found to be 6.1µm and 4.4µm respectively (Fig. 14B). On plotting the spindle lengths of the large budded cells, we observed that the majority of the cells of the wild-type population had a spindle size between 4-9 µm, whereas in *atg11* there were an equal proportion of cells with spindle size in the range of 5-10 µm and 1-3 µm. Presence of a population of cells harboring short spindle implies that SAC is getting activated (Figure 13C) and preventing the progression of the cell cycle. This indicates a lack of proper tension between the KT and MT in absence of Atg11.

(A)



(B)

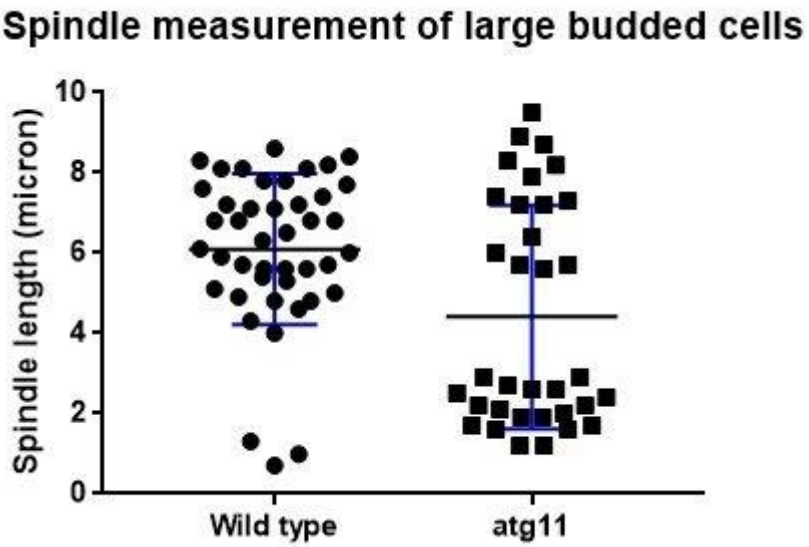


Figure 17: *atg11* has a short anaphase spindle. (A) *atg11* has a shorter spindle than the wild type in some of its large-budded cells. *TUB1* has been tagged with GFP. Arrowheads indicate spindle in large budded cells. **(B)** Average spindle size in large budded cells of *atg11* was lesser as compared to the isogenic wild-type strain. Large budded cells of *atg11* have an equal population of shorter and normal sized spindle.

The observation that *atg11* and the wild-type produced similar levels of β-galactosidase suggested that absence of Atg11 is not be perturbing the inner kinetochore assembly at the centromere. Whereas, the shorter anaphase spindle in *atg11* indicates a lack of proper tension between kinetochore and microtubules.

Discussion

In this work, we have explored the role of autophagy proteins in mediating faithful chromosome segregation in *S. cerevisiae*, a study first of its kind in this organism. Previous studies have reported the role of autophagy proteins in regulating kinetochore assembly in humans (Fremont et al. 2013) and halting of the cell cycle by autophagy proteins during S phase checkpoint in *S. cerevisiae* (Eapen et al. 2017). These reports indicate that the role of autophagy proteins in maintaining genome stability might be conserved from yeast to humans.

3.1 Autophagy proteins interact physically and genetically with proteins of the segregation machinery in *S. cerevisiae*

A genetic interaction implies that the genes share a functional relationship. The interacting partners may be involved in the same or compensatory biological pathways. Genetic interactions can be positive or negative. Negative interactions aggravate the defective phenotype. A synthetic lethal interaction is an extreme case of a negative interaction, where on deleting the two genes the cell is no more viable. Positive interactions mitigate the defects arising due to deletion of one of the interacting partners. Physical interactions explain how a protein executes its function, i.e. to perform its function inside the cell, what all partners does the protein interact with.

On interrogating the database of all the reported interactions in *S. cerevisiae* (*Saccharomyces* genome database), both physical and genetic interactions (positive, negative and synthetic lethal) were found between autophagy proteins and proteins of the

segregation machinery (Figure 9 A and B). Physical interactions were intriguing given that the budding yeast undergoes closed mitosis. This could mean that the two sets of proteins interact to regulate each others function. As autophagy is largely a degradative process, therefore it was hypothesised that autophagy proteins could be regulating the turnover of the proteins of the segregation machinery. If the hypothesis was correct, then the absence of autophagy proteins would lead to segregation defects in the cell, or in extreme cases lethality.

3.2 *atg1* and *atg17* do not show nuclear segregation defects.

As Atg1 and Atg17 had the maximum number of reported interacting partners with the proteins of the segregation machinery, their roles in chromosome segregation was investigated first.

Atg1 is a serine/threonine kinase which regulates macromolecular flux through the autophagy pathway. It is encoded by the non-essential gene *ATG1* in yeast and its absence leads to impaired autophagy. Its human homolog is *ULK* (Unc-51-like kinases 1 and 2). It is essential for cell cycle progression from G2/M to subsequent G1 under nitrogen starvation (Mijaljica et al. 2012; Matsui et al. 2013).

Atg17 is a scaffold protein which organizes the PAS and stimulates the kinase activity of Atg1 (Liu and Klionsky 2016). It is encoded by the non-essential gene *ATG17* in yeast and its absence leads to defective autophagy. Its human homolog is *RB1CC1/FIP200.*

Deletion of *ATG17* in *S. cerevisiae* has been reported to result in increased loss of heterozygosity at the *MET15* locus, but not at the *MAT* or *SAM2* loci (Andersen et al. 2008).

No previous work reports the role of *ATG1/ULK* or *ATG17/RB1CC1/FIP200* in maintaining genome stability under normal conditions of growth. Based on our results of sensitivity to microtubule de-polymerising drugs and the mitotic stability of a monocentric plasmid in *atg1* and *atg17* (Figure 11), we concluded that these two proteins do not play any essential role in genome stability in *S. cerevisiae*. Hence, our results thus far do not establish the basis of the physical interactions between Atg1 or Atg17 and proteins of the segregation machinery.

3.3 *atg6, atg11* and *atg15* are sensitive to microtubule de-polymerising drugs.

When the sensitivity of 36 autophagy deletion mutants to microtubule de-polymerising drugs was assayed *atg6, atg11* and *atg15* were found to be more sensitive as compared to the wild-type (Figure 12). This drug sensitivity could be attributed to impairment of the basic DNA metabolic processes like replication, repair or/and segregation.

Atg6/Apg6/Vps30/Vpt30 is a protein essential for the processes of autophagy and vacuolar protein sorting (Cao and Klionsky 2007; Mei et al. 2016) and is encoded by the non-essential gene *ATG6/VPS30* in yeast. Its human homolog is *BECLIN1. beclin1*$^{-/-}$ mice die early in embryogenesis, whereas aging *beclin1*$^{+/-}$ mice have an increased incidence of

lymphoma and carcinomas of the lung and liver (Qu et al. 2003; Yue et al. 2003). Beclin1 controls retro-transposon RNA levels and retro-transposon mediated genetic change in mice (Guo et al. 2014); it also plays a role in chromosome congression and proper outer kinetochore assembly (Fremont et al. 2013). In our experiment, *atg6* showed sensitivity to benomyl but not to thiabendazole.

Atg15/Aut5/Cvt17 is a phospholipase required for the lysis of autophagic and Cvt bodies inside the vacuole (Ramya and Rajasekharan 2016). It is encoded by the non-essential gene *ATG15* in yeast. Earlier, *atg15*$^{-/-}$ has been reported to be sensitive to benomyl (Brown et al. 2006). We found *atg15* to be sensitive to both benomyl and thiabendazole.

Atg11/Cvt3/Cvt9 is a scaffold protein which localizes other autophagy proteins to PAS (Backues and Klionsky 2012) and is encoded by the non-essential gene *ATG11/CVT3/CVT9* in yeast. *atg11* exhibits decreased selective autophagy. No mammalian homolog of this protein is known. *atg11* and *atg11*$^{+/-}$ have been reported to be benomyl sensitive (Parsons et al. 2004; Strome et al. 2008) and exhibited a greater than 2-fold increase in the rate of chromosome loss. Absence of Atg11 leads to reduced telomere length (Askree et al. 2004). Together these results indicate that Atg11 plays a role in chromosome maintenance. Among all the autophagy deletion mutants screened, *atg11* showed the maximum sensitivity to both benomyl and thiabendazole.

3.4 *atg11* has defects in chromosome segregation.

A defect in the DNA replication and/or chromosome segregation machinery in the cell will result in decreased mitotic stability of a monocentric plasmid (Maine et al. 1984). Based on the reduced mitotic stability of a monocentric plasmid (Figure 14A) and the defective nuclear segregation patterns (Figure 14B), we concluded that the segregation machinery is unable to function properly in the absence of Atg11. Similar results were obtained for *ctf19* which plays an important but non-essential role in the assembly of the kinetochore. Absence of Ctf19 leads to chromosome segregation defects (Hyland et al. 1999).

Improper microtubule-kinetochore attachments, which lead to defects in chromosome segregation, can be attributed to a defective kinetochore assembly or/and spindle dynamics. We tested the two possibilities one by one. Based on our results of the transcription read-through assay (Figure 16) and analysis of the spindle dynamics in *atg11* (Figure 17) we concluded that absence of Atg11 leads to problems in spindle dynamics but not on inner kinetochore assembly.

Atg11 is known to localize in the cytoplasm next to the vacuole. Hence, we suspect that it could be playing a role in the cytoplasmic regulation of nuclear segregation in budding yeast. Nuclear migration and alignment of the mitotic spindle along the bud neck require associations between astral microtubules and the cortex of the cell, which is mediated by microtubule based motors (Yeh et al. 2000). Motor proteins Kar9, Bim1 and Num1 localize to the cortical tip and capture astral microtubule ends which helps in the nuclear movement to the bud-neck (Adames and Cooper 2000). Whereas, Dyn1, Arp1 and

Nip100 are required for spindle movement into the bud neck via sliding of astral microtubules along the surface of the cortex (Moore and Cooper 2010). Interestingly, Atg11 has been reported to connect actin cables and PAS elements (Monastyrska 2006). Hence, future work will involve determining the role of Atg11 in conjunction with motor proteins to understand how Atg11 is regulating spindle dynamics while being present in the cytoplasm. As Atg11 is a huge protein harboring four coiled-coil domains, it could be acting as a scaffold to mediate interactions between other proteins to bring about faithful chromosome segregation. However, we do not rule a previously unknown localization of Atg11 in the nucleus.

We would also like to emphasise the role of Atg11 in maintaining genome stability and not only in the process of chromosome segregation, in the light of the sensitivity of *atg11* to the DNA damaging drug hydroxyurea (Figure 13) and the recent report about the role of Atg11 in DNA damage repair (Eapen et al. 2017). These two results very clearly indicate that apart from its role in segregation, Atg11 plays a role upstream of the process of segregation in *S. cerevisiae.* Hence, we propose that the autophagy protein, Atg11 plays a role in mediating genome stability in the cell.

Materials and Methods

4.1 Media, transformation and growth conditions.

S. cerevisiae strains were grown in YEPD (1% yeast extract, 2% peptone and 2% dextrose) at 30°C. Transformation was performed by high-efficiency Lithium-acetate protocol (Gietz and Schiestl 2007).

4.2 Drug sensitivity assay.

Equal numbers of exponential phase cells were spotted onto drug containing plates. Stock solutions of benomyl (10mg/mL in dimethyl sulfoxide) and thiabendazole (25mg/ml in dimethyl formamide) were used to prepare plates containing the desired concentration of drug. Control plates not containing the drug (DMSO for benomyl and DMF for thiabendazole) were also spotted with the same number of cells.

4.3 Plasmid stability assay.

Mitotic stability of a monocentric plasmid is the ability of a cell to retain a centromeric plasmid after several rounds of cell division. It is determined by counting the fraction of cells harboring the plasmid in the total cell population.

The plasmid pRS313 (yeast centromere vector with a *HIS3* marker and an MCS derived from pBLUESCRIPT) was transformed into the mutant and the isogenic wild type strain. Transformants were obtained on synthetic dextrose media (2% dextrose, 1% YNB and auxotrophic supplements) lacking histidine (SD-His). The transformants were then

inoculated in non-selective media (YEPD) and allowed to grow for 7-10 generations. Again, these were streaked on non-selective media to obtain single colonies. Single colonies (200 of each transformant) were subsequently patched onto SD-His (selective media) and YEPD (non-selective media). Mitotic stability of the monocentric plasmid was calculated by:

$$\% \text{ mitotic stability} = \frac{\text{Number of colonies growing on selective media}}{\text{Number of colonies growing on non-selective media}} \times 100$$

4.4 DAPI staining and microscopy.

DAPI staining is performed to determine the location of the nuclear mass in the cell. DAPI (4', 6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to AT rich regions in DNA. As DAPI can pass through an intact cell membrane, it can be used to stain both live and fixed cells, though it passes through the membrane less efficiently in live cells.

DAPI staining was performed by the following the method (Kikuchi and Toh-e 1986). Exponential phase cells were washed with 25% ethanol, 15mM $MgCl_2$ and re-suspended in the same solution for fixation at room temperature, post which they were washed with water. The fixed cells were suspended in 0.1µg/mL DAPI and then illuminated at a wavelength of 340-360nm. Cells were imaged using fluoroscence microscope (Olympus BX51) and processed using Image J software.

4.5 FACS analysis

Cells were harvested and processed as described in Koshland Lab protocols (http://mcb.berkeley.edu/labs/koshland/Protocols/YEAST/facs.html). Prior to injection of the sample into the flow cytometer, the cell suspension was sonicated briefly (30% amplitude, 5 s pulse). The sonicated sample was diluted to desired cell density using 1X PBS and injected into the flow cytomer (BD FACS Calibur) for analysis. The output was analysed using BD CellQuest Pro software.

4.6 Transcription read-through assay.

Transcription read-through assay is performed to determine the binding of the kinetochore at the centromere. A *CEN* is placed in between a strong promoter (*GAL1*) and a reporter gene *lacZ α*. The amount of β-galactosidase produced is used as readout for kinetochore assembly onto the centromere (Reynolds and Lundblad 1995). A transcript originating at the *GAL1* promoter has to pass *CEN* to reach the *lacZ* gene. Kinetochore assembled over *CEN* will act as a physical hindrance resulting in termination of transcription. If a mutation affects kinetochore assembly, transcription machinery can move through the centromere, thus giving higher levels of β-galactosidase.

pAKD06+*CEN4* (carrying the *URA3* marker) was transformed into autophagy deletion mutants and the isogenic wild-type strain. The construct is designed to direct the integration of the repoter via homologous recombination to the *ura3* locus on chromosome V of *S. cerevisiae.* The transformants were obtained on SD media lacking uracil (SD-Ura).

Single colony was inoculated in 5 ml of synthetic media lacking uracil, containing 2% galactose and 0.3% raffinose (instead of dextrose) and was grown to OD_{600}=1. Cell pellet was washed with water and re-suspended in 1ml of Z buffer (60mM Na_2HPO_4, 40mM $NaH_2PO_4.2H_2O$, 10mM KCl, 5mM β-ME). 0.1ml of this was taken for determination of OD_{610}. 0.1ml of Z buffer was added to the remaining 0.9ml of cells. The cells were then permeabilised by adding 50 µl of 0.1% SDS and 100 µl of chloroform (incubated at 30°C for 15 minutes). 0.2ml of 4mg/ml ONPG (ortho-nitrophenyl-β-galactoside) was added to the cell suspension and incubated at 30°C till yellow colour developed. Reaction was stopped by adding 0.5ml 1M Na_2CO_3. Cells were spun down at 10,000rpm and the clear supernatant was transferred to a fresh tube. The optical density of this solution was measured at 420 and 550 nm. The enzyme activity was normalized with respect to cell density. Units of β galactosidase were measured as:

$$\text{Miller units} = \frac{1000\,[(OD_{420})-1.75\times(OD_{550})]}{t \times v \times OD_{610}}$$

t= time of reaction (minutes)

v= volume of cell suspension used in the assay (ml)

4.7 Strains, plasmids and primers

Strains, plasmids and primers used in the study have been listed below:

Table 1: *S. cerevisiae* strains used in the study.

S.No.	Strain	Genotype/ Background	Source
1.	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF
1.	atg1Δ::*KanMX*	BY4741	EUROSCARF
2.	atg2Δ::*KanMX*	BY4741	EUROSCARF
3.	atg3Δ::*KanMX*	BY4741	EUROSCARF
4.	atg4Δ::*KanMX*	BY4741	EUROSCARF
5.	atg5Δ::*KanMX*	BY4741	EUROSCARF
6.	atg6Δ::*KanMX*	BY4741	EUROSCARF
7.	atg7Δ::*KanMX*	BY4741	EUROSCARF
8.	atg8Δ::*KanMX*	BY4741	EUROSCARF
9.	atg9Δ::*KanMX*	BY4741	EUROSCARF
10.	atg10Δ::*KanMX*	BY4741	EUROSCARF
11.	atg11Δ::*KanMX*	BY4741	EUROSCARF
12.	atg12Δ::*KanMX*	BY4741	EUROSCARF
13.	atg14Δ::*KanMX*	BY4741	EUROSCARF
14.	atg15Δ::*KanMX*	BY4741	EUROSCARF
15.	atg16Δ::*KanMX*	BY4741	EUROSCARF
16.	atg17Δ::*KanMX*	BY4741	EUROSCARF
17.	atg18Δ::*KanMX*	BY4741	EUROSCARF

18.	atg19Δ::*KanMX*	BY4741	EUROSCARF
19.	atg20Δ::*KanMX*	BY4741	EUROSCARF
20.	atg21Δ::*KanMX*	BY4741	EUROSCARF
21.	atg22Δ::*KanMX*	BY4741	EUROSCARF
22.	atg23Δ::*KanMX*	BY4741	EUROSCARF
23.	atg24Δ::*KanMX*	BY4741	EUROSCARF
24.	atg26Δ::*KanMX*	BY4741	EUROSCARF
25.	atg27Δ::*KanMX*	BY4741	EUROSCARF
26.	atg29Δ::*KanMX*	BY4741	EUROSCARF
27.	atg31Δ::*KanMX*	BY4741	EUROSCARF
28.	atg32Δ::*KanMX*	BY4741	EUROSCARF
29.	atg32Δ::*KanMX*	BY4741	EUROSCARF
30.	atg33Δ::*KanMX*	BY4741	EUROSCARF
31.	atg34Δ::*KanMX*	BY4741	EUROSCARF
32.	atg36Δ::*KanMX*	BY4741	EUROSCARF
33.	atg38Δ::*KanMX*	BY4741	EUROSCARF
34.	atg39Δ::*KanMX*	BY4741	EUROSCARF
35.	atg40Δ::*KanMX*	BY4741	EUROSCARF
36.	atg41Δ::*KanMX*	BY4741	EUROSCARF
37.	nvj1Δ::*KanMX*	BY4741	EUROSCARF

Table 2: Plasmids used in the study.

S.No.	Plasmid	Construct	Source
1.	pRS313	Yeast centromere vector with a *HIS3* marker and an MCS derived from pBLUESCRIPT.	Addgene Vectors
2.	pAFS125	GFP-Tub1 expression cassette for integration at the chromosomal *URA3* locus.	Straight *et al*., 1997
3.	pAKD06+CENIV	Yeast centromere vector with a *URA3* marker and another *CEN* between *GAL1* promoter and *lacZα* sequence.	Dr. Santanu Kumar Ghosh, IIT-B

Table 3: Primers used in the study.

S.No.	Primer name	Sequence	Purpose
1.	Sc-Atg1-FP	AAGTTAAGTACCAAGGCCAT	Forward primer to confirm atg1Δ::KanMX
2.	Sc-Atg11-FP	ATGTCCAATGGCTTGTACAC	Forward primer to confirm atg11Δ::KanMX
3.	Sc-Atg17-FP	CTTGAATTATTATCTTCCTC	Forward primer to confirm atg17Δ::KanMX
4.	Sc-Atg39-FP	ATCGAGCCATAAAAATTGAT	Forward primer to confirm atg39Δ::KanMX
5.	Sc-KanMX-RP	ATTACGCTCGTCATCAAAATCA	Reverse primer to confirm all deletions with KanMX

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