

# The role of autophagy in maintenance of genomic integrity

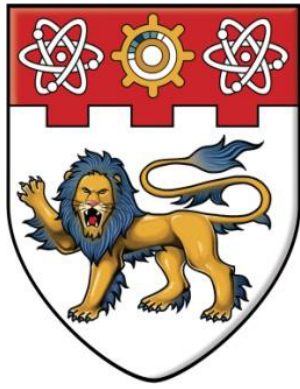
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**NANYANG**  
**TECHNOLOGICAL**  
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**THE ROLE OF AUTOPHAGY IN MAINTENANCE OF  
GENOMIC INTEGRITY**

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**SCHOOL OF BIOLOGICAL SCIENCES**  
**2015**

# **THE ROLE OF AUTOPHAGY IN MAINTENANCE OF GENOMIC INTEGRITY**

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## LIST OF ABBREVIATION

°C	Degree Celsius
hrs	Hours
kDa	KiloDalton
mg	Milligram
mins	Minutes
mm	Millimeter
mM	Millimolar
nM	Nanomolar
%	Percent
µg	Microgram
µl	Microlitre
APS	Ammonium Persulfate
ATG	Autophagy-related
ATP	Adenosine Triphosphate
β-TRCP	Beta-transducin repeat containing protein
BSA	Bovine Serum Albumin
CENP-A	Centromere protein A
CENP-E	Centromere protein E
CDKs	Cyclin dependent kinases
CMA	Chaperone-mediated autophagy
CREST	Calcium responsive transactivator protein
DAPI	4',6-diamidino-2-phenylindole, nuclei marker
DMEM	Dulbecco's Modified Eagle Media
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ER	Endoplasmic reticulum
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
H3	Histone H3
HA	Hemagglutinin
HECT	Homologous to E6-associated protein C-terminus

HDAC6	Histone deacetylase 6
HSC70	Heat shock-cognate protein of 70 kDa
IF	Immunofluorescence
INCENP	Inner Centromere Protein
IP	Immunoprecipitation
LAMP-2A	Lysosome-associated membrane protein type 2A
LB	Lysogeny Broth
LC3	Microtubule-associated protein 1A/1B-light chain 3
Lys	Lysine
MPS1	Monopolar spindle 1 kinase
mRNA	Messenger Ribonucleic Acid
NAC	N-acetyl l-cysteine
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAGE	Polyacrylamide Gel Electrophoresis
PALA	N-(phosphonoacetyl)-L-aspartate
PARP-1	Poly(ADP-ribose) polymerase 1
PAS	Pre-autophagosomal structure
PBS	Phosphate Buffered Saline
PE	Phosphatidylethanolamine
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamines
Plk1	Polo-like Kinase 1
UPS	Ubiquitin proteasome system
QPCR	Quantitative real-time polymerase chain reaction
RING	Really interesting new gene
ROS	Reactive oxygen species
SDS	Sodium Dodecyl Sulfate
siRNA	Short-interfering RNA
SMC	Structural maintenance of chromosome
TBST	Tris buffered saline with tween-20
TEMED	Tetramethylethylenediamine
WB	Western Blot

## **ABSTRACT**

Autophagy is an intracellular degradation pathway that is important for sustaining cellular metabolism. It is a catabolic process whereby cytoplasmic components such as damaged proteins and organelles are delivered to the lysosomes for degradation. Beyond autophagy's housekeeping functions, recent studies have uncovered a myriad of physiological and pathophysiological roles played by autophagy, such as the modulation of host defenses, ageing and tumorigenesis. In particular, my interest lies in autophagy's role in maintenance of genomic integrity. However, the mechanisms underlying this role remain elusive. Here, autophagy inhibition resulted in reduced protein levels of condensin-II and Kif4a. This negatively affected the amount of condensin-II and Kif4a localized on the chromosomes and in turn, impeded proper chromosome condensation and kinetochore-microtubule attachments during mitosis. As a result, there was increased incidence of lagging chromosomes from aberrant chromosome segregation, thus compromising genomic integrity. These indicated a pathway where autophagy enables proper chromosome segregation through its regulation on the protein levels of condensin-II and Kif4a.

# 1 INTRODUCTION

## 1.1 Intracellular protein degradation

The stability of proteins is critical to the health of the cell and eventually affects the lifespan of the organism. Composition of the proteome is constantly changing as the cell progresses through the cell cycle and adapts to its varying environments. With the steady-state levels of proteins being determined by the rate of synthesis and degradation, protein turnover is a dynamic process that is highly regulated (Saric and Goldberg 2001).

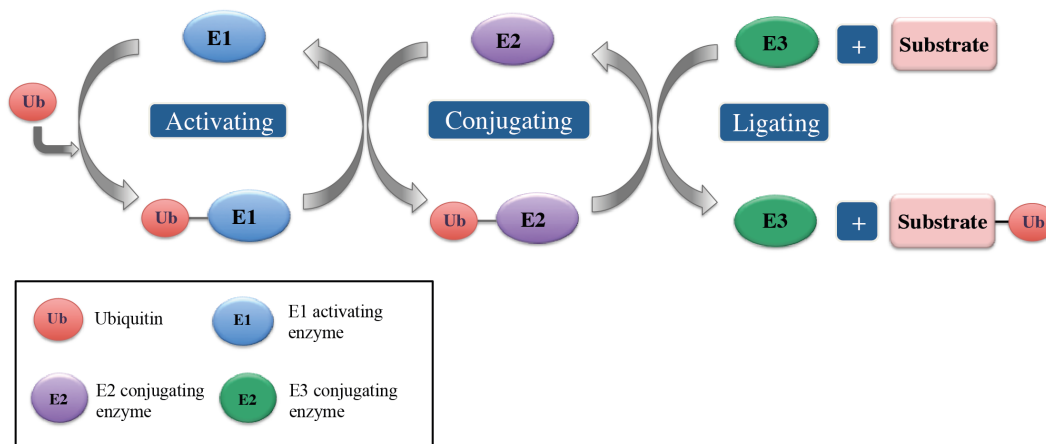
Some proteins are relatively stable while others possess very short half-lives. Such wide range of protein stability defines the roles played by different proteins in the cell. Regulatory proteins such as the cyclins are classic examples of proteins with low stability. Cyclins are rapidly degraded, thus enabling cyclin-dependent kinases (CDKs) to activate a set of processes that drive the cell cycle in a timely manner (Bloom and Cross 2007). In addition, proteins that are impaired and/or misfolded need to be swiftly removed in order to prevent formation of toxic aggregates that may cause irreversible damage to the cell. For long-lived proteins, they are usually components of larger complexes such as the ribosomes but are metabolically unstable as individual subunits (Toyama and Hetzer 2013).

### 1.1.1 Ubiquitin tags protein for degradation

To allow recognition of proteins to be sent for degradation, the cell uses ubiquitin as a tag to mark proteins for destruction (Clague and Urbé 2010). Ubiquitin is highly conserved and consists of 76 amino acids. The conjugation of ubiquitin to its target proteins is selective. Ubiquitin is first activated by binding to E1 enzyme with the formation of a thioester bond between cysteine residue at the active site of E1 and the C-terminus glycine of ubiquitin. The activated ubiquitin is then transferred to E2 conjugating enzyme, which then forms a thioester bond between its active-site cysteine residue and the C-terminus glycine of ubiquitin. Lastly, ubiquitin is covalently attached to the target protein through an isopeptide bond

between the C-terminus glycine of ubiquitin and the  $\epsilon$  amino group of an internal lysine residue of the target protein. E3 ubiquitin ligase may catalyze this step directly or act as a scaffolding protein to facilitate the reaction (Fig. 1.1.1) (Tu, Chen et al. 2012). Together with the deubiquitinases (DUBs) that cleave and remove ubiquitin conjugates, proteins may end up being monoubiquitinated at one/more places or be polyubiquitinated by having subsequent rounds of ubiquitination on lysines of the attached ubiquitins to produce different types of polyubiquitin chains.

Since degradation is irreversible, high level of substrate specificity is crucial to prevent erroneous protein proteolysis. Such specificity is achievable thanks to the availability of a wide range of E3 ubiquitin ligases and DUBs, as well as the distinct types of post-translational modifications or sequence motifs on substrates that are recognizable by different E3 ligases and DUBs (Weissman 2001).



**Figure 1.1.1. The role of E1, E2 and E3 enzymes in ubiquitination.**

#### *1.1.1.1 E3 ubiquitin ligase – The effector of ubiquitination*

The human genome encodes approximately 1000 E3 ligases that can be classified into three major types according to the type of conserved structural domains they possess, namely a RING (really interesting new gene) finger, HECT (homologous to E6-associated protein C-terminus), or U-box (a modified RING motif without

the full complement of Zn<sup>2+</sup>-binding ligands) domain. Among the three classes of E3 ligases, those with the HECT domains are the only class that plays a direct catalysis role in protein ubiquitination while those with RING finger and U-box domains act as scaffolding proteins that facilitate the interaction of ubiquitin-loaded E2 enzymes with the substrates (Ardley and Robinson 2005).

In recent years, compelling amount of evidence implicates E3 ligases in tumorigenesis. They are frequently overexpressed in human cancers and correlated with increased chemoresistance (Sun 2006). Therefore, studies have started to focus on understanding the involvement of E3 ligases in various cellular pathways. For instance, a ubiquitin ligase, RING finger protein 4, was found to prevent the loss of intact chromosomes, thus ensuring the maintenance of chromosome integrity (Hirota, Tsuda et al. 2014). The HECT E3 ligase, Smurf2, was also revealed as an important regulator of Mad2-dependent spindle assembly checkpoint (Osmundson, Ray et al. 2008). In particular, Skp-1-Cullin1-F-box protein (SCF)<sup>beta-transducin repeat containing (β-TRCP)</sup> E3 ligase is said to contribute to malignant transformation of cells (Karin, Cao et al. 2002, Richmond 2002). SCF<sup>β-TRCP</sup> E3 ligase belongs to the RING finger family of E3 ligases and is involved in regulating important signaling pathways like that of β-catenin/Tcf and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). As such, any perturbation to its levels will inevitably contribute towards cancer development.

#### *1.1.1.2 DUB - The protein that counteracts the actions of E3 ligase*

DUBs are components of the UPS that catalyze removal of ubiquitin by cleaving isopeptide or peptidic bonds between target proteins and ubiquitin, hence resulting in altered signaling or protein stability (D'Arcy, Wang et al. 2015).

The human genome encodes ~80 functional DUBs that are divided into six classes based on structural and sequence similarity: ubiquitin-specific proteases, ubiquitin carboxy-terminal hydrolases, Machado–Joseph disease protein domain proteases, ovarian-tumor proteases, monocyte chemotactic protein-induced protein and JAMM/MPN domain-associated metallopeptidases (Komander, Clague et al. 2009, Fraile, Quesada et al. 2012, D'Arcy, Wang et al. 2015). By regulating

protein stability and controlling the amount of free ubiquitins available, DUBs are undoubtedly important players in biological processes such as DNA repair, cell-cycle control, chromatin remodeling and signaling pathways, all of which are frequently altered in cancers (Hussain, Zhang et al. 2009, Komander, Clague et al. 2009).

Their activities are broadly divided into three types. Firstly, DUBs contribute to the pool of free ubiquitins by processing inactive ubiquitin precursors that either exist in the form of fusions with ribosomal proteins or head-to-tail-linked ubiquitin polymers with an additional amino acid attached to the last ubiquitin. Such processing is essential to produce active ubiquitins capable of attaching to proteins. Furthermore, DUBs maintain the pool of active ubiquitins by keeping them from being rapidly titrated by small intracellular nucleophiles such as polyamines (Amerik and Hochstrasser 2004). In addition, proteasome-associated DUBs remove polyubiquitin chains from the substrates prior to degradation in the proteasome to enable recycling of ubiquitins. Secondly, DUBs form a layer of proofreading by assessing the ubiquitinated proteins and removing ubiquitin chains for those that are inappropriately targeted to the proteasome, thus rescuing them from the fate of degradation (Lam, Xu et al. 1997). Thirdly, DUBs serve to modify the type of ubiquitin conjugates attached to proteins, which may alter the type of regulation conferred by the ubiquitin conjugates (Amerik and Hochstrasser 2004).

#### *1.1.1.3 Types of ubiquitination*

As mentioned earlier, ubiquitins can be conjugated to each other to produce a variety of polyubiquitin chains. Within the ubiquitin molecule, there are seven lysine (Lys) residues, namely Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63. Lys48 and Lys63 are most often used in polyubiquitin chain formation and are better studied (Varadan, Walker et al. 2002).

Structural studies suggested that ubiquitins attached to different lysine residues may adopt distinct conformations recognizable by specific ubiquitin-binding proteins for different purposes (Ikeda and Dikic 2008). For Lys48, it is most

commonly used for but not limited to, as a signal for proteasomal degradation (Jacobson, Zhang et al. 2009). In contrast, polyubiquitin chains linked through Lys63 is more associated with non-proteolytic signals such as protein-protein interaction, DNA repair and kinase activation (Deng, Wang et al. 2000, Kawadler and Yang 2006, Zeng, Sun et al. 2010, Komander and Rape 2012). However in recent years, it was discovered that Lys-63 is also used as a signal for autophagic-lysosomal degradation (Seibenhener, Babu et al. 2004, Olzmann and Chin 2008, Tan, Wong et al. 2008). To complicate matters, both Lys48 and Lys63 were later revealed to be capable of signaling proteins for degradation via proteasomal as well as autophagic-lysosomal pathways (Zhang, Xu et al. 2013).

As with the case of Lys63, ubiquitination are capable of conferring regulatory instead of destructive signals on the proteins they are attached to. This kind of modification is monoubiquitination where there is an attachment of only one ubiquitin to the protein (Hicke and Dunn 2003). For instance, monoubiquitination of histones and transcription factors regulates gene transcription instead of marking them for proteasomal degradation (Muratani and Tansey 2003). Monoubiquitination at lysine-164 residue of proliferating cell nuclear antigen (PCNA) is also critical to the Rad6-Rad18-dependent repair processes that are triggered in response to the DNA-damaging treatments (Stelter and Ulrich 2003). Exceptions to such regulatory functions of monoubiquitination exist as well, with the monoubiquitination of integral membrane proteins serving as a destructive signal for internalization into the endocytic pathway and their subsequent degradation in lysosomes. Therefore, the role of ubiquitin is more ubiquitous and complex than once thought.

#### 1.1.2 Degradative pathway for short-lived proteins – The ubiquitin-proteasomal system (UPS)

It was first thought that most proteins would be directed to the lysosomal compartment for degradation upon ubiquitination. However this view was abolished when the stability of many cellular proteins is unaffected by the alkalization of lysosomes to inhibit autophagic-lysosomal degradation.

Subsequently, UPS was discovered as the other major site for protein degradation, thus accounting for the proteins that are not substrates of autophagic-lysosomal degradation (Clague and Urbé 2010).

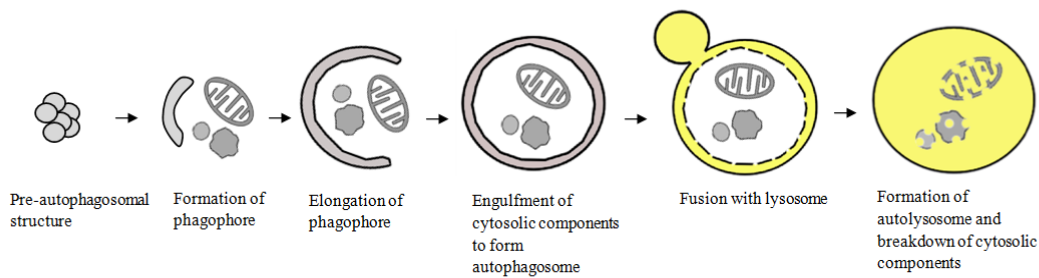
The UPS serves as the primary route for degradation of short-lived proteins, enabling rapid adjustments to steady-state levels of regulatory proteins for normal functioning of cellular pathways. UPS consists of a proteasome that is a multi-subunit complex that comprises two 19S lid complexes flanking a 20S proteolytic core. The 19S complexes bind the cargo-transporting proteins and channel the cargo through the proteolytic core, where proteins will be degraded into small peptides or amino acids by various catalytic activities (Tu, Chen et al. 2012). However, polyubiquitin chains on these proteins will not be degraded and are instead, released by proteasome-associated DUBs.

#### 1.1.3 Degradative pathway for long-lived proteins - The autophagy-lysosomal pathway

In addition to the UPS, autophagy is the other major pathway for protein degradation. The word “autophagy” is derived from the Greek word for “self-eating”. This is rightly so, as autophagy involves catabolic processes responsible for turning over intracellular proteins and organelles via the lysosomal degradation pathway (Yang and Klionsky 2010). This process includes double-membrane vesicles named autophagosomes, which engulf long-lived proteins, damaged organelles, and even invasive pathogens, and direct them to the lysosomes for degradation. The resulting metabolites are then used as energy sources and for the synthesis of new proteins for cell growth and survival.

Macroautophagy is the most prevalent type of autophagy characterized by a double-membrane structure termed phagophore. These phagophores will eventually mature and enclose their substrates within it. Upon autophagy induction, autophagy-related (Atg) proteins assemble at the pre-autophagosomal structure (PAS) with the addition of membrane derived from sources such as the plasma membrane, Golgi apparatus and endoplasmic reticulum to form the

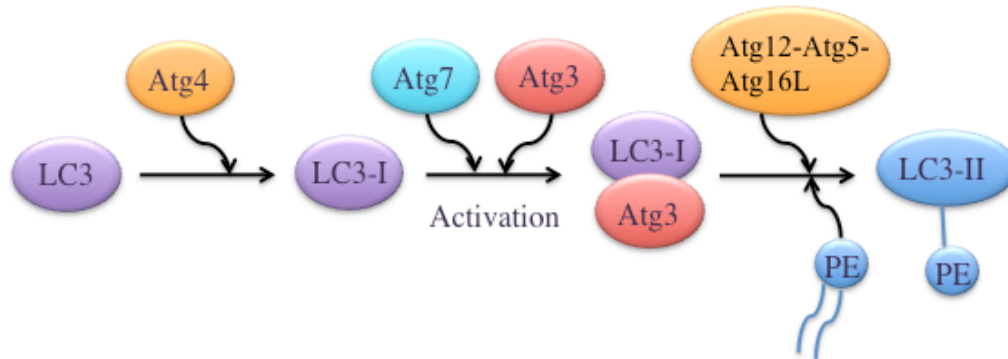
phagophore (Rubinsztein, Shpilka et al. 2012). The phagophore membrane then proceeds to engulf cytosolic components with the elongation and closure of the phagophore ends to form an enclosed autophagosome. Complete autophagosomes subsequently fuse with the lysosomes to form autolysosomes, exposing their contents to the hydrolases within. This is followed by the degradation of cytosolic components, releasing the products for recycling in the cytosol (Fig. 1.1.3).



**Figure 1.1.3. The process of macroautophagy in mammalian cells.**

During the formation of autophagosomes to engulf cytosolic components, microtubule-associated protein 1A/1B-light chain 3 (LC3) is cleaved by cysteine protease, Atg4, at the carboxy-terminal arginine to expose the glycine. This is the LC3-I form that will then be activated by E1-like enzyme, Atg7 (Kabeya, Mizushima et al. 2004). The resultant LC3-I will be transferred to E2-like enzyme, Atg3. Here, an E3-like complex that consists of Atg5, Atg12 and Atg16L will catalyze the transfer of LC3-I to phosphatidylethanolamines (PE) to form LC3-II (Fig. 1.1.3.1) (Ichimura, Kirisako et al. 2000). LC3 is a soluble protein that is expressed ubiquitously in mammalian cells and the lipidation of LC3-I to LC3-II is required for the recruitment of LC3-II to autophagosomal membranes. The subsequent fusion of autophagosomes with lysosomes then results in LC3-II being degraded along with the other contents within the autolysosomal lumen. As the presence of LC3-II largely reflects autophagic activity, detection of LC3-II via western blotting or immunofluorescence is commonly used to monitor autophagy. Many mouse models have been generated with targeted deletions of Atg genes such as Atg5 and Atg7. As these Atg proteins are essential during the formation of

autophagosomal membranes, such mouse models exhibit defective autophagy that is instrumental in the elucidation of autophagy's functions.



**Figure 1.1.3.1. LC3 conjugation system mediated by the various Atg proteins.**

Macroautophagy was originally regarded as a non-selective process, but compelling evidence now shed light on the specificity of macroautophagy for its substrates. p62, also known as Sequestosome-1, is a protein that targets specific substrates for macroautophagy (Lamark, Kirkin et al. 2009). When macroautophagy is perturbed in different cell types, p62 accumulated with ubiquitinated protein aggregates that disappeared upon depletion of p62 (Nezis, Simonsen et al. 2008). Therefore, p62 functions like a double-edge knife that aids in the selective removal of potentially harmful proteins, but promotes the formation of aggregates once autophagy is disturbed, a condition reminiscent to that of neurodegenerative disorders.

Macroautophagy can be further subdivided into “basal autophagy” and “induced autophagy”. Basal autophagy is occurring at all times with the constitutive turnover of intracellular proteins and organelles. Induced autophagy typically occurs when there is nutrient starvation such as the lack of total amino acids, hormones or growth factors, in order to provide amino acids for nutrient-deprived cells via the degradation of cellular components (Ravikumar, Vacher et al. 2004, Mizushima 2005). It should be noted, however, that such excess production of amino acids via induced autophagy is only an acute response to support cell survival for a short period of time.

Other than macroautophagy, there are two other types of autophagy pathways termed microautophagy and chaperone-mediated autophagy (CMA). Cargo from all three pathways will ultimately be delivered to the lysosomes for degradation, but they differ in the pathways taken to transport their cargo to the lysosomes.

Microautophagy can be a selective or non-selective process that involves direct invagination of lysosomal membrane into autophagic tubes, followed by the formation of vesicles that bud into the lumen for degradation by hydrolases (Shintani and Klionsky 2004, Li, Li et al. 2012). On the other hand, CMA is a selective process where specific substrates are recognized and bound by a chaperone, the heat shock-cognate protein of 70 kDa (hsc70) for delivery to lysosomes. The transport of substrates through the lysosomal membrane is then mediated by their interaction with membrane protein lysosome-associated membrane protein type 2A (LAMP-2A) in the presence of hsc70 and its co-chaperones (Cuervo and Dice 1996, Agarraberes and Dice 2001).

From this point onwards, the term “autophagy” refers to macroautophagy unless otherwise specified.

#### *1.1.3.1 Functions of autophagy - Its role in genomic stability*

In recent years, studies have revealed many physiological and pathophysiological roles of autophagy besides its widely known functions in turnover of intracellular components and ensuring cell survival under conditions of metabolic stress. These include roles in development, defense against extracellular microorganisms, ageing, tumorigenesis, tissue remodeling, programmed cell death, antigen presentation and maintenance of genome integrity (Mizushima 2007, Cecconi and Levine 2008). However, a large part of these functions remain unclear and more studies need to be done. In particular, autophagy's role in maintaining genomic stability remains poorly understood.

As autophagy is known for its housekeeping function in homeostasis and protein quality control, defective autophagy may result in chromosomal instability through the accumulation of damaged proteins, including those that are important

in critical cellular processes such as chromosome segregation. For example, autophagy is critical for timely clearance of damaged mitochondria, thus preventing deoxyribonucleic acid (DNA) damage and consequent tumorigenesis (Mathew, Kongara et al. 2007). In addition, functional autophagy is crucial for activation of apoptosis in response to H<sub>2</sub>O<sub>2</sub> treatment. Such autophagy-dependent apoptosis prevents cells with DNA damage from participating in subsequent rounds of cell divisions, thus contributing to genomic stability (Castino, Bellio et al. 2010, Rubinsztein, Marino et al. 2011). Impaired autophagy also exacerbated susceptibility of cells to metabolic stress and increased resistance to N-(phosphonoacetyl)-L-aspartate (PALA) treatment that indicated higher gene amplification rates. This eventually led to aneuploidy, thus supporting the proposed role of autophagy in suppressing genomic instability (Mathew, Kongara et al. 2007). Interestingly, the addition of N-acetyl l-cysteine (NAC) to quench reactive oxygen species (ROS) delayed aneuploidy and increased survival rates of the cells with defective autophagy. Therefore, ROS appeared to be the main contributor behind genomic instability in these cells (Karantza-Wadsworth, Patel et al. 2007).

The tumor suppressive roles played by autophagy also hint at its importance in maintaining genomic stability. For instance, maintenance of autophagic activity is critical to prevent formation of malignant tumors that arise due to gene amplification and DNA damage (Gozuacik and Kimchi 2004, Mathew, Kongara et al. 2007). Intriguingly, DNA damage induces autophagy, thus indicating that autophagy may be involved in DNA damage response and/or DNA repair as well (Polager, Ofir et al. 2008, Munoz-Gamez, Rodriguez-Vargas et al. 2009). This was later confirmed when autophagy was found to regulate DNA repair by recycling key proteins needed in the processing of DNA lesions. One of such protein is Sae2, an endonuclease that is required for initiating the end-resection step of repair. Clearance of acetylated Sae2 by autophagy serves to control DNA repair of double-stranded breaks and prevent extensive resection that may be harmful to cells (Robert, Vanoli et al. 2011).

As mentioned above, most of the studies were centered on autophagy's involvement in timely degradation of mitochondria and DNA repair, thereby preventing the accumulation of ROS that would induce DNA damages and promoting repair of any prevailing DNA lesions. For the rest, only a correlation between autophagy and genomic instability were found without establishing the mechanisms or pathways linking them together. Since autophagy is a major degradation system with a myriad of substrates, it will definitely have either direct or indirect control over many cellular pathways through its regulation on the protein levels of its substrates. Yet, many substrates of autophagy remained uncovered, especially those potentially involved in preventing chromosomal instability.

#### 1.1.4 Cross talk between UPS and autophagy

For a long time, UPS and autophagy were viewed as two independent degradation systems without any overlap of signaling pathways. However, recent findings shed light on the presence of cross talk between the two systems with ubiquitin as the unifying factor. Both UPS and autophagy use ubiquitination to mark proteins that are to be degraded. Many proteins are also known to be substrates of both pathways, where ubiquitinated proteasomal substrates are sometimes degraded via autophagy and vice versa (Fuertes, Martin De Llano et al. 2003, Fuertes, Villarroya et al. 2003, Wooten, Geetha et al. 2008). However, the type of ubiquitination employed by UPS mostly differs from autophagy. As mentioned before, while UPS recognizes mainly Lys48-linked ubiquitin chains, autophagy's substrates are mostly tagged with Lys63-linked ubiquitin chains or monoubiquitins (Welchman, Gordon et al. 2005, Kuang, Qi et al. 2013).

Perturbation of either degradation system has been reported to affect the activity of the other system. Inhibition of UPS's activity in proteasome mutants was found to induce histone deacetylase 6 (HDAC6)-dependent autophagy as a compensatory mechanism to relieve the cells of the accumulated UPS substrates (Pandey, Nie et al. 2007). In addition, upregulation of autophagy via the use of an autophagy-inducing drug, rapamycin, was found to protect against cell death from defective proteasomal functions (Pan, Kondo et al. 2008). In contrast, impaired

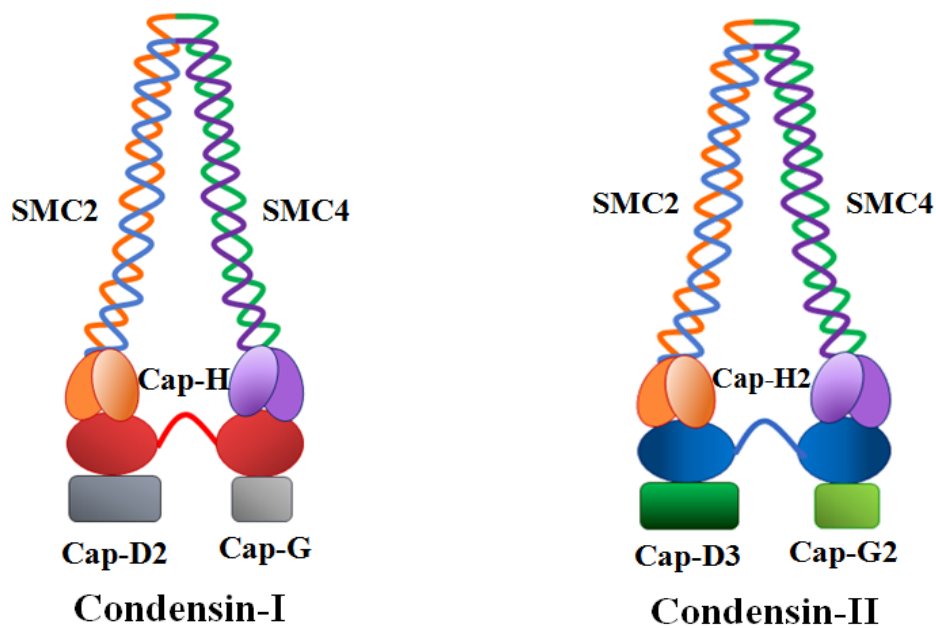
autophagy led to defective proteasomal degradation instead of an upregulation in proteasomal degradation (Pickart and Eddins 2004). The reason behind UPS's blockage was revealed to be the accumulation of p62 when autophagy is impaired. This was shown when the knockdown of p62 resumed the degradation of UPS substrates in autophagy-compromised cells (Korolchuk, Menzies et al. 2009). It is speculated that the elevated p62 levels may out-compete ubiquitin-binding proteins that are involved in proteasomal degradation such as p92/valosin-containing protein (VCP). As such, the accumulated p62 bind ubiquitinated UPS substrates and prevent their delivery to proteasomes (Korolchuk, Mansilla et al. 2009, Korolchuk, Menzies et al. 2009). The accumulation of UPS substrates may thus contribute towards the toxic effects that arise due to defective autophagy.

## **1.2 Condensins - The architects of chromosomes**

Other than autophagy, condensins also contribute towards chromosomal stability by preventing the formation of lagging chromosomes, chromosomal bridges and micronuclei (Green, Kalitsis et al. 2012, Cuylen, Metz et al. 2013).

Being the key regulators governing the architecture of metaphase chromosomes, condensins are multi-subunit protein complexes that are highly conserved and responsible for condensation of the chromatin to form individual chromosomes during mitosis. This process is essential for proper segregation of sister chromatids so as to prevent situations of aneuploidy or polyploidy that create genomic instability (Ono, Fang et al. 2004).

It is currently known that there are two types of condensin complexes, namely condensin-I and -II. They share the same core heterodimer, structural maintenance of chromosome (SMC) 2/4, but differ in the three non-SMC subunits i.e. Cap-D2/G/H for condensin-I and Cap-D3/G2/H2 for condensin-II (Fig. 1.2) (Hirano 2012).



**Figure 1.2. Condensin-I and II have the same SMC2/4 subunits but differ in their non-SMC subunits.**

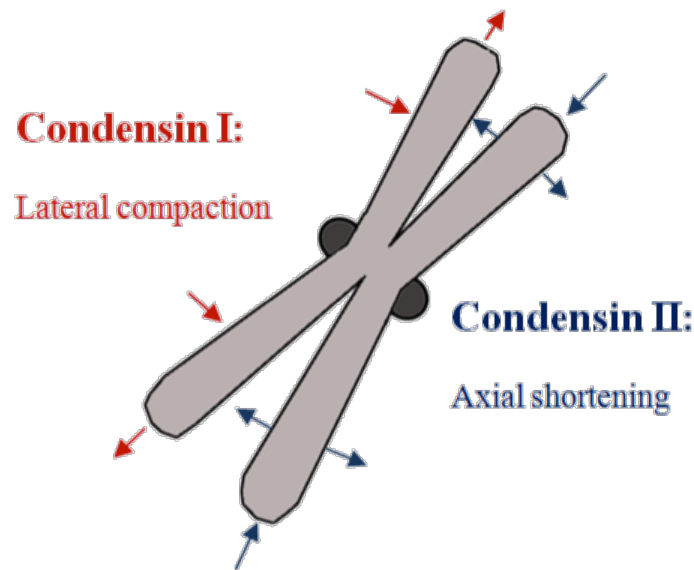
### 1.2.1 Differential contribution of condensin-I & -II to chromosome architecture

Based on immunofluorescence microscopy experiments, condensin-I is cytoplasmic until nuclear envelope breakdown (NEBD), whereas condensin-II remains in the nucleus throughout the cell cycle and starts binding to chromosomes in prophase (Hirota, Gerlich et al. 2004). Their binding to the chromosomes are also independent of each other as seen by the persistent enrichment of condensin-II on chromosomes in condensin-I depleted HeLa cells and vice versa (Hirota, Gerlich et al. 2004).

It was reported that condensin-I and -II exhibit distinct distributions along the length of chromosomes with the depletion of condensin-I or -II specific subunits giving rise to different structural defects in metaphase chromosomes. In *Xenopus* egg extract depleted of condensin-I, sperm chromatin was unable to assemble into rod-shaped chromosomes and was instead converted into a mass of decondensed chromatin. In contrast, sperm chromatin assembled into chromosomes with a curly appearance in condensin-II depleted extract. Similar observations were

noted in HeLa cells as well (Ono, Losada et al. 2003, Ono, Fang et al. 2004). These data suggested that while condensin-II is crucial in determining the final chromosome shape, condensin-I is the critical component needed to support the assembly of chromosomes.

Apart from knowing how condensin-I and -II function individually, a study explored further to determine their effects on chromosomes relative to each other. This was done by adjusting the levels of condensin-I and -II by quantitative immunodepletion in *Xenopus* egg extracts (Shintomi and Hirano 2011). The ratio of condensin-I to condensin-II was manipulated to be 1:1, compared to control that had a ratio of 5:1 (Shintomi and Hirano 2011). Interestingly, the assembled chromosomes in 1:1 extract were wider and shorter than the chromosomes that assembled in 5:1 extract. When condensin-II was further depleted in the 1:1 extract such that the ratio became 1:0, the lengths and widths of the assembled chromosomes then resembled those in control extract (5:1). Such results clearly indicated that condensin-I is important for lateral compaction of chromosomes whereas condensin-II is important for axial shortening of chromosomes (Fig. 1.2.1) (Shintomi and Hirano 2011).



**Figure 1.2.1. A model that depicts how the balancing action of condensin-I & -II can determine the shape of metaphase chromosomes.**

Red arrows indicate the action of condensin-I and blue arrows indicate that of condensin-II on the chromosome. (Figure modified from Shintomi et. al.)

### 1.2.2 Contribution of condensins to chromosomal stability

Other than their obvious roles in chromosome condensation, condensins also function in the maintenance of chromosomal stability. They achieve this via various ways, such as the regulation of gene expression, DNA repair and ensuring proper chromosome segregation.

Many studies have uncovered the involvement of condensins in regulating gene expression (Bhalla, Biggins et al. 2002, Rawlings, Gatzka et al. 2011). For instance, yeast condensins bound at rDNA is important in preserving the status of rDNA as a silent chromatin domain, by maintaining the appropriate balance of nucleolar and telomeric silencing protein, Sir2p (Machín, Paschos et al. 2004). Additionally, chromosome condensation mediated by condensin-II, Cap-H2, was found to be essential in maintenance of T cells in the quiescent state (Rawlings, Gatzka et al. 2011). The interaction between murine homolog of condensin-II subunit, Cap-G2 (MTB), the stem cell leukemia transcription factor and E12

transcription factor also enabled MTB to repress transcription during erythroid cell development (Xu, Leung et al. 2006).

Another area that involves condensins lies in DNA repair. This was first suggested in the yeasts where DNA damage accumulated in condensin mutants (Aono, Sutani et al. 2002). It was later revealed that condensin-I forms part of the DNA damage response for double-strand breaks (DSB) by interacting with DNA nick-sensor, poly(ADP-ribose) polymerase 1 (PARP-1) (Heale, Ball et al. 2006). Involvement of condensin-II in homologous recombination repair was also evident (Wood, Liang et al. 2008).

Notably, condensins were discovered to be crucial in ensuring proper chromosome segregation during anaphase. This arose due to the observations of lagging chromosomes as a prominent phenotype in condensin-depleted cells (Coelho, Queiroz-Machado et al. 2003, Hudson, Vagnarelli et al. 2003, Green, Kalitsis et al. 2012). Lagging chromosomes are chromosomes that are left behind at the metaphase plate during anaphase, due to errors in chromosome segregation. Such errors commonly arise due to abnormalities in the attachment of spindle microtubules to the kinetochores.

Indeed, the depletion of condensins was found to affect back-to-back orientation of kinetochores by altering the geometry of calcium responsive transactivator protein (CREST) and centromere protein E (CENP-E) at the centromere/kinetochore region (Ono, Fang et al. 2004). Furthermore, localization of centromere protein A (CENP-A) at the kinetochores was also severely disrupted (Yong-Gonzalez, Wang et al. 2007). Such perturbations in kinetochore assembly compromised kinetochore tension and gave rise to aberrant kinetochore-microtubule attachments. In addition, condensin-II subunits are needed to recruit polo-like kinase 1 (PLK1) to the kinetochores and chromosome axes during mitosis, such that proper chromosome condensation and subsequent segregation can take place (Abe, Nagasaka et al. 2011, Kim, Shim et al. 2014). In fact, the loss of any condensin subunit has been shown to affect chromosome segregation in all models, though to a less extent in higher eukaryotes where an alternate condensin-

independent condensation exists. Such condensation, however, produces defective chromosomes that are unable to withstand the physical stress associated with sister-chromatid separation (Bazile, St-Pierre et al. 2010). Therefore, this underscores the importance of condensins in producing functionally condensed chromosomes.

### **1.3 Kif4a – The molecular motor in mitosis**

In addition to autophagy and condensins, Kinesin Family Member 4A (Kif4a) also contributes towards chromosomal stability.

Kif4a is a chromokinesin that belongs to the kinesin superfamily of motor proteins (Lawrence, Dawe et al. 2004). It is required in various aspects of mitosis by ensuring different protein cargoes are accurately transported to the appropriate locations. During mitosis, Kif4a is mostly enriched on the chromosomal arms. When mitosis progresses from anaphase to cytokinesis, Kif4a also accumulates at the mid-body as two ring-like structures connecting the two opposing spindle microtubules. There, it remains until the cell pinches off into two daughter cells (Mazumdar, Sundareshan et al. 2004).

The mitotic roles of Kif4a start at the condensation of chromosomes. It contributes to higher order organization by possibly acting as a molecular linker between the DNA and proteins needed for chromosome condensation. Therefore, its depletion is expected to cause a collapse of the chromosomal structure. From prometaphase to metaphase, Kif4a is required for proper organization and alignment of chromosomes at the metaphase plate. After segregation of chromosomes, Kif4a contributes towards the formation of spindle midzone and subsequent cytokinesis by transporting the appropriate protein to the midbody (Mazumdar, Sundareshan et al. 2004). Due to its various roles in mitosis, Kif4a helps to ensure smooth mitotic progression.

#### **1.4 Aims of this study**

We know that autophagy plays an important role in maintenance of genomic integrity, thanks to the numerous studies that have been done ever since autophagy was discovered as a critical regulator of cellular homeostasis. While many studies focused on characterization of autophagy by identifying its functions in different physiological pathways, few managed to unravel the mechanisms employed by autophagy to carry out those functions. As such, our current knowledge regarding autophagy is mostly limited to their functions, without truly understanding how they are executed in the cells.

As chromosomal instability is one of the main forces driving the development of tumors, I am interested to elucidate the mechanisms underlying autophagy's role in maintaining genomic integrity. Since chromosome mis-segregation has been a major cause of aneuploidy and chromosomal instability in cancer cells, this study focuses on autophagy's contribution to proper chromosome segregation.

## 2 MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Antibodies

##### 2.1.1.1 Primary Antibodies

Antibodies	Host	Dilution	Application	Company
$\alpha$ -Tubulin-fluorescein isothiocyanate (FITC)	Mouse	1:1000	WB	Sigma-Aldrich (St. Louis, MO, USA)
Beta-TRCP2	Mouse	1:500	WB	Santa Cruz Biotechnology (Dallas, TX, USA)
GFP mAb	Rabbit	1:500	WB	Santa Cruz Biotechnology (Dallas, TX, USA)
H2B	Rabbit	1:1000	WB	Abcam (Cambridge, UK)
HA (Y-11) pAb	Rabbit	1:1000	WB	Santa Cruz Biotechnology (Dallas, TX, USA)
LC3	Rabbit	1:1000	WB	Abcam (Cambridge, UK)
NCAPD2	Mouse	1:500	WB	Novus Biologicals (Southpark way, USA)
NCAPD3	Rabbit	1:500	WB	Novus Biologicals (Southpark way, USA)
NCAPG	Mouse	1:500	WB	Abcam (Cambridge, UK)
NCAPG2	Rabbit	1:500	WB, IF	Sigma-Aldrich (St. Louis, MO, USA)
NCAPH	Rabbit	1:500	WB, IF	Novus Biologicals (Southpark way, USA)
NCAPH2	Rabbit	1:500	WB, IF	Bethyl Laboratories, Inc. (Montgomery, TX, USA)
p53	Rabbit	1:1000	WB	Cell Signaling Technology (Beverly, MA, USA)

P62	Rabbit	1:1000	WB	Cell Signaling Technology (Beverly, MA, USA)
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Table 2.1.1.1 Details of primary antibodies.

### 2.1.1.2 Secondary Antibodies

Goat anti-mouse horseradish peroxidase	Santa Cruz Biotechnology (Dallas, TX, USA)
Goat anti-rabbit horseradish peroxidase	Molecular Probes, Life Technologies, (Carlsbad, CA, USA)
Goat anti-rabbit Alexa Fluor 594	Molecular Probes, Life Technologies, (Carlsbad, CA, USA)
Rabbit anti-human Rhodamine	Sigma-Aldrich (St. Louis, MO, USA)

### 2.1.1.3 Antibody conjugated to agarose beads for immunoprecipitation

Antibody	Host	Company
GFP-binding protein (Not a mAb)	NIL	ChromoTek Inc. (Hauppauge, NY, USA)
HA mAb	Mouse	Sigma-Aldrich (St. Louis, MO, USA)

Table 2.1.1.3 Details of antibody-conjugated beads

### 2.1.2 Chemicals

Acetic acid	Merck Millipore (Darmstadt, Germany)
Acrylamide/Bis Solution	Bio-Rad (Hercules, CA, USA)
Agarose	Vivantis (Selangor, Malaysia)
Ammonium Persulphate	Promega (Madison, WI, USA)
Ampicillin	USB Corporation (Cleveland, OH, USA)
Beta-mercaptoethanol	Merck Millipore (Darmstadt, Germany)
Bovine Serum Albumin (BSA)	Sigma-Aldrich (St. Louis, MO, USA)
Bradford Assay Dye Solution	Bio-Rad (Hercules, CA, USA)
Bromophenol blue	USB Corporation (Cleveland, OH, USA)
Complete EDTA-free protease inhibitor	Roche (Basel, Switzerland)
Dithiothreitol (DTT)	Sigma-Aldrich (St. Louis, MO, USA)
DMSO	Sigma-Aldrich (St. Louis, MO, USA)
Dulbecco's Modified Eagle Medium (DMEM)	Gibco, Life Technologies (Carlsbad, CA, USA)
EDTA	Sigma-Aldrich (St. Louis, MO, USA)
EGTA	Sigma-Aldrich (St. Louis, MO, USA)
Fetal Bovine Serum (FBS)	Hyclone, Thermo Scientific (Waltham, MA, USA)
Glycerol	USB Corporation (Cleveland, OH, USA)
Glycine	1 <sup>st</sup> Base, Axil Scientific (Singapore)
HEPES	USB Corporation (Cleveland, OH, USA)
Kanamycin	A.G. Scientific (San Diego, CA, USA)
Lipofectamine 2000 Transfection Reagent	Gibco, Life Technologies (Carlsbad, CA, USA)

Low fat milk powder	Anlene, Fonterra (New Zealand)
Luria-Bertani (LB) Agar	Difco, Becton Dickinson (Franklin Lakes, NJ, USA)
Luria-Bertani (LB) Broth	Difco, Becton Dickinson (Franklin Lakes, NJ, USA)
M-PER Mammalian Protein Extraction Reagent	Thermo Scientific (Waltham, MA, USA)
Magnesium chloride	Merck Millipore (Darmstadt, Germany)
Methanol	Fisher Scientific
Opti-MEM I Reduced Serum Medium	Gibco, Life Technologies (Carlsbad, CA, USA)
Paraformaldehyde	Alfa Aesar, Johnson Matthey (Lancashire, UK)
Pencillin-Streptomycin	Gibco, Life Technologies (Carlsbad, CA, USA)
PhosSTOP phosphatase inhibitor	Roche (Basel, Switzerland)
Potassium chloride	USB Corporation (Cleveland, OH, USA)
Potassium phosphate monobasic	Sigma-Aldrich (St. Louis, MO, USA)
Prolong Gold antifade reagent with DAPI	Molecular Probes, Life Technologies (Carlsbad, CA, USA)
Sodium chloride	Merck Millipore (Darmstadt, Germany)
Sodium dodecyl sulphate (SDS)	Hoefer (San Francisco, CA, USA)
Sodium phosphate dibasic	Sigma-Aldrich (St. Louis, MO, USA)
TEMED	Bio-Rad (Hercules, CA, USA)
Tris base	USB Corporation (Cleveland, OH, USA)
Triton X-100	USB Corporation (Cleveland, OH, USA)
Tween 20	USBiological (Salem, MA, USA)

### 2.1.3 Drugs

Cytochalasin B	Sigma-Aldrich (St. Louis, MO, USA)
Leupeptin	Sigma-Aldrich (St. Louis, MO, USA)
MG132	Sigma-Aldrich (St. Louis, MO, USA)
NH <sub>4</sub> Cl	Sigma-Aldrich (St. Louis, MO, USA)
Paclitaxel (Taxol)	Sigma-Aldrich (St. Louis, MO, USA)
PR-619	Sigma-Aldrich (St. Louis, MO, USA)
Thymidine	Sigma-Aldrich (St. Louis, MO, USA)

### 2.1.4 Enzymes

Leupeptin	Sigma-Aldrich (St. Louis, MO, USA)
RevertAid Reverse Transcriptase	Thermo Scientific (Waltham, MA, USA)
T4 DNA ligase	New England BioLabs (Ipswich, MA, USA)
T4 Polynucleotide kinase	New England BioLabs (Ipswich, MA, USA)
Trypsin	Gibco, Life Technologies (Carlsbad, CA, USA)

### 2.1.5 Restriction enzymes

BamHI	New England BioLabs (Ipswich, MA, USA)
EcoRI	New England BioLabs (Ipswich, MA, USA)
HindIII	New England BioLabs (Ipswich, MA, USA)
KpnI	New England BioLabs (Ipswich, MA, USA)
XhoI	New England BioLabs (Ipswich, MA, USA)

### 2.1.6 Kits

Chemiluminescent ECL Substrate	Pierce, Thermo Scientific (Waltham, MA, USA)
Chemiluminescent ECL Prime Substrate	Amersham, GE Healthcare (Little Chalfont, UK)
DNA Plasmid Mini Extraction Kit	Qiagen (Venlo, Limburg, Netherlands)
DNA Plasmid Midi Extraction Kit	Qiagen (Venlo, Limburg, Netherlands)
DNA Gel Extraction Kit	Machery-Nagel (Duren, Germany)
KAPA Taq DNA polymerase kit	Kapa Biosystems (Wilmington, MA, USA)
KAPA HiFi DNA polymerase kit	Kapa Biosystems (Wilmington, MA, USA)

### 2.1.7 Mammalian cell lines

HeLa human cervix adenocarcinoma epithelial cells	American Type Cell Culture
Human telomerase reverse transcriptase immortalized in human retinal pigmented epithelial cells (hTERT RPE-1)	American Type Cell Culture
Mouse embryonic fibroblast cells (MEFs)	Dr Esther Wong's lab
Mouse embryonic fibroblast cells with stable knockout of ATG5 (ATG5KO MEFs)	Dr Esther Wong's lab

### 2.1.8 Other materials

Konica Minolta AX NIF Blue film	Konica Minolta (Tokyo, Japan)
Nitrocellulose membrane	Whatman, GE Healthcare (Little Chalfont, UK)
PVDF membrane	Whatman, GE Healthcare (Little Chalfont, UK)

### 2.1.9 Primers

All primers were obtained from Sigma-Aldrich (St. Louis, MA, USA).

	<b>DNA</b>	<b>Primers</b>	<b>5' to 3'</b>
1	Cap-D2	NCAP-D2 (F)	GTATGCTGCCTCTCATCTGGTC
2	Cap-D2	NCAP-D2 (R)	AGGCATCCACTAGCAGCAGAGA
3	Cap-D3	NCAP-D3 (F)	GAAAGCCAGGAACTGAGCCGAT
4	Cap-D3	NCAP-D3 (R)	AGCCAGCAATCTTGGAGAGCAG
5	Cap-G	NCAP-G (F)	GACGAACAGGAGGTGTCAGACT
6	Cap-G	NCAP-G (R)	TGCTGCGGTTTTGGCTCGTCTT
7	Cap-G2	NCAP-G2 (F)	GGAAGTGGCATTGACACGAGC
8	Cap-G2	NCAP-G2 (R)	GCTGCTCTAACAATGGGTGGCT
9	Cap-H	NCAP-H (F)	CCTCAATGTCTCCGAAGCAGATC

10	Cap-H	NCAP-H (R)	TGTAGTCCTGGCAGTGGAGAGT
11	Cap-H2	NCAP-H2 (F)	TGACTCCTTGGAGTCTAAGCCC
12	Cap-H2	NCAP-H2 (R)	TACCACTGGTGGGAAGTCCTGCA
13	GAPDH	GAPDH (F)	TGATGACATCAAGAAGGTGGTGAAG
14	GAPDH	GAPDH (R)	TCCTTGGAGGCCATGTGGGCCAT
15	Integrin Beta 1	IGTB1 (F)	TCAGAATTGGATTTGGCTCATT
16	Integrin Beta 1	IGTB1 (R)	CCTGAGCTTAGCTGGTGTGTG

Table 2.1.9 Sequence of primers for quantitative real-time polymerase chain reaction

	<b>DNA</b>	<b>Vector</b>	<b>Primers</b>	<b>5' to 3'</b>
1	Cap-D3	PXJ40-HA	CAP-D3 (F)	TAGGGCGCGGCCGCTATG GTGGCGTTG
2	Cap-D3	PXJ40-HA	CAP-D3 (R)	TAGGGCGTCGACTTAGTT GGCTGTTTT
3	Cap-G2	PXJ40-HA	CAP-G2 (F)	TAGGGCCTCGAGATGGAA AAACGTGAG
4	Cap-G2	PXJ40-HA	CAP-G2 (R)	TAGGGCGGTACCTTTATG AATTCAAAGTTC
5	Cap-H2	PXJ40-HA	CAP-H2 (F)	TAGGGCAAGCTTATGAAC TTCATTGAG
6	Cap-H2	PXJ40-HA	CAP-H2 (R)	TAGGGCGCGGCCGCTCAG GGCTGGGCCATGGA
7	Ubiquitin	pEGFP-C1	GFP-Ub (F)	TAGGGCGAATTCAATGCA GATCTTCGTGAAAACCCT TACCGGC
8	Ubiquitin	pEGFP-C1	GFP-Ub (R)	TAGGGCGGATCCTTAACA GCCACCCCT

Table 2.1.9.1 Sequence of primers for molecular cloning

## 2.2 Methods

### 2.2.1 Mammalian cell culture & transfection

#### Reagent & chemicals

Cell culture:	DMEM
	F12/DMEM
	FBS
	Penicillin-Streptomycin
	Phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.8 mM KH <sub>2</sub> PO <sub>4</sub> , pH 7.4)
	Trypsin-EDTA
Transfection:	Lipofectamine 2000
	Opti-MEM I Reduced Serum Media

HeLa, MEF and ATG5KO MEF cell lines were grown in DMEM while hTERT RPE-1 cell line was maintained in F12/DMEM. Both DMEM and F12/DMEM were supplemented with 10% FBS and 1% Penicillin-Streptomycin. The cells were kept at a constant temperature of 37°C in an incubator with humidified atmosphere containing 5% CO<sub>2</sub>. Cells were passaged once every 2 days via trypsinization with trypsin-EDTA. Transfection was done by incubating DNA plasmid/s with lipofectamine 2000 at a ratio of 1(μg):2(μL) in Opti-MEM according to manufacturers' instructions. Transfection of ATG5 siRNA (Ambion® *Silencer*® Select siRNA, Cat. No. 4392420, Invitrogen) into RPE-1 cells was performed using Lipofectamine<sup>TM</sup> RNAiMAX reagent according to manufacturer's instructions (Invitrogen). Serum-starvation of cells was achieved by growing cells in DMEM, 1% Penicillin/Streptomycin, with leupeptin and NH<sub>4</sub>Cl added to a final concentration of 100μM and 10mM respectively without FBS.

### 2.2.2 Drug treatment

<b>Drug</b>	<b>Final Concentration</b>	<b>Treatment duration</b>
Cytochalasin B	4.5 µg/mL	24 hrs
Leupeptin	100 µM	6 hrs
MG132	5 nM	18 hrs
NH <sub>4</sub> Cl	10 mM	24 hrs/ 48 hrs
Paclitaxel (Taxol)	10 nM	24 hrs
PR-619	5 µM	48 hrs
Thymidine	2 mM	16 hrs

Table 2.2.2. Details of different drug concentrations and treatment durations

### 2.2.3 Protein lysate collection and quantification

#### Reagent & chemicals

Protein lysate collection: M-PER Mammalian Protein Extraction Reagent  
PhosStop Phosphatase inhibitor  
Complete EDTA-free Protease inhibitor

Protein lysate quantification: BSA  
Bradford Assay Dye Reagent

Whole cell lysates were collected by lysing the cells with M-PER mammalian protein extraction reagent supplemented with protease & phosphatase inhibitors. Relative protein concentrations were obtained with Bradford assay dye reagent and BSA protein standards.

## 2.2.4 SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis:	5x Laemmli Sample Buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 5% $\beta$ -mercaptoethanol, 0.01% bromophenol blue) Acrylamide/Bis solution Resolving gel buffer (1.5 M Tris-HCl pH 8.8 with 0.4% SDS) Stacking gel buffer (0.5 M Tris-HCl pH 6.8 with 0.4% SDS) APS TEMED Running buffer (25 mM Tris-base, 192 mM Glycine, 0.1% SDS)
Transfer of protein from gel to membrane:	Transfer buffer (24 mM Tris-base, 77 mM Glycine, 20% Methanol) Nitrocellulose membrane
Chemiluminescence of protein:	BSA Low-fat milk powder TBS-T: Tris-buffered saline (TBS) (137 mM NaCl and 20 mM Tris base, pH 7.6) with 0.1% (v/v) Tween 20 Chemiluminescence substrate kit

The percentage of acrylamide in SDS gel was determined based on the protein size to be resolved while the composition of the resolving gel, APS and TEMED, remained the same for all gels casted. Protein samples were loaded into the casted gels and subjected to gel electrophoresis in running buffer with constant voltage of 125V.

Protein samples were transferred from polyacrylamide gels onto nitrocellulose membranes using the wet transfer method, with a constant voltage of 100V for 1 hour (hr) for protein sizes below 50 kDa, and 1 hr 30 minutes (mins) for those above that size.

For detection of proteins with antibodies, membranes were incubated in low-fat milk powder or BSA dissolved in TBST containing the appropriate primary antibodies. Chemiluminescent reagents were used for the detection of proteins of interest, with the detection of luminescent signal by Konica Minolta AX NIF Blue films. Development of films was done with a Kodak X-OMAT 2000 processor.

#### 2.2.5 Immunoprecipitation (IP)

##### Reagents and Chemicals

Immunoprecipitation:	Tris Wash Buffer (50mM Tris-HCl, pH7.4, 150mM NaCl)
	HA-conjugated agarose beads
	Pierce Lysis Buffer
	PhosStop Phosphatase inhibitor
	Complete EDTA-free Protease inhibitor
	N-Ethylmaleimide (NEM) 10mM
	6M urea

Protein samples were lysed using Pierce IP lysis buffer supplemented with protease inhibitors, phosphatase inhibitors, NEM and 6M of urea. The lysates collected were incubated with Hemagglutinin (HA)-conjugated beads in Tris wash buffer at 4°C overnight with gentle agitation. Beads were spun down via centrifugation and the supernatant is collected as unbound fraction. Protein samples were eluted from the beads and analyzed via SDS-PAGE.

### 2.2.6 Molecular cloning

#### Reagents, chemicals and kits

Reverse Transcription: Thermo Scientific RevertAid Reverse Transcriptase

PCR: KAPA HiFi DNA Polymerase kit

Molecular Cloning: Restriction Endonucleases  
T4 DNA ligase

Agarose Gel Electrophoresis: Agarose  
TAE buffer  
Ethidium Bromide  
6x Loading Dye  
Gene Ruler 1kb DNA Ladder

Gel Extraction: Machery-Nagel gel extraction kit

DNA Plasmid Purification: LB Broth  
LB Agar Plates  
Antibiotics  
Qiagen Mini- or MIDI-prep kit

Cap-D3, Cap-H2 and Cap-G2 are first amplified from hTERT RPE-1 cDNA using KAPA HiFi DNA polymerase kit and cloned into PXJ40-HA vectors, which were a kind gift from Dr Koh Cheng Gee's lab. The primers were designed to include specific restriction endonuclease sites in the resulting PCR product (Table 2.1.9.1).

PCR product was analyzed via agarose gel electrophoresis and extracted with a gel extraction kit. Extracted DNA fragments and PXJ40-HA vectors were

digested with appropriate restriction endonucleases according to the manufacturer's instructions. Digested cDNAs and vectors were analyzed via agarose electrophoresis and purified with a gel extraction kit. After purifying the cleaved DNA fragments and vectors with a clean-up kit, they were ligated using T4 DNA ligase. All DNA plasmids obtained from molecular cloning were sent to 1<sup>st</sup> Base, Axil Scientific (Singapore) for verification via DNA sequencing.

#### 2.2.7 Immunofluorescence & time-lapse imaging

##### Reagents and chemicals

Cell fixation:	4% Paraformaldehyde in PBS 0.5% Triton X-100 in PBS 0.1% Triton X-100 in XBE2 (10 mM HEPES, pH 7.7, 2 mM MgCl <sub>2</sub> , 100 mM KCl and 5 mM EGTA) 2% Paraformaldehyde in XBE2
Cell Immunostaining:	5% BSA in TBST TBS-T: Tris-buffered saline (137 mM NaCl and 20 mM Tris base, pH 7.6) with 0.1% (v/v) Tween 20
Coverslip Mounting:	Prolong Gold antifade reagent with DAPI
Time lapse imaging:	Leibovitz's L-15 medium with 10% FBS

##### Immunofluorescence:

hTERT RPE-1 cells were seeded on glass coverslips and cultured as per mentioned in the cell culture methods mentioned above. Cells were fixed with 4% paraformaldehyde for 10 mins at RT. Thereafter, cells were permeabilized with 0.5% Triton X-100 for 3 mins. Alternatively, cells were pre-extracted with 0.1% Triton X-100 in XBE2 buffer at RT for 2 mins and then fixed with 2% paraformaldehyde in XBE2 buffer for 15 mins. Cells were blocked with 1% BSA in TBST for 1 hr at RT, followed by immunostaining with primary and secondary antibodies diluted in 1% BSA in TBST. Washing of cells following

immunostaining with primary and secondary antibodies were done with TBST. Coverslips were then mounted on glass slides with ProLong gold antifade mounting media added with DAPI.

Time-lapse imaging:

hTERT RPE-1 cells were seeded onto 35mm glass bottom dishes (World Precision Instruments, Sarasota, Florida). After incubation the cells in 10mM of  $\text{NH}_4\text{Cl}$  for 48 hrs, the media was changed into Leibovitz's L-15 medium that was supplemented with 10% FBS and 10mM of  $\text{NH}_4\text{Cl}$ . Time-lapse imaging was carried out in a 37°C humidified atmosphere at 10-min intervals on a Zeiss Axiovert microscope (Carl Zeiss Microscopy, Jena, Germany) with a 20x 0.75NA objective. Time-lapse videos of cells were captured through differential interference contrast (DIC) / fluorescence microscopy and analyzed using the Axiovision software (Carl Zeiss Microscopy, Jena, Germany).

## 2.2.8 Chromosome spread preparation

### Reagents and chemicals

Cell preparation: Hypotonic buffer (75mM KCl)

Cell fixation: Fixative solution (Methanol:Acetic acid at 3:1 ratio)

Coverslip Mounting: Prolong Gold antifade reagent with DAPI

Mitotic cells were collected via mechanical shake-off and incubated in hypotonic buffer at 37°C for 20 mins. The cells were then fixed and washed with ice cold fixative solution and stored overnight at 4°C. The fixed cells were dropped onto glass slides, dried and mounted with Prolong Gold antifade reagent with DAPI the following day. Chromosome spread images were acquired using an Axiovert 200M inverted microscope equipped with a 100x/1.3NA Plan Apo oil immersion objective (Carl Zeiss, Germany).

### 3 RESULTS

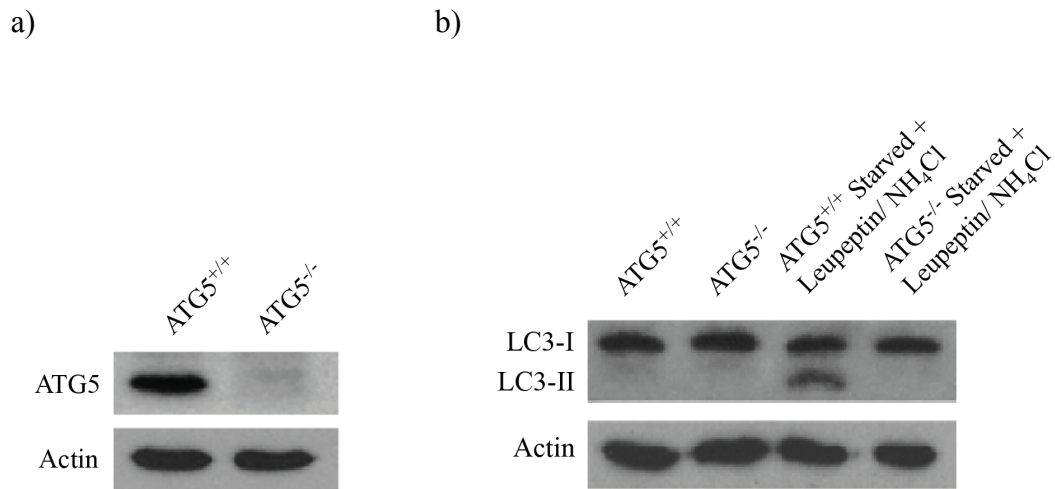
#### 3.1 ATG5<sup>-/-</sup> MEFs sustained greater DNA damage after genomic insult

As it is still unclear how autophagy maintains genomic integrity, we sought to identify the proteins that are regulated by autophagy. To do this, we used a MEF cell-line that has a stable knockout of ATG5, an essential gene of autophagy (Fig. 3.1a). To confirm that the loss of ATG5 did render the cells incapable of undergoing autophagy, ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs were serum-starved to induce autophagy and western blot analysis was performed to detect the processing of LC3-I to LC3-II. The formation of LC3-II will signify the accumulation of autophagosomes, thus indicative of autophagy. Both ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs showed negligible level of LC3-II under normal growth conditions, whereas serum starvation induced the formation of LC3-II in ATG5<sup>+/+</sup> MEFs but not in ATG5<sup>-/-</sup> MEFs (Fig. 3.1b). This showed that ATG5<sup>-/-</sup> MEFs were unable to undergo autophagy, thus allowing us to examine autophagy-dependent functions.

Initial examination of ATG5<sup>-/-</sup> MEFs detected a higher frequency of micronuclei compared to ATG5<sup>+/+</sup> MEFs. As micronuclei result from the loss of whole chromosome or chromosome breakage, they could indicate the presence of DNA damage. This observation thus suggested that impaired autophagy might increase the cells' susceptibility to DNA damage.

To confirm this, ATG5<sup>-/-</sup> and ATG5<sup>+/+</sup> MEFs were treated with 1 $\mu$ M etoposide (VP16) to induce DNA damage. Cytokinesis-block micronucleus (CBMN) assay was then used to measure and compare the DNA damage in the form of micronuclei, sustained by ATG5<sup>-/-</sup> and ATG5<sup>+/+</sup> MEFs. CBMN assay is currently the preferred method for quantifying micronuclei in mammalian cells with high reliability and reproducibility. In the CBMN assay, cytochalasin B, an inhibitor of cytokinesis, is used after the addition of the drug of interest so as to restrict the scoring of micronuclei only in cells that have divided once i.e. in binucleated cells (Fig. 3.1.1a). This prevents the inclusion of non-dividing cells that are unable to form micronuclei (Fenech 2007). Therefore, this assay is more accurate than the usual micronucleus assay and was chosen for this experiment to quantify the

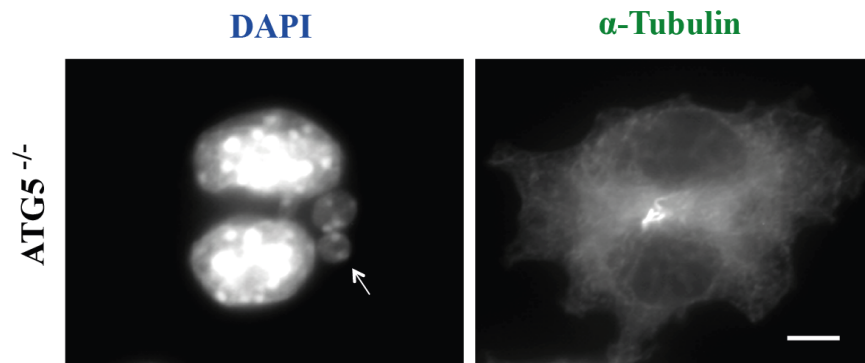
micronuclei. Based on this assay, ATG5<sup>-/-</sup> MEFs was found to exhibit a higher level of DNA damage as indicated by a higher percentage of micronuclei scored (Fig. 3.1.1).



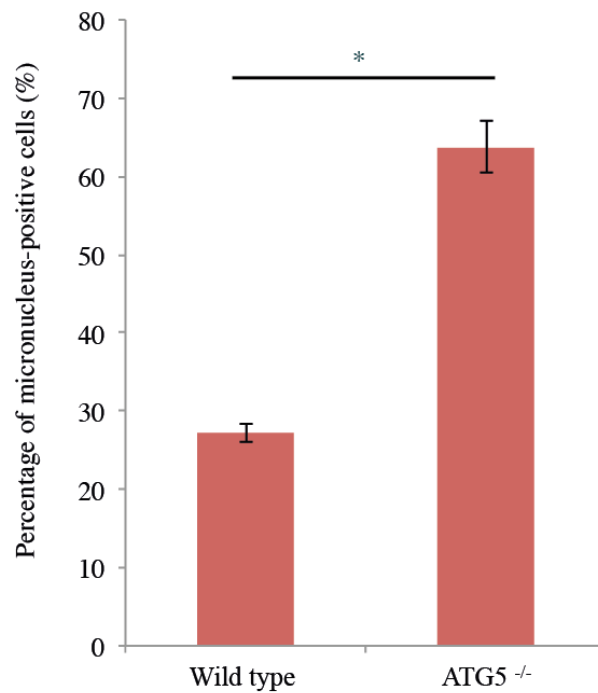
**Figure 3.1. Protein levels of ATG5 and LC3 in ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs.**

a) Western blots showing protein levels of ATG5 in ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs.  
b) Western blots displaying protein levels of LC3-I and LC3-II in ATG5<sup>+/+</sup>, ATG5<sup>-/-</sup>, serum-starved ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> that were added with leupeptin and NH<sub>4</sub>Cl. Leupeptin (protease inhibitor) and NH<sub>4</sub>Cl were added to inhibit lysosomal degradation and accumulate autophagosomes. The formation of LC3-II will signify the accumulation of autophagosomes, thus indicative of autophagy.

a)



b)



**Figure 3.1.1. Higher percentages of micronucleus-positive cells were quantified in ATG5<sup>-/-</sup> MEFs.**

a) Images of an ATG5<sup>-/-</sup> binucleated cell stained for DNA (blue) and  $\alpha$ -tubulin (green). White arrow indicates a micronucleus. Scale bars: 5  $\mu$ m. b) Graphical representation showing percentages of micronucleus-positive binucleated cells in wild type and ATG5<sup>-/-</sup> MEFs. 1000 binucleated cells were analyzed from each of the 3 independent experiments. \*P=0.0045. Error bars = standard deviation.

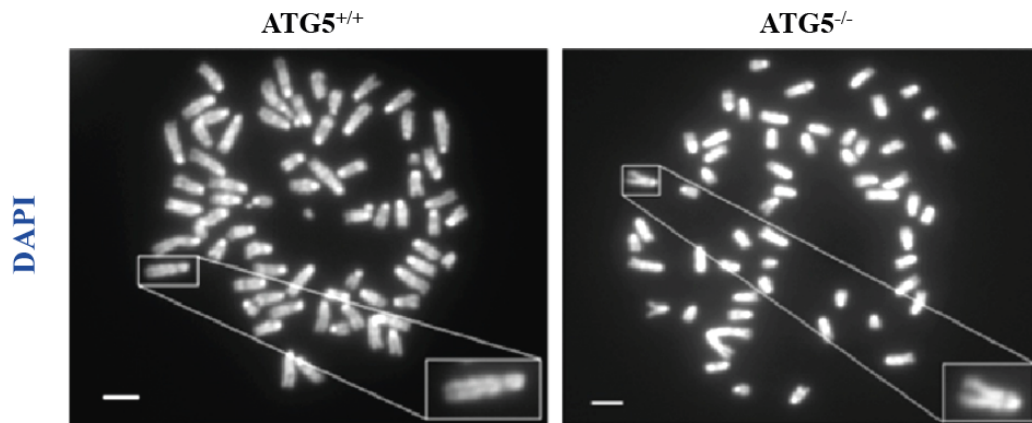
### 3.2 Impaired autophagy led to altered chromosomal length and width

In order to find out if ATG5<sup>-/-</sup> MEFs exhibit any abnormalities in the chromosome structures, metaphase chromosome spreads of ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs were prepared and metaphase chromosomes were stained with DAPI. It was found that chromosomes prepared from ATG5<sup>-/-</sup> MEFs showed slight longitudinal shortening and widening when compared to the control MEFs (Fig. 3.2a). The average length of control chromosomes was  $4.06 \pm 1.18 \mu\text{m}$  while the average width was  $0.73 \pm 0.12 \mu\text{m}$ . In contrast, chromosomes prepared from ATG5<sup>-/-</sup> MEFs were on average  $3.61 \pm 0.83 \mu\text{m}$  long and  $0.91 \pm 0.16 \mu\text{m}$  wide (Fig. 3.2b). The differences in both variables were found to be statistically significant ( $P < 0.0001$ ).

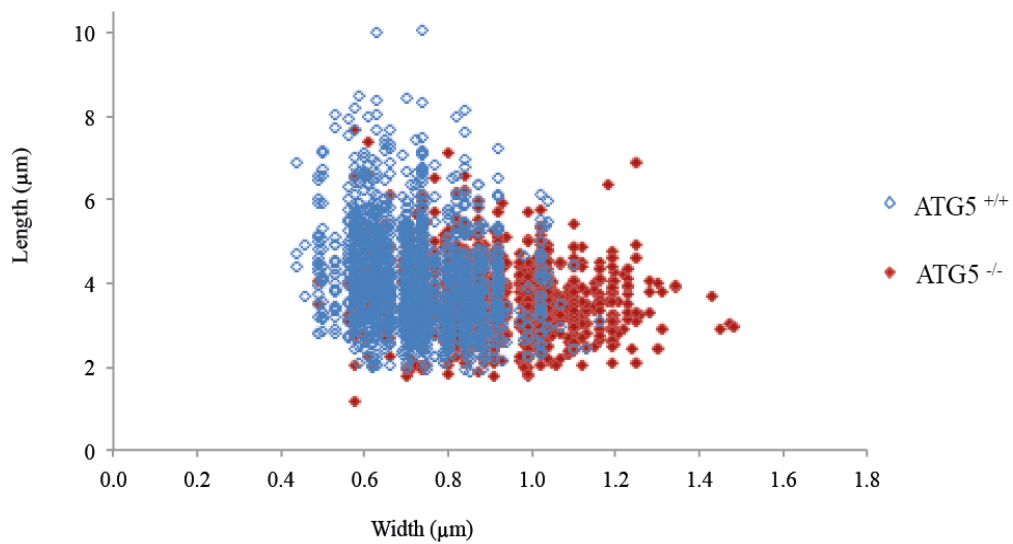
As condensin-I and -II complexes are responsible for determining the architecture of metaphase chromosomes, we were prompted to examine the protein levels of condensin-I and -II subunits in Atg5<sup>-/-</sup> MEFs. Western blot analysis was carried out using antibodies against different non-SMC subunits of condensins. Protein level of Cap-G (condensin-I) was found to be much lower in ATG5<sup>-/-</sup> MEFs. On the contrary, Cap-D2 (condensin-II) was higher in ATG5<sup>-/-</sup> MEFs. Similarly, the protein level of Cap-D3 (condensin-II) was also found to be higher in ATG5<sup>-/-</sup> MEFs (Fig. 3.2.1).

As condensin-I functions in lateral compaction while condensin-II functions in axial shortening, a decrease in condensin-I (Cap-G) and increase in condensin-II (Cap-D3) is consistent with previous observation of shorter and wider chromosomes. However, there is a discrepancy in the levels of different condensin-I subunits where Cap-D2 is present at a higher level but Cap-G is at a lower level in ATG5<sup>-/-</sup> MEFs.

a)



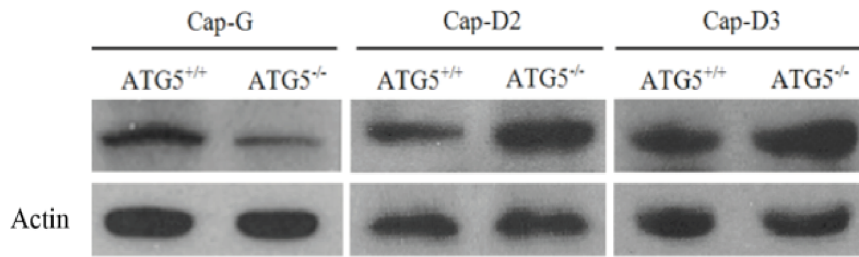
b)



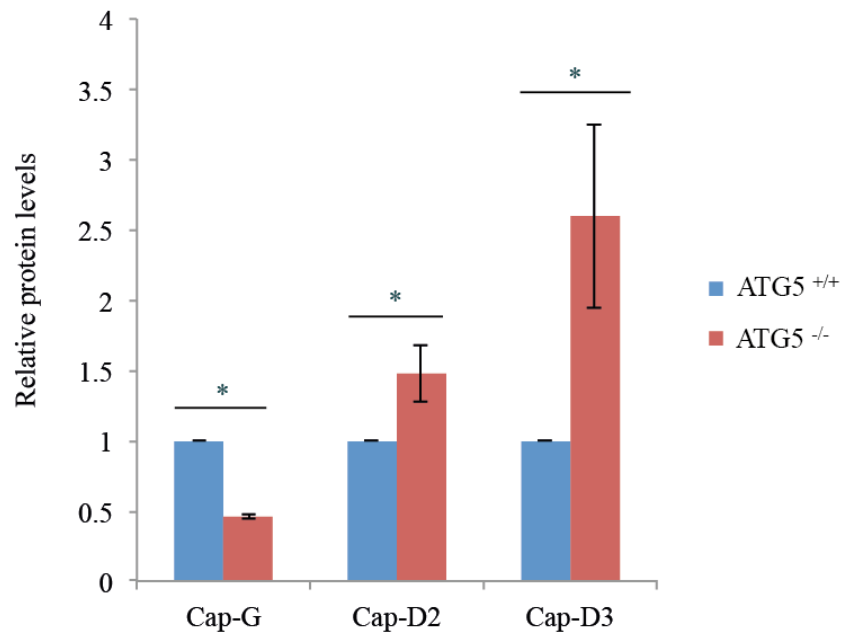
**Figure 3.2. Chromosomes from ATG5<sup>-/-</sup> MEFs exhibited longitudinal shortening and widening.**

a) Images of metaphase chromosome spreads prepared from ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> cells. DAPI (blue) was used to stain for DNA. Scale bars: 5 μm. b) A scatter plot showing the lengths and widths of chromosomes from ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> MEFs. At least 1400 chromosomes were analyzed from each of the 3 independent experiments.  $P < 0.0001$ .

a)



b)



**Figure 3.2.1. Differences in protein levels of condensin-I & -II subunits in ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> cells.**

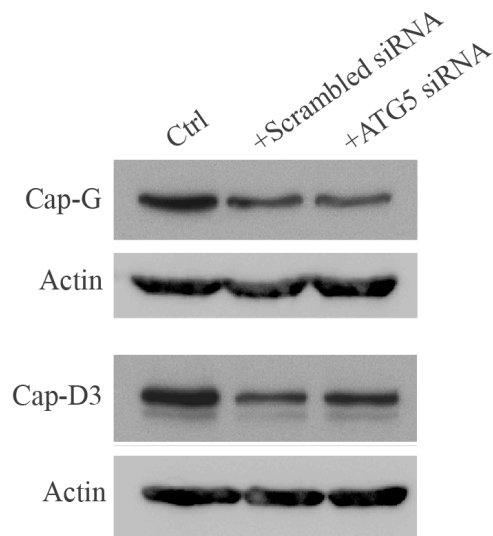
a) Western blots showing the difference in protein levels of Cap-G, Cap-D2, and Cap-D3 in total cell lysates of ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> cells. b) Graphical representation showing the difference in relative protein levels of Cap-D2, Cap-G and Cap-D3 in ATG5<sup>+/+</sup> and ATG5<sup>-/-</sup> cells. Data were derived from 3 independent experiments. \*P<0.01.

### **3.3 Examination of MEF-derived results in human cells**

Thus far, the removal of ATG5 gene resulted in a greater susceptibility of cells to DNA damage with their chromosomes exhibiting longitudinal shortening and widening. Moreover, protein levels of condensin-I and -II subunits were found to be different in ATG5 knockout MEFs.

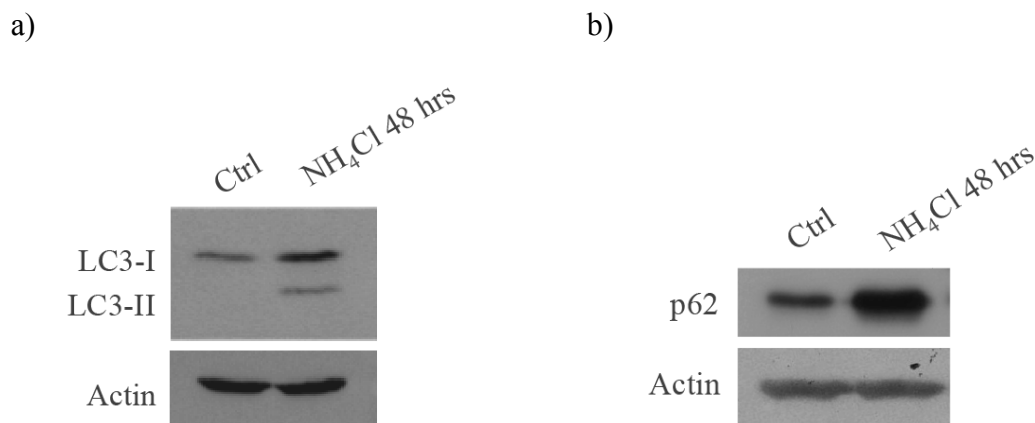
In order to examine if autophagy exerts similar effects on chromosomes and condensin I & II subunits in human cells, I performed RNAi knockdown using ATG5 specific siRNA in hTERT RPE-1 cells. Surprisingly, I found that control scrambled siRNA exerted non-specific effects on endogenous condensins, Cap-G and Cap-D3 (Fig. 3.3). As such, I concluded that RNAi knockdown experiment is not suitable to detect the changes of condensin subunits in the absence of ATG5. To further elucidate the effect of autophagy on the level of condensin subunits, I employed NH<sub>4</sub>Cl to inhibit autophagy and examined the level of condensin subunits. NH<sub>4</sub>Cl is a weak base that prevents acidification of lysosomes, which is required for digestion of its contents. Hence, NH<sub>4</sub>Cl can inhibit the progression of autophagy and degradation of its substrates.

RPE-1 cells were treated with 10 mM of NH<sub>4</sub>Cl for 48 hours or buffer as control prior to western blot analysis using anti-LC3-II and anti-p62 antibodies. Protein level of LC3-II increased in NH<sub>4</sub>Cl-treated RPE-1 cells, but not in control cells. This indicated that autophagosomes accumulated as a result of NH<sub>4</sub>Cl treatment (Fig. 3.3.1a). Similarly, p62, a known substrate of autophagy accumulated in NH<sub>4</sub>Cl-treated cells. These data suggested that autophagy progression is indeed blocked by NH<sub>4</sub>Cl treatment (Fig. 3.3.1b).



**Figure 3.3. Non-specific targeting of control scrambled siRNA on condensin-I (Cap-G) and -II (Cap-D3) subunits.**

Western blot showing the protein levels of condensin-I (Cap-G) and -II (Cap-D3) subunits in lysates of cells added with buffer as control (Ctrl), transfected with scrambled siRNA or ATG5 siRNA.



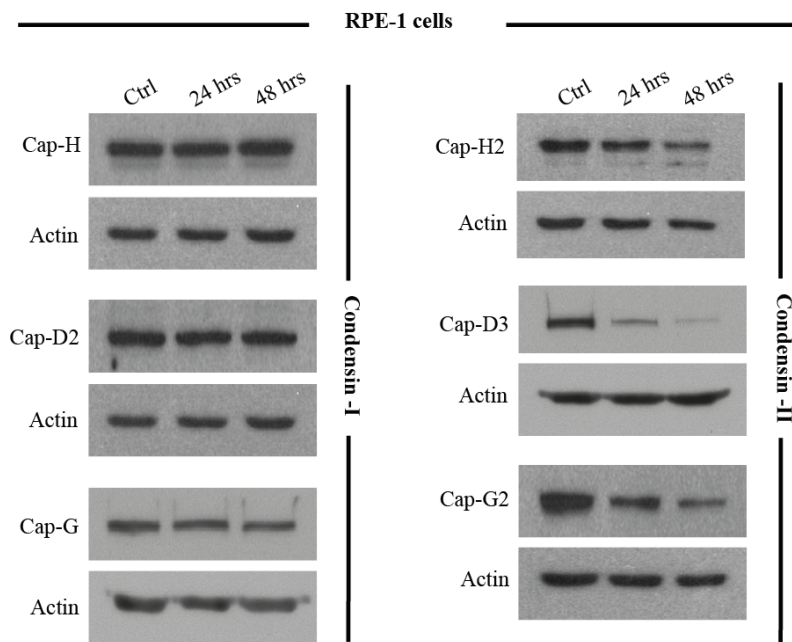
**Figure 3.3.1. Treatment of RPE-1 cells with 10mM NH<sub>4</sub>Cl for 48 hrs inhibited the progression of autophagy.**

Immunoblots showing protein levels of LC3-I, LC3-II and p62 in control (Ctrl) and NH<sub>4</sub>Cl-treated cells. The formation of LC3-II indicated the accumulation of autophagosomes due to inhibition of autophagy progression.

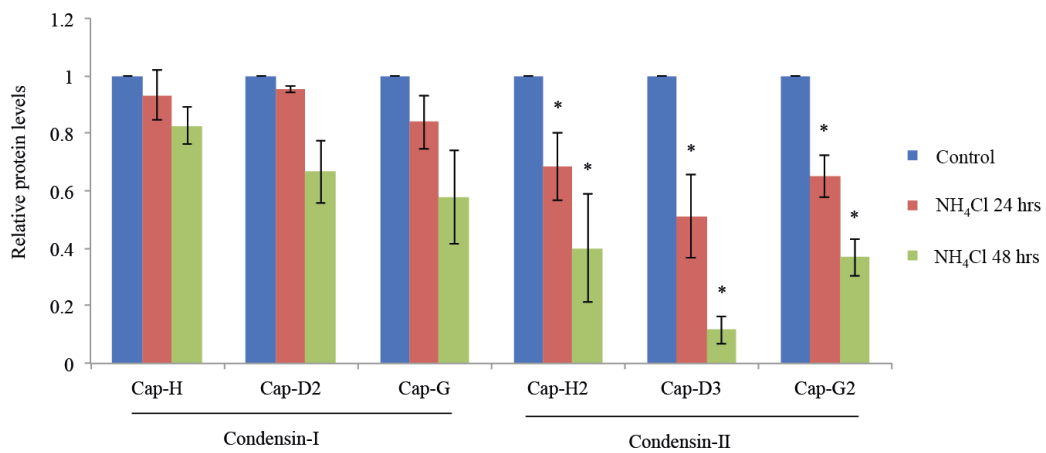
### **3.4 Autophagy inhibition reduced the protein levels of condensin-II**

First, I examined the protein levels of condensin subunits when autophagy was inhibited. Both RPE-1 and HeLa cells were treated with  $\text{NH}_4\text{Cl}$  or buffer as control for 24 hours and 48 hours. Protein levels of condensin subunits were then examined via western blot analysis. Unlike in MEFs where impaired autophagy resulted in decreased protein levels of condensin-I subunit, Cap-G, and increased levels of condensin-II subunit, Cap-D3, autophagy inhibition by  $\text{NH}_4\text{Cl}$  treatment in RPE-1 and HeLa cells led to a significant decrease in condensin-II subunits but not in condensin-I subunits (Fig. 3.4 & 3.4.1). This disparity suggested that autophagy inhibition might exert different effects on condensin complexes in human cells compared to that in mouse cells.

a)



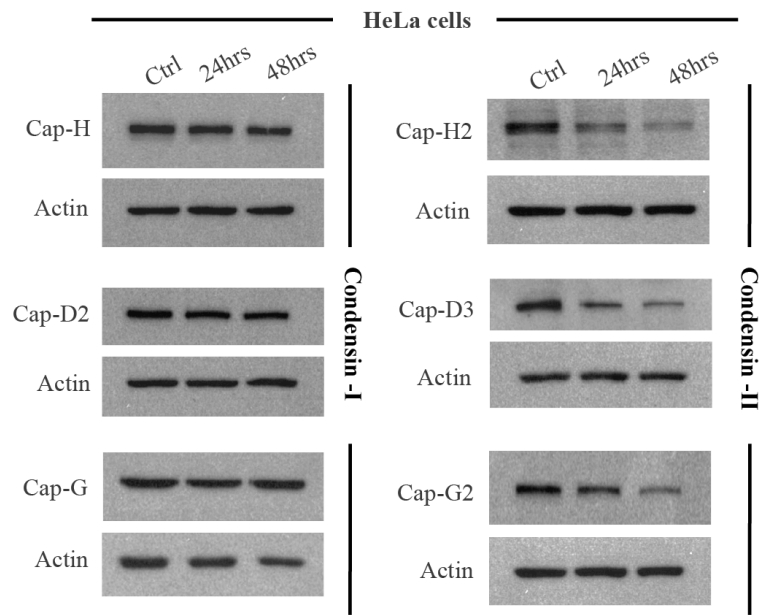
b)



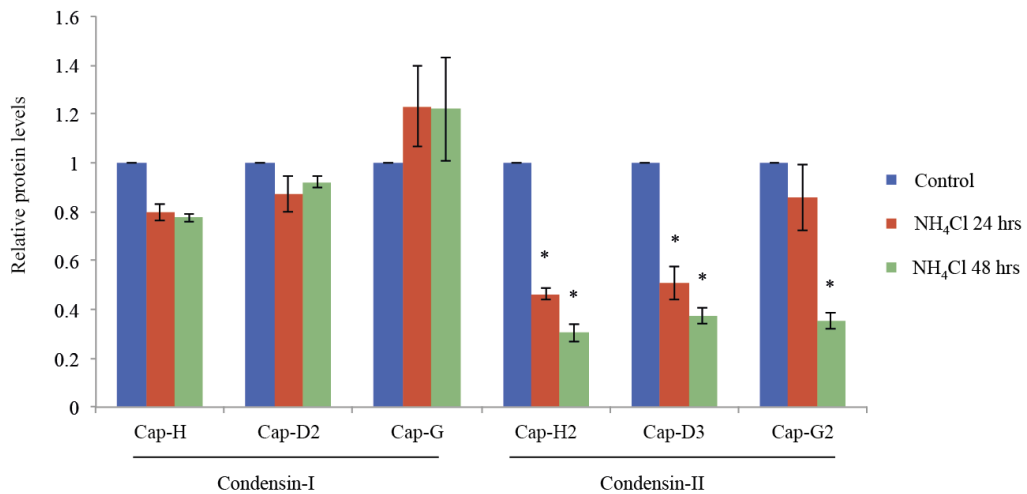
**Figure 3.4. Autophagy inhibition resulted in decreased protein levels of condensin subunits in hTERT RPE-1 cells.**

a) Immunoblots of control (Ctrl), NH<sub>4</sub>Cl 24 hours-treated (24 hrs) and NH<sub>4</sub>Cl 48 hours-treated (48 hrs) RPE-1 cells probed for the different condensin subunits. b) Graphical representation showing the relative protein levels of condensins in control, NH<sub>4</sub>Cl 24 hours-treated and NH<sub>4</sub>Cl 48 hours-treated cells. Data were derived from 3 independent experiments. \*P< 0.04.

a)



b)



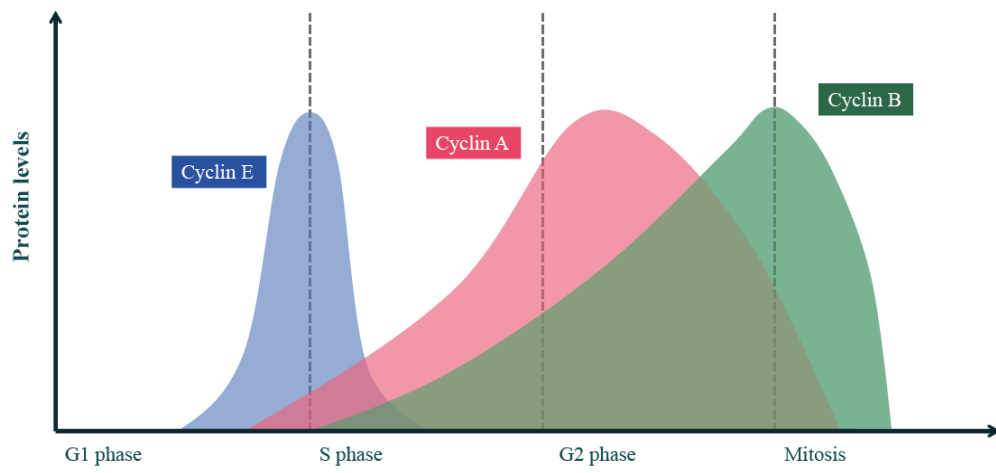
**Figure 3.4.1. Autophagy inhibition resulted in decreased protein levels of condensin subunits in HeLa cells.**

a) Immunoblots of control (Ctrl), NH<sub>4</sub>Cl 24 hours-treated (24 hrs) and NH<sub>4</sub>Cl 48 hours-treated (48 hrs) HeLa cells probed for the different condensin subunits. b) Graphical representation showing the relative protein levels of condensins in control, NH<sub>4</sub>Cl 24 hours-treated and NH<sub>4</sub>Cl 48 hours-treated cells. Data were derived from 3 independent experiments. \*P< 0.03.

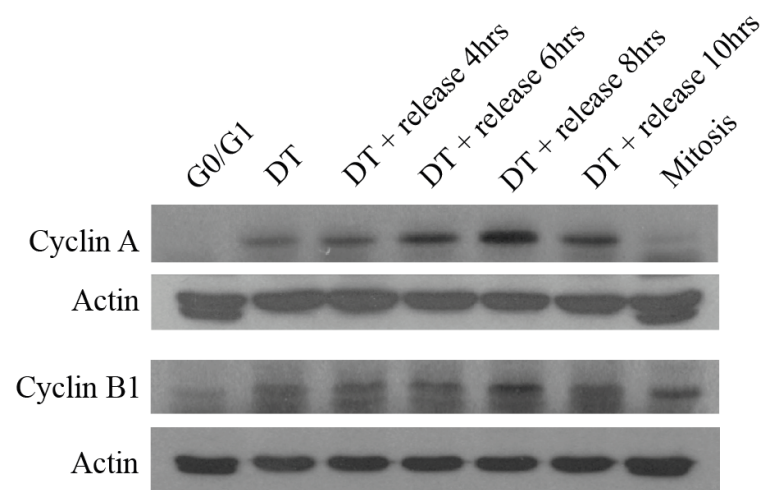
### **3.5 Protein level of condensin-II is relatively constant in different cell cycle phases**

The difference in the amount of condensin-II subunits detected after autophagy inhibition could be cell cycle dependent. To rule out this possibility, the protein level of condensin-II subunit, Cap-G2, was examined in different cell cycle phases. I first standardized the protocols to synchronize cells in different phases of the cell cycle. Double thymidine block (DT) was used to synchronize the cells at G1/S phase (cells were treated with 2 mM thymidine for 16 hours, followed by a release from thymidine for 9 hours prior to another round of thymidine treatment for 16 hours). Cells were then released from DT block for 4, 6, 8 and 10 hours. Both western blot and fluorescence-activated cell sorting (FACS) analysis were carried out using anti-Cyclin A, anti-Cyclin B1 and anti-phosphorylated histone H3 (H3) antibodies to verify the cell cycle phases of cells under different treatment conditions (Fig. 3.5 and Fig 3.5.1). To collect the cells in G0/G1 phase, they were serum-starved for 18 hours. For collection of cells in S and G2 phases, they were subjected to DT treatment before being released for 4 and 6 hours respectively. Mitotic cells were collected via mechanical shake-off and the presence of phosphorylated H3 indicated that the cells were in mitosis (Fig. 3.5.1a). The protein level of Cap-G2 (condensin-II subunit) was then determined in cells that were synchronized in G0/G1, S, G2 phases and cells isolated from mitotic shake-off. The protein levels of Cap-G2 remained relatively constant and only decreased slightly in mitosis (Fig. 3.5.1c). Since  $\text{NH}_4\text{Cl}$  treatment did not arrest the cells in mitosis, these data indicated that the detected decrease in condensin-II subunits following autophagy inhibition was not cell cycle dependent.

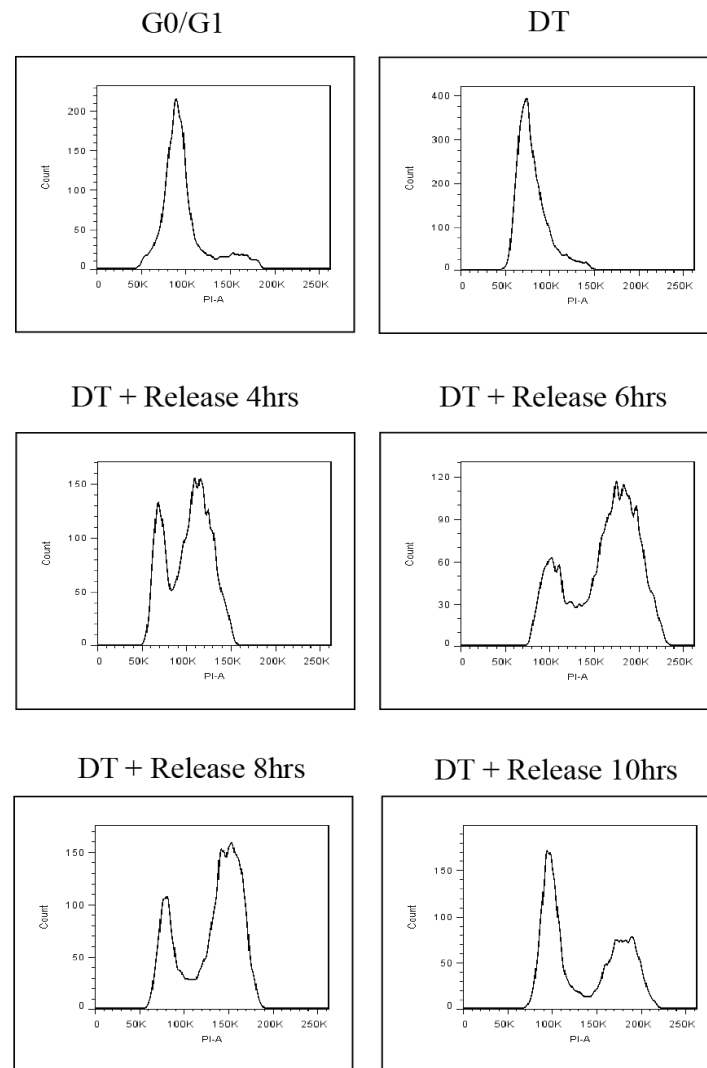
a)



b)



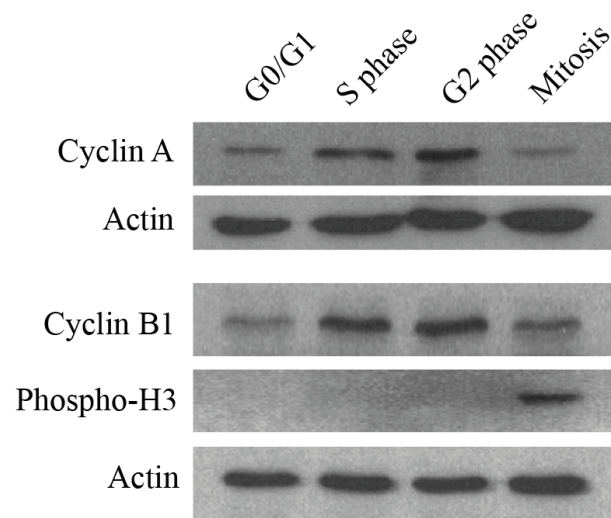
c)



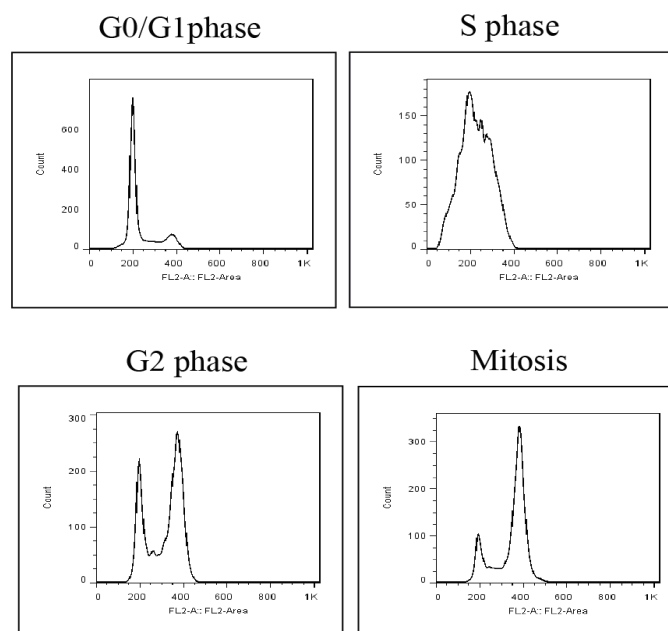
**Figure 3.5. Protein levels of cyclin A and B1 were indicative of the different cell cycle phases.**

a) A graphical representation showing the typical changes in protein levels of cyclin A and B1 during the different cell cycle phases. b) Immunoblots showing the difference in protein levels of cyclin A and B1 after undergoing serum starvation for 18 hrs, DT block, release from DT block for 4 hours, 6 hours, 8 hours, 10 hours and during mitosis. c) A series of graphical representations derived from FACS analysis, showing the cell count in different cell cycle phases under different treatments. The first peak indicates the number of cells in G0/G1 phase, the second peak indicates that in G2/M phase and those in between the two peaks indicates that in S phase.

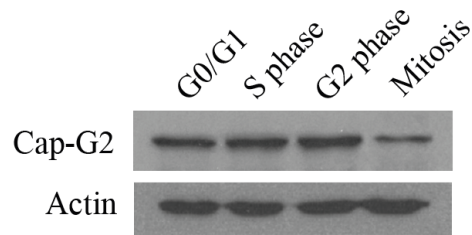
a)



b)



c)

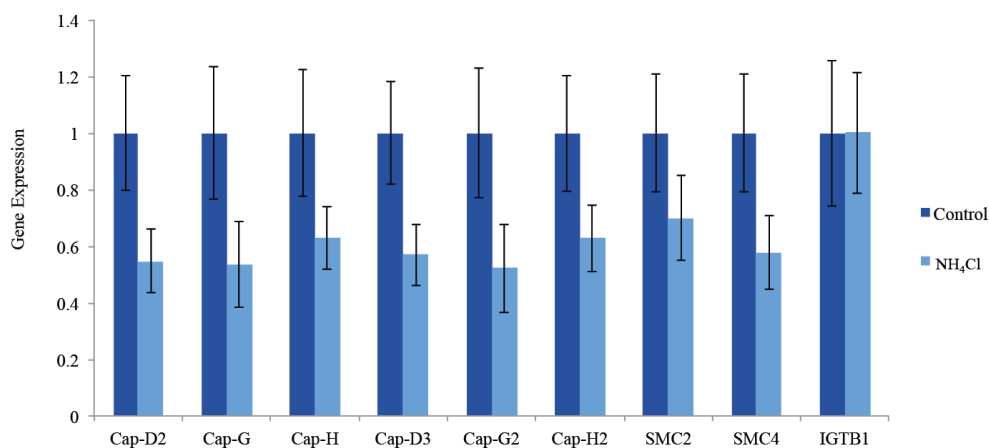


**Figure 3.5.1. Protein level of Cap-G2 is relatively constant in the different cell cycle phases.**

a) Immunoblots showing the protein levels of cyclin A, B1 and phosphorylated-H3 in G0/G1, S, G2 phases and mitosis. b) Graphical representations derived from FACS analysis, displaying the cell count in different cell cycle phases for cells in G0/G1, S, G2 phases and mitosis. The first peak indicates the number of cells in G0/G1 phase, the second peak indicates that in G2/M phase and those in between the two peaks indicates that in S phase. c) Immunoblots displaying the protein level of Cap-G2 (condensin-II subunit) in G0/G1, S, G2 phases and mitosis.

### 3.6 Reduced levels of condensin-II was not caused by lowered transcriptional activity

The decrease in condensin-II observed may be caused by a lowered level of transcription or increased rate of protein degradation. To investigate if the transcription of condensin subunits is affected by autophagy inhibition, I did QPCR to detect changes in the amount of messenger RNA (mRNA) of condensin subunits after  $\text{NH}_4\text{Cl}$ -mediated autophagy inhibition. After 48 hours of  $\text{NH}_4\text{Cl}$  treatment, cells exhibited significant reduction in their mRNA levels in all the condensin subunits as compared to control cells (Fig. 3.6). This showed that autophagy inhibition adversely affected the transcription level of condensin subunits.

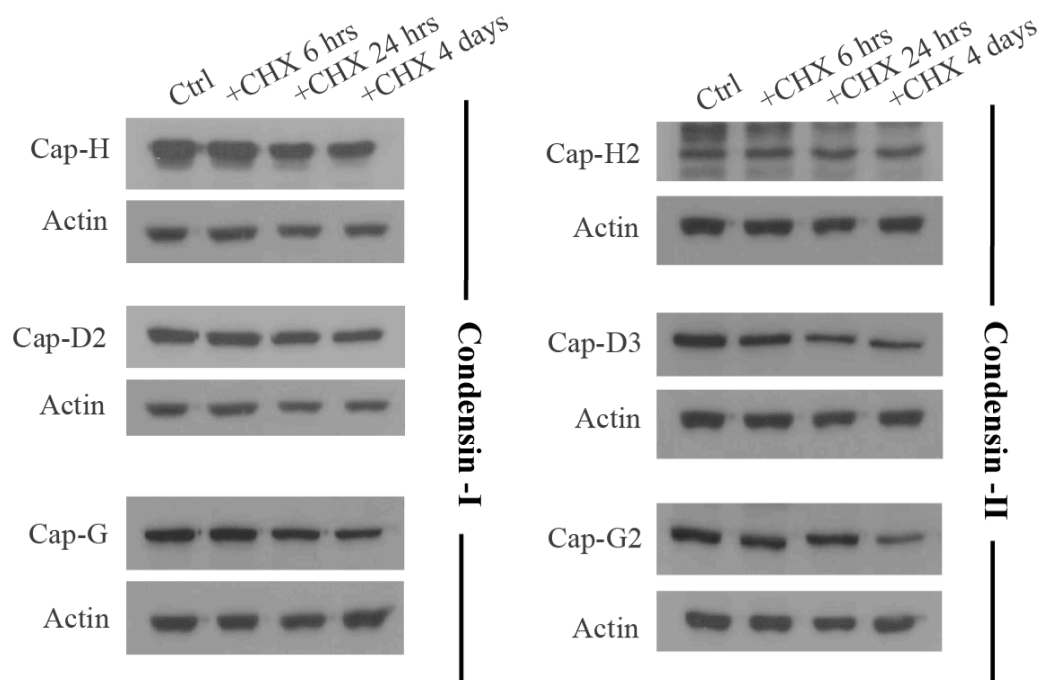


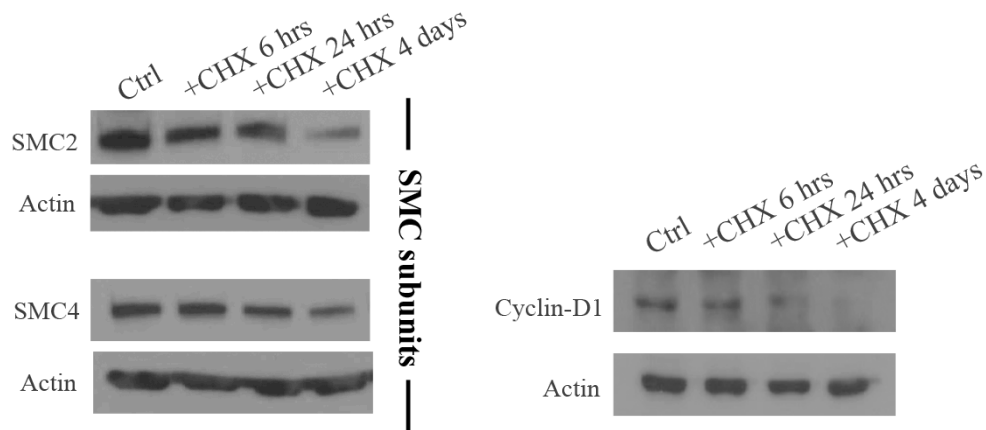
**Figure 3.6. Autophagy inhibition adversely affected the mRNA levels of condensins.**

Graphical representation showing the relative mRNA levels of the different condensin subunits normalized with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Integrin-Beta 1 (IGTB1) served as a control. Data were derived from 3 independent experiments.  $P < 0.01$ , except for IGTB1.

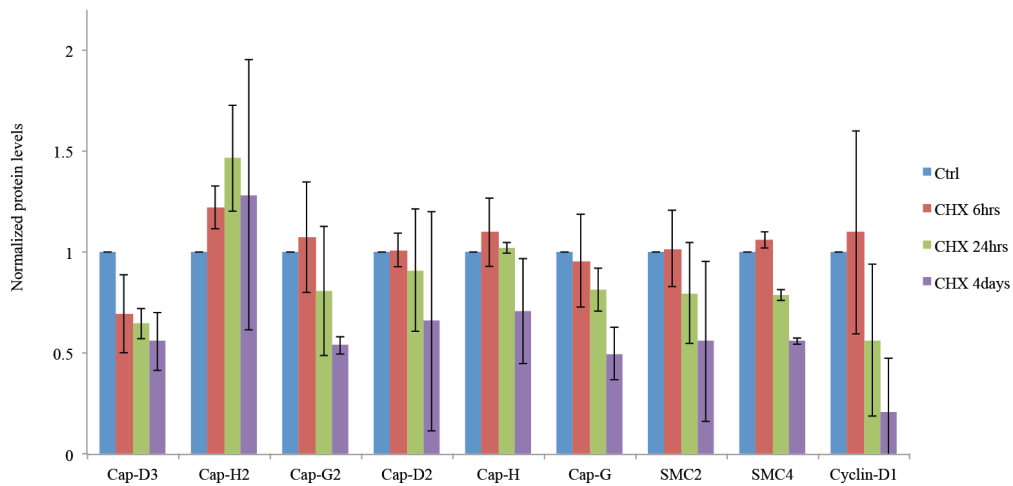
This finding of a reduced transcriptional activity of condensin-I and SMC subunits was unexpected since it did not translate into a decline in protein levels as detected via western blot analysis. This could be due to condensins and SMC subunits being one of the long-lived proteins with higher stability, thus a short period of low transcriptional activity did not affect the overall available condensin-I and SMC subunits. I then tested the stability of condensin subunits by inhibiting protein synthesis using cycloheximide (CHX). RPE-1 cells were subjected to CHX treatment for 6 hours, 24 hours and 4 days, followed by western blot analysis. As shown in figure 3.7.1, subunits of condensin-I, -II and SMC remained albeit being present at a lower level on the 4<sup>th</sup> day of CHX treatment. In contrast, known short-lived protein, cyclin D1, was almost fully degraded by the end of 24 hours (Fig. 3.6.1). This showed that condensin subunits are indeed highly stable. Since condensins are long-lived proteins, it confirmed that a short period of low gene transcription does not affect the overall available condensin-I, -II and SMC subunits. Therefore, the decrease in condensin-II observed after autophagy inhibition was not caused by lowered transcriptional activity. Rather, an increased rate of protein turnover was the more plausible cause.

a)





b)

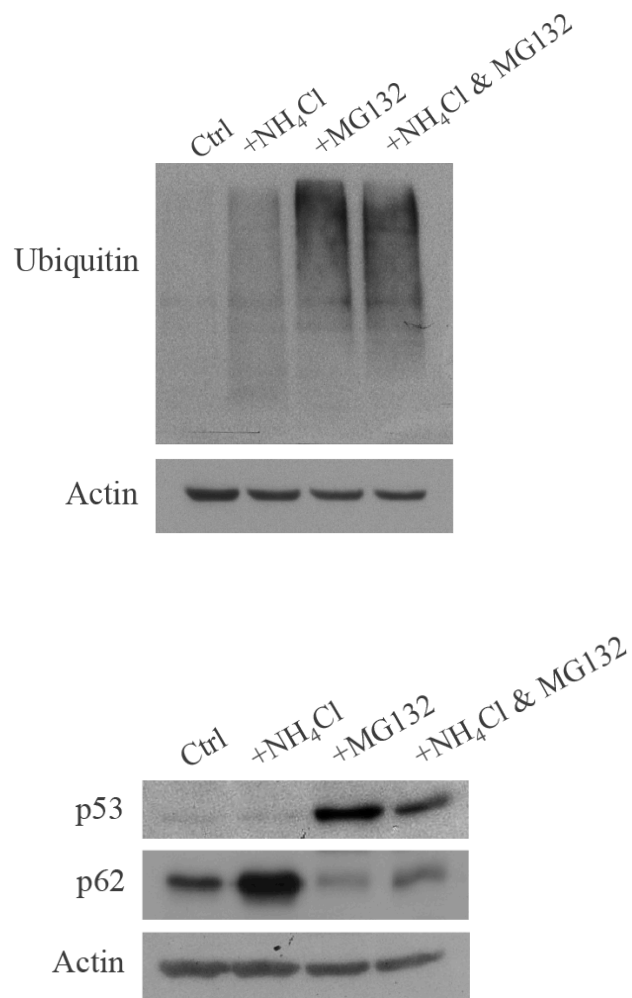


**Figure 3.6.1. Condensin subunits are proteins with high stability.**

a) Immunoblots showing the protein levels of condensin-I, -II, SMC subunits and Cyclin-D1 after cells were being subjected to 0 hour (Ctrl), 6 hours (+CHX 6 hrs), 24 hours (+CHX 24 hrs) and 4 days (+CHX 4 days) of CHX treatment. b) Graphical representation showing the protein levels of cyclin-D1, condensin-I, -II and SMC subunits D1 after cells were being subjected to 0 hour (Ctrl), 6 hours (CHX 6 hrs), 24 hours (CHX 24 hrs) and 4 days (CHX 4 days) of CHX treatment. Data were obtained from two independent experiments.

### **3.7 Depletion of condensin-II was achieved via the proteasomal degradation pathway**

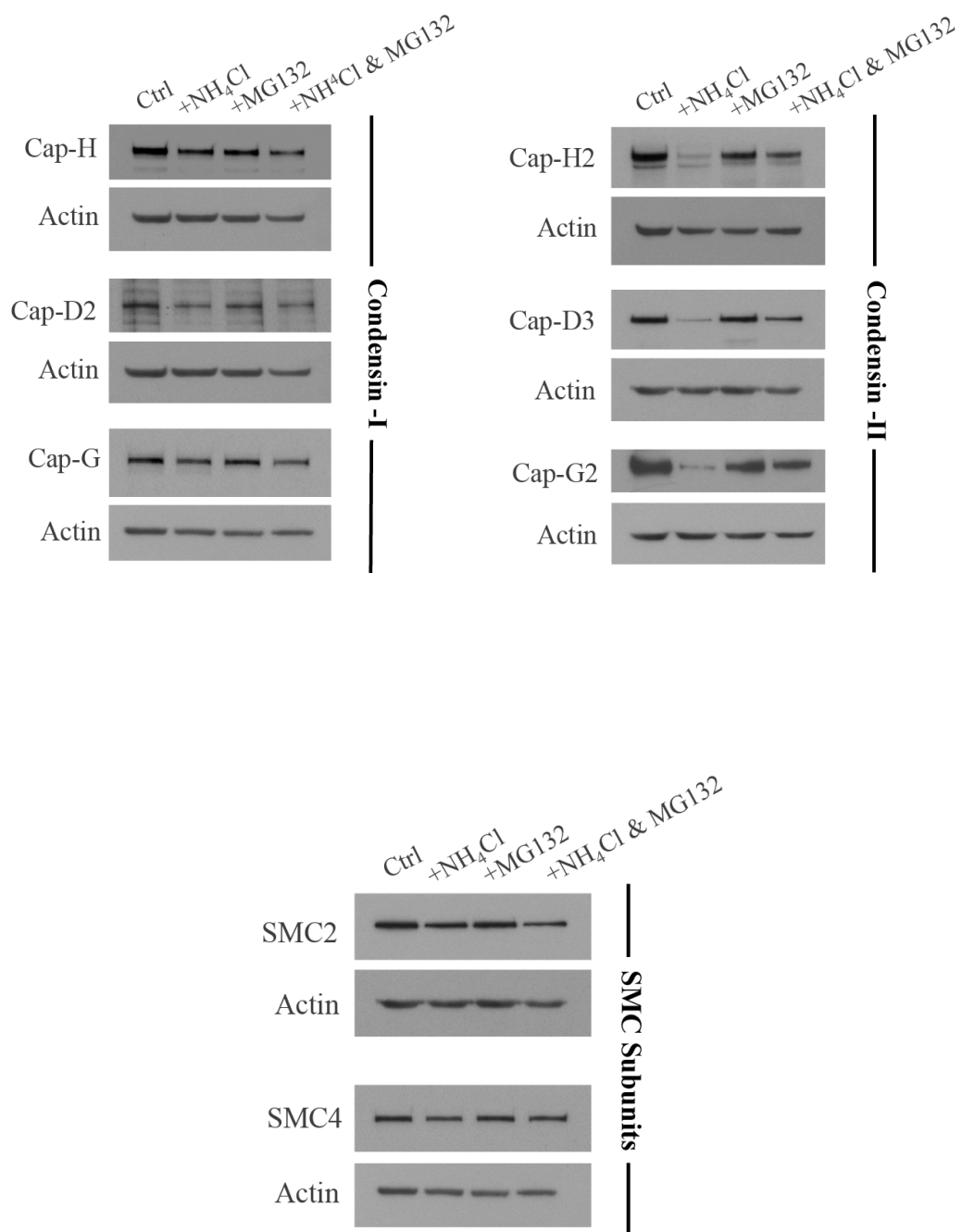
I then proceeded to examine whether the depletion of condensin II subunits was a result of proteasome-dependent protein turnover. MG132 was used in this experiment to inhibit proteasomal activity with a treatment duration of 18 hours. To confirm that  $\text{NH}_4\text{Cl}$  treatment caused the degradation of condensin-II via the proteasomal pathway, rescue experiment was carried out with a combined treatment of  $\text{NH}_4\text{Cl}$  (48 hours) and MG132 (last 18 hours). Inhibition of proteasomal activity was verified by western blot analysis using anti-ubiquitin antibodies. Ladders/smear of protein bands with high molecular weights were detected in lysates treated with MG132, indicating an accumulation of ubiquitinated proteins (Fig. 3.7). Moreover, a known substrate of proteasomal degradation pathway, p53, also accumulated upon treatment with MG132. This showed that the concentration and treatment duration of MG132 was sufficient to inhibit proteasomal activity. Thereafter, the same experiment was repeated and western blot analysis was carried out to compare the protein levels of different condensin subunits in lysates from control,  $\text{NH}_4\text{Cl}$ -treated, MG132-treated and  $\text{NH}_4\text{Cl}$  & MG132-treated cells. For condensin-I and SMC subunits, there was no significant difference in the protein levels between  $\text{NH}_4\text{Cl}$ -treated and  $\text{NH}_4\text{Cl}$  & MG132-treated cells. In contrast, partial rescue in protein levels of condensin-II subunits was observed after MG132 treatment (Fig. 3.7.1). Hence, this indicated that the decrease in condensin-II subunits after autophagy inhibition was due to degradation via the proteasomal pathway.



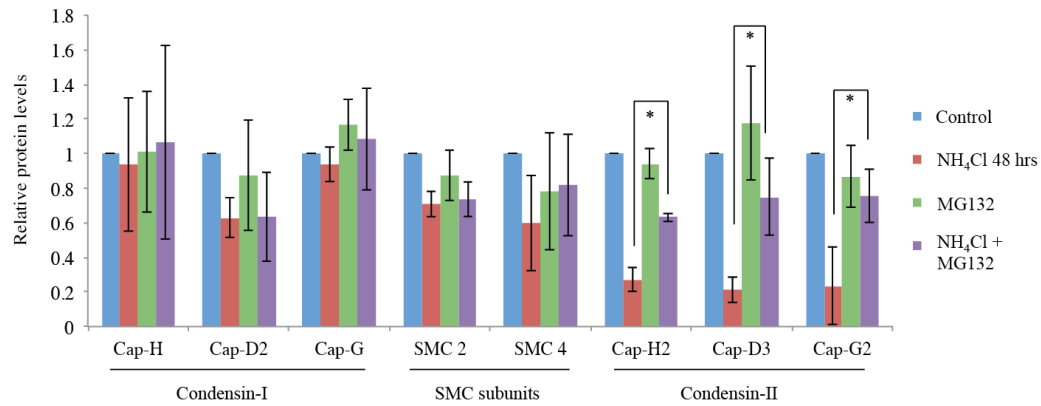
**Figure 3.7. Treatment of cells with NH<sub>4</sub>Cl, MG132 and a combination of both drugs inhibited autophagy progression, proteasomal degradation and both autophagy and proteasomal degradation together respectively.**

Immunoblots showing the protein levels of ubiquitin, p53 and p62 in lysates of control (Ctrl), NH<sub>4</sub>Cl-treated (+NH<sub>4</sub>Cl), MG132-treated (+MG132) and NH<sub>4</sub>Cl & MG132-treated (+NH<sub>4</sub>Cl & MG132) cells.

a)



b)



**Figure 3.7.1. A rescue in protein levels of condensin-II subunits was observed after inhibition of proteasomal degradation.**

a) Immunoblots showing the protein levels of condensin subunits in control (Ctrl), NH<sub>4</sub>Cl-treated (+NH<sub>4</sub>Cl), MG132-treated (+MG132) and NH<sub>4</sub>Cl & MG132-treated (+NH<sub>4</sub>Cl & MG132) cells. b) Graphical representation displaying the relative protein levels of condensin-I, -II and SMC subunits in control, NH<sub>4</sub>Cl-treated, MG132-treated and NH<sub>4</sub>Cl & MG132-treated cells. Data were obtained from 3 independent experiments. \*P<0.01.

### **3.8 $\beta$ -TRCP2 is not the perpetrator of condensin-II's degradation via the proteasomal pathway**

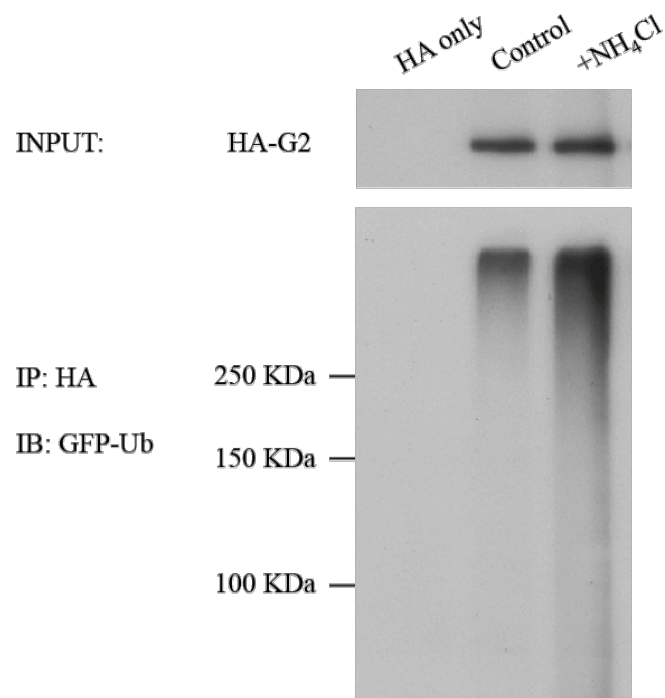
Thus far, it was found that proteasome-dependent degradation caused the decrease in the level of condensin-II when autophagy was inhibited by  $\text{NH}_4\text{Cl}$ . In other words, the presence of autophagy could have a positive effect to protect/prevent excessive degradation of condensin-II subunits. I then focused on uncovering the mechanism of how autophagy may play a role in regulating the degradation of condensin-II subunits. To address this, I aimed to confirm that condensin-II is indeed ubiquitinated. As such, the ubiquitination level of condensin-II was determined. Briefly, cells were transfected with HA-tagged Cap-G2 (HA-G2) and GFP-tagged ubiquitin (GFP-Ub). Immunoprecipitation of HA-G2 was then performed in control and  $\text{NH}_4\text{Cl}$ -treated cells and the presence of ubiquitinated HA-G2 was determined by western blot analysis using both anti-HA and anti-GFP antibodies. Protein bands/smears of high molecular weight species representing ubiquitinated HA-G2 were detected in both samples, indicating that Cap-G2 is indeed ubiquitinated (Fig. 3.8). In addition, a significantly higher level of ubiquitinated HA-G2 was present in  $\text{NH}_4\text{Cl}$ -treated cells compared to control cells, indicating that the impairment of autophagy led to an increase in ubiquitination of condensin-II.

Since E3 ligase and DUB play important roles in the regulation of ubiquitination, it was then hypothesized that autophagy may control the protein level of condensin-II subunits via its regulation on E3 ligase or DUB alone, or on both enzymes. A quick literature search revealed that in *Drosophila*, the SCF<sup>Slimb</sup> (fly homologue of human  $\beta$ -TRCP2) E3 ligase targets fly condensin-II subunit, Cap-H2, for ubiquitination and subsequent degradation (Buster, Daniel et al. 2013). This prompted my investigation on human SCF <sup>$\beta$ -TRCP2</sup> E3 ligase ( $\beta$ -TRCP2) to find out if it is responsible for the degradation of condensin-II subunits following autophagy impairment.

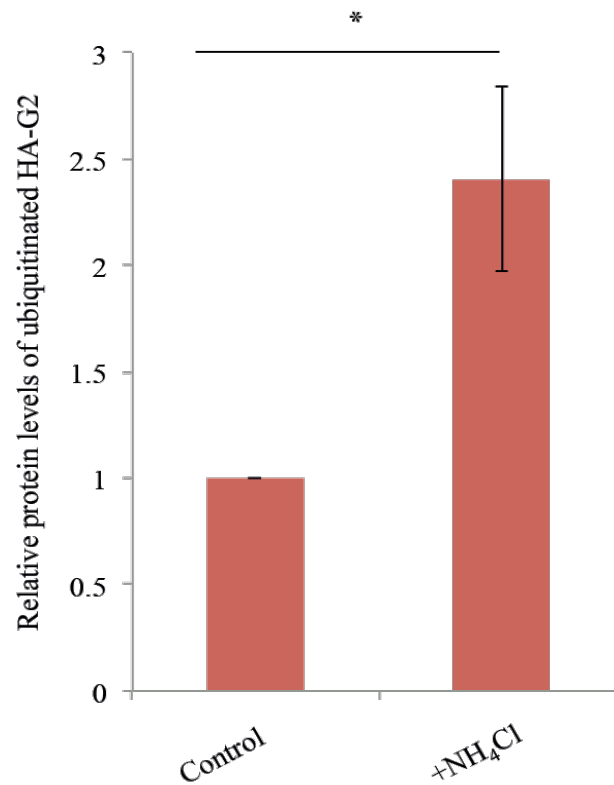
To assess if  $\beta$ -TRCP2's protein level is regulated by autophagy, causing it to affect condensin-II subunits' protein levels, western blot analysis was carried out to compare the protein levels of  $\beta$ -TRCP2 in lysates from control,  $\text{NH}_4\text{Cl}$ -treated,

MG132-treated and  $\text{NH}_4\text{Cl}$  & MG132-treated cells. As shown, there was negligible change in the protein level of  $\beta$ -TRCP2 even when autophagy was inhibited (Fig. 3.8.1). This showed that  $\beta$ -TRCP2 was not regulated by autophagy.

a)



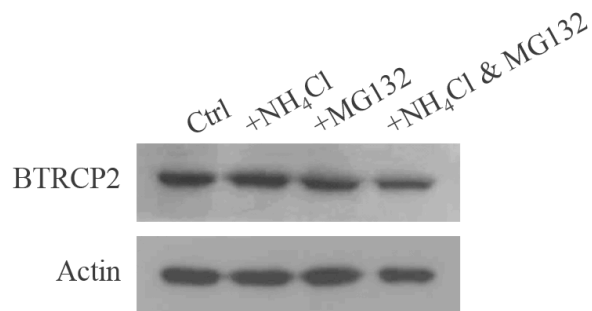
b)



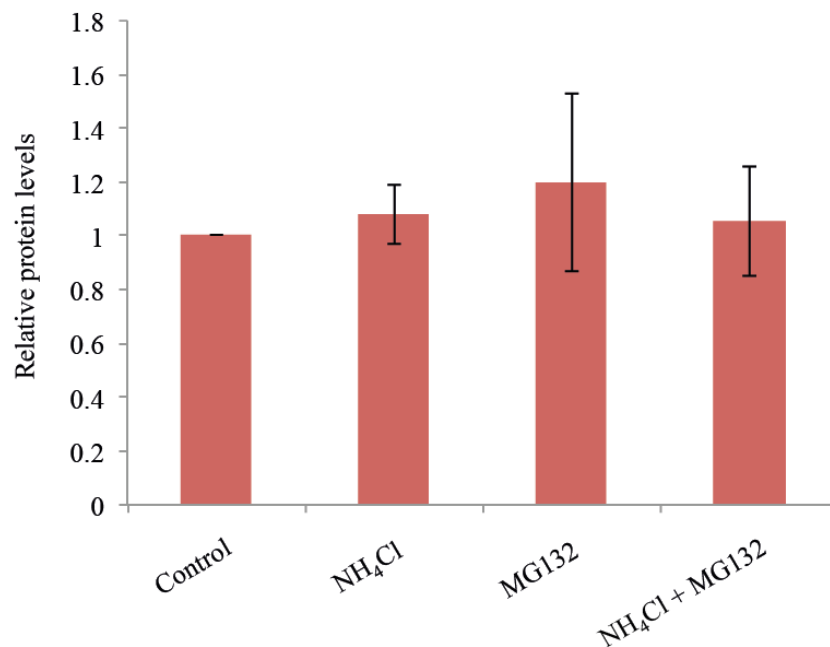
**Figure 3.8. Higher amount of ubiquitinated HA-G2 was present after autophagy inhibition.**

a) Western blot images showing the IP of HA-G2 and immunoblotting (IB) for the presence of GFP-Ubs that were attached to ubiquitinated HA-G2 pulled down in cells with transfected HA without treatment (HA only), cells with transfected HA-G2 without treatment (Control), and cells with transfected HA-G2 treated with NH<sub>4</sub>Cl (+NH<sub>4</sub>Cl). b) Graphical representation showing the relative protein levels of ubiquitinated HA-G2 in Control and NH<sub>4</sub>Cl-treated cells. \*P<0.0452.

a)



b)



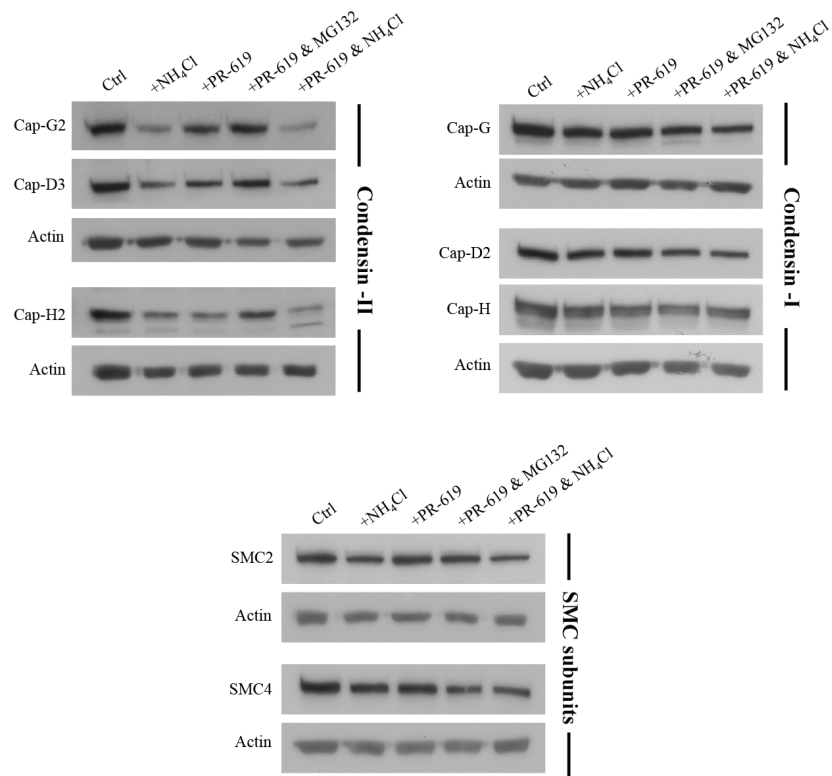
**Figure 3.8.1. Protein level of  $\beta$ -TRCP2 is unaffected by defective autophagy and/or proteasomal pathways.**

a) Immunoblots showing the protein levels of  $\beta$ -TRCP2 in control (Ctrl), NH<sub>4</sub>Cl-treated (+NH<sub>4</sub>Cl), MG132-treated (+MG132) and NH<sub>4</sub>Cl & MG132-treated (+NH<sub>4</sub>Cl & MG132) cells. b) Graphical representation displaying the relative protein levels of  $\beta$ -TRCP2 in control, NH<sub>4</sub>Cl-treated, MG132-treated and NH<sub>4</sub>Cl & MG132-treated cells. Data were obtained from 3 independent experiments.

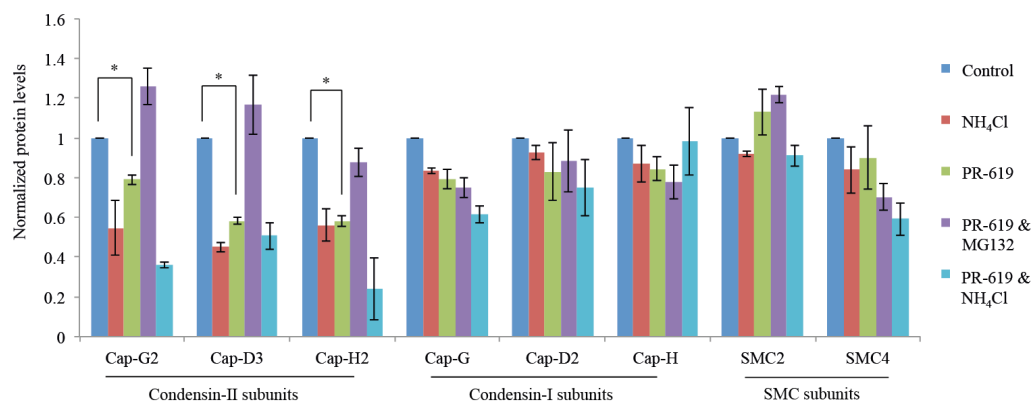
### **3.9 DUBs may be likely candidates that bring about degradation of condensin-II after autophagy inhibition**

As mentioned earlier, autophagy could be regulating both E3 ligase and DUB, thus controlling the protein level of condensin-II. Hence, I moved on to explore the possibility of insufficient DUBs being present during impaired autophagy. To test this idea, PR-619, a broad-spectrum DUB inhibitor, was employed to inhibit the activity of DUBs in RPE-1 cells. Protein levels of condensin-I, -II and SMC subunits were then examined for a decrease that is similar to those observed during autophagy inhibition via western blot analysis. As seen in the blots, there was a comparable drop in protein levels of condensin-II subunits after 48 hours of 5 $\mu$ M PR-619 treatment (Fig. 3.9). Likewise, this decrease in condensin-II subunits was rescued with the inhibition of proteasomal degradation via the use of MG132 (Fig. 3.9). In contrast, the decrease in condensin-I and SMC subunits after treatment with PR-619 was negligible. These findings indicated that if the impairment of autophagy does lead to a loss of function or decreased protein levels of DUBs, it would induce a decrease in condensin-II's stability that parallels what we have observed after autophagy inhibition.

a)



b)

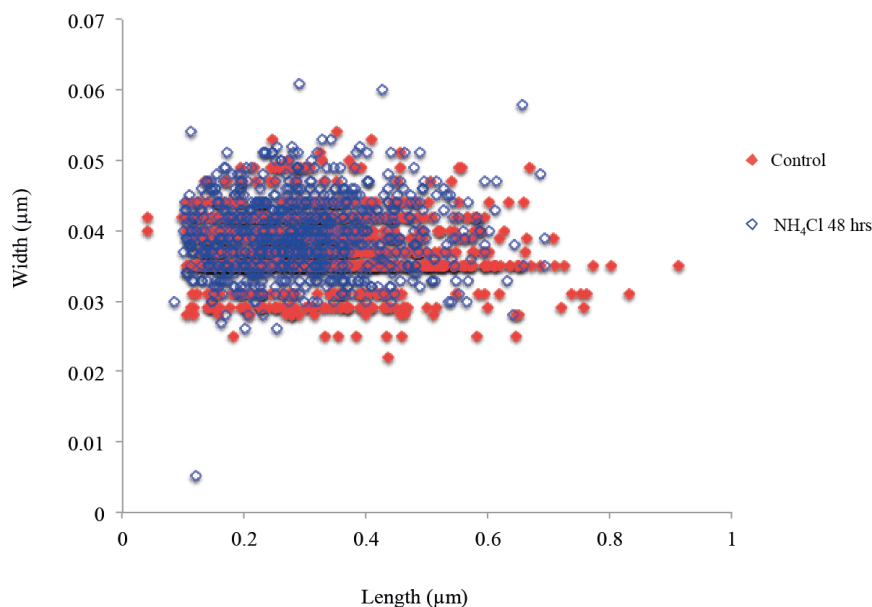


**Figure 3.9. Inhibition of DUBs led to a decrease in condensin-II.**

a) Immunoblots showing the protein levels of Condensin-I, -II and SMC subunits in lysates from control (Ctrl), NH<sub>4</sub>Cl-treated (+NH<sub>4</sub>Cl), PR-619-treated (+PR-619), PR-619 & MG132-treated (+PR-619 & MG132) and PR-619 & NH<sub>4</sub>Cl-treated (+PR-619 & NH<sub>4</sub>Cl) cells. b) Graphical representation showing relative protein levels of Condensin-I, -II and SMC subunits in lysates from control, NH<sub>4</sub>Cl-treated, PR-619-treated, PR-619 & MG132-treated and PR-619 & NH<sub>4</sub>Cl-treated cells. Data were obtained from 2 independent experiments. \* P<0.007.

### 3.10 Autophagy inhibition does not affect the length and width of chromosomes in human cells

Earlier in the thesis, I showed that mitotic chromosomal integrity was altered in the ATG5 knockout MEF cells. In addition, inhibition of autophagy by  $\text{NH}_4\text{Cl}$  resulted in down-regulation of condensin-II in RPE-1 cells. Since condensin-II is essential for the integrity of mitotic chromosomes, I then examined the length and width of mitotic chromosomes when autophagy was inhibited by  $\text{NH}_4\text{Cl}$ . However, there was no apparent difference in the lengths and widths of chromosomes in  $\text{NH}_4\text{Cl}$ -treated cells compared to those in control cells. The average length of control chromosomes was  $0.327 \pm 0.141 \mu\text{m}$  while the average width was  $0.0372 \pm 0.005 \mu\text{m}$ . Similarly, chromosomes from  $\text{NH}_4\text{Cl}$ -treated cells were on average  $0.297 \pm 0.12 \mu\text{m}$  while the average width was  $0.0395 \pm 0.0052 \mu\text{m}$ . Lengths and widths of control and  $\text{NH}_4\text{Cl}$ -treated chromosomes were then plotted into a scatter plot (Fig. 3.10). As seen in the plot, majority of the measured values from control and  $\text{NH}_4\text{Cl}$ -treated chromosomes overlapped, suggesting that there is little to no change in their lengths and widths, although the P value for the measured lengths and widths were less than 0.0001.



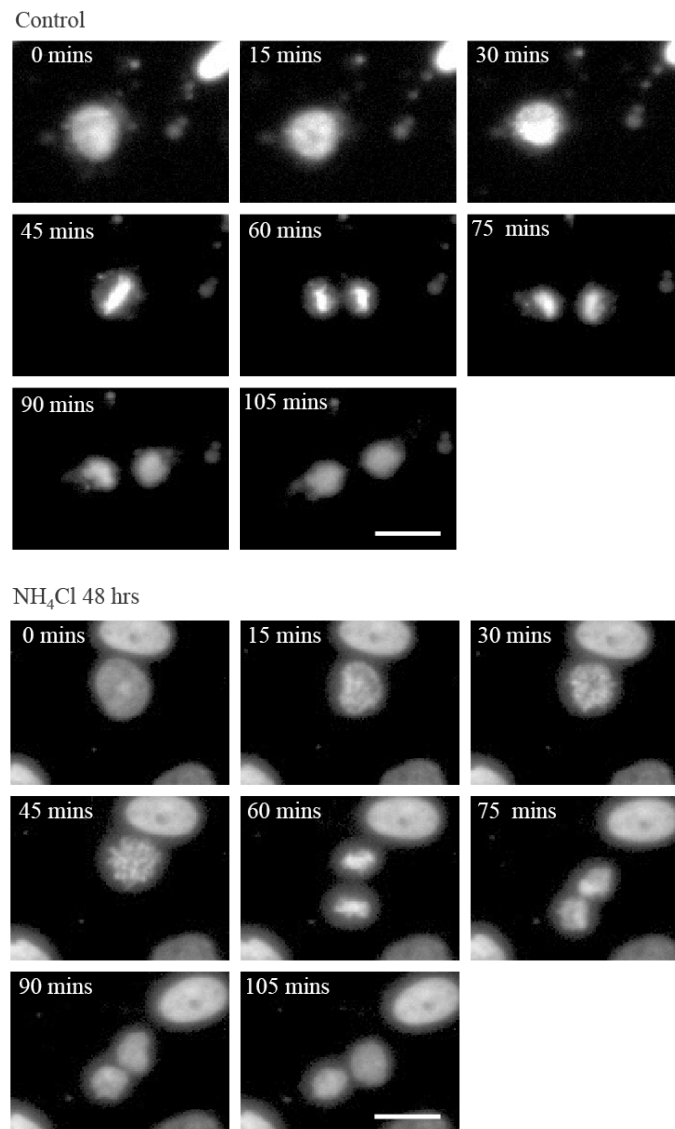
**Figure 3.10. Autophagy inhibition in RPE-1 cells did not induce a change in chromosomal length and width.**

Scatter plot showing measured lengths and widths of chromosomes from control and  $\text{NH}_4\text{Cl}$ -treated cells. Length:  $P < 0.0001$ , Width:  $P < 0.0001$ .

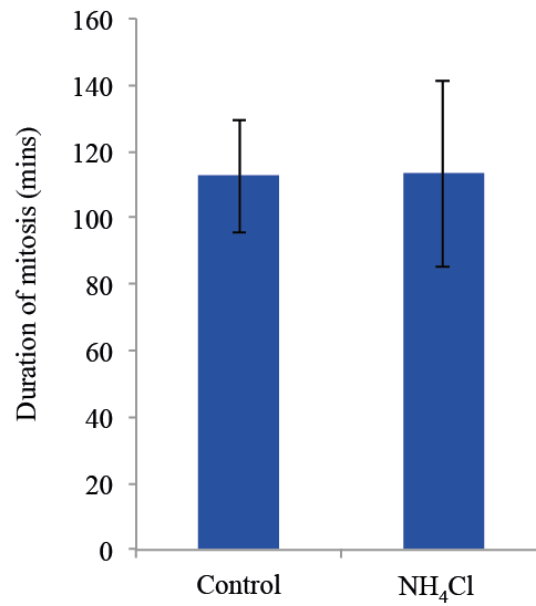
### 3.11 Autophagy inhibition does not affect mitotic progression

Since the protein levels of condensin subunits were affected by autophagy inhibition and condensin complexes play an important role in mitosis, I hypothesized that the inhibition of autophagy pathway could lead to defects in mitosis. To test this hypothesis, time-lapse imaging was carried out using mCherry-histone H2B (H2B) transfected RPE-1 cells with or without treatment of  $\text{NH}_4\text{Cl}$ . mCherry-H2B enables visualization of DNA in the cell and thus mitotic progression can be monitored. As shown in the time-lapse images, both control and  $\text{NH}_4\text{Cl}$ -treated cells progressed through mitosis at the same rate without any significant delay (Fig. 3.11).

a)



b)



**Figure 3.11. The duration of mitosis remained unchanged after autophagy inhibition in RPE-1 cells.**

a) Time-lapse images showing the duration of mitosis in control and  $\text{NH}_4\text{Cl}$ -treated cells. Scale bars: 10  $\mu\text{m}$ . b) Graphical representation comparing the duration of mitosis in control and  $\text{NH}_4\text{Cl}$ -treated cells. Data were obtained from 20 mitotic cells in each of the 2 independent experiments.

### 3.12 Impaired autophagy gives rise to lagging chromosomes

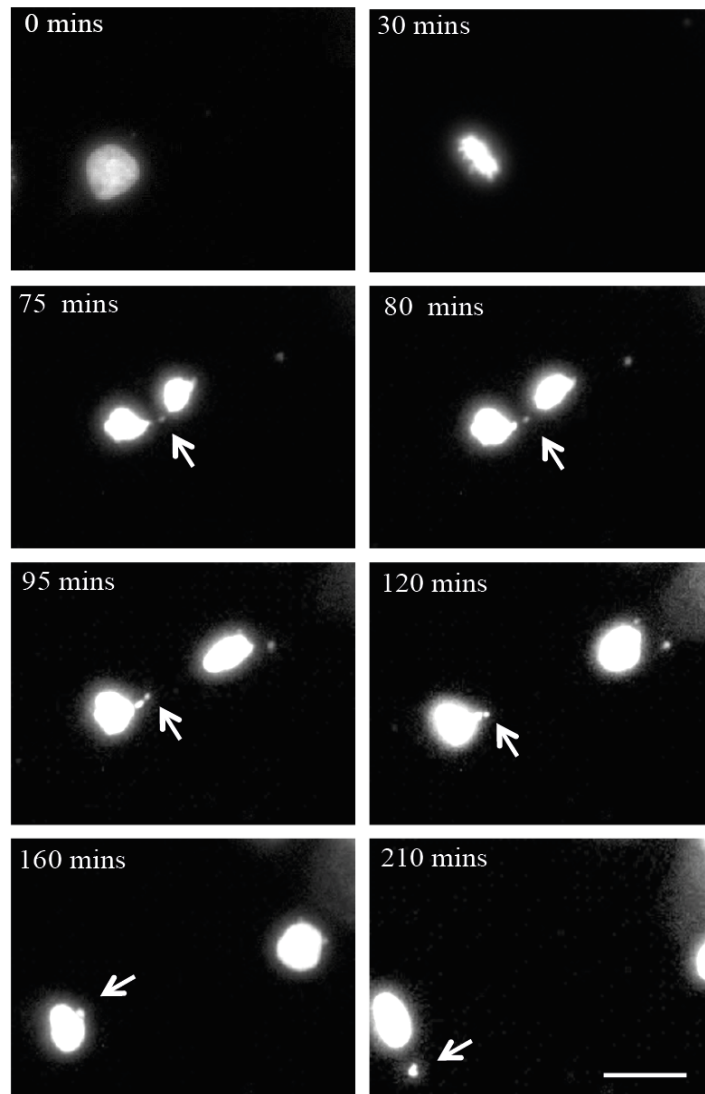
Although mitotic progression was unaffected by autophagy inhibition via  $\text{NH}_4\text{Cl}$  treatment, I observed a higher frequency of  $\text{NH}_4\text{Cl}$ -treated anaphase cells containing lagging chromosome when it was compared to control (Fig. 3.12a). Quantification of the percentage of mitotic cells (cells in anaphase and telophase) that contained lagging chromosomes was then carried out. I found that a significantly higher percentage of mitotic cells displayed lagging chromosomes in  $\text{NH}_4\text{Cl}$ -treated anaphase/telophase cells (6.8%) compared to control anaphase/telophase cells (0.83%) (Fig. 3.12c). Time-lapse imaging using mCherry-H2B was repeated to visualize the formation and fate of lagging chromosome during anaphase in the absence of autophagy. The lagging chromosome was not incorporated into the divided daughter cell nuclei after completion of cell division (Fig. 3.12b). This observation could well explain why a high percentage of ATG5 knockout MEFs exhibited micronuclei.

a)

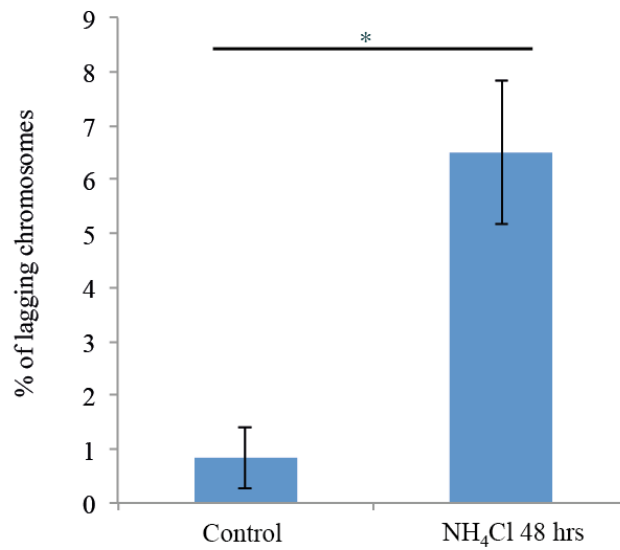


b)

$\text{NH}_4\text{Cl}$  48 hrs



c)



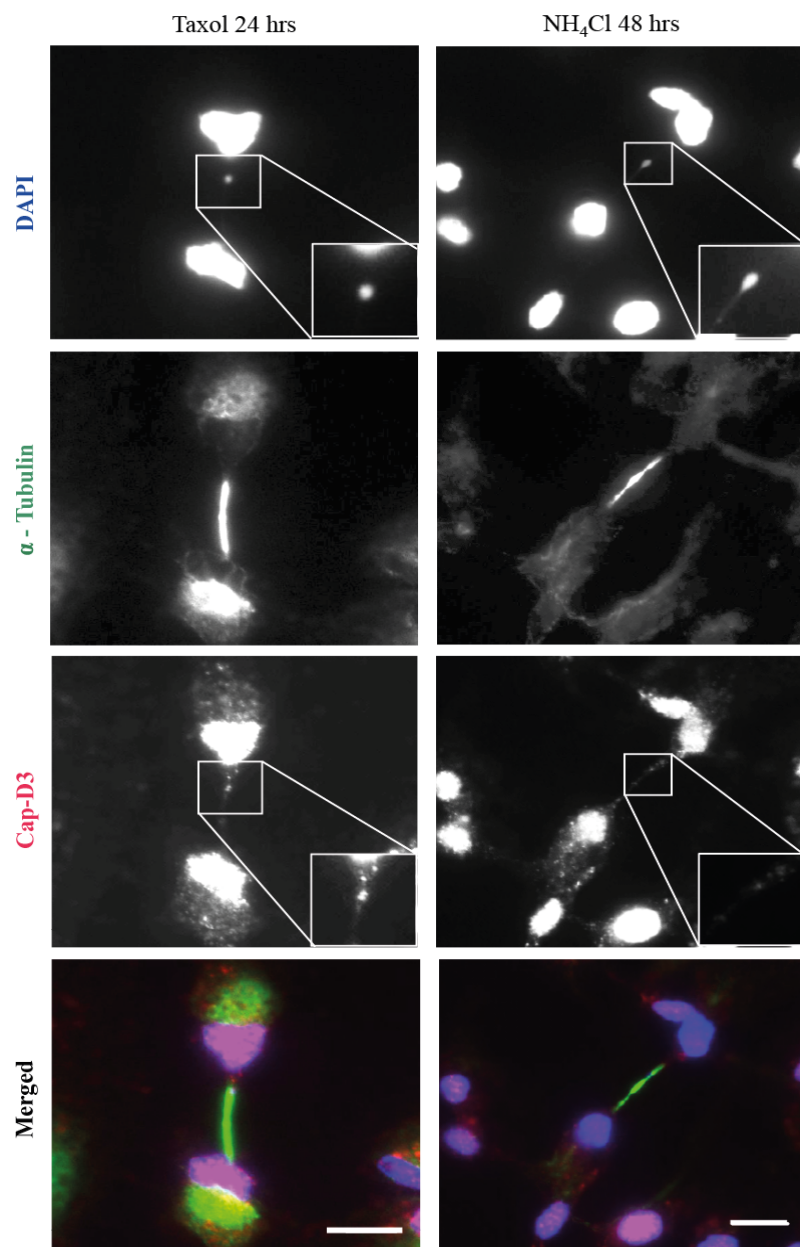
**Figure 3.12. Autophagy inhibition increased occurrence of lagging chromosomes.**

a) Immunofluorescence images of a lagging chromosome during anaphase after treatment of  $\text{NH}_4\text{Cl}$  for 48 hours. White arrow points to the lagging chromosome. Scale bar: 5  $\mu\text{m}$ . b) Time-lapse images of a  $\text{NH}_4\text{Cl}$ -treated cell undergoing mitosis, giving rise to lagging chromosome parts. White arrows point to lagging chromosome parts. Scale bars: 10  $\mu\text{m}$ . c) Graphical representation showing the percentage of lagging chromosomes in control and  $\text{NH}_4\text{Cl}$ -treated cells. \* $P = 0.0236$ . 200 mitotic cells in anaphase/telophase were analyzed in each of the 3 independent experiments.

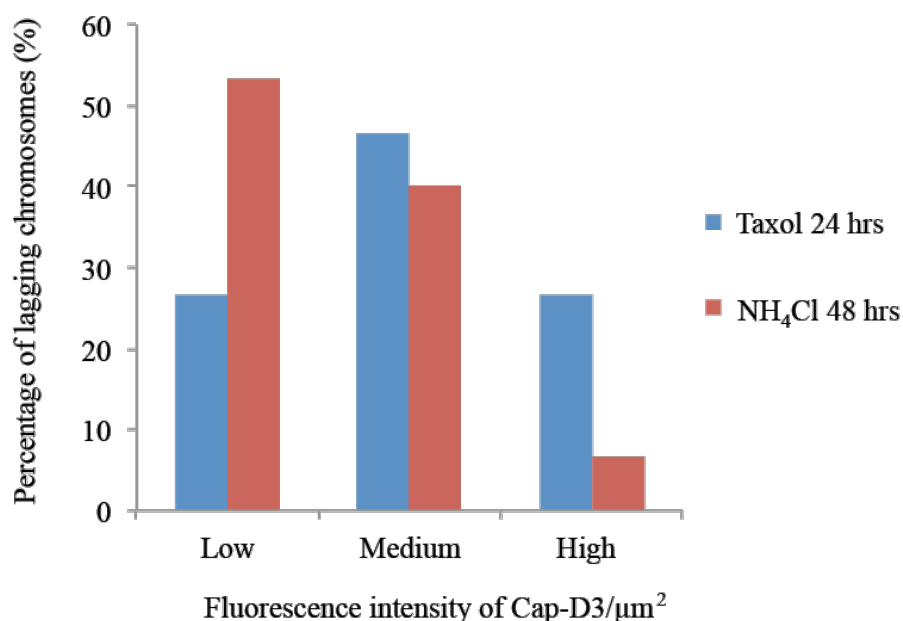
### **3.13 Lower amount of condensin-II remained chromosome-bound after autophagy inhibition**

Inhibition of autophagy resulted in higher occurrence of lagging chromosomes and lower amount of condensin-II. I therefore speculated that there might be a lower amount of condensin-II localized on a population of chromosomes, causing them to be mis-segregated and lagged behind at the equator during anaphase. To test this possibility, Taxol was utilized to induce lagging chromosomes that occurred due to defects in mitotic spindle assembly and not lack of chromosomal condensin-II, thus acting as a positive control for chromosomal condensin-II staining. Immunofluorescence staining was then carried out to compare the fluorescence intensity of condensin-II subunits on lagging chromosomes induced by autophagy inhibition, to that of lagging chromosomes induced by 10 nM Taxol treatment for 24 hours. As expected, higher percentage of lagging chromosomes from NH<sub>4</sub>Cl-treated cells were found to have lower fluorescence intensity of condensin-II subunits, Cap-G2 and Cap-D3, as compared to that in Taxol-treated cells (Fig. 3.13, 3.13.1).

a)



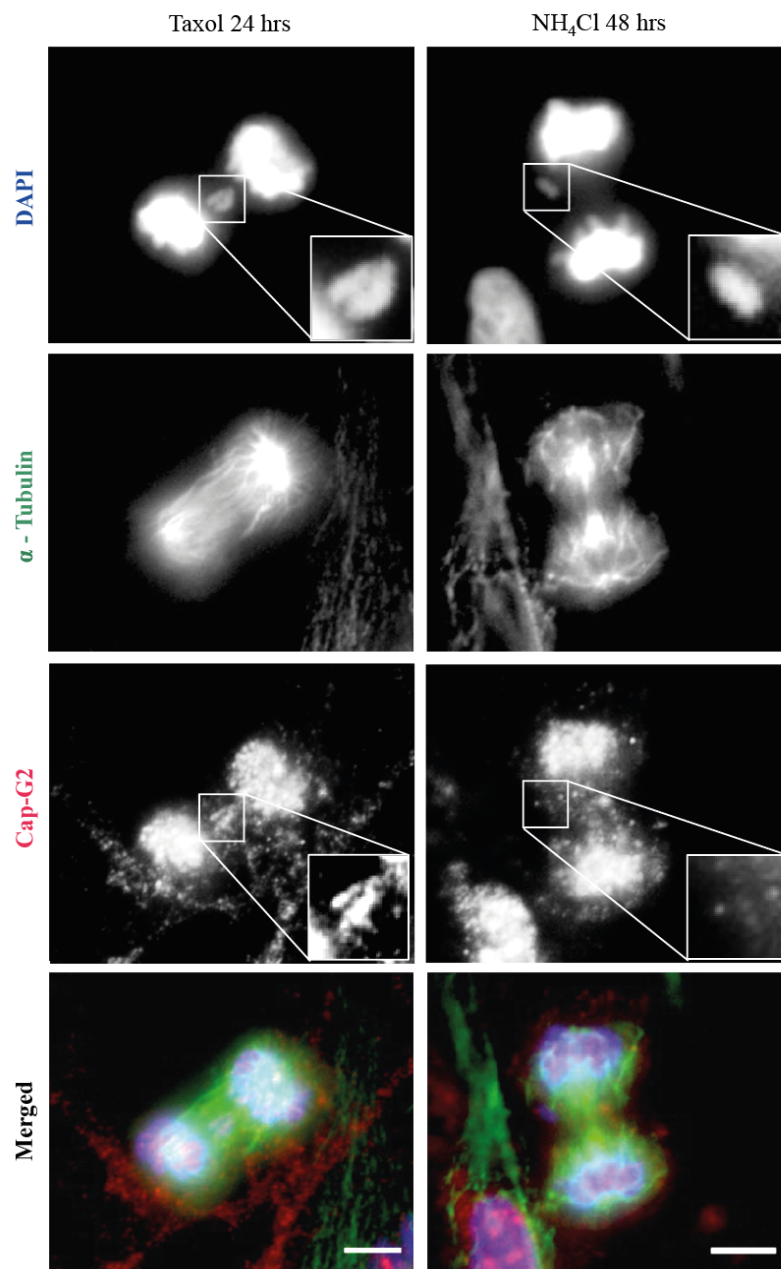
b)



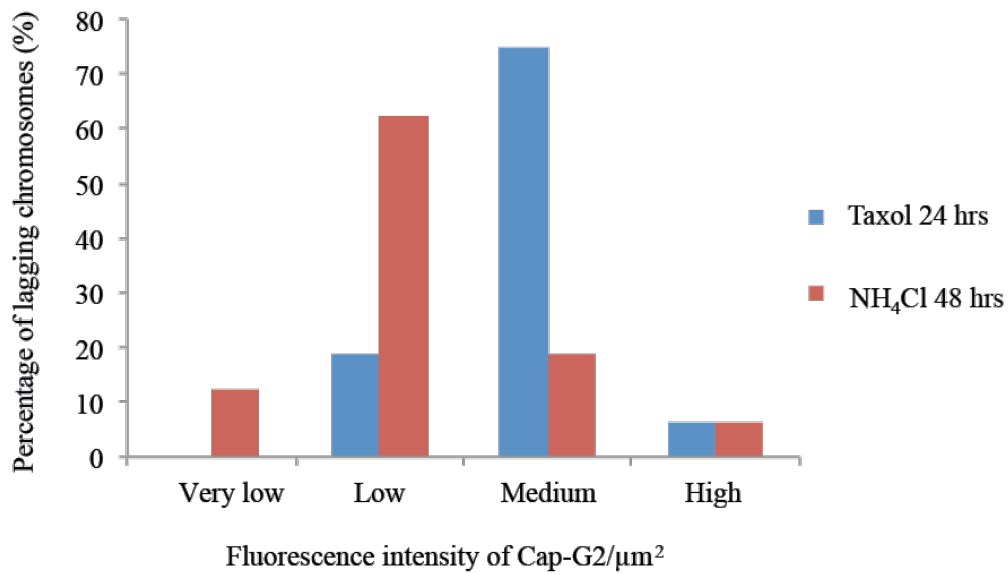
**Figure 3.13. Autophagy inhibition disrupted the localization of Cap-D3 on chromosomes.**

a) Immunofluorescence images of chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells that were stained for Cap-D3 (red),  $\alpha$ -tubulin (green) and DNA (blue). Insets: enlarged images of lagging chromosome parts. Scale bars: 5μm b) Graphical representation displaying the percentages of lagging chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells, in different categories of Cap-D3's fluorescence intensity.

a)



b)

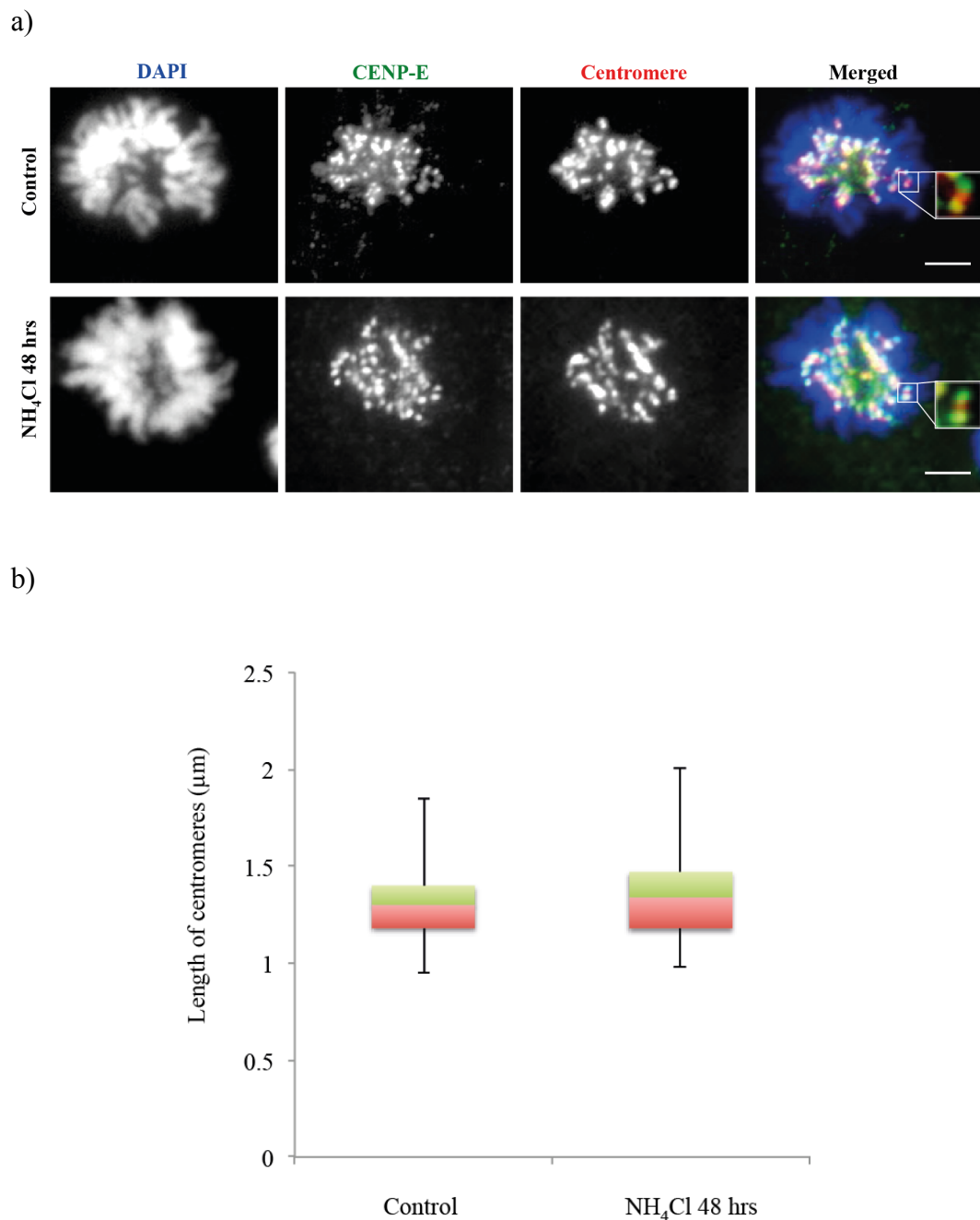


**Figure 3.13.1. Autophagy inhibition adversely affects the localization of Cap-G2 on chromosomes.**

a) Immunofluorescence images of chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells that were stained for Cap-G2 (red),  $\alpha$ -tubulin (green) and DNA (blue). Insets: enlarged images of lagging chromosome parts. Scale bars: 5μm b) Graphical representation displaying the percentages of lagging chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells, in different categories of Cap-G2's fluorescence intensity. Data were obtained from 15 cells exhibiting lagging chromosomes in each of the 2 independent experiments. \*P<0.0467.

### **3.14 Decrease in condensin-II does not affect the integrity of kinetochore/ centromere**

A lower amount of chromosomal condensin-II is now correlated with a higher incidence of lagging chromosomes. However, the mechanism is still unclear. Lagging chromosomes commonly arise from improper attachment of spindle microtubules to kinetochores. Furthermore, it is known that the loss of condensin-I and -II through siRNA-depletion of SMC2 caused kinetochore defect in the form of centromere stretching, consequently leading to chromosome mis-segregation (Samoshkin, Arnaoutov et al. 2009). As autophagy inhibition resulted in decreased levels of condensin-II, it might induce centromere stretching as well. Therefore, I examined the centromeres after treatment of cells with  $\text{NH}_4\text{Cl}$  for 48 hours. Immunofluorescence imaging was carried out with anti-centromere and anti-CENP-E antibodies and centromeres' lengths were measured for presence of stretching (Fig. 3.14a). There was no significant difference between the length of centromeres from control and  $\text{NH}_4\text{Cl}$ -treated chromosomes (Fig. 3.14b). This indicated that condensin-II alone might not be crucial for maintaining mitotic centromere rigidity.



**Figure 3.14. Reduced amount of condensin-II did not induce centromere stretching.**

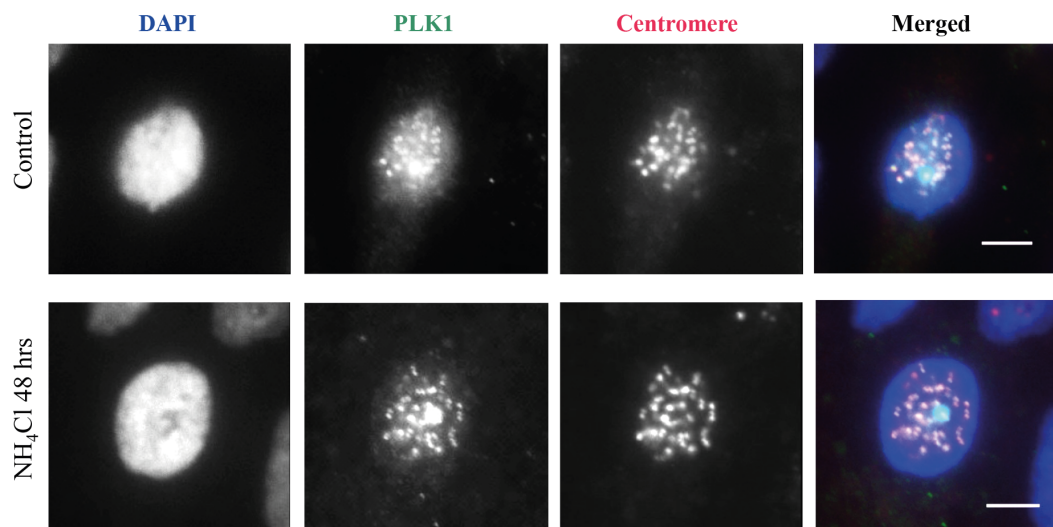
a) Immunofluorescence images showing chromosomes from control and NH<sub>4</sub>Cl-treated cells that were stained for the centromeres (red), CENP-E (green) and DNA (blue). Scale bars: 5 µm. b) Graphical representation comparing the length of centromeres from control and NH<sub>4</sub>Cl-treated chromosomes in the form of a box plot. Data were derived from at least 140 chromosomes in each of the 2 independent experiments.

### **3.15 Autophagy inhibition has no effect on PLK1's protein levels and localization.**

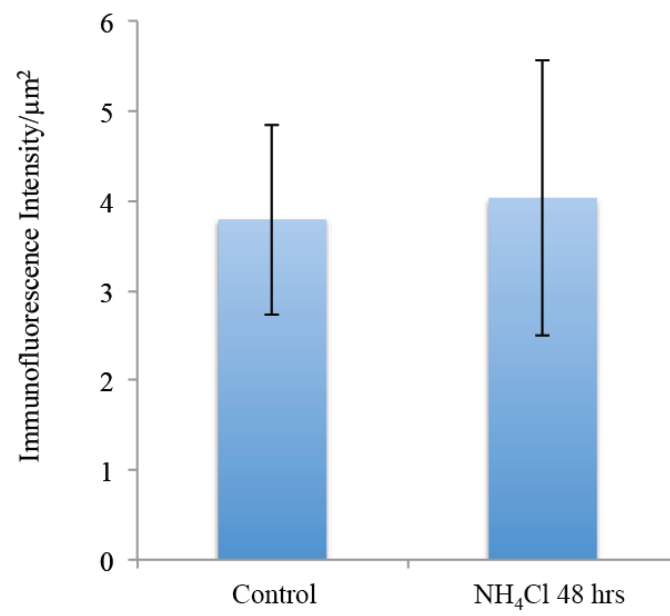
Condensin complexes are important regulators of mitotic chromosome condensation and Cap-G2 (condensin-II subunit) is crucial in chromosome segregation through the recruitment of PLK1 to prometaphase kinetochores (Kim, Shim et al. 2014). As such, it is possible that the decrease in levels of condensin-II subunits caused by autophagy inhibition affected the interaction between Cap-G2 and PLK1. In turn, recruitment of PLK1 at the kinetochores would be disrupted and thus be unable to ensure proper spindle attachments are made before onset of anaphase.

To test this possibility, cells were treated with  $\text{NH}_4\text{Cl}$  for 48 hours, followed by immunostaining for PLK1 to check if its localization at the kinetochores is disrupted. As shown in the immunofluorescence images, PLK1 remained localized at the kinetochores and there was no significant difference in the fluorescence intensity of PLK1 between control and  $\text{NH}_4\text{Cl}$ -treated cells (Fig. 3.15a, b). Protein levels of PLK1 was also examined via western blot analysis and found to be unchanged even after 48 hours of  $\text{NH}_4\text{Cl}$  treatment (Fig. 3.15c, d). This suggested that the depletion of condensin-II at the chromosomes did not affect both the protein level of PLK1 and its localization at the kinetochores. Therefore, PLK1 is not a downstream effector of Cap-G2 in the formation of lagging chromosomes.

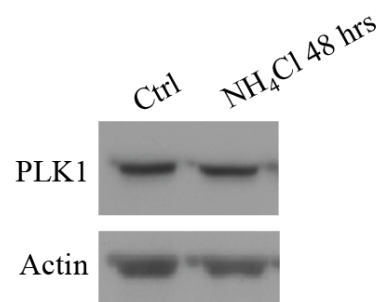
a)



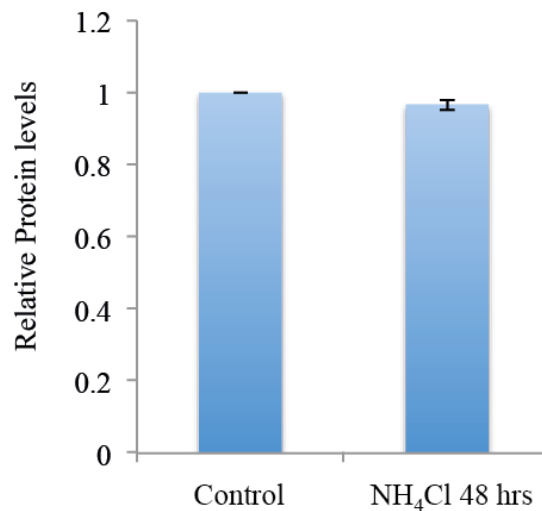
b)



c)



d)



**Figure 3.15. Depletion of chromosomal condensin-II mediated by autophagy inhibition did not affect PLK1's protein level and localization.**

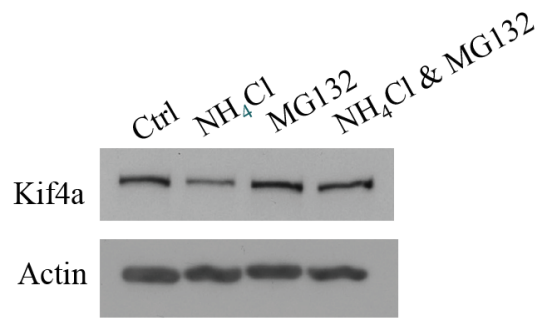
a) Immunofluorescence images of a prometaphase cell in control and NH<sub>4</sub>Cl-treated cells that were stained for the centromeres (red), PLK1 (green) and DNA (blue). Scale bars: 5  $\mu$ m. b) Graphical representation showing immunofluorescence intensity of PLK1 at the kinetochores in control and NH<sub>4</sub>Cl-treated cells. At least 150 kinetochores were analyzed in each of the 2 independent experiments. c) Western blot images showing protein levels of PLK1 in control and NH<sub>4</sub>Cl-treated lysates. d) Graphical representation showing relative protein levels of PLK1 in control and NH<sub>4</sub>Cl-treated cells. Data were derived from 3 independent experiments.

### **3.16 Autophagy impairment resulted in reduced amount of kif4a**

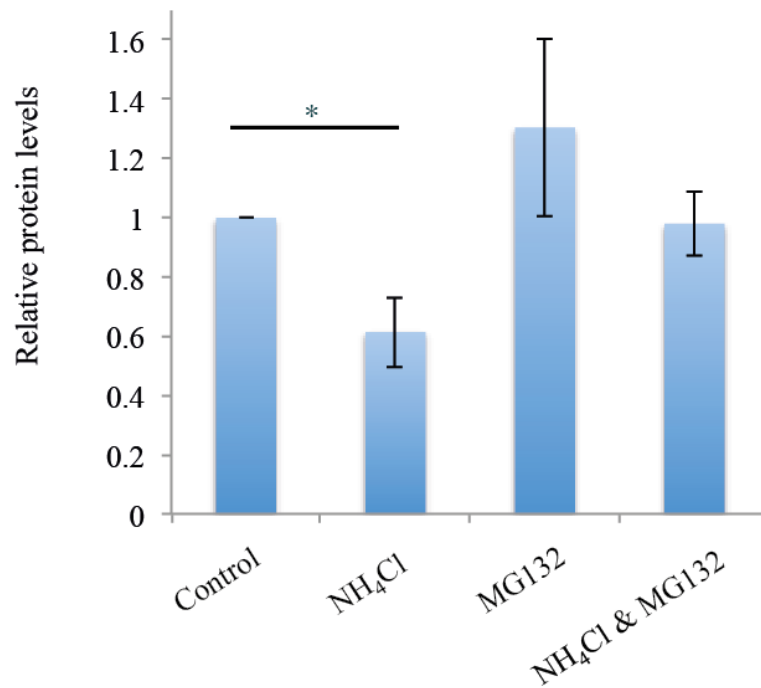
Another protein that is critical for proper chromosome segregation is Kinesin Family Member 4A (Kif4a). Kif4a interacts with condensin-I and -II and its depletion was found to affect the localization of condensins, causing the collapse of chromatin fibers and the occurrence of lagging chromosomes (Mazumdar, Sundareshan et al. 2004). In addition, total abolishment of condensin-I and -II at the chromosome axis was found to disrupt the localization of Kif4a (Green, Kalitsis et al. 2012). In my study, the decrease in chromosomal condensin-II induced by autophagy inhibition may also affect the localization of Kif4a, thus leading to mis-segregation and formation of lagging chromosomes. Furthermore, autophagy inhibition may also alter the protein level of Kif4a. If so, the treatment with DUBs inhibitor, PR-619, may induce a similar decrease in protein levels of Kif4a as well.

In order to investigate these, western blot analysis was carried out to compare the protein levels of Kif4a in control, NH<sub>4</sub>Cl-treated, MG132-treated and NH<sub>4</sub>Cl/MG132-treated cells. Autophagy inhibition was found to decrease the protein level of Kif4a in a manner similar to condensin-II subunits (Fig. 3.16). Addition of MG132 to inhibit proteasomal activity also rescued the amount of Kif4a, indicating that Kif4a's degradation is achieved via the proteasomal pathway. This was followed by immuno-staining using anti-Kif4a antibodies to compare the amount of Kif4a on lagging chromosomes in Taxol-treated and NH<sub>4</sub>Cl-treated cells. Interestingly, there was a significantly lower level of Kif4a staining the lagging chromosomes in NH<sub>4</sub>Cl-treated cells compared to control Taxol-treated cells (Fig. 3.16.1). In addition, inhibition of DUBs with PR-619 resulted in reduced protein levels of Kif4a that was recoverable with the addition of MG132 to inhibit proteasomal degradation (Fig. 3.16.2).

a)



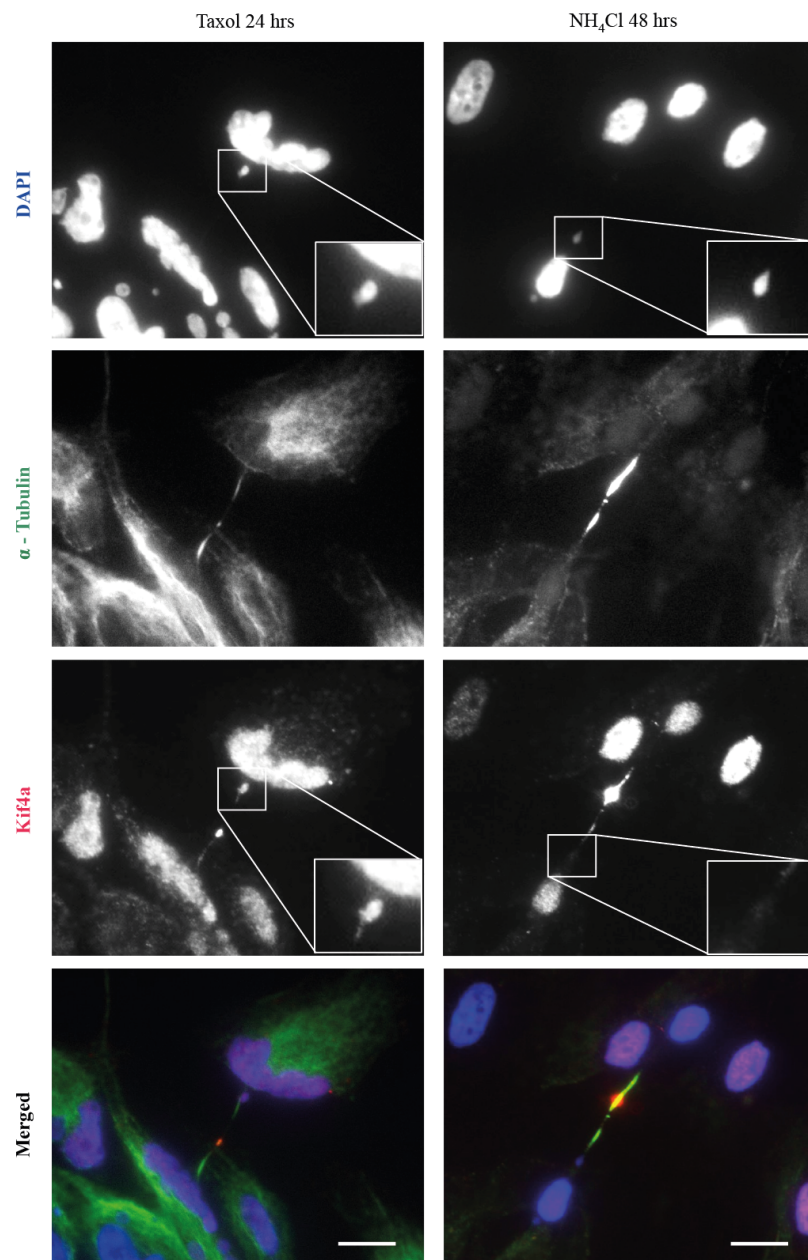
b)



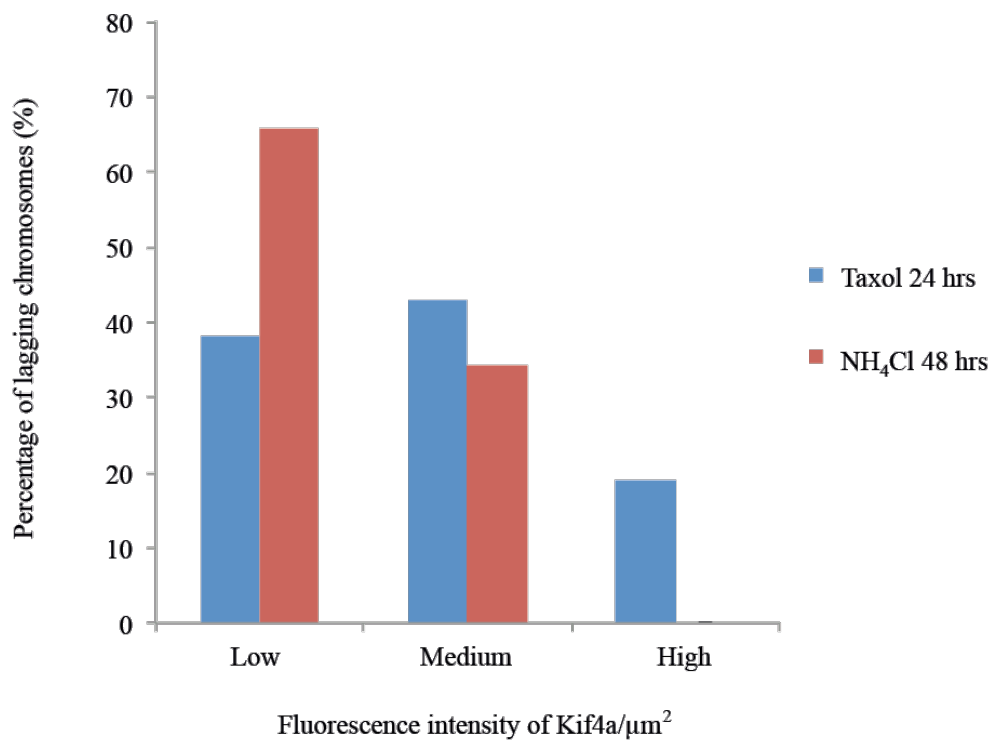
**Figure 3.16. Autophagy inhibition adversely affected the amount of Kif4a.**

a) Immunoblots showing the protein levels of Kif4a in control, NH<sub>4</sub>Cl-treated, MG132-treated and NH<sub>4</sub>Cl & MG132-treated cells. b) Graphical representation showing the protein levels of Kif4a in control, NH<sub>4</sub>Cl-treated, MG132-treated and NH<sub>4</sub>Cl & MG132-treated cells. Data were derived from 3 independent experiments. \*P = 0.0047.

a)



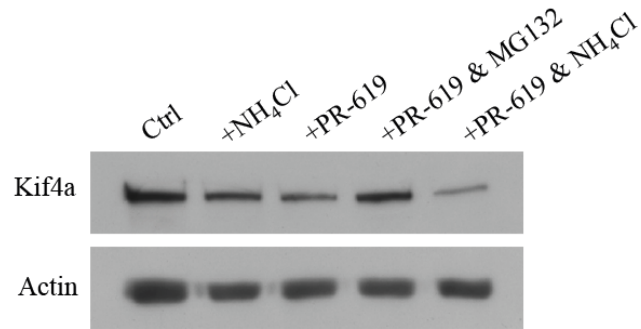
b)



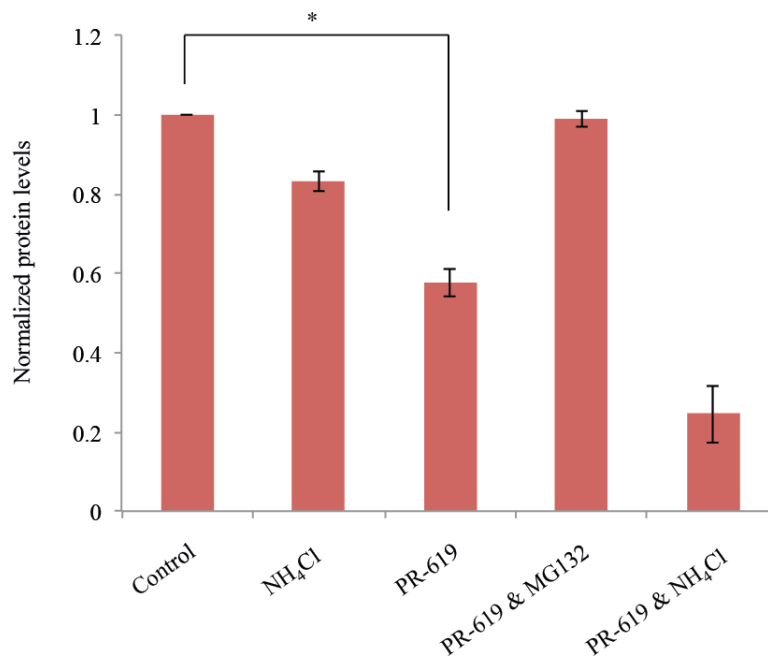
**Figure 3.16.1. Defective autophagy disrupted the localization of Kif4a at the chromosomes.**

a) Immunofluorescence images of chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells, stained for Kif4a (red), α-tubulin (green) and DNA (blue). Insets: enlarged images of lagging chromosome parts. Scale bars: 5μm b) Graphical representation displaying the percentages of lagging chromosomes from Taxol-treated and NH<sub>4</sub>Cl-treated cells, in different categories of Kif4a's fluorescence intensity. Data were obtained from 15 cells exhibiting lagging chromosomes in each of the 2 independent experiments. \*P<0.0543.

a)



b)



**Figure 3.16.2. Inhibition of DUBs led to a decrease in Kif4a.**

a) Immunoblots showing the protein levels of Kif4a in lysates from control (Ctrl), NH<sub>4</sub>Cl-treated (+NH<sub>4</sub>Cl), PR-619-treated (+PR-619), PR-619 & MG132-treated (+PR-619 & MG132) and PR-619 & NH<sub>4</sub>Cl-treated (+PR-619 & NH<sub>4</sub>Cl) cells. b) Graphical representation showing relative protein levels of Kif4a in lysates from control, NH<sub>4</sub>Cl-treated, PR-619-treated, PR-619 & MG132-treated and PR-619 & NH<sub>4</sub>Cl-treated cells. Data were obtained from 2 independent experiments. \* P<0.003.

## **4 DISCUSSION**

Autophagy is known to play fundamental roles in cellular homeostasis by degrading proteins and organelles that are damaged. This process is also crucial to ensuring cell survival at times when energy resources are low, by providing substrates for energy production. In addition to these, autophagy is responsible for a myriad of other functions, such as gene regulation, DNA repair and maintenance of genomic integrity. Despite discovering roles of autophagy in different cellular pathways, little to no studies were made on finding the mechanisms underlying such roles. In particular, I am interested in elucidating the steps taken by autophagy to prevent genomic instability.

### **4.1 Effects of autophagy-induced degradation**

With autophagy being a major degradation system, many of its substrates are involved in pathways that are critical for cellular well-being. In my study, I identified two important proteins whose levels are indirectly regulated by autophagy, namely condensin-II and Kif4a. I demonstrated that when there is autophagy impairment, condensin-II and Kif4a are negatively regulated, causing a decline in their protein levels. This reduction in availability of both proteins correlated with an elevated frequency of lagging chromosomes.

Lagging chromosomes are symptomatic of defects in chromosome condensation and/or dynamics of kinetochore-microtubule attachment (Thompson and Compton 2011). As condensin-II is a protein regulator that is crucial to maintain the shape and structural integrity of chromosomes, the decrease in its level possibly generated insufficient chromosome condensation. The resultant chromosomes will thus be poorly resolved, leading to formation of lagging chromosomes. This is in line with the findings made by several groups where defects in chromosome condensation as a result of condensins depletion, manifest as lagging chromosomes during anaphase (Hirota, Gerlich et al. 2004, Ono, Fang et al. 2004, Samoshkin, Arnaoutov et al. 2009).

The other identified protein regulated by autophagy is Kif4a. It is a chromokinesin that is equipped with the ability to carry molecular cargoes along microtubules. By carrying out its primary function of transporting molecular cargoes, Kif4a contributes to smooth progression of mitosis as well as condensation of mitotic chromosomes (Mazumdar, Sundareshan et al. 2004).

Kif4a's interaction with condensin complexes was found to be essential for both of their localizations on chromosomes, in order for proper chromosomal condensation to occur. This therefore, led to the deduction that Kif4a may function as a molecular linker between condensins, and together with other non-histone proteins, constitute the structural framework of mitotic chromosome (Hudson, Vagnarelli et al. 2003, Gassmann, Vagnarelli et al. 2004). Without Kif4a, chromosomal defects in the form of lagging chromosomes, chromatin bridges and aneuploidy appeared. This is consistent with my finding where reduced availability of Kif4a induced by autophagy inhibition, led to higher frequency of lagging chromosomes.

In addition, Kif4a is important for maintaining the structural integrity of spindle microtubules, spindle microtubule flux and regular kinetochore movements (Mazumdar, Sundareshan et al. 2004, Wandke, Barisic et al. 2012). As these are all critical factors that affect the kinetochore-microtubule attachments, any disruption will result in aberrant attachments such as syntelic and merotelic attachments.

Therefore, I speculate that the decrease in Kif4a and condensin-II, especially the chromosome-bound populations, may disrupt the structural framework of mitotic chromosomes and kinetochore-microtubule attachments, thus encouraging the formation of lagging chromosomes. However, I was unable to determine the exact perturbation caused by a deficiency in Kif4a and condensin-II that manifested as lagging chromosomes. In order to clarify that, I need to narrow down the possibilities by first examining the structural integrity of chromosomes depleted of condensin-II and Kif4a after autophagy impairment. As the depletion of these two proteins are partial and not complete abolishment, the possible defects in

chromosomes are not detectable under normal live-cell imaging. As such, I intend to utilize the intrinsic metaphase structure (IMS) assay developed by Hudson et al to compare the structural integrity of chromosomes treated with  $\text{NH}_4\text{Cl}$  with that of control chromosomes (Hudson, Vagnarelli et al. 2003). This assay allows one to effectively monitor the structural integrity of metaphase chromosomes after different perturbations. The IMS assay is based on the structural “memory” possessed by chromosomes, where they can be swollen beyond recognition and then restored to their original appearance. This is achieved by subjecting them to a first structural disturbance in hypotonic TEEN buffer (1 mM triethanolamine-HCl [pH 8.5], 0.2 mM NaEDTA, and 25 mM NaCl). As there is inadequate positive charge on the histone to fully neutralize the negative charges on the DNA, cations like  $\text{Mg}^{2+}$  are required to achieve complete neutralization. The hypotonic TEEN buffer serves to chelate such cations with EDTA, thus resulting in loosening of the chromatin’s higher-order structure down to the level of 10 nm fiber (Earnshaw and Heck 1985). Subsequently, the change in buffer from TEEN to RSB buffer (10 mM Tris [pH 7.4], 10 mM NaCl, and 5 mM  $\text{MgCl}_2$ ) restores the higher-order structure by resupplying the swollen chromosomes with  $\text{Mg}^{2+}$ . This is followed by a repetition of structural perturbation and recovery. Chromosomes with compromised structural integrity will not be able to recover to their initial morphology and instead, appear as disorganized and decondensed chromatin. Thereafter, length and flux of spindle microtubules, as well as kinetochore movements, will be examined via immunofluorescence microscopy for any changes after autophagy inhibition.

#### 4.1.1 Intermediary proteins that drive condensin-II & Kif4a’s degradation

The partial recovery in protein levels of condensin-II and Kif4a after inhibition of proteasomes was indicative of a UPS-mediated degradation. This was also supported by the increased ubiquitination levels detected on condensin-II after autophagy inhibition. Since degradation of a protein begins at the step of ubiquitin labeling, the proteins responsible for initiating degradation of condensin-II and Kif4a are most likely E3 ligases and DUBs.

I began by examining the possibility of  $\beta$ -TRCP2 ligase being the protein that drives the degradation of condensin-II and Kif4a, especially since SCF<sup>Slimb</sup> E3 ligase, the fly homologue of  $\beta$ -TRCP2, was found to ubiquitinate condensin-II subunit for subsequent degradation in *Drosophila* (Buster, Daniel et al. 2013). In order to generate the elevated ubiquitination of condensin-II, it was expected that  $\beta$ -TRCP2 would increase after autophagy inhibition. However, my results proved otherwise as the amount of  $\beta$ -TRCP2 remained the same upon autophagy inhibition. Therefore, it is unlikely that  $\beta$ -TRCP2 is the one ubiquitinating condensin-II. However, more evidence is needed to truly eliminate  $\beta$ -TRCP2 as the culprit. For example, the examination of condensin-II's ubiquitination status and protein levels with and without autophagy inhibition after RNAi-mediated depletion of  $\beta$ -TRCP2 will provide convincing evidence to the presence or absence of its involvement.

On the other hand, investigation of DUBs suggested that they might be the ones driving condensin-II's degradation. DUBs have the ability to rescue proteins that are targeted for degradation by altering the ubiquitin conjugates attached to them. As such, I hypothesize that if defective autophagy brings about a decrease in DUBs, it may increase the degradation of condensin-II and Kif4a. As expected, DUBs inhibition via the addition of PR-619 gave rise to a reduced level of condensin-II subunits and Kif4a that was recoverable via the inhibition of proteasomal degradation. This result thus signified that if impaired autophagy does lead to a loss of functional DUBs, it would induce a decrease in protein levels of condensin-II and Kif4a that resembles what we have observed after autophagy inhibition. However, more experiments have to be done to locate the link between autophagy and DUBs in order to verify if they are truly the intermediary proteins that drive condensin-II and Kif4a's degradation following the blockage of autophagy. In addition, it is important to note the possibility that the degradation of condensin-II and Kif4a is the interplay effect exerted by both E3 ligases and DUBs.

## **4.2 Autophagy's regulation on gene expression**

In this study, my findings suggested a role of autophagy in transcriptional regulation of condensins where autophagy inhibition negatively regulated the mRNA levels of condensins. Although autophagy is known to be involved in a plethora of pathways such as development and antigen presentation, this is the first time autophagy is implicated to play a role in regulating gene expression (Mizushima 2007, Cecconi and Levine 2008).

As autophagy is a degradation system, most of these functions are based on its ability to adjust the levels of different proteins and the indirect effects exerted by such regulations. This may be the same for my finding where autophagy's regulation on transcriptional activity of condensins is indirect and most likely involves intermediary proteins whose levels are sensitive to autophagy. However, there are too many possible candidates that may be modulated by autophagy to act on the gene expression of condensins, such as transcription factors, chromatin remodeling complexes and small non-coding RNAs. Therefore, a transcriptome array may be employed for a comprehensive analysis of the human transcriptome in order to measure relative abundance of many different genes at once. This will help to narrow down possible candidates that are regulating condensins in response to impaired autophagy and allow easy identification of genes that are indirectly regulated by autophagy in addition to condensins.

## **4.3 Role of condensins and Kif4 in tumorigenesis**

By having multiple roles in addition to determining the architecture of chromosomes, condensins are implicated in tumorigenesis. Mutations in SMC subunits, SMC2 and SMC4, have been identified in leukemia-lymphoma cell-lines and tumor samples derived from patients suffering from pyothorax-associated lymphoma (Ham, Takakuwa et al. 2007). A study also found an association between the loss of heterozygosity in the chromosomal region containing the Cap-D3 locus and breast cancer (Gentile, Wiman et al. 2001). In addition, the expression of Cap-D3 was associated with reduced tumor recurrence in prostate cancer patients (Lapointe, Malhotra et al. 2008). In support of

condensin-II's role in suppressing tumorigenesis, it was revealed recently that condensin-II subunit, Cap-H2, also functions to drive cell senescence via nuclear reorganization. The knockdown of Cap-H2 was found to inhibit oncogene-induced senescence and thus promoted tumorigenesis (Yokoyama, Zhu et al. 2015).

Similarly, the expression of Kif4a is associated with different types of cancers such as small-cell lung cancer, breast cancer, oral squamous cell carcinomas and cervical cancer. Overexpression of Kif4a was found in these cancers accompanied with enhanced invasiveness of the cancer cells. Furthermore, studies done on Kif4a have revealed its potential as a prognostic biomarker and possible drug target for cancer treatments (Narayan, Bourdon et al. 2007, Taniwaki, Takano et al. 2007, Rouam, Moreau et al. 2010, Minakawa, Kasamatsu et al. 2013).

These taken together with past studies where perturbations to condensins and Kif4a resulted in genomic instability, it is important to elucidate the cellular pathways controlling condensins and Kif4a, such as their upstream regulators and downstream effectors, so as to better understand their involvement in carcinogenesis. As in the case of my study where autophagy is an upstream regulator for condensin-II and Kif4a, it presented new insights as to how they became aberrantly regulated in conditions like prostate and breast cancers. In addition, autophagy-associated proteins may serve as potential biomarkers for prognosis of such cancers.

Although many studies have been done on condensins, this is the first time that they have been shown to be long-lived proteins where condensin-II's levels are regulated through ubiquitination and degradation via the proteasomal pathway. Prior to this, there is only one paper that has found a mode of degradation for condensin-I subunit, Cap-H, which is via caspase 3-mediated cleavage in human cells under conditions of prolonged mitotic arrest (Lai, Wong et al. 2011). With more in-depth understanding of how condensins are regulated, treatments that are based on manipulating pathways involving condensins may be better fine-tuned to avoid unwanted side effects.

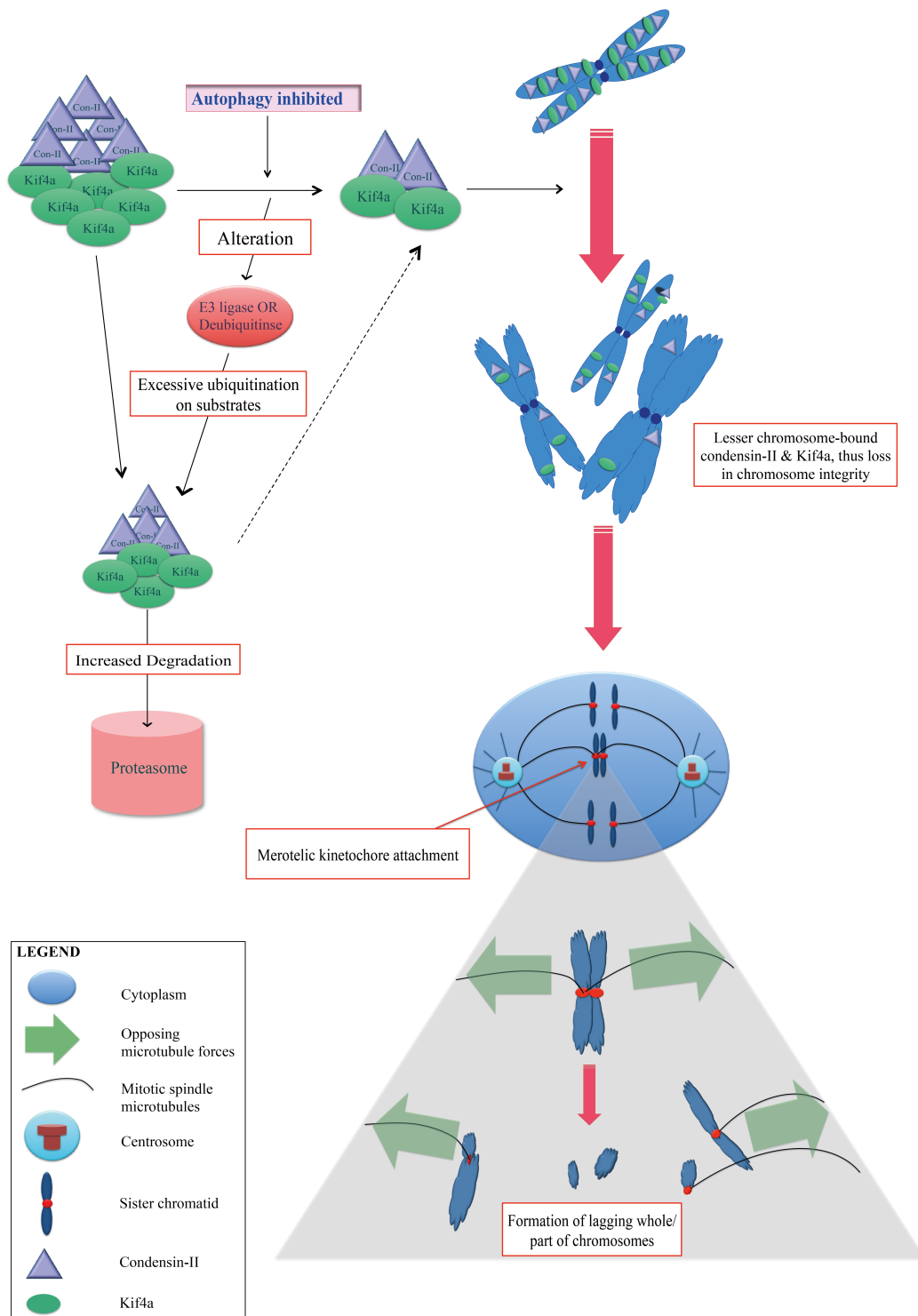
#### **4.4 Role of autophagy in tumorigenesis**

In recent years, there has been increasing interest on autophagy's role in suppressing cancer. Autophagy was found to limit chromosomal instability where defective autophagy in an apoptosis-deficient background resulted in chromosomal loss and gain, centrosome abnormalities and presence of double-minute chromosomes, all of which are hallmarks of cancer. Murine cells with defective autophagy also suffered from greater extent of DNA damage when subjected to metabolic stress (Mathew, Kongara et al. 2007). As such, autophagy is said to limit tumor initiation and progression. Furthermore, autophagy is believed to be necessary for oncogene-induced senescence that serves as a tumor-suppressive mechanism (Young, Narita et al. 2009). This is similar to the proposed role for condensin-II subunit, Cap-H2, to drive oncogene-induced senescence, thus suggesting a possibility where this is achieved with autophagy being the upstream regulator and Cap-H2 being the downstream effector.

Similarly, my study revealed a previously unknown pathway used by autophagy to suppress genomic instability where condensins and Kif4a are the downstream effectors (Fig. 4.3). When autophagy is defective, proteins such as E3 ligases or DUBs are affected which may/may not cooperate to generate elevated ubiquitination of condensin-II. This will then result in excessive degradation of condensin-II. Similarly, this is the most likely scenario for Kif4a, where autophagy inhibition led to a decrease in overall protein level of Kif4a that is achieved via proteasomal degradation. Consequently, such rapid decline in availability of condensin-II and Kif4a will lead to insufficient amount of both proteins that are bound to chromosomes. Since the amount of chromosome-bound kif4a is crucial to maintain the localization of condensin-I and -II, and vice versa, the depletion of both kif4a and condensin-II creates a vicious cycle of reduced localization of both proteins on the chromosomes (Mazumdar, Sundareshan et al. 2004). As such, the structural integrity of chromosomes will most likely be adversely affected. Together with possible defects in kinetochore-microtubule attachments, these eventually increase the occurrence of lagging chromosomes, thus compromising genomic integrity. All in all, such findings will build up our understanding of autophagy's functions and hopefully shed light on unanswered

questions regarding autophagy-related issues such as ageing, neurological disorders and tumorigenesis.

However, there are instances where autophagy is exploited by tumor cells for survival (Degenhardt, Mathew et al. 2006). Solid tumors usually consist of poorly vascularized areas that are hypoxic, filled with damaged organelles and plagued with oxidative stress. Since hypoxia activates autophagy, tumor cells are able to make use of autophagy to remove reactive oxidative species (ROS) together with damaged organelles and the resulting products are used to fuel their growth (Pavlides, Whitaker-Menezes et al. 2009, Martinez-Outschoorn, Whitaker-Menezes et al. 2011, Mah and Ryan 2012). As the mechanisms utilized by autophagy to exert its anti/pro-tumorigenic functions are still largely unknown, it warrants more work to improve our understanding of autophagy, particularly on the cellular events that trigger the switch between its anti- and pro-tumorigenic roles.



**Figure 4.3.** A schematic diagram depicting the proposed model for autophagy's role in maintaining genomic integrity through preventing chromosome mis-segregation.

## **5 CONCLUSION**

Extensive studies have been done on autophagy in recent decades, with an exponential increase in knowledge on the functional importance of this pathway. While many physiological functions performed by autophagy have been identified, detailed understanding of the mechanisms underlying such functions is still inadequate. In particular, autophagy's function in preserving genomic integrity is of paramount importance since chromosomal instability is one of the main driving forces generating the genetic diversity needed to expedite the acquisition of cancer hallmarks. Current findings in my study highlighted the steps taken by autophagy to maintain genomic integrity. This is achieved by monitoring the levels of its substrates such that aberrant degradation is prevented, thereby ensuring proper chromosome segregation. However, more work is needed to identify the functional interplay exerted by autophagy's regulation on its different substrates, in order to navigate the complexity that plagued diseases associated with defective autophagy. As such, it is imperative that studies start focusing on unraveling the exact pathways implicated in each of autophagy's functions.

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