



Regulatory Interaction of the Class III PI3 Kinase Complex and p53

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Regulatory Interaction of the Class III PI3 Kinase Complex and p53

Abstract

Autophagy is a catabolic pathway utilized by cells to maintain homeostasis. Dysregulation of this pathway often leads to various diseases, such as cancers and neurodegeneration. Therefore, autophagy must be tightly regulated by the extracellular environment or signaling pathways. The class III PI3 kinase complex, a lipid kinase complex functioning in converting phosphatidylinositol to phosphatidylinositol-3-phosphate, is a key regulator of autophagy that functions as a signaling hub where multiple regulatory signals converge.

Here, we demonstrate that the class III PI3 kinase complex is negatively regulated by cyclin-dependent kinases (Cdks). The catalytic subunit of the kinase complex, Vps34, is phosphorylated by Cdk1 in mitotic cells and by Cdk5 in postmitotic cells. Phosphorylation on Vps34 results in its dissociation from a regulatory subunit Beclin 1, leading to decreased lipid kinase activity. As a result, autophagy is inhibited in dividing cells and postmitotic neuronal cells with elevated Cdk5 activity. Since dysfunction of autophagy has been shown to be implicated in cancers and neurodegeneration, which are characterized by abnormal activity of Cdk1 and Cdk5, respectively, our study provides a mechanism by which autophagy is modulated in those diseases.

To further discover the regulatory mechanisms of autophagy, we used a novel autophagy inhibitor, spautin-1, identified in a small molecule screening. Spautin-1 inhibits autophagy by inhibiting Usp10/Usp13, which deubiquitinate and stabilize the class III PI3 kinase complex. Interestingly, Usp10/Usp13 are also stabilized by the class III PI3 kinase complex, suggesting that they are reciprocally regulated. These results led us to the observation that p53, a substrate of Usp10 is regulated by the class III PI3 kinase complex and spautin-1. We also report that A70, a more potent derivative of spautin-1, leads to the degradation of mutant p53 through the chaperone-mediated autophagy, whereas the wild-type p53 is degraded by the ubiquitin-proteasome system. Our study demonstrates an important regulatory interaction between the class III PI3 kinase complex and p53, suggesting a novel tumor suppressive function of the class III PI3 kinase complex.

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Chapter 1

Introduction

Autophagy and Its Significance

Overview: Autophagy

Autophagy is a catabolic pathway utilized by cells to recycle intracellular materials in order to generate energy and building blocks for macromolecules. This pathway is well conserved throughout the eukaryotes ranging from yeast to human. There are three different types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (Figure 1.1A-C). In macroautophagy, double membrane structures called autophagosomes engulf some portion of cytoplasm that include cellular components, and deliver them to the lysosomes for degradation. Lysosomes can also engulf small components of the cytosol through the invagination of the membrane, which is called microautophagy (Li *et al.*, 2012). Chaperone-mediated autophagy is distinct from the others in that the substrates of this pathway are escorted to the lysosomes with the help of chaperone protein Hsc70 (heat shock cognate protein 70), and then bind to the lysosomal membrane protein Lamp-2A (lysosomal-associated membrane protein 2A) to translocate (Orenstein and Cuervo, 2010). These pathways share the lysosomal degradation of cellular components in common, whereas the protein machinery involved in the delivery of the components to the lysosomes differs. Among these pathways, macroautophagy (hereafter simply referred to as autophagy) has been most intensively studied thus far.

Although autophagy has been discovered as a survival mechanism under starvation conditions, it plays a crucial role to maintain homeostasis, especially in higher eukaryotes (Mizushima *et al.*, 2008). Protein aggregates that can no longer be degraded by proteasomes, damaged organelles, and intracellular pathogens can be cleared by autophagy. Therefore,

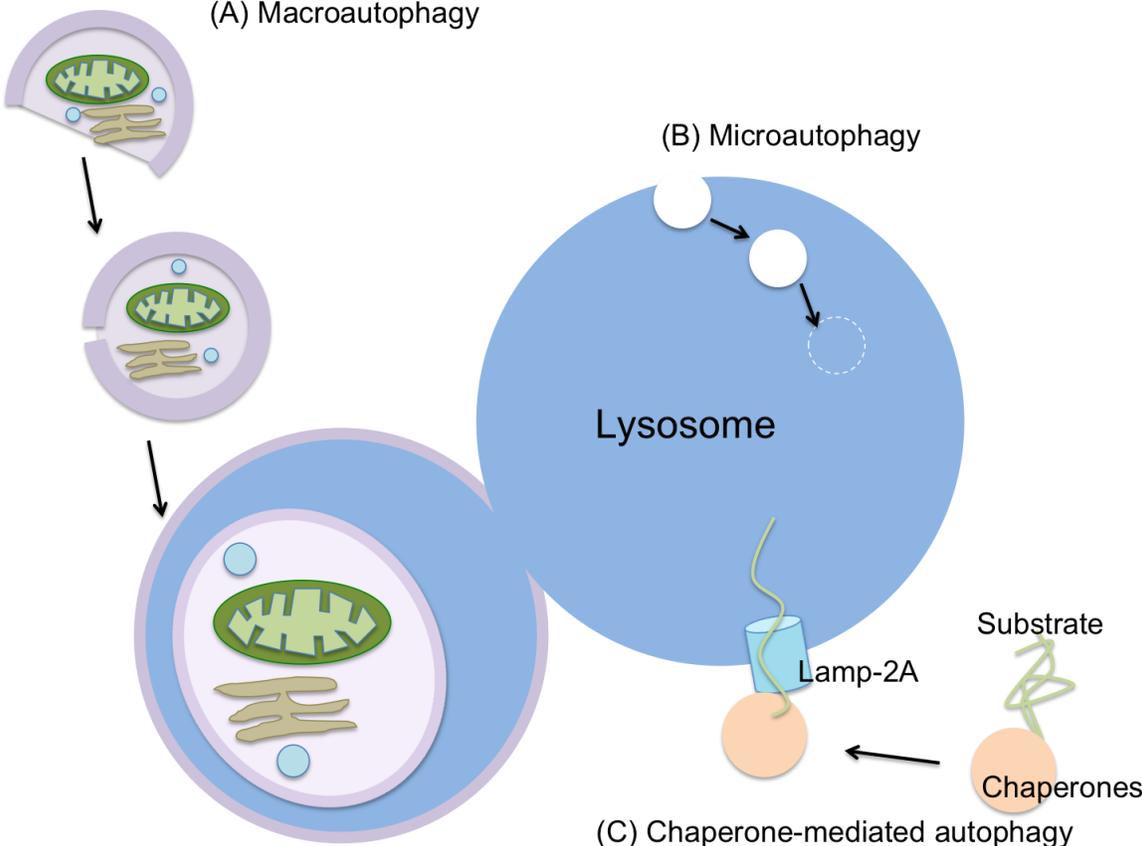
Figure 1.1. Three Different Types of Autophagy

(A) In macroautophagy, bulky cytoplasmic components such as cellular organelles can be enclosed by a double membrane structure. After the membrane is elongated and closed, the outer membrane of the autophagosome is fused with lysosome, and the cargoes of the autophagosome including the inner membrane are degraded by the lysosomal hydrolases.

(B) In microautophagy, lysosomal membrane is invaginated, capturing a small portion of cytosol that mostly consists of soluble components. Engulfed vesicle is then degraded by lysosomal hydrolases.

(C) In chaperone-mediated autophagy, substrates with specific targeting sequence are recognized by Hsc70 and co-chaperones. The chaperones then bind to Lamp-2A to mediate the translocation of unfolded substrates into the lysosome.

Figure 1.1 (Continued).



dysregulation of this pathway may lead to the accumulation of pathogenic materials in the cells and cause various diseases. For example, impairment of basal level autophagy in the brain results in the accumulation of protein aggregates and neurodegeneration in mice (Hara *et al.*, 2006; Komatsu *et al.*, 2006). *Drosophila* mutants that carry autophagy deficiency show increased susceptibility to the infection by certain pathogens (Shelly *et al.*, 2009; Yano *et al.*, 2008). Moreover, autophagy is required for proper differentiation and development process in multicellular organisms (Mizushima and Levine, 2010).

However, over-activation of autophagy may lead to deleterious consequences. Excess autophagy has been shown to induce muscle wasting and implicated in muscle atrophy (Mammucari *et al.*, 2007; Zhao *et al.*, 2007). Moreover, autophagosomes can be used for some pathogens such as hepatitis B virus and coronaviruses to replicate (Sir *et al.*, 2010; Reggiori *et al.*, 2010). Therefore, understanding the mechanism of autophagy is important for developing methods to modulate autophagy for therapeutic purposes.

Autophagosome Formation

The mechanism of autophagy can be divided into different stages: nucleation, elongation, closure, and recycling (Levine and Yuan, 2005; Codogno *et al.*, 2011). Each stage is carried out by different sets of genes. The genes involved in autophagy have first been identified in autophagy-deficient yeast mutants, and they are highly conserved in mammals (Tsukada and Ohsumi, 1993). In mammals, mTOR (mammalian target of rapamycin) and AMPK (AMP-activated protein kinase) are regulated by the nutrients around the cell, and phosphorylates ULK1 (unc-51-like kinase 1), which is a positive regulator of autophagy (Kim *et*

al., 2011). When the cells are under nutrients-deprived conditions, AMPK is activated and phosphorylates Ser317/Ser777 on ULK1 to activate it. In contrast, under rich nutrition, mTOR is activated and phosphorylates Ser757 on ULK1 to inactivate it. Active mTOR also disrupts the regulation of ULK1 by AMPK. This regulation describes how autophagy can be triggered by orchestrated nutrition-dependent signaling pathways.

Upon induction of autophagy, autophagosomal membrane, derived from various intracellular membrane sources, forms a structure called phagophore. Endoplasmic reticulum (ER), Golgi, mitochondria, and even plasma membranes have been reported as membrane donors for autophagosome, although phagophore formation takes place at the close distance from ER (Mizushima *et al.*, 2011). Activated ULK1, together with its binding partners such as Atg13, FIP200 (focal adhesion kinase family interacting protein of 200kDa), and Atg101, translocates to ER, and positively regulates the class III phosphatidylinositol-3-kinase (PI3 kinase) complex (Ganley *et al.*, 2009; Jung *et al.*, 2009; Chang and Neufeld, 2009; Hosokawa *et al.*, 2009). Conversion of PI to PI3P is a key event in the nucleation step of autophagy, and PI3P recruits DFCP1 (double FYVE-containing protein 1) and WIPI (WD repeat domain phosphoinositide-interacting protein) proteins which contain domains specifically recognizing PI3P over other phosphoinositides to promote the phagophore formation (Matsunaga *et al.*, 2010; Polson *et al.*, 2010).

Once the autophagosome is nucleated, vesicle elongation is mediated by the ubiquitin-like conjugation system. LC3 (microtubule-associated protein 1 light chain 3) and Atg12 are ubiquitin-like proteins conjugated to E1-like Atg7 and transferred to E2-like Atg10 and Atg3, respectively. Atg12 is then conjugated to the substrate, Atg5, which is bound to Atg16L1 to

form an 800kDa oligomeric complex. The complex functions as an E3-like enzyme to link LC3 protein to phosphatidylethanolamine (PE), which is used as a marker of autophagy (Noda *et al*, 2008). Lipidated LC3 (LC3-II) is separatable from cytosolic LC3 (LC3-I) by SDS-PAGE. Moreover, LC3-II appears as puncta under fluorescence microscope, whereas LC3-I is diffused in the cytosol. The mammalian paralogues of LC3, such as GABARAP (g-aminobutyric acid receptor-associated protein) isoforms, are expressed as precursors, which require Atg4 cysteine protease for activation (Kabeya *et al.*, 2004). Autophagosomes are decorated with the LC3 family both on inner and outer membrane, and inhibition of LC3 lipidation by knockout of any conjugating enzyme or expressing dominant-negative form of Atg4 leads to the accumulation of abnormal phagophores, suggesting that LC3 lipidation is a crucial step for the expansion and closure of the autophagosome (Fujita *et al.*, 2008). Moreover, LC3 binds to the adaptor proteins that specifically escort cargoes to the autophagosome. Among the adaptors, p62/SQSTM1 (sequestosome 1) and NBR1 (neighbor of BRCA1 gene 1) have the ubiquitin-associated domain that allows the proteins to recognize ubiquitinated species and target them to the autophagosome (Lamark *et al.*, 2009; Kirkin *et al.*, 2009). Nix (also known as BNIP3L) mediates autophagic degradation of mitochondria by physically linking the mitochondria to GABARAP (Novak *et al.*, 2010; Schwarten *et al.*, 2009). The presence of the adaptors is thought to facilitate selective autophagic degradation of polyubiquitinated protein aggregates and damaged organelles over normal cytoplasmic constituents.

After the closed autophagosome is formed, the outer membrane of autophagosome is fused with the lysosome to form autolysosome. Then, lysosomal hydrolases, such as proteases and nucleases, degrade the cargoes inside the autophagosome as well as the inner membrane.

At the termination step of autophagy, lysosomal membrane generates small tubules and vesicles, which in turn produce new lysosomes (Yu *et al.*, 2010). This is an important step to maintain lysosomal homeostasis.

Class III PI3 Kinase Complexes

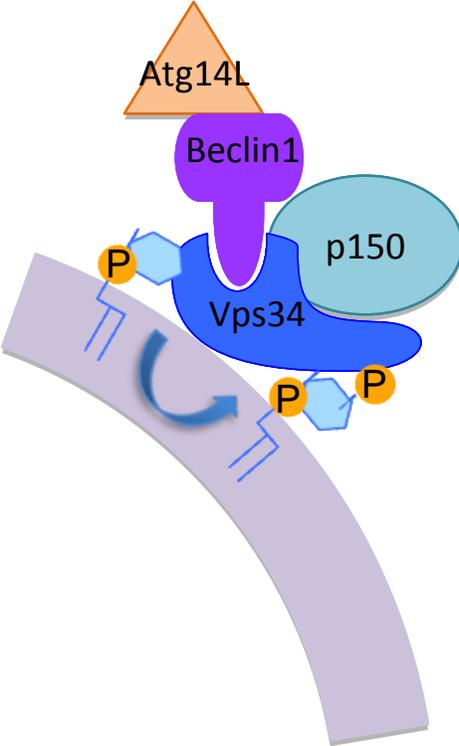
The class III PI3 kinase was first discovered in screening of yeast cells defective in vacuolar protein sorting and named Vps34 (Herman and Emr, 1990). However, the function of Vps34 had not been known until the class I PI3 kinase was identified as a lipid kinase (Hiles *et al.*, 1992). The catalytic subunit of class III PI3 kinase is homologous to Vps34, suggesting that Vps34 is also a lipid kinase. More importantly, the lipid kinase activity of Vps34 is required for its function in vacuolar protein sorting (Schu *et al.*, 2003). Different classes of PI3 kinases have different substrate specificities. Vps34 preferentially phosphorylates PI to produce PI3P, whereas class I PI3K prefers PI4,5P₂ to generate PI3,4,5P₃ (Vanhaesebroeck *et al.*, 2001). While Vps34 is the sole PI3 kinase in yeast, different classes of PI3 kinases exist in mammalian cells, rendering it more difficult to study the function of Vps34 genetically and biochemically. Moreover, different kinds of PIPs and lipid phosphatases imply that there will be multiple pathways to generate PI3P.

Vps34 forms different complexes with different partners to be functionally active. In yeast, the binding partners of Vps34 determine the functions of the complex, such as endosomal sorting and autophagy, and many of the binding partners are conserved in mammals (Figure 1.2). Vps15, a membrane anchored regulatory subunit of Vps34 is required for the activity of Vps34 (Stack *et al.*, 1995). Although p150 has been identified as a Vps15

Figure 1.2. The Class III PI3 Kinase Complex

In the initiation step, the class III PI3 kinase complex plays a crucial role. Vps34, the catalytic subunit of the kinase complex, converts PI, a kind of membrane phospholipids, to PI3P by phosphorylating the hydroxyl group on the 3rd carbon position of the inositol ring. This step is required to recruit the downstream effectors that specifically bind to the PI3P. For its kinase activity, Vps34 requires regulatory subunits, such as Beclin 1, p150, and Atg14L, altogether forming the class III PI3 kinase complex. Although there are more known subunits that bind to the kinase complex, and multiple complexes with different combination of the subunits exist, they are not shown in this figure.

Figure 1.2 (Continued).



homologue in mammalian cells, its role still remains elusive (Panaretou *et al.*, 1997). p150 is a protein kinase with a N-terminal myristoylation consensus sequence. p150 is composed of N-terminal kinase domain followed by multiple HEAT motifs and WD40 domain repeats which are known to be involved in protein interaction. In yeast, the HEAT domains of Vps15 are shown to interact with the C-terminal conserved sequence of Vps34 (Budovskaya *et al.*, 2002). However, whether this interaction is conserved in mammals is not known. Vps15 seems to play a key role in multicellular organisms as well. In flies, Vps15 homologue ird1 is required for removal of protein aggregates and antibacterial immunity (Lindmo *et al.*, 2008; Wu *et al.*, 2006).

Vps30/Atg6, another subunit of Vps34 complex in yeast, is involved in both vesicular trafficking and autophagy. Beclin 1, a mammalian homologue of Vps30/Atg6, is a haplo-insufficient tumor suppressor, which is first identified as a Bcl-2 interacting protein with its BH3 domain (Liang *et al.*, 1998). Since Beclin 1 interacts with Bcl-2/Bcl-xL, Beclin 1 functions as a mediator of crosstalk between apoptosis and autophagy (Levine *et al.*, 2008). Moreover, BH3 domain of Beclin 1 implies that other BH3 containing proteins can also be involved in autophagy as well as apoptosis by outcompeting and liberating Beclin 1.

In addition to p150 and Beclin 1, other mammalian counterparts of the yeast Vps34 complex have been identified. Atg14L, a homologue of yeast Atg14, is a positive regulator of autophagy, and recognizes the membrane curvature of the phagophore (Fan *et al.*, 2011). UVRAG (UV radiation resistance associated gene), a homologue of yeast Vps38, is involved in the later step of autophagy and endosomal trafficking. In contrast to yeast, mammals have Rubicon, as a negative regulator of autophagy (Itakura *et al.*, 2008; Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). Moreover, there are other known regulators of autophagy in human cells

such as Ambra1 (activating molecule in Beclin 1-regulated autophagy) and Bif-1 (bax-interacting factor-1), which are also required (Fimia *et al.*, 2007; Takahasi *et al.*, 2007). However, the detailed molecular mechanisms by which these subunits contribute to autophagy, and their regulation have yet to be studied.

In Chapter 2, we report that the class III PI3 kinase complex is regulated by the cyclin-dependent kinases (Cdks). Using quantitative microscopy as well as biochemical studies, we demonstrate the regulation of the catalytic subunit Vps34 by two different Cdks, namely Cdk1 and Cdk5. Since Cdk1 and Cdk5 primarily function in dividing cells and post-mitotic neuronal cells, respectively, our work describes how autophagy is regulated in different cell types.

Autophagy and Cell Death

Although autophagy plays a protective role for cell survival, it has also been known to be involved in specialized type of cell death, namely autophagic cell death. Autophagic cell death, also known as type II cell death, was first defined by the morphology of various dying cells (Clarke, 1990). In many dying cells during development, large portions of cytoplasm are filled with double or multiple membrane vesicles. However, autophagy is also induced in other types of cell death, such as apoptosis or necrosis, there is growing opinion that meaning of autophagic cell death should be narrowed down to indicate cell death caused by autophagy. In this case, autophagic cell death is inhibited, when autophagy genes are down-regulated or autophagy inhibitors are used.

Autophagic cell death is thought to happen under defined context in specific cell types rather than a general phenomenon, and it is best studied in developing insect cells. For

example, removal of salivary glands during development in flies is inhibited by mutations in autophagy genes (Berry and Baehrecke, 2007). Germline cell death during oogenesis in female flies is also ablated by similar mutations (Nezis *et al.*, 2009). In mammals, autophagic cell death is well understood in mouse fibroblast L929 cells. When caspase-8 is inhibited, autophagy is induced to selectively degrade catalase, leading to the accumulation of reactive oxygen species (ROS) and cell death (Yu *et al.*, 2004; Yu *et al.*, 2006). Inhibition of autophagy by treatment of 3-methyladenine (3-MA) that blocks the kinase activity of Vps34 reduces this cell death. Consistently, knockdown of autophagy genes leads to similar results. This autophagic cell death also involves the secretion of tumor necrosis factor α (TNF α), which is dependent on the kinase activity of RIPK1 (receptor interacting protein kinase 1) and an E3 ligase EDD (Christofferson *et al.*, 2012). However, the mechanism by which death signals such as pan-caspase inhibitor zVAD or TNF α induce autophagy is still elusive.

In the Appendix I, I discuss the interaction between RIPK1/EDD complex and the class III PI3 kinase complex. Although the detailed mechanism has not been demonstrated, the interaction and the E3 ligase activity of EDD on UVRAG, one of the subunits of the class III PI3 kinase complex, suggest that autophagy is directly regulated by RIPK1/EDD complex rather than indirectly induced by metabolic stress which is caused during the cell death pathway.

Autophagy and Cancer

In cancers, autophagy plays a dual role depending on the stage or the nature of the cancers. In some cases, autophagy functions as a tumor suppressive mechanism, especially in the early stage of tumorigenesis. As discussed above, autophagy is important for the quality

control of the cells such as removing damaged mitochondria. Under metabolic stress, cells are more likely to have defects on the mitochondria, leading to increased levels of ROS which result in the modification of proteins and nucleic acids and eventually tumorigenesis. Consistently, cells with reduced autophagy are more sensitive to metabolic stress (Karantza-Wadsworth *et al.*, 2007). They acquire more DNA damage and genomic instability than normal cells.

Moreover, autophagy-defective tumor cells have accumulated p62/SQSTM1, which is an adaptor for ubiquitinated proteins as well as a substrate for autophagy itself (Mathew *et al.*, 2009). Surprisingly, p62 has been shown to regulate cell defense mechanism at the transcriptional level, which may also be involved in tumorigenesis (Komatsu *et al.*, 2010).

Consistent with the observation that autophagy protects from tumor formation, many of the autophagy genes have been reported as tumor suppressor genes, and they are frequently mutated in various types of cancers. Among them, Beclin 1 is most widely studied and has been shown to be a haploinsufficient tumor suppressor (Qu *et al.*, 2003; Yue *et al.*, 2003). It is often monoallelically deleted in some types of human cancers, such as breast and ovarian cancers, and Beclin 1 heterozygous mice spontaneously develop tumors. Since the deletion of Atg7 only leads to the formation of benign tumors in the liver (Takamura *et al.*, 2011), the aggressive tumor development as a result of mutation in subunits of the class III PI3 kinase complex, e.g. the tumor phenotype of Beclin 1 heterozygosity, cannot be explained by autophagy deficiency alone. This may suggest that Atg5/Atg7-independent autophagy plays a role in tumor suppression, or the subunits of the class III PI3 kinase complex have a function totally separate from the PI3 kinase activity (Nishida *et al.*, 2009). Notably, UVRAG is shown to

contribute to chromosomal stability by promoting DNA double strand break repair, as well as assisting proper chromosome segregation (Zhao *et al.*, 2012).

However, once the tumors progress to a certain stage, autophagy can promote the survival and resistance of the tumors. Since cancer cells reside in a microenvironment that provides cells with limiting nutrients and oxygen, utilization of autophagy to generate energy for cell survival is crucial. Moreover, most chemotherapeutic agents specifically target cancer cells by inducing cytotoxicity to highly proliferative cells, suggesting that autophagy as a cellular defense mechanism may reduce the effect of such treatments. Therefore, inhibition of autophagy by using small molecules or knockdown of autophagy genes often causes cancer cell death. In tumors with oncogenic Ras, defective autophagy reduces tumorigenicity (Guo *et al.*, 2011). Similarly, autophagy inhibitors such as chloroquines are being tested for combination treatment with currently known anti-cancer drugs (White and DiPaola, 2009). Interestingly, autophagy has also been shown to contribute to the aggressiveness of the cancers by facilitating metastasis (Fung *et al.*, 2008). Nevertheless, inducing cancer cell death by blocking autophagy is not always beneficial because apoptosis-incompetent cells undergo necrotic cell death, leading to further proliferative environment by triggering pro-inflammatory responses (Degenhardt *et al.*, 2006).

As discussed above, the effect of autophagy on cancers is dependent on multiple factors such as the microenvironment, stage, and genetic background. Thus, autophagy targeting therapeutic interventions must be considered carefully, and preceded by the studies to identify the nature of the cancers to be treated.

Chaperone-mediated Autophagy

Chaperone-mediated autophagy (CMA) is distinct from other types of autophagy in that CMA does not involve membrane invagination. The substrate proteins for CMA are targeted to the lysosome directly by binding to the chaperone Hsc70 and co-chaperones through the recognition sequences (KFERQ or biochemically related sequences) on the substrates (Chiang *et al.*, 1989). This suggests that CMA has the highest selectivity among different types of autophagy. Then, the lysosomal membrane protein Lamp-2A binds to the substrates to translocate them into the lysosomes where the substrates are degraded (Cuervo and Dice, 1996).

CMA has been reported to be activated after prolonged starvation or oxidative stress (Cuervo *et al.*, 1995; Kiffin *et al.*, 2004). It is also constitutively active when macroautophagy is compromised (Kaushik *et al.*, 2008). More importantly, CMA is implicated in proteotoxicity caused by adverse alterations or modifications of proteins. Conversely, excess modifications or aggregation of proteins prevent the substrate proteins from being degraded by CMA. For example, mutation on α -synuclein impairs its degradation by CMA, which at least in part, contributes to the pathogenesis in Parkinson's disease (Cuervo *et al.*, 2004). Interestingly, artificial delivery of mutant huntingtin to the lysosomes using CMA ameliorates the disease phenotype of Huntington's disease (Bauer *et al.*, 2010). Notably, the activity of CMA declines with age, suggesting that the age-dependent increasing incidence of neurodegenerative diseases may be attributed to decreased protein quality control mechanism by CMA (Kiffin *et al.*, 2007).

p53 as a Tumor Suppressor

Function and Regulation of p53

p53 was identified as a binding protein of simian virus 40 (SV40) large T-antigen in mediating the transformation of normal cells to tumors (Lane and Crawford, 1979; Linzer and Levine, 1979). For a long time, p53 was believed to be oncogenic due to its elevated expression in transformed cells (Sarnow *et al.*, 1982). Consistent with an oncogenic role, several clones of p53 actually enhanced tumorigenesis (Eliyahu *et al.*, 1984; Jenkins *et al.*, 1984; Parada *et al.*, 1984). However, the tumor suppression role of wild-type p53 was revealed after extensive molecular biology and genetic studies. Consistently, deletion mutations in p53 gene were found in cancers (Baker *et al.*, 1989). Overexpression of wild-type p53 suppressed tumor formation induced by other known oncogenes such as Ras and Myc (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989). Most importantly, loss of p53 in mice results in spontaneous tumor formation, suggesting that p53 is a *bona fide* tumor suppressor (Donehower *et al.*, 1992).

Extensive studies have demonstrated that p53 functions as a guardian of the genomic stability. Under normal conditions, the activity of p53 remains low. However, when the cells are under various stresses such as genotoxic stress or oxidative stress, p53 becomes activated and mediates multiple stress responses. The most intensively studied function of p53 is as a transcription factor with the DNA-binding domain flanked by N-terminal transactivation domain and C-terminal regulatory domain. Since p53 was shown to bind to the specific sequence of DNA, many target genes have been identified including p21 that is a cyclin-dependent kinase inhibitor (Menendez *et al.*, 2009). Notably, many target genes of p53 are involved in cell cycle arrest, senescence, and programmed cell death.

The function of p53 is not limited in the nucleus. It also plays an important role in the cytoplasm, contributing to it being a tumor suppressor. Interestingly, in addition to activating the expression of apoptotic genes, p53 directly regulates the execution of apoptosis in the cytoplasm. When p53 is accumulated in the cytosol, it is localized to the mitochondria and induces permeabilization of the outer membrane (Mihara *et al.*, 2003). p53 also activates proapoptotic proteins that are sequestered by antiapoptotic proteins under normal condition (Chipuk *et al.*, 2004). In addition to the proapoptotic functions, cytoplasmic p53 is involved in centrosome duplication to ensure proper chromosome segregation. In the absence of p53, multiple centrosomes are generated in a single cell cycle, leading to chromosomal instability (Fukasawa *et al.*, 1996).

Decades of studies have demonstrated the mechanisms by which p53 is regulated. p53 is constitutively ubiquitinated and rapidly degraded through the proteasome under normal condition. Although this regulatory mechanism was first shown by viral oncogene, several endogenous regulators have also been reported (Scheffner *et al.*, 1993). Among the different regulators of p53, Mdm2 (mouse double-minute 2) has been best characterized. Mdm2 gene was amplified in a spontaneously transformed cell line, suggesting its oncogenic property (Fakharzadeh *et al.*, 1991). Molecular studies has revealed that Mdm2 physically interacts with p53 and inhibits its activity (Momand *et al.*, 1992; Oliner *et al.*, 1992). Moreover, this inhibition is attributed to the ubiquitination-dependent degradation of p53 due to the function of Mdm2 as an E3 ligase for p53 (Haupt *et al.*, 1997; Kubbutat *et al.*, 1997; Honda *et al.*, 1997). Interestingly, low levels of Mdm2 mediate monoubiquitination of p53 that leads to its nuclear export, whereas high levels of Mdm2 promote polyubiquitination and degradation (Li *et al.*,

2003). Furthermore, the expression of Mdm2 is positively regulated by p53, generating a negative feedback loop (Barak *et al.*, 1993; Wu *et al.*, 1993). Therefore, when p53 is activated, Mdm2 expression increases to maintain the levels of p53 low after the cell overcomes the stress and no longer requires p53. In contrast, Mdm2 regulates p53 by monoubiquitination under normal condition. Regulation of p53 by Mdm2 is indispensable for proper embryonic development, and loss of Mdm2 results in embryonic lethality that can be rescued by loss of p53 (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995).

In contrast to the low levels of p53 in normal conditions, p53 protein is highly accumulated upon genotoxic stress, such as DNA damage induced by UV radiation. This accumulation is achieved either by inhibition of ubiquitination or by inducing deubiquitination. After DNA damage, the posttranslational modifications on p53 inhibit ubiquitination by Mdm2. For example, acetylation on the C-terminal lysine residues of p53 blocks ubiquitination because acetylated lysines can no longer serve as substrates for ubiquitination (Li *et al.*, 2002). In the same sense, deacetylation of p53 promotes its degradation by Mdm2-mediated ubiquitination (Ito *et al.*, 2002). In addition to acetylation, phosphorylation of p53 on its N-terminus also leads to the activation by inhibiting the interaction with Mdm2 (Shieh *et al.*, 1997). Moreover, p53 can be stabilized by deubiquitination, which is mediated by multiple deubiquitinases (DUBs). HAUSP (herpes virus-associated ubiquitin specific protease) regulates p53 activity by deubiquitinating both p53 and Mdm2 depending on the expression levels (Li *et al.*, 2004). Usp10 is activated by DNA damage and stabilize p53 by deubiquitination (Yuan *et al.*, 2010). Therefore, the stability and activity of p53 is systematically regulated by coordinated modifications and/or removal of modifications.

Regulation of Autophagy by p53

As a protective mechanism that helps cells deal with different kinds of stress, autophagy is regulated by p53. Interestingly, p53 can both activate and suppress autophagy depending on its cellular localization and mediators. Many of the genes transcriptionally activated by p53 are inducers of autophagy. Among the target genes is ULK1 that is required for autophagy (Gao *et al.*, 2011). Upon DNA damage, p53 directly activates the transcription of ULK1, which in part mediates p53-dependent DNA damage responses. p53 also negatively regulates mTOR signaling pathway, resulting in the activation of autophagy (Feng *et al.*, 2005). Notably, p53-dependent transcription of Sestrin1 and Sestrin2 has been reported to activate AMPK (Budanov and Karin, 2008). Activated AMPK then phosphorylates and activates TSC2 (tuberous sclerosis protein 2) that inhibits mTOR. Loss of Sestrin2 diminishes the ability of p53 in inhibiting the activity of mTOR, suggesting that Sestrin2 is the major mediator of p53-dependent mTOR regulation.

Moreover, p53 activates the transcription of many proapoptotic genes that also induce autophagy. Since Beclin 1 is a BH3-only protein that binds to Bcl-2/Bcl-xL, other BH3-only proteins can induce autophagy by liberating Beclin 1 from Bcl-2/Bcl-xL (Maiuri *et al.*, 2007). DRAM (damage-regulated autophagy modulator) is another example of autophagy-inducing proapoptotic gene transcriptionally activated by p53 (Crighton *et al.*, 2006). DRAM is a lysosomal protein that is required for DNA damage induced autophagy, and loss of DRAM leads to reduced apoptotic cell death induced by DNA damage. Therefore, consistent with its stress-responsive roles, p53 functions as a positive regulator of autophagy regarding its transcriptional activity.

Paradoxically, genetic deletion, siRNA-mediated knockdown, and pharmacological inhibition of p53 all result in induction of autophagy (Tasdemir *et al.*, 2008). Rescuing experiments of the activity of p53 in different subcellular compartments have shown that cytoplasmic p53 inhibits autophagy, in contrast to that of the nuclear p53. p53 also negatively regulates autophagy by reducing the levels of mature LC3B mRNA after prolonged starvation (Scherz-Shouval *et al.*, 2010). These results suggest that wild-type p53 of physiological expression level inhibits autophagy, and either positive or negative regulation of p53 leads to increased autophagy. Since p53, as a stress sensor of the cell, may be perturbed in different ways upon various types of stress, this provides a coordinated crosstalk between p53 and autophagy to relieve cellular stress or induce programmed cell death.

Mutant p53

Since p53 functions as a tumor suppressor, mutations on p53 are often implicated in tumorigenesis. Either as a driving mutation or by increased genomic instability, p53 is affected in nearly half of human cancers (Hollstein *et al.*, 1991). However, in contrast to most of other tumor suppressors, the majority of the p53 mutations are missense mutations caused by single base substitutions (Petitjean *et al.*, 2007). Thus, although loss of p53 tumor suppressor function promotes oncogenesis, the missense mutations frequently occurring in p53 may provide a gain-of-function as a strong positive selection to further facilitate tumor development (Brosh and Rotter, 2009). Several lines of evidence report that there are multiple effects of p53 mutations (Figure 1.3A-C). In additions to the lack of wild-type p53 activity, mutant p53 contributes to tumor progression in various ways (Kato *et al.*, 2003). When some mutant p53 proteins are co-

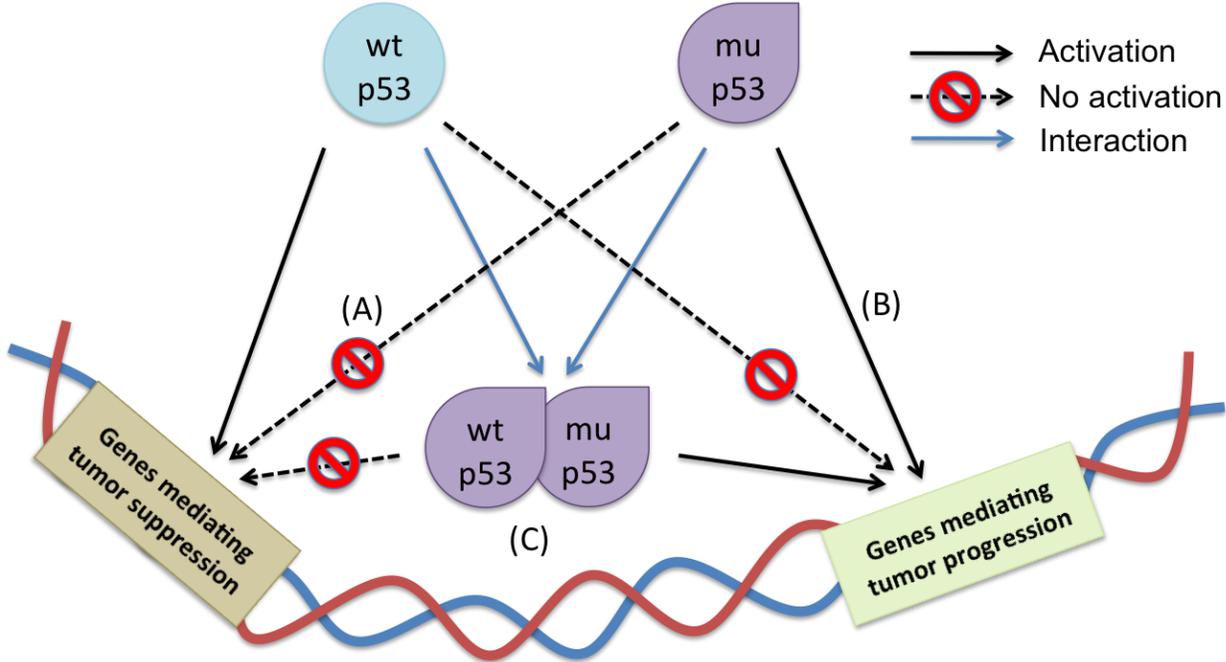
Figure 1.3. The Effects of Mutations on p53

(A) Loss of function: Mutant p53 (labeled as mu p53) does not bind to the region of DNA where the wild type (labeled as wt p53) normally binds, and thus has no ability to activate the target genes of wild-type p53 involved in tumor suppression.

(B) Gain of function: Mutant p53 can bind to the region of DNA where the wild type does not bind, possibly activating or repressing the expression of a different set of genes from the wild-type p53, which contributes to the oncogenic property of the mutant p53.

(C) Dominant-negative effect: Mutant forms of p53 have different conformation from the wild type, which can explain the functions of mutant p53 discussed in (A) and (B). Moreover, some of the mutations on p53 can change the conformation of wild type to that of mutants, when expressed together. Therefore, when these mutations are generated in one copy of the genome leaving the other copy unaffected, the mutant p53 can repress the activity of functional p53.

Figure 1.3 (Continued).



expressed with the wild-type p53, the mutant interacts with the wild type to change its conformation in a cooperative manner into the conformation of the mutant p53 (Milner and Medcalf, 1991). The mutant p53 is aggregated using the nucleating sequence in the DNA-binding domain, and it is co-aggregated with the wild-type p53 (Xu *et al.*, 2011). This aggregation resembles amyloid structures that prion proteins generate (Ano Bom *et al.*, 2012). Conversely, wild-type p53 can function as a chaperone for the mutant p53, maintaining a dynamic equilibrium between wild type and mutant p53 (Gogna *et al.*, 2012). This suggests that monoallelic mutation of p53 can result in complete loss of wild-type p53 activity. Moreover, increased mutation rate in cancers often leads to the mutation on remaining wild-type p53 allele, which is another mechanism of tumor progression by p53 mutation. Surprisingly, mutations on p53 can also render it gain novel oncogenic properties. For example, mutant p53 can activate the transcription of genes involved in cell proliferation, such as TERT (telomerase reverse transcriptase) and Myc (Scian *et al.*, 2004; Frazier *et al.*, 1998). Furthermore, it interacts with other cytosolic proteins that promote tumorigenesis. Therefore, mutant p53 reorganizes the architecture of the cells by regulating a variety of targets in an orchestrated manner.

Mutant p53 is often accumulated in cancer cells due to its inability to be degraded by Mdm2 (Lukashchuk and Vousden, 2007). Neither does it activate the transcription of Mdm2, suggesting that the negative feedback loop between p53 and Mdm2 does not exist for mutant p53, in contrast to the wild type. In normal tissues, mutant p53 is not significantly accumulated, as shown in mutant p53 knock-in mice (Lang *et al.*, 2004; Olive *et al.*, 2004). However, loss of Mdm2 leads to the accumulation of mutant p53 both in normal and cancer cells, raising the possibility that the mutant p53 is regulated differently in cancer cells compared with normal

cells. Due to its conformational properties, the stability of mutant p53 has been known to be regulated by chaperone proteins. Hsp90 (heat shock protein 90) contributes to the stability of mutant p53, and inhibition of Hsp90 results in reduced levels of mutant p53 (Lin *et al.*, 2008). The mutant p53 is also known to be associated with other chaperones, such as Hsc70 and Hsp84 (Hinds *et al.*, 1987; Sepehrnia *et al.*, 1996). These results suggest that the regulation of the mutant p53 may be dependent on the cellular quality control.

In Chapter 3, we utilize a small molecule inhibitor of autophagy to demonstrate the regulation of p53. Using the compound, we show that the class III PI3 kinase complex and p53 is coordinately regulated through deubiquitination. Interestingly, mutant p53 is also regulated by autophagy inhibitor, but the mode of regulation may be unique, involving chaperone-mediated autophagy.

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Chapter 2

Negative Regulation of Vps34 by Cdk Mediated Phosphorylation

Attribution of experimental contributions

The work presented in Chapter 2 has been published in *Molecular Cell* 38, 500–511. The contents of the publication are presented here as published but in dissertation format.

Tsuyoshi Furuya performed Figure 2.2A, 2.3-2.5, 2.6B-C, and 2.7A-C.

Marta Lipinski performed Figure 2.1.

Minsu Kim performed all other experiments, did two rounds of revision.

Junying Yuan supervised all of the work.

Other co-authors contributed comments or reagents, but did not perform experiments.

Negative Regulation of Vps34 by Cdk Mediated Phosphorylation

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Summary

Vacuolar protein sorting 34 (Vps34) complexes, the class III PtdIns3 kinase, specifically phosphorylate the D3 position of PtdIns to produce PtdIns3P. Vps34 is involved in the control of multiple key intracellular membrane trafficking pathways including endocytic sorting and autophagy. In mammalian cells, Vps34 interacts with Beclin 1, an ortholog of Atg6 in yeast, to regulate the production of PtdIns3P and autophagy. We show that Vps34 is phosphorylated on Thr159 by Cdk1, which negatively regulates its interaction with Beclin 1 during mitosis. Cdk5/p25, a neuronal Cdk shown to play a role in Alzheimer's disease, can also phosphorylate Thr159 of Vps34. Phosphorylation of Vps34 on Thr159 inhibits its interaction with Beclin 1. We propose that phosphorylation of Thr159 in Vps34 is a key regulatory mechanism that controls the class III PtdIns3 kinase activity in cell-cycle progression, development, and human diseases including neurodegeneration and cancers.

Introduction

Vacuolar protein sorting 34 (Vps34), a class III PtdIns3 kinase (phosphatidylinositol 3-kinase), was first identified as a regulator of vacuolar hydrolase sorting in yeast (Herman and Emr, 1990). Vps34 specifically phosphorylates the D-3 position on the inositol ring of phosphatidylinositol (PtdIns) to produce PtdIns3P (Schu *et al.*, 1993). In yeast, Vps34 is present in two complexes that are involved in the regulating autophagy (complex I) and vacuolar protein sorting (complex II) (Kihara *et al.*, 2001b). In mammalian cells, Vps34 is present in multiple protein complexes that include regulatory proteins Beclin 1 and p150 as well as one or more of the following proteins: Atg14L, UVRAG, and a negative regulator Rubicon (Itakura *et al.*, 2008; Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). Dynamic regulation of Vps34 complexes may provide an important regulatory mechanism to control multiple vesicular trafficking pathways. Although the class III PI3 kinase has been recognized to play an important role in regulating many important intracellular and extracellular signaling events in mediating membrane trafficking including endocytosis and autophagy, we still know very little about the molecular mechanisms that regulate the interaction of Vps34 with its partners.

Cyclin-dependent kinases (Cdks) are critical regulators of multiple cellular processes that include cell-cycle progression, development, and intracellular signaling in response to external stimuli. Their activity is tightly regulated and restricted to specific stages of the cell cycle. Cdk5, which is closely related to Cdk1 but not a part of the core cell-cycle machinery, normally functions during the development of nervous systems by regulating neuronal migration and neuritic outgrowth as well as neurotransmitter signaling in the mature nervous system (Dhavan and Tsai, 2001). Cdk5 was found to be abnormally activated by p25, a proteolytic product of

p35, the normal partner of Cdk5, to aberrantly hyperphosphorylate tau to contribute to the formation of neurofibrillary tangles, an important pathological event in Alzheimer's disease (AD) (Patrick *et al.*, 1999).

In this study, we examined the mechanism that regulates the Vps34 complexes by Cdk5. We show that Thr159 of Vps34 can be phosphorylated by Cdk1 and Cdk5, which inhibit its interaction with Beclin 1. We show that phosphorylation of Thr159 in Vps34 occurs specifically in mitotic cells and in p25 transgenic (Tg) mice, a model of AD (Cruz *et al.*, 2006). Our results demonstrate that the phosphorylation of Thr159 in Vps34 is an important regulatory event in the membrane trafficking in mammalian cells and may contribute to neurodegeneration in human diseases such as AD.

Results

Regulation of Autophagy and PtdIns3P in Mitotic Cells

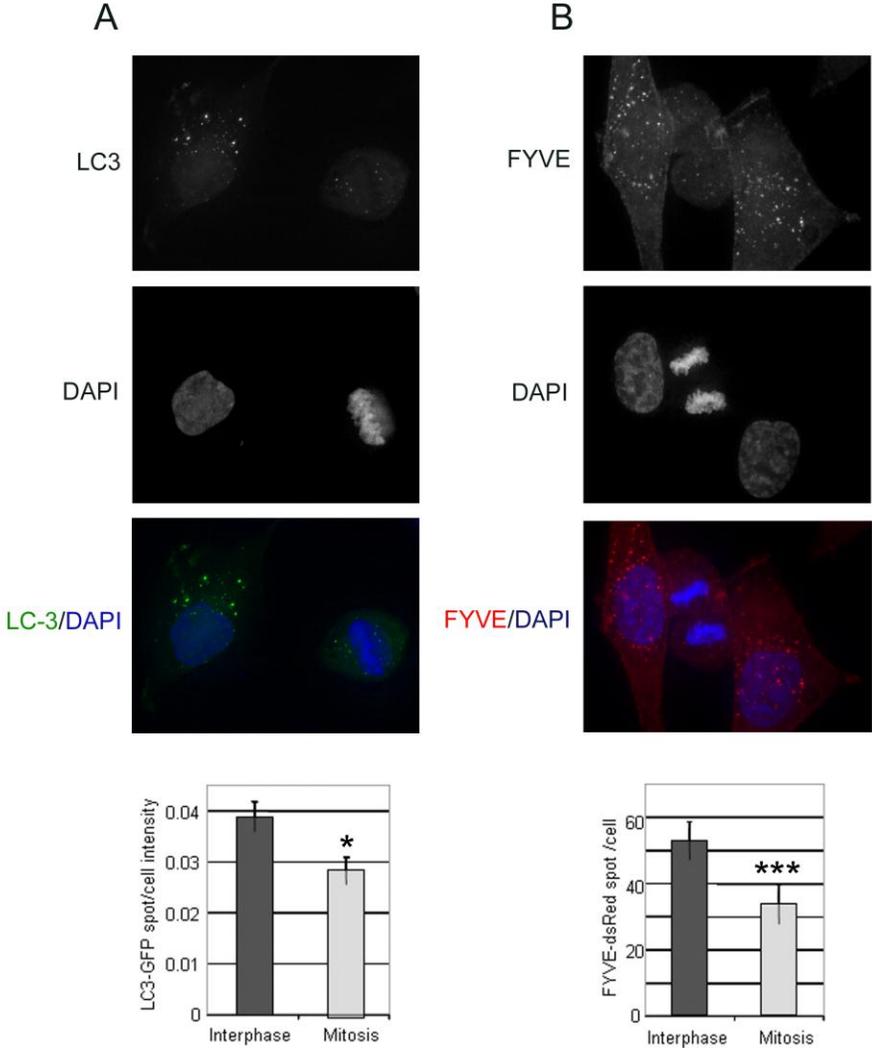
Eskelinen *et al.* reported that the number of autophagosomes was reduced in nocodazole-arrested mitotic cells and proposed that autophagy might be inhibited during mitosis (Eskelinen *et al.*, 2002). To determine if the levels of autophagy are indeed reduced during mitosis in an asynchronously proliferating cell population, we used human glioblastoma H4 cells expressing LC3-GFP, a marker of autophagosomes (Kabeya *et al.*, 2000). We first observed the numbers and intensity of LC3-GFP dots in the mitotic versus interphase cells using fluorescent microscopy. We found that the cells in the interphase contained significantly more LC3-GFP-positive autophagosomes than the mitotic cells (Figure 2.1A). We quantified the intensity of LC3-GFP present on the autophagosomes versus the total intensity of LC3-GFP

Figure 2.1. The Levels of Autophagy and PtdIns3P Are Decreased during Mitosis

(A) Asynchronously growing H4 cells stably expressing LC3-GFP were counterstained with Hoechst dye to visualize nuclei and fixed with 4% paraformaldehyde. The Z series were acquired at 603 magnification on a wide-field microscope, and deconvolved. Maximum projection images are shown. The levels of autophagy were assessed in interphase and mitotic cells by quantifying the translocation of LC3-GFP from diffuse cytosolic to punctate autophagosomal location from the pictures and expressed as a ratio of LC3-GFP intensity in autophagosomal (spot signal) versus cytosolic (diffused signal) location per cell. The data represent an analysis of 13 mitotic and 28 interphase cells from two independent experiments. Error bars indicate standard deviation. * $p = 0.04$.

(B) Asynchronously growing H4 cells stably expressing FYVE-dsRed were counterstained with DAPI to visualize nuclei and fixed with 4% paraformaldehyde. The Z series were acquired on a wide-field microscope at 603 magnification and deconvolved. Maximum projection images are shown. The levels of PtdIns3P were assessed in interphase versus mitotic cells by quantifying the amount of FYVE-dsRed from the pictures and expressed as number of FYVE-dsRed spots per cell. The data represent an analysis of 14 mitotic and 20 interphase cells from two independent experiments. Error bars indicate standard deviation. *** $p = 0.0007$.

Figure 2.1 (Continued).



expression in the mitotic and interphase cells under normal asynchronously proliferating state using fluorescent microscopy with Z stack analysis. Our data indicate that the fraction of LC3-GFP localized to autophagosomes is significantly decreased in the mitotic as compared to the interphase cells ($p = 0.04$ in two-tailed equal variance Student's t test) (Figure 2.1A). From these results, we conclude that autophagy is indeed significantly reduced in mitotic cells.

To study the mechanism by which autophagy is inhibited in mitotic cells, we measured the changes in the levels of phos-phatidylinositol-3-phosphate (PtdIns3P), a key lipid messenger required for autophagy (Kametaka *et al.*, 1998) during cell cycle using H4 cells expressing a PtdIns3P-binding reporter protein FYVE fused with a fluorescent marker protein dsRed (H4-FYVE-dsRed) (Gaullier *et al.*, 1998; Gillooly *et al.*, 2000; Kutateladze *et al.*, 1999). Analysis of asynchronously proliferating H4-FYVE-dsRed cells using 3D fluorescent microscopy showed a significant reduction in the FYVE-dsRed dots in mitotic cells as compared to the interphase cells ($p = 0.0007$), suggesting a significant reduction in the levels of PtdIns3P in mitotic cells (Figure 2.1B). Taken together, our results indicate that a reduction in autophagic activity in mitotic cells is associated with a reduction in the levels of PtdIns3P and suggest that the activity of Vps34 complex, the class III PtdIns3 kinase responsible for the production of PtdIns3P, might be reduced in mitotic cells.

Vps34 Is a Substrate of Cdk1

Since the levels of PtdIns3P are reduced specifically in mitotic cells, we hypothesize that the mitotic kinase Cdk1 might negatively regulate the activity of the class III PtdIns3 kinase Vps34 complex. Based on an analysis of the amino acid sequence of Vps34 using the Scansite

(<http://scansite.mit.edu/>), Thr159 of Vps34 is a strongly predicted phosphorylation site for Cdk1. To examine if Cdk1 can directly phosphorylate Vps34, we incubated immunoprecipitated Vps34 protein with or without recombinant Cdk1/cyclin B complex in the presence of [γ - 32 P]-ATP. The levels of Vps34 phosphorylation were significantly increased when incubated with recombinant Cdk1/cyclin B complex but reduced in the presence of alsterpaullon, a specific inhibitor of Cdk1 (Figure 2.2A). Consistent with the phosphorylation of T159 by Cdk1, the phosphorylation of T159A mutant in the same reaction was significantly lower than that of WT.

To determine if Thr159 of Vps34 is phosphorylated by Cdk1, we generated an antibody that specifically recognizes phosphorylated Thr159 region (anti-pT159-Vps34). As shown in Figure 2.2B, recognition of Vps34 by this antibody was significantly enhanced following incubation with Cdk1/cyclin B complex but was inhibited in the presence of roscovitine. Consistent with a high specificity of the anti-pThr159 Vps34 antibody used, the anti-pT159 of Vps34 signal was significantly reduced after phosphatase treatment. To further confirm that Cdk1 is the specific kinase for Vps34 phosphorylation, we immunodepleted Cdk1 from the mitotic cell lysate. The levels of Vps34 phosphorylation in vitro were higher after incubation with mitotic cell lysates than that of asynchronized lysate. Furthermore, the phosphorylation of T159 Vps34 in mitotic lysates was significantly reduced after immunodepletion of Cdk1 compared to that of mock-depleted mitotic lysate (Figure 2.2C). From these results, we conclude that Cdk1/cyclin B can phosphorylate Thr159 of Vps34.

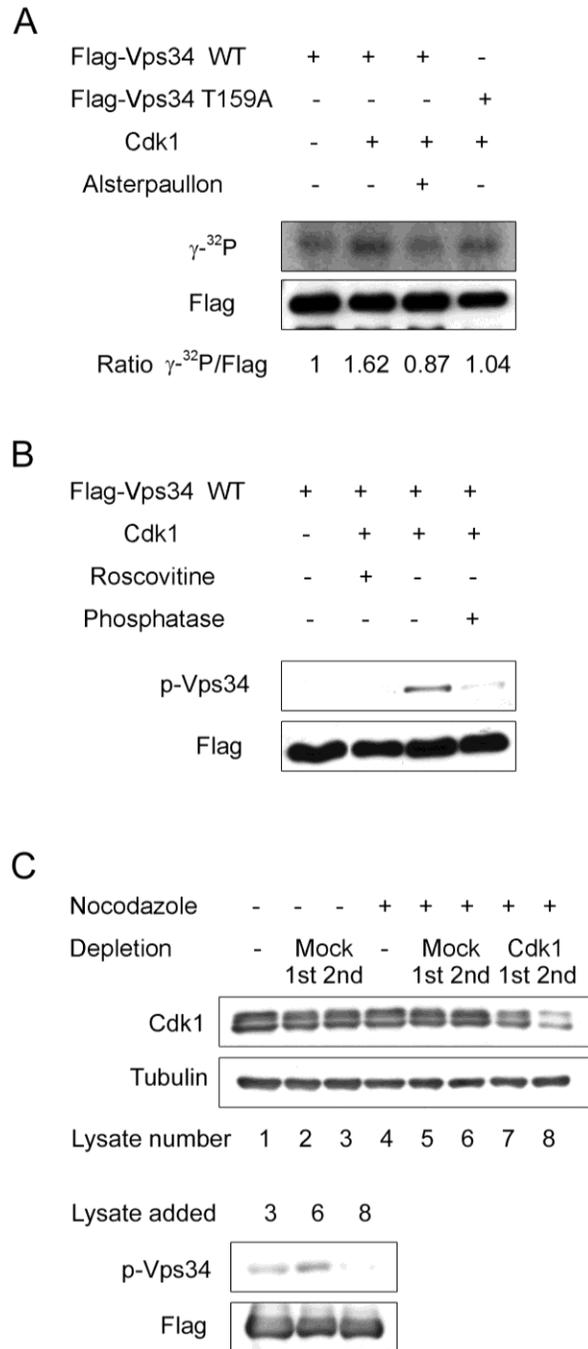
Figure 2.2. Vps34 Is Phosphorylated by Cdk1

(A) Equal amounts of purified Flag-tagged Vps34 WT or T159A protein complexes were incubated with active Cdk1/cyclin B1 complex in an in vitro phosphorylation assay with [γ - 32 P]-ATP in the absence or presence of alsterpaullon (0.1 μ M), and phosphorylation of Vps34 protein was detected by autoradiography after proteins were resolved on SDS-PAGE. The ratios of 32 P signal versus anti-Flag signal were indicated below.

(B) Equal amounts of purified Flag-tagged Vps34 WT complexes were incubated with active Cdk1/cyclin B1 complex in an in vitro phosphorylation assay with ATP in the absence or presence of roscovitine (1 μ M). The samples were either mock treated or phosphatase treated as indicated. Phosphorylation of Vps34 protein was detected with anti-pThr159 Vps34 antibody by western blotting.

(C) Flag-Vps34 was incubated with mitotic extract after two rounds of Cdk1 depletion using anti-Cdk1, or mock-depleted asynchronous or mitotic 293T extracts using a control antibody. The amount of residual Cdk1 was measured by western blotting. Phosphorylation of Vps34 was measured by western blotting with anti-pThr159 Vps34 antibody.

Figure 2.2 (Continued).



Vps34 Is Phosphorylated at T159 in Mitotic Cells

Since Cdk1/cyclin B is specifically activated during the mitotic phase, we hypothesize that Thr159 of Vps34 may be phosphorylated specifically during mitosis. Exposure of proliferating cells to a microtubule destabilizing agent, nocodazole, induces mitotic arrest. 293T, H4, and HeLa cells were treated with nocodazole (200 ng/ml) to synchronize these cell lines in the mitotic phase and the cell lysates were analyzed by western blotting using anti-pT159-Vps34 antibody. As shown in Figure 2.3A, phosphorylation of Thr159 in Vps34 was increased in a time-dependent manner upon nocodazole treatment. Treatment with Cdk1 inhibitors roscovitine (10 μ M) or alsterpaullon (1 μ M) dramatically reduced nocodazole-induced Thr159 phosphorylation on Vps34 (Figure 2.3B). Moreover, treatment of phosphatase after synchronizing H4 and HeLa cells in mitosis also resulted in decreased phosphorylation of Vps34 as recognized by anti-pT159-Vps34 antibody (Figure 2.3C).

To determine whether the increase of Vps34 Thr159 phosphorylation was caused by the stress elicited by interference with microtubule stability or a normal cell-cycle event associated with mitosis, we monitored the levels of Vps34 Thr159 phosphorylation in synchronized H4 cells after releasing serum-starved cells from the G0/G1 block by serum addition. While the levels of total Vps34 protein remained relatively constant during cell cycle, the maximum level of Vps34 Thr159 phosphorylation was detected at 36 hr post-serum addition, which coincided with the time when the levels of cyclin B reached its peak (Figure 2.3D). To further confirm this result, we analyzed the levels of Vps34 Thr159 phosphorylation in HeLa cells synchronized using double thymidine block procedure. As shown in Figure 2.3E, the levels of phosphorylated Vps34 were significantly elevated at 10 hr after the release when cells were in the mitotic phase.

Figure 2.3. Vps34 Is Phosphorylated in the Mitotic Phase

(A) Treatment of proliferating 293T, H4, and HeLa cells with nocodazole for indicated amount of times led to a gradual increase of Vps34 Thr159 phosphorylation as detected by western blotting using anti-pT159 antibody. Anti-tubulin was used as a loading control.

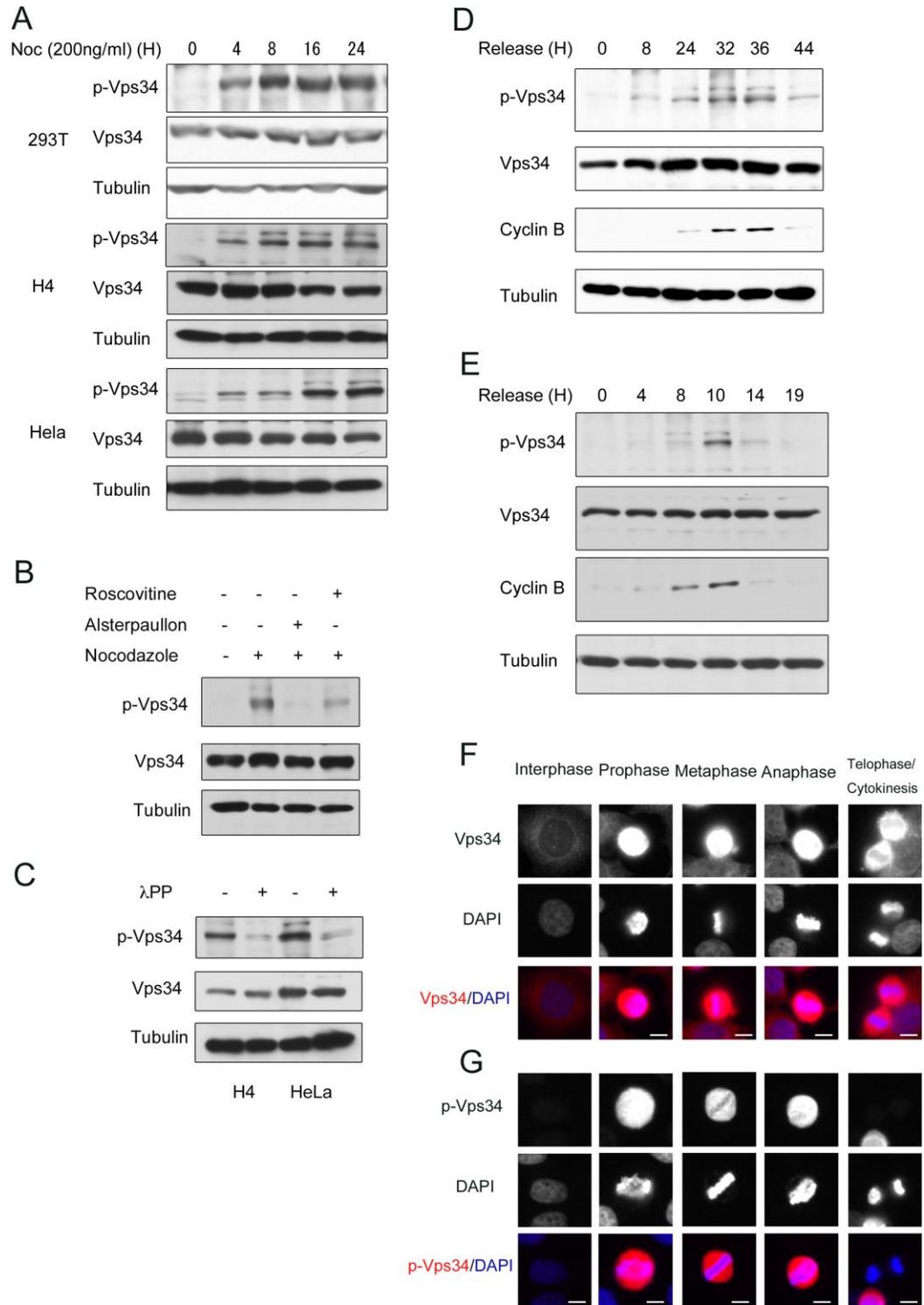
(B) HeLa cells were arrested in mitosis using 200 ng/ml nocodazole for 16 hr. Two different Cdk1 inhibitors, alsterpaullone (1 μ M) and roscovitine (10 μ M), reduced nocodazole-induced Vps34 Thr159 phosphorylation. Phosphorylation of Vps34 was detected by western blotting using anti-pT159 antibody.

(C) H4 and HeLa cells were treated with nocodazole for 16 hr, and the lysates were analyzed by western blotting with anti-pT159 antibody with or without I phosphatase treatment as indicated.

(D and E) HeLa cells were harvested after serum addition to induce synchronous cell cycle re-entering after 3 days of serum deprivation (D) or release from double thymidine block (E). The total lysates were analyzed at the indicated time points by western blotting with anti-cyclin B1 and anti-pT159 antibody.

(F and G) Asynchronously proliferating HeLa cells were fixed by paraformaldehyde and immunostained with anti-Vps34 (F) or affinity-purified anti-pThr159 Vps34 (G) antibodies and DAPI. Vps34, phosphorylated Vps34, DAPI, and merged images were shown in each stage of the cell cycle. These images were magnified from Figures S2.1A and S2.1B. Scale bar, 10 μ m.

Figure 2.3 (Continued).



Taken together, we conclude that the phosphorylation of Vps34 at Thr159 is a normal mitotic phase- associated event.

We next examined the expression and subcellular localization of Thr159-phosphorylated Vps34 in asynchronously proliferating HeLa cells during interphase and mitotic phase by immunofluorescence microscopy. In human cells, Vps34 was localized in the perinuclear area in the interphase (Kihara *et al.*, 2001a). In the mitotic cells, Vps34 was evenly distributed in the cells after nuclear envelope breakdown (Figure 2.3F and Figure A2.1A). On the other hand, the signal for p-Thr159 Vps34 was largely absent in nonmitotic cells but significantly increased in the cells during early mitotic phase (prophase, metaphase, and anaphase) and dramatically reduced in the late mitotic phase (telophase/cytokinesis) (Figure 2.3G and Figure A2.1B). This result is consistent with the western blotting analysis as shown in Figure 2.3E: the levels of Thr159-phosphorylated Vps34 were significantly decreased at 14 hr after the release when cyclin B expression was also decreased. These data indicate that in HeLa cells, Vps34 is phosphorylated on Thr159 in the early mitotic phase, but dephosphorylated in the late mitotic phase.

Cdk5 Can Also Phosphorylate Vps34

Although Cdk5, a member of the Cdk family, does not play a role in the regulation of cell cycle as Cdk1, Cdk5 has also been reported to phosphorylate certain substrates of Cdk1 (Smith and Tsai, 2002). To test if Cdk5 can phosphorylate Vps34, we incubated immunoprecipitated Vps34 protein with or without recombinant Cdk5/p25 complex in the presence of [γ - 32 P]-ATP. As shown in Figure 2.4A, the levels of 32 P-labeled Vps34 were increased after the incubation

Figure 2.4. Vps34 Is Phosphorylated by Cdk5/p25

(A) Vps34 was immunoprecipitated using anti-Flag antibody from 293T cells transfected with Flag-Vps34 vector and incubated in the absence or with different amounts of Cdk5/p25 complex and [γ - 32 P] ATP. The mixtures were resolved with 8% SDS/PAGE and subjected to autoradiography. Relative ratios of the 32 P signals divided by the amount of protein are indicated.

(B) A schematic representation of phosphorylation sites on Vps34. The upper side shows phosphorylation sites detected without incubation with Cdk5/p25. The under side shows two additional phosphorylation sites detected only after incubation with Cdk5/p25.

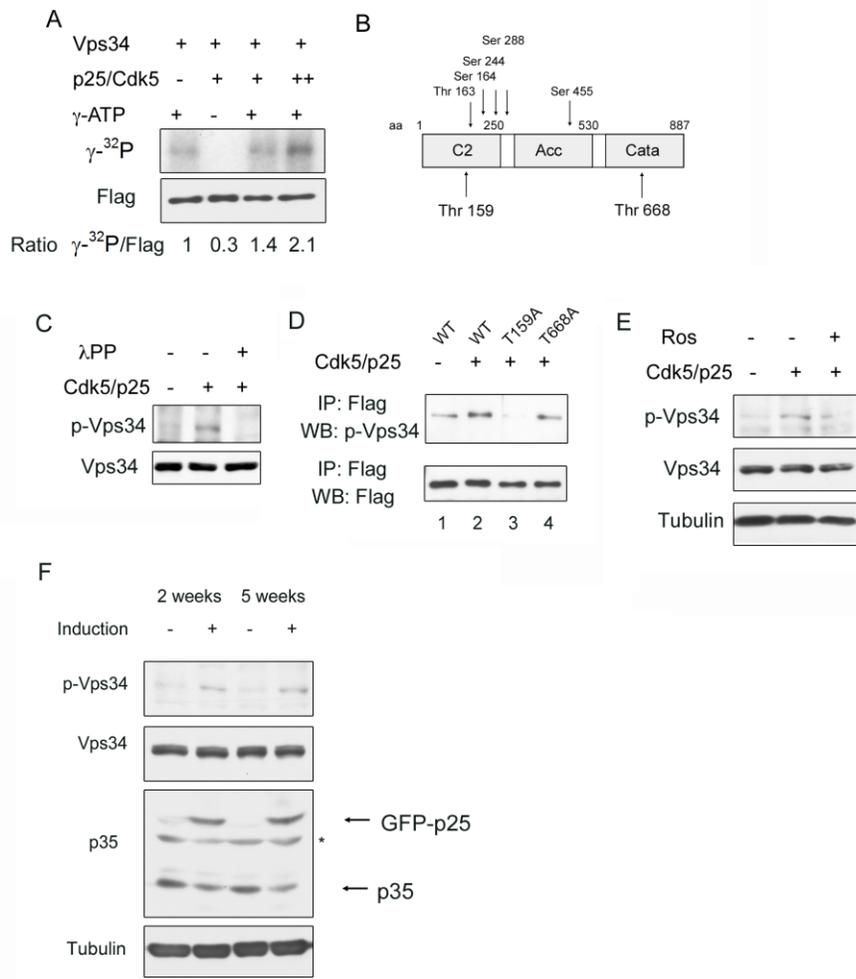
(C) Lysates from H4 cells expressing Cdk5/p25 were either untreated or treated with IPP prior to western blotting with anti-pThr159 Vps34 and total Vps34 antibodies.

(D) 293T cells were transfected with indicated expression constructs of Vps34 with or without that of Cdk5/p25. The immunoprecipitants with anti-Flag antibody were analyzed by western blotting using anti-pThr159 Vps34 and anti-Flag antibodies.

(E) H4 cells were transfected with Cdk5/p25 vectors with or without 10 μ M roscovitine (Ros). After 20 hr, the whole-cell lysates were analyzed by western blotting using anti-pThr159 Vps34 and total Vps34 antibodies.

(F) Western blotting analysis of CK-p25 Tg mouse forebrain lysates after induced for 2 or 5 weeks (Tg-On) or not induced (control) (Tg-Off) using anti-pT159 Vps34, total Vps34, anti-p35, and anti-tubulin (as a loading control).

Figure 2.4 (Continued).



with recombinant Cdk5/p25 complex.

To identify the in vivo phosphorylation sites of Vps34 by Cdk5/p25, we immunoprecipitated Vps34 from cells cotransfected with Cdk5/p25 or vector controls and analyzed the sites of phosphorylation by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Phosphorylated Thr163, Ser164, Ser244, Ser282, and Ser455 of Vps34 were detected without cotransfection with Cdk5/p25. However, two additional amino acids in Vps34, Thr159 and Thr668, were found to be phosphorylated only after cotransfection with Cdk5/p25 (Figure 2.4B).

We next tested the ability of anti-pT159-Vps34 antibody to detect Vps34 phosphorylated by Cdk5/p25. Lysates of H4 cells transfected with Cdk5/p25 expression vector were prepared and either left untreated or treated with lambda protein phosphatase (IPP) prior to immunoblotting. Anti-pThr159-Vps34 antibody detected phosphorylated Thr159 Vps34 only in the lysates of cells expressing Cdk5/p25. The signal was completely removed by the phosphatase treatment. In contrast, the addition of phosphatase had no effect on the total levels of Vps34 (Figure 2.4C).

We further confirmed the phosphorylation of Thr159 by Cdk5/p25 using Vps34 mutants. We analyzed the immunoprecipitates isolated with anti-Flag antibody from 293T cells transfected with vectors expressing Flag-tagged wild-type (WT), T159A, or T668A mutant forms of Vps34 in the presence or absence of Cdk5/p25 by immunoblotting using anti-pT159-Vps34 and Flag antibodies. Although the expression levels of total Vps34 were not appreciably different, anti-pT159-Vps34 antibody detected a stronger signal in cells expressing Cdk5/p25 than those without Cdk5/p25 (lanes 1 and 2, Figure 2.4D). Mutation of Thr159 to Ala (T159A)

resulted in a complete loss of recognition by anti-pT159-Vps34 antibody. On the other hand, T688A mutation in Vps34 did not change its recognition by anti-pT159-Vps34 antibody or the levels of phosphorylation in the presence of cdk5/p25 (lanes 3 and 4, Figure 2.4D). These results demonstrate that Cdk5/p25 can phosphorylate Thr159 of Vps34.

To further examine if Cdk5/p25 can mediate the phosphorylation of endogenous Vps34 in cells, we analyzed the lysates of H4 cells transfected with Cdk5/p25 or vectors control with or without the treatment with roscovitine using anti-pT159-Vps34 antibody. As expected, Thr159-phosphorylated Vps34 was detected in Cdk5/p25-expressing cells, and the phosphorylation was attenuated by roscovitine (Figure 2.4E).

To confirm the phosphorylation of Vps34 *in vivo*, we used anti-pT159-Vps34 antibody to examine the phosphorylation status of Thr159 site in CK-p25 Tg mouse brains after inducing p25 expression which activates endogenous Cdk5 (Cruz *et al.*, 2003). As shown in Figure 2.4F, a significant increase in Thr159 phosphorylated Vps34 was observed after 2 and 5 weeks p25 induction, which was when the earliest biochemical changes could be observed in this line of Tg mice after inducing p25 expression (Cruz *et al.*, 2003). Taken together, we conclude that Thr159 Vps34 can be phosphorylated by Cdk5/p25 *in vitro* and *in vivo*.

Since Vps34 is known to be positively regulated by p150 (Panaretou *et al.*, 1997; Yan *et al.*, 2009), we tested whether the expression of p150 had any effect on Thr159 phosphorylation of Vps34. We found that overexpression of p150 did not lead to T159 phosphorylation of Vps34, nor did it have any effect on T159 phosphorylation by Cdk5/p25 (Figure A2.2). Thus, it is most likely that Cdks directly target and phosphorylate Vps34.

Thr159 Phosphorylation Negatively Regulates the Interaction of Vps34 with Beclin 1

The activity of type III PI3 kinase is determined by the interaction of Vps34 with its regulatory subunits, including Beclin 1. Therefore, we evaluated the effect of T159 phosphorylation on Beclin 1/Vps34 complex formation. To identify the Vps34 domain that binds to Beclin 1, we coexpressed individual Vps34 domains (Figure A2.3A) with domains of Beclin 1 in 293T cells. Consistent with previous reports (Furuya *et al.*, 2005; Liang *et al.*, 2006), the C2 domain of Vps34, where T159 is localized, bound to Beclin 1 (Figure A2.3B). Conversely, the coiled-coil domain (CCD) and the ECD of Beclin 1 were required for binding of Vps34 (Figure A2.3C). To determine whether Cdk5/p25 can influence the Beclin 1/Vps34 complex, we cotransfected 293T cells with Flag-Vps34 and GFP-Beclin 1 in the presence or absence of Cdk5/p25 expression. GFP-tagged Beclin 1 could be coimmunoprecipitated with Flag-tagged Vps34 by anti-Flag antibody in the absence of Cdk5/p25. When p25 and Cdk5 were coexpressed, coimmunoprecipitation of GFP-Beclin 1 with Flag-Vps34 was drastically reduced (Figure 2.5A). However, the interaction of Vps34 and Beclin 1 was partially rescued in the Cdk5/p25-expressing cells in the presence of roscovitine (Figure 2.5A), suggesting that the kinase activity of Cdk5/p25 was necessary for the disruption of the Beclin 1/Vps34 complex. Similarly, HA-tagged Vps34 could be coimmunoprecipitated with Flag-tagged Beclin 1 by anti-Flag antibody in the absence of Cdk5/p25 expression. This interaction was significantly reduced in Cdk5/p25-expressing cells (Figure 2.5B).

To confirm that endogenous Beclin 1/Vps34 complex is regulated by Cdk5/p25, we transfected p25 expression vector into H4 cells and immunoprecipitated Vps34 complex using anti-Beclin 1 antibody. We found that the endogenous Vps34 was coimmunoprecipitated with

Figure 2.5. Cdk5/p25 Disrupts Beclin 1/Vps34 Complex

(A) 293T cells were transfected with Flag-Vps34 and GFP-Beclin 1 with or without Cdk5/p25 expression vectors. Flag-Vps34 was immunoprecipitated with anti-Flag antibody from the lysates. The immunoprecipitates were blotted with anti-GFP antibody and subsequently probed with anti-Flag, p35, and Cdk5 antibodies.

(B) HA-Vps34 and Flag-Beclin 1 with or without Cdk5/p25 expression vectors were transfected into 293T cells. The protein complexes were immunoprecipitated using anti-Flag antibody and analyzed by western blotting using anti-HA antibody.

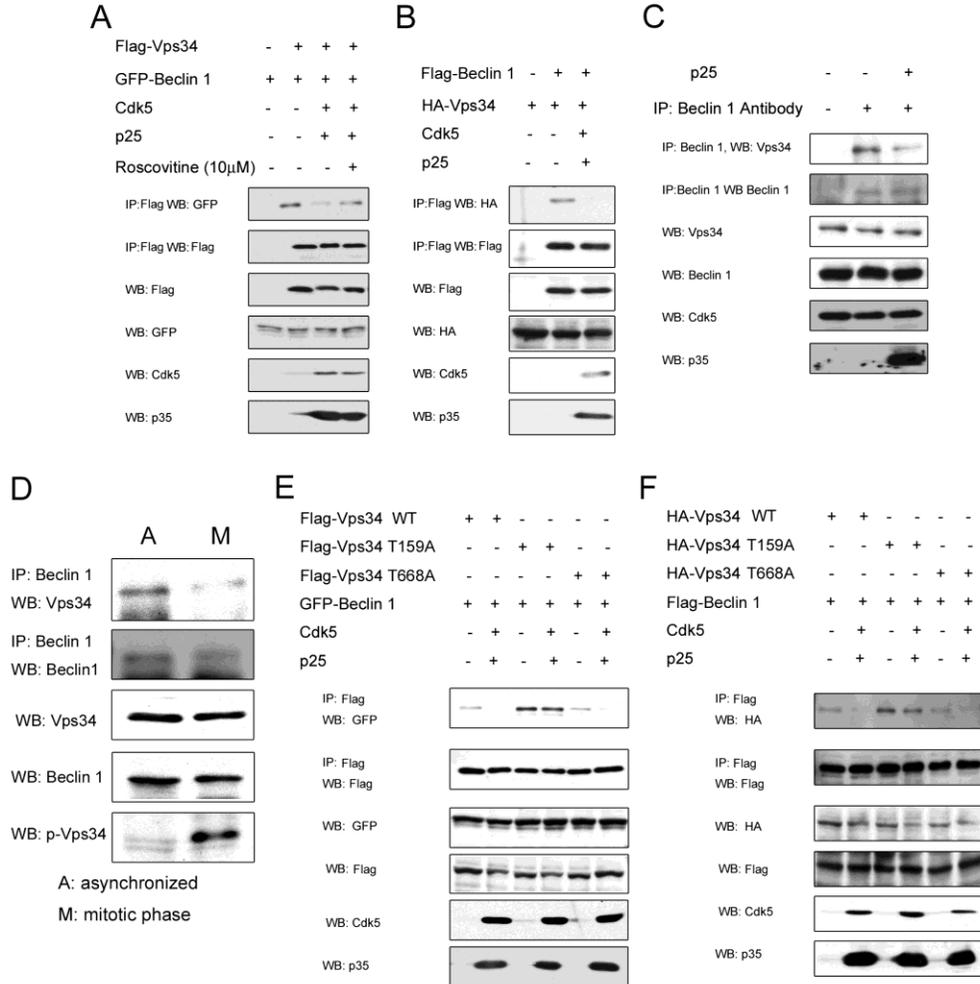
(C) H4 cells were transfected with or without p25 expression vector. Beclin 1 was immunoprecipitated with anti-Beclin 1 antibody from the lysates. The immunoprecipitates were analyzed by western blotting using anti-Vps34 antibody.

(D) HeLa cells were synchronized in mitotic phase with nocodazole. Beclin 1 was immunoprecipitated with anti-Beclin 1 antibody from lysates. The immunoprecipitates were analyzed by western blotting using anti-Vps34 and pThr159 Vps34 antibodies.

(E) 293T cells were transfected with Flag-tagged Vps34 WT, mutant T159A, T668A, and GFP-Beclin 1 with or without Cdk5/p25 expression vectors. Flag-Vps34 was immunoprecipitated with anti-Flag antibody from the lysates. The immunocomplexes were analyzed by western blotting using anti-GFP and Flag antibodies.

(F) 293T cells were transfected with Flag-tagged Beclin 1 with HA tagged Vps34 WT, mutant T159A, and T668A with or without Cdk5/p25 expression vectors. Flag-Beclin 1 was immunoprecipitated with anti-Flag antibody from the lysates. The immunocomplexes were analyzed using anti-HA and Flag antibodies.

Figure 2.5 (Continued).



endogenous Beclin 1 in the absence of p25 expression, but this interaction was disrupted by p25 expression (Figure 2.5C). To examine if the formation of Vps34 /Beclin 1 complex is also disrupted in the mitotic phase due to the Cdk1/cyclin B activity, we synchronized HeLa cells with 16 hr nocodazole treatment and immunoprecipitated with Beclin 1 antibody. As expected, this interaction was significantly reduced in mitotic as compared with asynchronous cells (Figure 2.5D). Taken together, we conclude that the interaction of Beclin 1/Vps34 is disrupted in the presence of active Cdk5/p25 or Cdk1.

To examine if the phosphorylation of Thr159 and/or Thr668 in Vps34 by Cdk5/p25 is responsible for the observed reduction of Beclin 1/Vps34 interaction, we compared the interaction of T159A, T668A, or WT Vps34 with Beclin 1 with or without Cdk5/p25. As shown in Figure 2.5E, coimmunoprecipitation of Flag-tagged WT or T688A Vps34 with GFP-tagged Beclin 1 was inhibited by the expression of Cdk5/p25, whereas that of T159A Vps34 with Beclin 1 was insensitive to cdk5/p25 expression. Similarly, coimmunoprecipitation of Flag-tagged Beclin 1 with HA-tagged WT or T688A Vps34 was reduced by Cdk5/p25 expression, while the interaction of flagged-Beclin 1 with T159A Vps34 was not affected by Cdk5/p25 expression (Figure 2.5F). Interestingly, in both experiments the phosphomutant Vps34 (T159A) showed an increased interaction with Beclin 1 as compared with WT Vps34 (Figures 2.5E and 2.5F). Taken together, these data provide strong evidence that Cdk5/p25 phosphorylation of Thr159 in the C2 domain responsible for interacting with Beclin 1 reduces the interaction of Vps34 with Beclin 1.

Thr159 Phosphorylation Negatively Regulates the PtdIns3 Kinase Activity of Vps34 and Autophagy

Since the experiments described above demonstrated an important role of Thr159 phosphorylation in regulating the interaction of Vps34 with Beclin 1, we wished to further evaluate the functional significance of this finding. We examined the levels of FYVE-dsRed dots in control vector or p25 expressing H4-FYVE-dsRed cells. Under nutrient-rich condition, H4-FYVE-dsRed cells showed no significant difference in the FYVE-dsRed dots with or without p25 expression. However, under starvation condition, known to induce autophagy, the FYVE-dsRed dot formation in p25-transfected H4-FYVE-dsRed cells was significantly lower than in control vector-transfected H4-FYVE-dsRed cells, which was recovered by roscovitine treatment (Figure 2.6A). Thus, the expression of p25 reduces the production of PtdIns3P under starvation condition by phosphorylating Thr159 of Vps34.

To further test if Thr159 phosphorylation of Vps34 might reduce its lipid kinase activity in converting PtdIns to PtdIns3P, we transfected 293T cells with vectors expressing Flag-tagged WT Vps34 and GFP-Beclin 1 in the presence or absence of Cdk5/p25 expression. We immunoprecipitated Vps34 protein with anti-Flag antibody and incubated it with purified bovine phosphatidylinositol in the presence of [γ - 32 P]-ATP. Extracted phospholipid products were separated by thin-layer chromatography. Consistent with a lower level of FYVE-dsRed dots in p25-expressing cells, Vps34 immunoprecipitated from cells expressing Cdk5 and p25 demonstrated a much lower level of PtdIns3 kinase activity in converting PtdIns to PtdIns3P in vitro (Figure 2.6B). As a control, treatment with wortmannin, a general PtdIns3 kinase inhibitor, completely inhibited the PtdIns3 kinase activity (Figure 2.6B).

Figure 2.6. Phosphorylation of Vps34 Negatively Regulates the Class III PI3 Kinase Activity

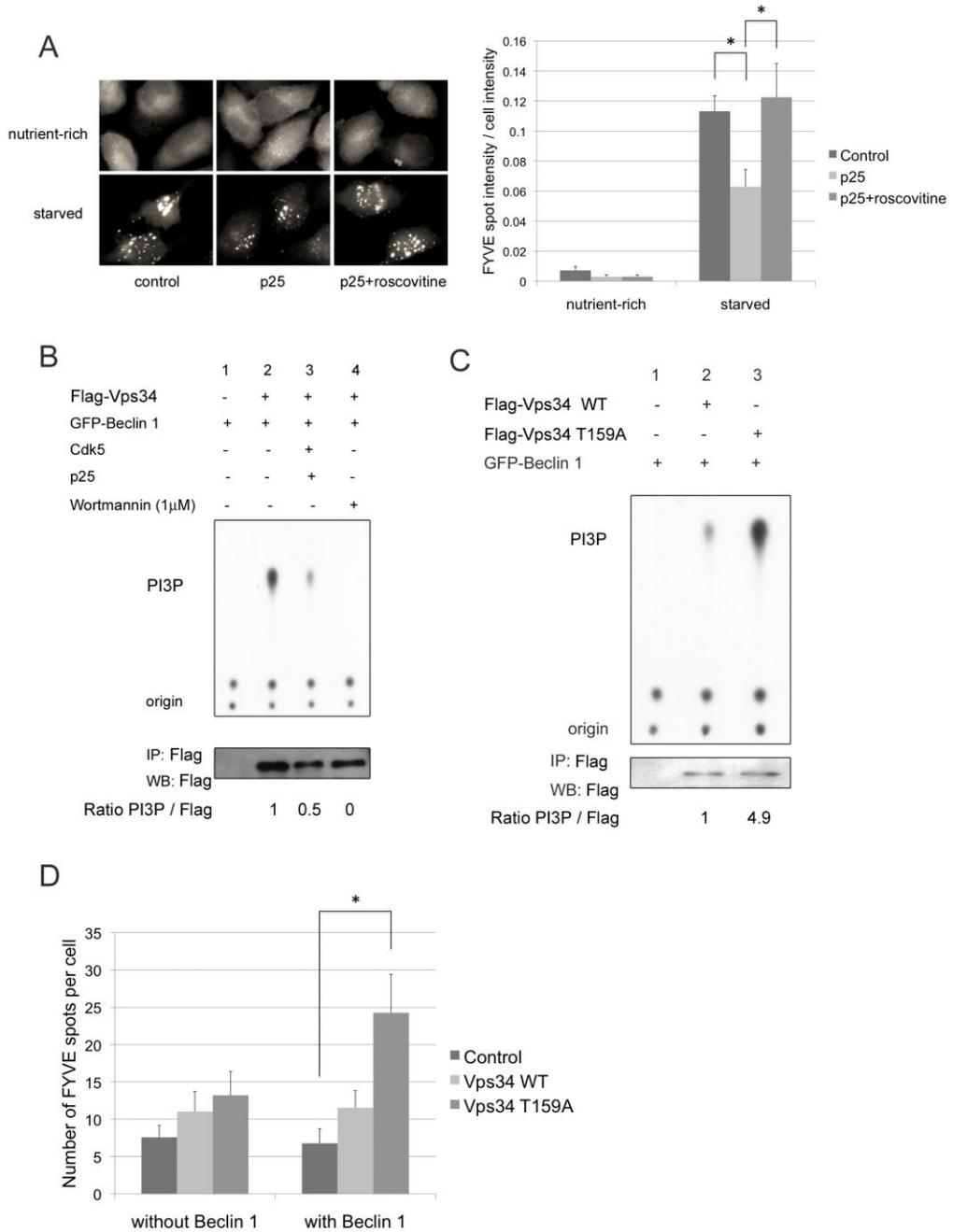
(A) H4 cells stably expressing FYVE-dsRed were transfected with p25 expression vector. FYVE-dsRed H4 cells were stimulated for 2 hr with HBSS as starvation condition or with roscovitine in HBSS to inhibit Cdk5 activity. The cells were fixed with 3.7% formaldehyde and used for quantifying the intensity of FYVE-dsRed dots with MetaMorph. Statistical analysis was performed by Student's t test. Error bars indicate standard error. * $p < 0.05$.

(B) 293T cells were transfected with vector control (lane 1), with Flag-Vps34 and GFP-Beclin 1 (lane 2 and 4), with Flag-Vps34, GFP-Beclin 1 and Cdk5/p25 (lane 3). The whole-cell lysates were used for immunoprecipitation using anti-Flag antibody followed by an assay for Vps34 lipid kinase activity. Wortmannin (10 μ M) was added prior to PtdIns3P kinase assay (lane 4) as a positive control.

(C) 293T cells were transfected with vector control (lane 1), with Flag-tagged WT Vps34 and GFP-Beclin 1 (lane 2), with Flag-tagged phosphomutant T159A Vps34 and GFP-Beclin 1 (lane 3). Flag-tagged Vps34 was immunoprecipitated with anti-Flag antibody and followed by an assay for Vps34 lipid kinase activity. Relative ratios of the 32 P signal divided by the amount of protein are indicated for (B) and (C).

(D) H4 cells stably expressing FYVE-dsRed were transfected with WT or phosphomutant T159A Vps34, with or without Beclin 1. Cells were fixed and the number of FYVE dots was quantified. Error bars indicate standard error. * $p < 0.01$.

Figure 2.6 (Continued).



As discussed above, the phosphomutant Vps34 (T159A) has shown an increased interaction with Beclin 1 as compared with that of WT Vps34 (Figures 2.5D and 2.5E). To evaluate whether this phosphomutant Vps34 T159A has increased PtdIns3 lipid kinase activity, we evaluated the class III PtdIns3 kinase activity in immunoprecipitated Vps34/Beclin 1 complexes from 293T cells expressing Flag-tagged WT or phosphomutant T159A Vps34 and GFP-Beclin 1. Interestingly, we found that the complex with phosphomutant T159A Vps34 exhibited dramatically higher class III PtdIns3 kinase activity than that of WT Vps34 (Figure 2.6C).

To determine the effect of Vps34 T159A mutant/Beclin 1 complex in vivo, we transfected H4-FYVE-dsRed cells with WT or Vps34 T159A mutant, with or without Beclin 1. Consistent with the lipid kinase assay data, FYVE-dsRed dot formation was significantly increased only when the cells were cotransfected with Vps34 T159A mutant and Beclin 1 (Figure 2.6D).

To further determine whether autophagy is regulated consistently with the lipid kinase activity of Vps34 by Cdk5 and the T159A mutant of Vps34, we used H4 cells expressing LC3-GFP. Under nutrient-rich condition in which a minimal level of autophagy occurs, transfection of p25 did not affect the basal levels of autophagy, which was very low already. However, p25 significantly reduced the level of starvation-induced autophagy, consistent with the decrease of PI3P formation by p25 (Figure 2.7A). We also tested the effect of Vps34 T159A mutant, which is more active than the WT Vps34. Under nutrient-rich condition, the level of autophagy was increased in the cells transfected with Vps34 T159A mutant. The difference of autophagy level between them was diminished in starved cells, suggesting that Vps34 T159A mutant partially mimics starvation condition (Figure 2.7B).

Figure 2.7. Phosphorylation of Vps34 Results in the Inhibition of Autophagy

(A) H4 cells expressing LC3-GFP were transfected with p25 expression vector. Twenty-two hours after the transfection, starvation was induced by culturing in HBSS only. The cells were fixed after 1 and 2 hr of starvation with 3.7% formaldehyde and the area of LC3-GFP dots was quantified using MetaMorph. Error bars indicate standard deviation. * $p < 0.05$.

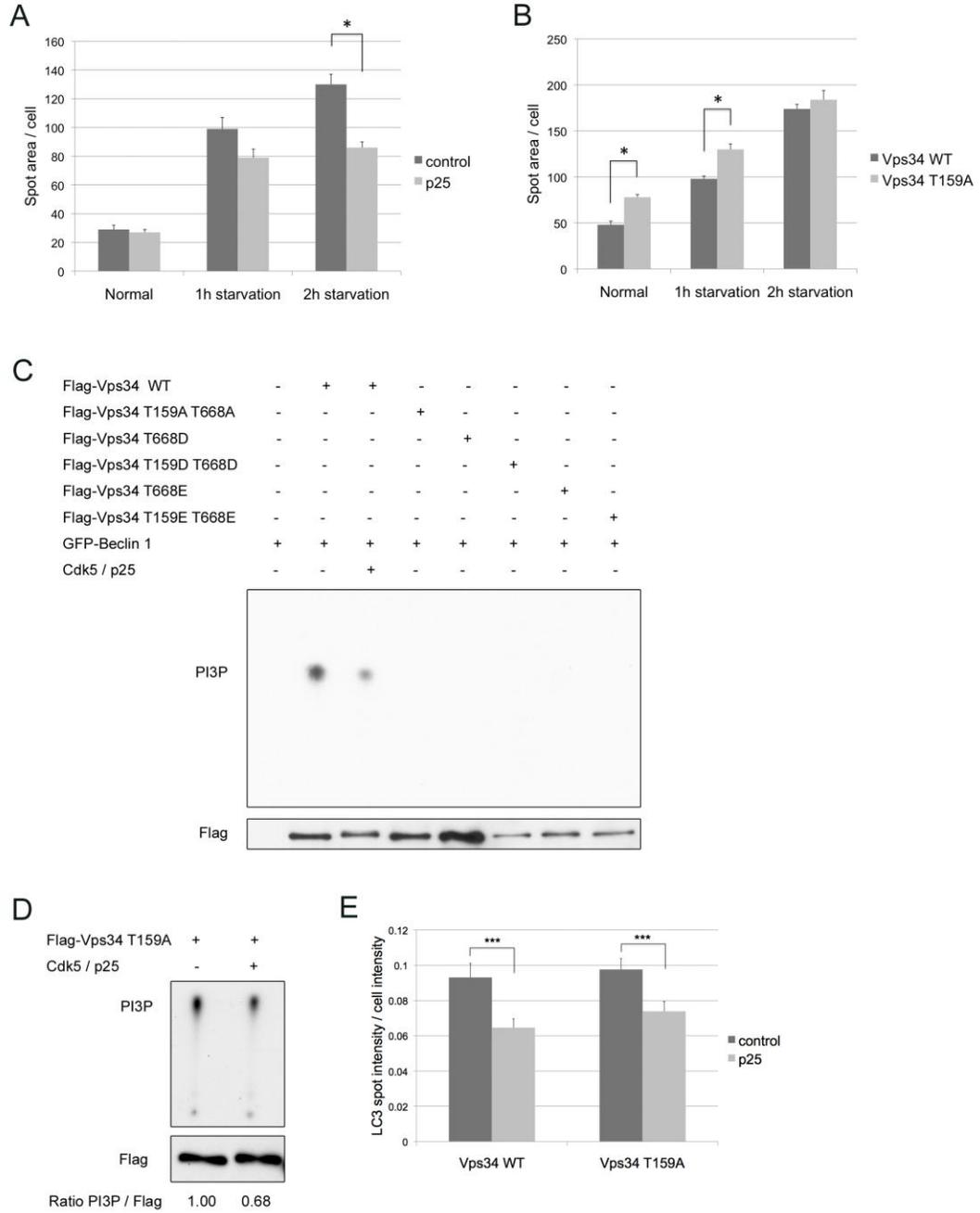
(B) H4 cells expressing LC3-GFP were transfected with WT or T159A Vps34 mutant. Twenty-two hours after the transfection, starvation was induced by culturing in HBSS only. The cells were fixed after 1 and 2 hr of starvation with 3.7% formaldehyde and analyzed as in (A). Error bars indicate standard deviation. * $p < 0.05$.

(C) 293T cells were transfected with different expression vectors of WT and T668A, T668D, and T668E mutants in different combination and lipid kinase assays were conducted as in Figure 2.6.

(D) 293T cells were transfected with indicated expression vectors, and lipid kinase assays were conducted as in Figure 2.6. Relative ratios of the ^{32}P signal divided by the amount of protein as measured by the densitometry are indicated.

(E) H4 cells expressing LC3-GFP were transfected with WT or T159A mutant Vps34 expression vector with or without p25 expression vector. Twenty-two hours after the transfection, starvation was induced by culturing in HBSS only for 2 hr. The cells were fixed with 4% paraformaldehyde, and the intensity of LC3-GFP dots was quantified using MetaMorph. Error bars indicate standard error. *** $p < 0.01$.

Figure 2.7 (Continued).



Finally, since we found that T668 can also be phosphorylated by Cdk5 (Figure 2.4B), we examined the requirement of T668 for the lipid kinase activity of Vps34. As shown in Figure 2.7C, T668A, T668D, or T668E Vps34 were all inactive in the in vitro lipid kinase assay. To determine the relative contribution of T159 and T668 phosphorylation by Cdk5 to inhibiting the lipid kinase activity, we tested the activity of T159A Vps34 protein immunoprecipitated from Cdk5/p25-expressing cells. The lipid kinase activity of T159A Vps34 mutant was also inhibited by Cdk5/p25 (Figure 2.7D); furthermore, overexpression of p25 in H4 cells resulted in inhibition of autophagy even in the presence of T159A Vps34 overexpression (Figure 2.7E). Thus, Cdks may have two mechanisms to negatively regulate Vps34: phosphorylation of T159, which interferes with its binding to Beclin 1, and phosphorylation of T668, a residue in the catalytic domain that is required for the lipid kinase activity.

Discussion

Our study demonstrates a mechanism that regulates the interaction of Vps34 with its key partner Beclin 1 by Cdks. It provides the first example of dynamic regulation of intracellular PtdIns3P production in mammalian cells through phosphorylation of the class III PI3 kinase. Since Cdk1 is a key mitotic kinase, while Cdk5 is involved in neural development by controlling axonal outgrowth and neuronal migration as well as neurodegeneration (Dhavan and Tsai, 2001) and abnormal regulation of Cdks has also been implicated in tumorigenesis (Malumbres and Barbacid, 2009), our study has implications for understanding of the regulation of PtdIns3P during cell-cycle progression, development, and in major human diseases including neurodegeneration and cancers.

Regulation of Vps34 and Beclin 1 Interaction

Our study provides a mechanism that regulates PtdIns3P under conditions that are not nutritionally limiting but when Cdks are activated. Since phosphorylation of Vps34 at Thr159 of the C2 domain by Cdk5 and Cdc2/Cdk1 inhibits its interaction with Beclin 1, a critical regulator subunit of the class III PI3 kinase complex, this mechanism may be utilized by the members of Cdk family to directly regulate the production and/or distribution of PtdIns3P under different physiological and pathological conditions. For example, phosphorylation of Vps34 in cancer cells by abnormally activated Cdks may provide a mechanism to lead to inhibition of class III PI3 kinase activity and autophagy, which may in turn contribute to genomic instability (Karantza-Wadsworth *et al.*, 2007). Since the stability of individual components in the Vps34 complex is highly dependent upon each other (Itakura *et al.*, 2008), phosphorylation of T159 may accelerate the degradation of Vps34 complex.

In the *S. cerevisiae*, the ATG6/VPS30 gene product is required for both autophagy and sorting of the vacuole resident hydrolase carboxypeptidase Y through the Vps pathway (Kametaka *et al.*, 1998). In mammalian cells, in addition to regulating autophagy and endosomal trafficking, Beclin 1 is known as a haploinsufficient tumor suppressor (Qu *et al.*, 2003; Yue *et al.*, 2003). Beclin 1 contains a BH3-only domain (Maiuri *et al.*, 2007; Oberstein *et al.*, 2007) and interacts with Bcl-2 (Pattingre *et al.*, 2005). Since increased interaction of Beclin 1 with Bcl-2 has been shown to negatively regulate autophagy by competing for binding with Vps34, phosphorylation of Thr159 on Vps34 may release Beclin 1, which in turn may increase its interaction with other cellular partners and positively regulate additional Beclin 1-mediated cellular processes.

Inhibition of Vps34 Kinase Activity by Cdk5 Mediated T668 Phosphorylation

From a mass spectrometric analysis, we have identified T668, a residue in the catalytic domain of Vps34, as a Cdk5 phosphorylation site. Any mutations we introduced in this site totally abolished Vps34 lipid kinase activity (Figure 2.7C). Although we do not yet have an antibody that can monitor the phosphorylation status of T668, this result suggests that T668 is required for the lipid kinase activity. This result also suggests that Cdk5 may have two mechanisms to negatively regulate Vps34 activity: phosphorylation of T668 to inhibit its lipid kinase activity and phosphorylation of T159 to interfere with its binding with Beclin 1. Since interfering with the Vps34 and Beclin 1 interaction may disrupt the Vps34 complex and in turn accelerate the degradation of individual components, inhibitory effect of T159 phosphorylation may be long lasting. On the other hand, phosphorylation of T668 may lead to a transient inhibition of Vps34 lipid kinase activity, which may be rapidly reactivated with an appropriate phosphatase. These possibilities may be directly examined by experiments in the future.

Changes in the Distributions of PtdIns3P in Mitosis

Inhibition of Vps34 activity has been shown to lead to multiple defects in vesicular trafficking such as membrane receptor degradation and multivesicular body formation. Phosphorylation of Vps34 by Cdks, however, may lead to a redistribution of Vps34 kinase activity by modulating its interaction with Beclin 1 and perhaps with other partners as well, rather than a total inhibition of Vps34 lipid kinase activity. Since suppression of Beclin 1 expression has been shown to lead to inhibition of autophagy (Liang *et al.*, 1999; Zeng *et al.*, 2006) and PtdIns3P is known to be important for autophagy signaling, inhibition of Vps34

activity may provide an important mechanism to regulate autophagy during mitosis after nuclear membrane breakdown. Phosphorylation of Vps34 during mitosis may function to selectively reduce the input to the autophagosome compartment during mitosis without affecting early endosomal trafficking. On the other hand, selective inhibition of membrane trafficking to the autophagosomes might be important to prevent the loss of the Golgi compartment, which undergoes a complete fragmentation during mitosis (for review, see Nelson, 2000). Although Beclin 1 may not be the partner for Vps34 in regulation of the vesicular trafficking (Zeng *et al.*, 2006), phosphorylation of Vps34 may affect its interaction with additional partner(s) mediating endosome to lysosome transport, which remains to be explored in future studies.

Cdks, Autophagy, and Neuronal Cell Death

Our study demonstrates a mechanism by which two members of the Cdk family of protein kinases, Cdk1 and Cdk5, negatively regulate the production of PtdIns3P, which may in turn negatively regulate autophagy. In yeast, Pho85p, a member of yeast Cdk family and a regulator of phosphate metabolism and glycogen synthase, has been shown to be a negative regulator of autophagy (Wang *et al.*, 2001). Increased autophagic activity, observed in WT cells entering the stationary phase where nutrient is limiting, was exaggerated in pho85 mutants. Thus, the Cdk family of protein kinases might have an evolutionarily conserved role in regulating cellular levels of autophagy.

Induction of G1-S cyclins and Cdks as well as evidence of S phase entry and DNA replication have been well documented in the neurons AD (Vincent *et al.*, 1997). Abnormal

activation of Cdks, perhaps as a part of aberrant cell-cycle reactivation in postmitotic neurons, has been proposed to be an important underlying cause for multiple neurodegenerative disorders including AD (Herrup and Yang, 2007). On the other hand, activation of Cdk5 by p25 as a result of calpain-mediated cleavage of p35 has been implicated in contributing to multiple pathological features of AD including tau hyperphosphorylation, formation of neurofibrillary tangles, and neurodegeneration (Cruz *et al.*, 2003). Autophagy has been demonstrated to play an important protective role in cellular and animal models of AD and other neurodegenerative diseases. The ability of Cdk1 and Cdk5 to phosphorylate Vps34 described here provides a new mechanism by which abnormal activation of Cdks contributes to neurodegeneration by negatively regulating autophagy.

Experimental Procedures

Chemicals and Antibodies

The sources of the antibodies used were as follows: monoclonal antibodies against tubulin and Flag (Sigma), cyclin B1 (sc-245, Santa Cruz Biotechnology), HA (HA.11) (Covance), GFP (Clontech), Ub (Dako), Beclin 1 (BD transduction), rabbit polyclonal antibodies against Beclin 1, Cdk5 (C-8), p35 (C-19) (Santa Cruz Biotechnology), and Vps34 (Zymed). Purified Cdk1/cyclin B and IPP were from New England Biochemical (Beverly, MA). Alsterpaullon was from A.G. Scientific (San Diego, CA). Chemicals were obtained from Sigma unless otherwise noted.

Cell Synchronization

For double thymidine block, HeLa cells were treated with 2 mM thymidine for 16–24 hr and released from G1/S phase in DMEM for 8 hr. HeLa cells were treated with thymidine again for 16–24 hr, then released from G1/S phase with DMEM for analyzing mitotic cells.

For serum starvation synchronization, H4 cells were treated with DMEM with 0.5% serum for 3 days. H4 cells were released from G0 phase with DMEM with 10% serum and then collected in indicated time.

Immunofluorescence

For immunofluorescence analysis, H4 and HeLa cells were grown on coverslips. The cells were fixed with 3.7% formaldehyde, permeabilized, and blocked with 3% bovine serum albumin (BSA). Blocked cells were incubated with indicated primary antibody overnight at 4°C. Cells were then incubated with Texas red-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch) for 1 hr at room temperature. Fluorescence imaging was done on Nikon Eclipse 80i fluorescent microscope and quantified using MetaMorph v.7.0 software.

Quantitative Fluorescent Microscopy for Mitosis

H4-LC3-GFP or H4-FYVE-dsRed cells were grown in asynchronous cultures on glass coverslips, counterstained with Hoechst dye for 1 hr, and fixed for 20 min in 4% paraformaldehyde/PBS. Z series (0.25 μm step size) were acquired on Nikon inverted TE2000E wide-field microscope using 603 1.4 na oil lens. The images were deconvolved using Autoquant

X AutoDeblur. Quantitation was performed on maximum projection from each Z series using MetaMorph v7.0 software.

Generation of Anti-Phosphor-Thr159-Vps34 Antibody

In brief, phosphopeptide (DGSEPTR/K(pT)PGRTSST) was synthesized, purified, and conjugated to KLH by Proteintech Group (Chicago, IL) and used to immunize two rabbits. Serum was collected from the two rabbits after four injections.

Vps34 Lipid Kinase Assay

Flag-tagged Vps34-expressed 293T cells were immunoprecipitated with anti-Flag M2-agarose affinity gel as immunoprecipitation assay and washed with NP-40 buffer, eluted with reaction buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) and then preincubated for 10 min at room temperature with 10 mM MnCl₂ and 2 mg sonicated phosphatidylinositol (Sigma). Finally we added 10 mCi [γ -³²P] ATP and 1 mM cold ATP for 15 min at room temperature. The kinase reactions were stopped by the addition of 20 ml 8M HCl and extracted with 160 ml chloroform:methanol (1:1). Extracted phospholipid products were separated on Silica Gel 60A (Merck). Plates were dried and exposed by autoradiography to visualize PtdIns3P production.

Mass Spectrometry

The Cdk5 phosphorylation sites were identified using MALDI-TOF MS in the Taplin Biological Mass Spectrometry Facility (<http://gygi.med.harvard.edu/facility/>).

Supplemental Information

Supplemental Information includes three figures and can be found at the end of the dissertation.

Acknowledgements

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Chapter 3

Regulation of p53 by a Small Molecule Inhibitor of Autophagy

Attribution of experimental contributions

The work presented in Chapter 3 is in part adapted from Cell *147*, 223–234.

Hongguang Xia performed Figure 3.1.

Helin Norberg performed Figure 3.3.

Minsu Kim performed all other experiments.

Junying Yuan supervised all of the work.

Abstract

Autophagy is an evolutionarily conserved catabolic pathway important for maintaining cellular homeostasis. Defects in autophagy have been implicated in a wide variety of diseases including cancers and neurodegenerative diseases. In a small molecule screening for the modulators of autophagy, we identified spautin-1 as an inhibitor of autophagy. Using this compound, we show that the class III PI3 kinase complex and Usp10/Usp13 are under reciprocal regulation, and thus destabilizing one leads to the degradation of the other. By controlling the levels of Usp10/Usp13, the class III PI3 kinase complex also regulates the levels of p53. In this Chapter, we investigated the effect of inhibiting autophagy on the levels of mutant p53. We show that the treatment of A70, a derivative of spautin-1, leads to the degradation of mutant p53 and the cell death of mutant p53 expressing ES-2 ovarian cancer cells, when the cells are under confluent or glucose-deprived conditions. By using inhibitors and siRNAs targeting different protein degradation pathways, we demonstrate that the degradation of mutant p53 induced by autophagy inhibition is primarily mediated by the chaperone-mediated autophagy, in contrast to that of the wild-type p53. This study describes a mechanistic distinction between the degradation of wild type and mutant p53.

Introduction

Class III PI3 kinase complex converts phosphatidylinositol (PI) to phosphatidylinositol-3-phosphate (PI3P) (Schu *et al.*, 1993). This is the only PI3 kinase complex conserved from yeast to human, playing a crucial role in autophagy and endosomal sorting pathway (Vanhaesebroeck *et al.*, 2001). Vps34, the catalytic subunit of the kinase complex, requires other regulatory subunits for its enzymatic activity (Backer, 2008). In mammalian cells, Vps34 forms two different complexes. In addition to the core complex including Vps34, Beclin 1, and p150, the complex I contains Atg14L and is involved in the initiation step of autophagy, whereas the complex II contains UVRAG and is involved in the maturation step of autophagy and endocytic pathway (Matsunaga *et al.*, 2009; Zhong *et al.*, 2009). Despite the need of specific inhibitors for Vps34 for research and clinical purposes, those currently used, such as 3-MA, also target other classes of PI3 kinases (Seglen and Gordon, 1982; Wu *et al.*, 2010).

p53 is one of the best-known tumor suppressors. It primarily functions as a stress-responsive transcription factor, regulating the expression of the wide variety of downstream genes (Bullock and Fersht, 2001). Those target genes work cooperatively to relieve the stress, for example, by repairing damaged DNA, or to induce cell cycle arrest and programmed cell death (Oren, 2003). Under normal condition, p53 expression remains low due to rapid degradation through the ubiquitin-proteasome pathway (Haupt *et al.*, 1997). However, under various kinds of stress, p53 is posttranslationally modified so that its degradation is inhibited. Since p53 functions as a guardian against genotoxic insults, deletions or point mutations on p53 often lead to tumorigenesis (Hollstein *et al.*, 1991). Such mutations result in loss of transcriptional activation of the target genes (Kato *et al.*, 2003). However, growing evidence

supports the idea that many mutant p53 forms acquire novel oncogenic functions (Wolf *et al.*, 1984). Furthermore, removing mutant p53 may reduce the malignancy of tumors (Bossi *et al.*, 2008).

In a high-throughput chemical screening to identify small molecules regulating autophagy, we identified MBCQ, a known phosphodiesterase 5 (PDE5) inhibitor, as an inhibitor of autophagy. By performing a diversity-oriented synthesis, we synthesized an MBCQ derivative, spautin-1, which potently inhibits autophagy without affecting PDE5. We report that spautin-1 inhibits Usp10 which deubiquitinates PI3 kinase complex, and thus also degrades p53, which is a substrate of Usp10 (Yuan *et al.*, 2010). We also show that a derivative of spautin-1 can be used to induce cell death of mutant p53 expressing cells through a caspase-independent mechanism. From the biochemical studies, we found that inhibition of autophagy promotes the degradation of the mutant p53 by chaperone-mediated autophagy. Our study demonstrates a novel pathway mediating the degradation of oncogenic mutant p53 proteins distinct from that of the wild-type p53.

Results

Identification and Characterization of Spautin-1 as an Inhibitor of Autophagy

Spautin-1 was identified in an image-based high-throughput screen for small molecules regulating autophagy. Treatment of spautin-1 significantly inhibited basal level autophagy as shown by decreased LC3 lipidation (Figure 3.1A). To further determine the mechanism by which spautin-1 inhibits autophagy, we tested whether it has any effect on the class III PI3 kinase complex that is a key mediator of autophagy. Interestingly, all subunits tested are degraded

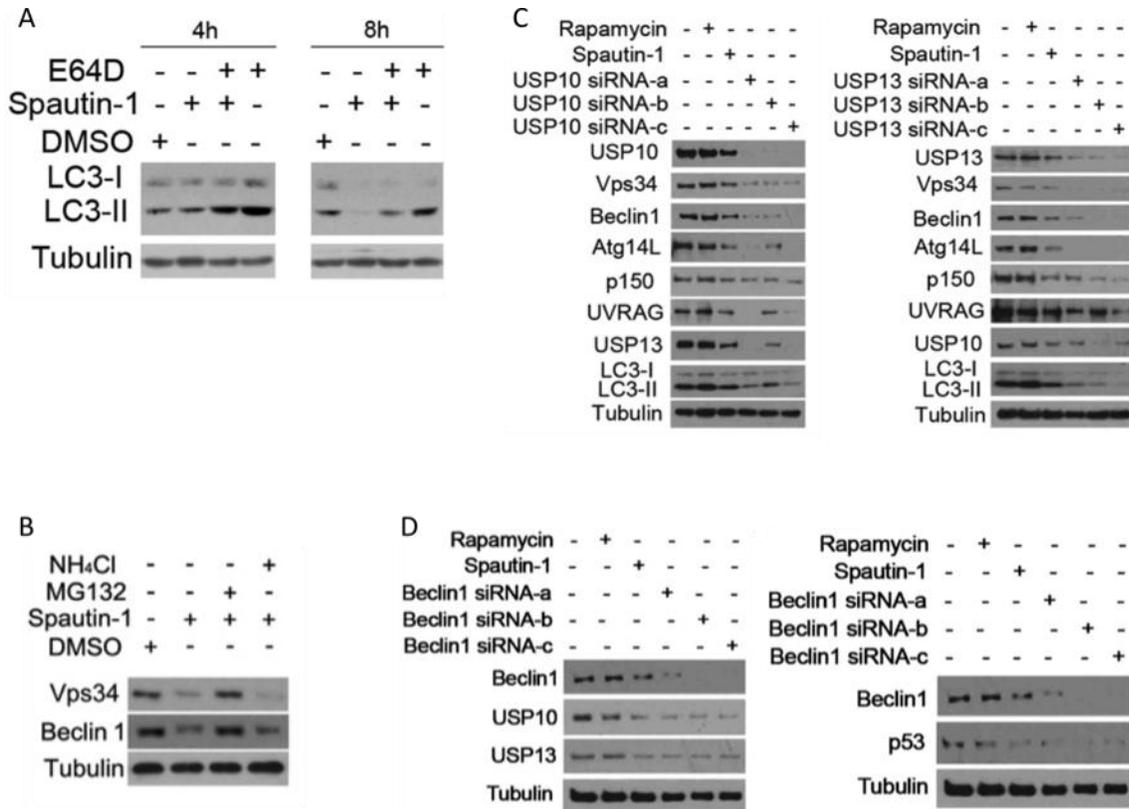
Figure 3.1. Spautin-1 Inhibits Autophagy by Degrading the Class III PI3 Kinase Complex

(A) H4-LC3-GFP cells were treated with spautin-1 (10 μ M) with or without E64D (5 μ M) for indicated periods of time. The cell lysates were analyzed by western blotting using anti-LC3 and anti- β -tubulin.

(B) H4-LC3-GFP cells were incubated with MG132 (10 μ M) or NH₄Cl (10 mM) with or without spautin-1 (10 μ M) for 6 hr. The cell lysates were analyzed by western blotting using indicated antibodies. β -tubulin was used as a control.

(C, D) H4-LC3-GFP cells were transfected with indicated siRNAs for 72 hr or treated with rapamycin (0.25 μ M) or spautin-1 (10 μ M) as indicated, the cell lysates were analyzed by western blotting using indicated antibodies. β -tubulin was used as a control.

Figure 3.1 (Continued).



upon the treatment of spautin-1, which can be blocked by inhibition of the proteasome, but not the lysosome, suggesting that it is degraded by the ubiquitin-proteasome system (Figure 3.1B). Given that most small molecules are inhibitors instead of activators, we hypothesized that spautin-1 inhibits a target deubiquitinase (DUB), which leads to the increased ubiquitination and degradation of the class III PI3 kinase complex through the proteasome. By testing siRNAs targeting DUBs, we identified Usp10 and Usp13 as regulators of autophagy. Spautin-1 leads to the degradation of Usp10/Usp13, and knockdown of Usp10/13 reduces the expression levels of the class III PI3 kinase complex as spautin-1 does (Figure 3.1C). Moreover, Usp10 and Usp13 stabilize each other. Interestingly, Usp10 and Usp13 are also degraded by knockdown of Beclin 1, suggesting that the class III PI3 kinase complex and Usp10/Usp13 are under reciprocal regulation (Figure 3.1D).

Regulation of p53 by the Class III PI3 Kinase Complex and Spautin-1

The interdependence of the class III PI3 kinase complex and Usp10/Usp13 raised a question if the levels of PI3 kinase complex affect the expression levels of p53, which is regulated by Usp10/Usp13. In the cell line tested, knockdown of Beclin 1 leads to the degradation of p53 (Figure 3.1D). This is also consistent with the finding that Beclin 1 is a haploinsufficient tumor suppressor, and the patterns of tumors in *BECN1*^{+/-} mice are similar to those in *p53*^{+/-} mice (Qu *et al.*, 2003; Jacks *et al.*, 1994). To test this hypothesis, an eight-week old *BECN1*^{+/-} mouse and its wild-type littermate were sacrificed, and the heart, lung, and liver tissues were harvested from them. Consistent with the previous report that the levels of the class III PI3 kinase complex are interdependent, all subunits of PI3 kinase complex tested are

reduced in BECN1^{+/-} mouse compared with BECN1^{+/+} mouse (Itakura *et al.*, 2008; Figure 3.2A). The expression levels of Usp10 and p53 are also decreased in BECN1^{+/-} mouse. However, autophagy is not significantly defective. Since the basal levels of autophagy as well as the expression levels of p53 are low in normal condition, lung fibroblasts were treated with rapamycin and MG132 to better visualize the difference of autophagy and p53, respectively. The accumulation of p53 by MG132 is significantly lower in BECN1^{+/-} fibroblasts, whereas rapamycin strongly induces autophagy in both cell lines, suggesting that the effect of Beclin 1 heterozygosity might be stronger on p53 levels than on autophagy (Figure 3.2B). To further analyze the effect of spautin-1 on DNA damage induced p53 activation, HT-1080 cells with wild-type p53 were treated with doxorubicin to induce DNA damage. Consistent with the previous observations, spautin-1 reduces p53 accumulation by doxorubicin treatment (Figure 3.2C).

A70, a Derivative of Spautin-1 Shows Specific Cytotoxicity on Confluent Cells with Mutant p53

Although spautin-1 has been shown to degrade wild-type p53, its expression level is very low in normal conditions, and thus it is difficult to see any p53 related physiological effect of spautin-1. In contrast, mutant p53 is more strongly expressed because it is frequently accumulated in cancer cells. Moreover, the expression levels of mutant p53 have an effect on various aspects of the cellular physiology to promote oncogenesis. From the structure-activity relationship, we identified a derivative of spautin-1, A70. It is about 100-fold more potent than spautin-1, with the working concentration of 100nM. Since it has been known that p53 mutation rate is high in breast and ovarian cancers, we tested the effect of A70 in some ovarian cancer cell lines with different p53 background (Schuijjer and Berns, 2003). Interestingly, A70

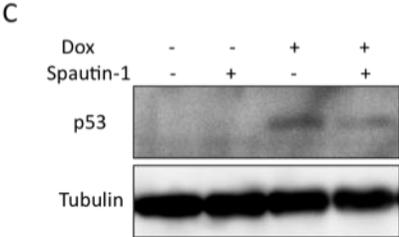
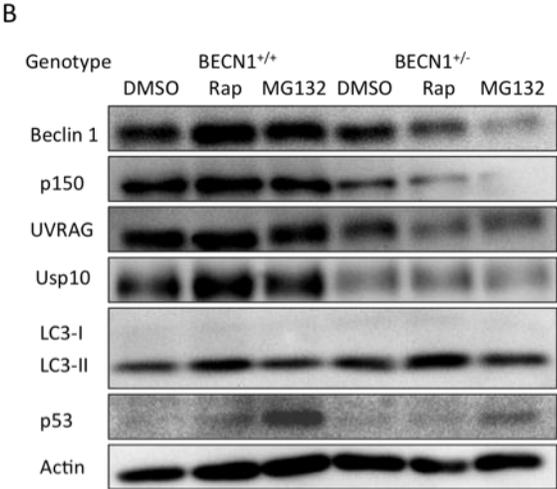
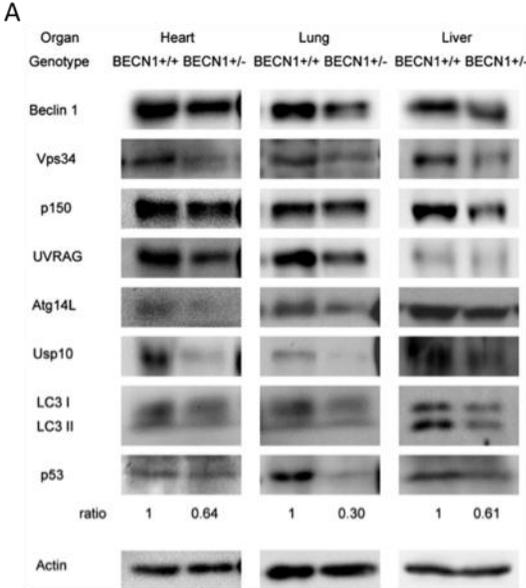
Figure 3.2. p53 is Reduced by Beclin 1 Heterozygosity or Spautin-1 Treatment

(A) Heart, lung and liver tissues of newborn BECN1^{+/+} and BECN1^{+/-} mice were isolated and analyzed by western blotting using indicated antibodies. Anti-actin was used as a loading control.

(B) Lung fibroblasts from BECN1^{+/+} and BECN1^{+/-} mice were treated with 100nM rapamycin (Rap) for 0.5 hrs or 10 μ M MG132 for 5 hrs, and cell lysates were analyzed by western blotting. Anti-actin was used as a loading control.

(C) HT-1080 fibrosarcoma cells with wild-type p53 background were treated with 1 μ M doxorubicin (Dox) to induce DNA damage and/or 10 μ M spautin-1 for 5 hrs, and cell lysates were analyzed by western blotting.

Figure 3.2 (Continued).



only induces cell death of confluent cells with mutant p53 background (Figure 3.3A, B). Dispersed cells or p53-null cells do not undergo cell death upon A70 treatment. Moreover, knockdown of the mutant p53 in ES-2 cells results in the resistance to A70 (Figure 3.3C). Consistently, overexpressing mutant p53 in SKOV-3 cells that do not express p53 renders the cells sensitive to A70, suggesting that expression of mutant p53 is necessary for the sensitivity to A70 (Figure 3.3D).

To explain the effect of A70 on mutant p53 expressing confluent cells, we chose ES-2 cells with a missense mutation on p53 (S241F) for molecular studies, based on the expression levels of p53, and the sensitivity to A70 (Figure 3.4A). In ES-2 cells, the degradation of mutant p53 and cell death are only induced under confluent condition after A70 treatment (Figure 3.4B). Interestingly, mutant p53 is degraded earlier than Usp13, suggesting that mutant p53 may not be direct substrate of Usp13 (Figure 3.4C). Deubiquitinating enzymes plays an important role in recycling free ubiquitins to maintain ubiquitin homeostasis in the cell, and thus A70-mediated degradation of multiple DUBs may render the cells suffer from the lack of available ubiquitins because the levels of free ubiquitin depend more on the recycling than synthesis or degradation. Consistent with this idea, the levels of free ubiquitin is lower in confluent cells, and even further depleted by A70 treatment (Figure 3.4D).

To determine how mutant p53 is degraded upon A70 treatment, we first checked the ubiquitination status of the mutant p53 because wild-type p53 is known to be constitutively ubiquitinated in normal conditions. However, there has not been much known about the ubiquitination of mutant p53 so far except for some selected mutations (Lukashchuk and Vousden, 2007). The mutant p53 in ES-2 cells is not as highly ubiquitinated as that of the wild-

Figure 3.3. A70 Specifically Induces Cell Death in Mutant p53 Expressing Cells

(A) Ovarian cancer cell lines with different genetic background of p53 were treated with A70, and then observed under phase contrast microscope. The magnification of the objective lens is 40X.

(B) Cell viability in (A) was analyzed by measuring cellular ATP levels. Error bars indicate standard deviation.

(C) ES-2 cells were infected with retroviruses expressing indicated shRNAs and treated with A70. Cell viability was analyzed by measuring cellular ATP levels. Error bars indicate standard deviation. p53 expression was confirmed by western blotting.

(D) SKOV-3 were infected with retroviruses expressing indicated constructs and treated with A70. Cell viability was analyzed by measuring cellular ATP levels. Error bars indicate standard deviation. p53 expression was confirmed by western blotting.

Figure 3.3 (Continued).

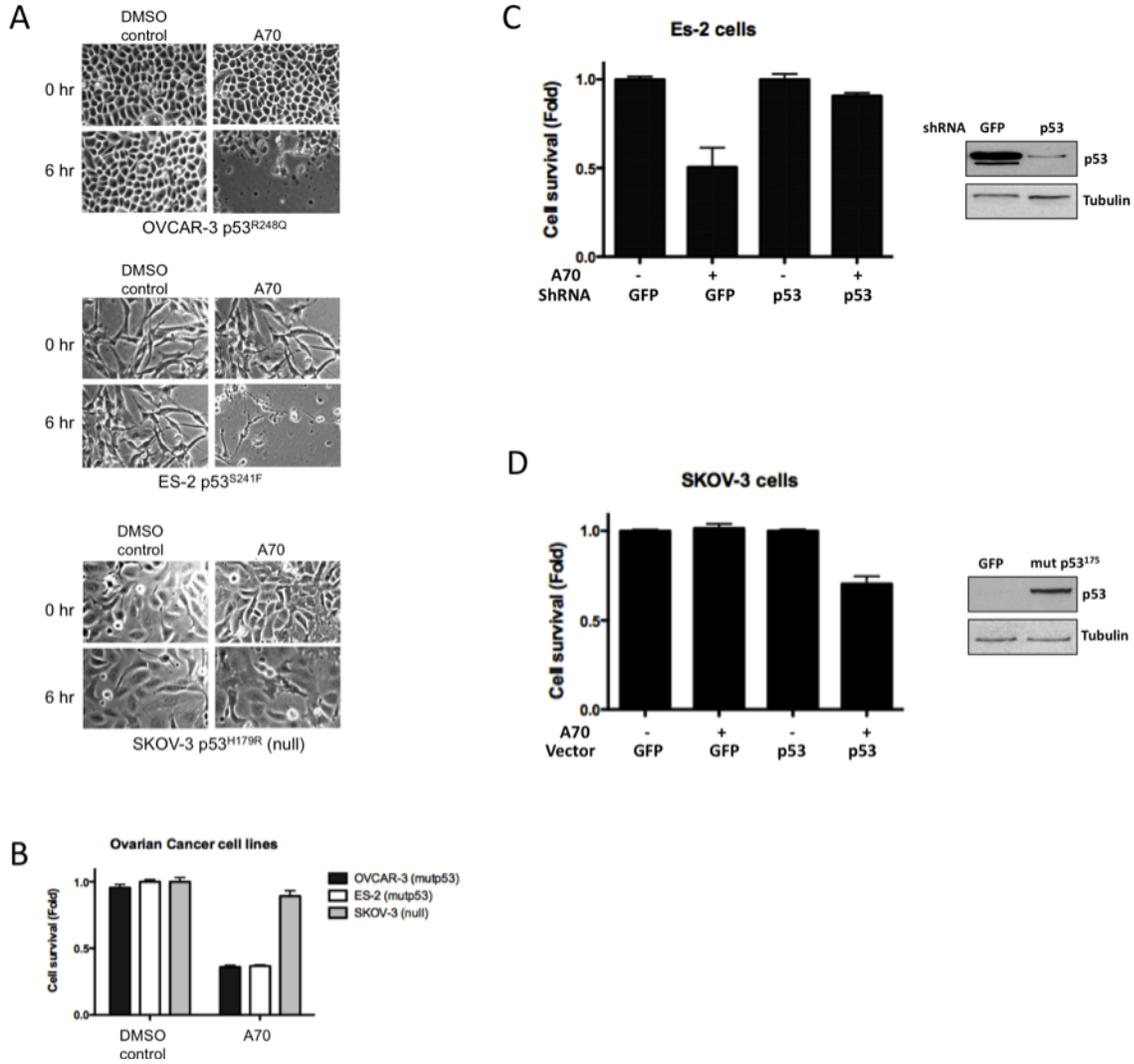


Figure 3.4. Mutant p53 Is Degraded by A70 in Confluent Cells

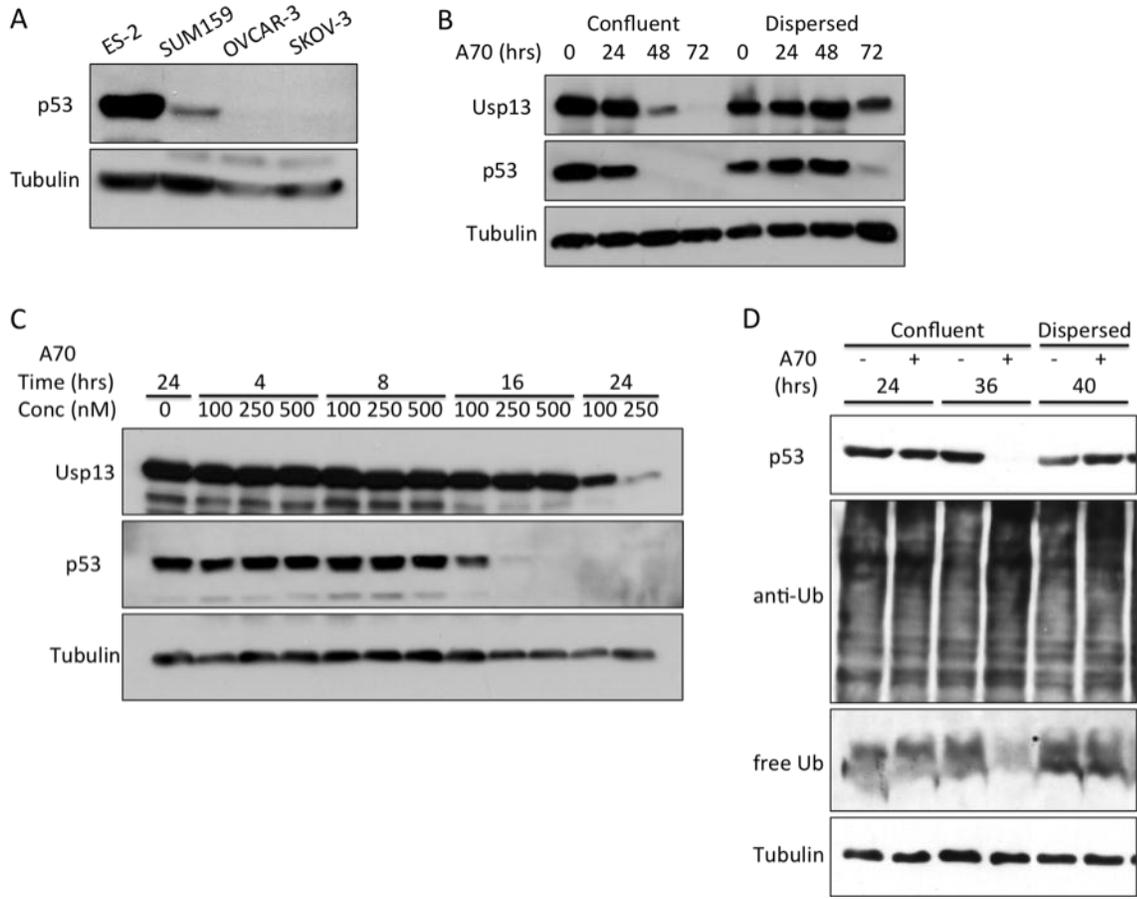
(A) Different cell lines were grown until confluence and harvested. Protein levels were analyzed by western blotting.

(B) ES-2 cells were treated with 100nM A70 and harvested at different time points as indicated. Protein levels were analyzed by western blotting.

(C) ES-2 cells were treated with different concentration of A70 and harvested at different time points. Protein levels were analyzed by western blotting.

(D) ES-2 cells were treated with 100nM A70 as indicated and harvested. Protein levels were analyzed by western blotting. The same anti-Ub antibody was used to detect both poly-ubiquitinated proteins and free monoubiquitins.

Figure 3.4 (Continued).



type p53 in HCT116 cells in the absence or in the presence of A70 (Figure 3.5A, B). Moreover, neither cell death nor the degradation of p53 by A70 is rescued by the inhibition of the proteasome (Figure 3.5C). In the cells with wild-type p53, the treatment of A70 leads to the degradation of p53 by inhibiting Usp10/Usp13, and thus either A70 or knockdown of Usp10/Usp13 is sufficient to remove p53. However, knockdown of Usp10 or Usp13 does not have any effect on the levels of the mutant p53 (Figure 3.5D). Interestingly, reduced levels of Usp10 or Usp13 delay A70 mediated degradation of mutant p53, suggesting that Usp10/Usp13 may have some indirect effect on A70 sensitivity.

Since A70 leads to the degradation of mutant p53 only in confluent cells, we asked what aspect of confluence sensitizes the cells to A70. We found that glucose in the media is almost used up in 24 hours when the cells are confluent and tested whether glucose deprivation has any effect on A70 sensitivity. Interestingly, p53 is degraded even in dispersed cells when the media is glucose-free (Figure 3.6A). In glucose-free media, p53 starts to be degraded in two hours after A70 treatment, as opposed to normal media in which p53 in confluent cells is degraded in 24 hours (Figure 3.6B). From these results, we suppose that when A70 is added in fresh media with glucose, A70 does not have any effect on p53 until glucose in the media is used up, which takes about 24 hours. To determine how mutant p53 is degraded in glucose-free media, we tested different inhibitors that block several known protein degradation pathways. E64d turned out to inhibit mutant p53 degradation suggesting that p53 is degraded through the lysosomal pathway (Figure 3.6C). Consistently, we observe mutant p53 is more rapidly degraded upon the treatment of proteasome inhibitors because inhibition of the proteasome leads to the activation of lysosomal pathway to compensate. However, none of the

Figure 3.5. Mutant p53 in ES-2 Cells Is Degraded by Proteasome-independent Pathway

(A) HCT116 and ES-2 cells were transfected with HA-Ub and harvested either when they were confluent, or still proliferating. MG132 was added 6 hours before harvesting, and p53 was immunoprecipitated. Ubiquitination of p53 was analyzed by western blotting.

(B) ES-2 cells were transfected with HA-Ub and treated with A70 for 24 hours followed by 6 hour MG132 treatment. Ubiquitination of p53 was analyzed as in (A).

(C) Increasing concentration of Velcade was treated with or without 100nM A70 on ES-2 cells. The cells were either harvested for western blotting or analyzed by ATP assay to measure cell viability. Error bars indicate standard deviation. Western blotting analysis was done only for the confluent cells.

(D) ES-2 cells were transfected with indicated siRNAs. After 48 hrs of transfection, cells were treated with 100nM A70 and harvested. Cell lysates were analyzed by western blotting.

Figure 3.5 (Continued).

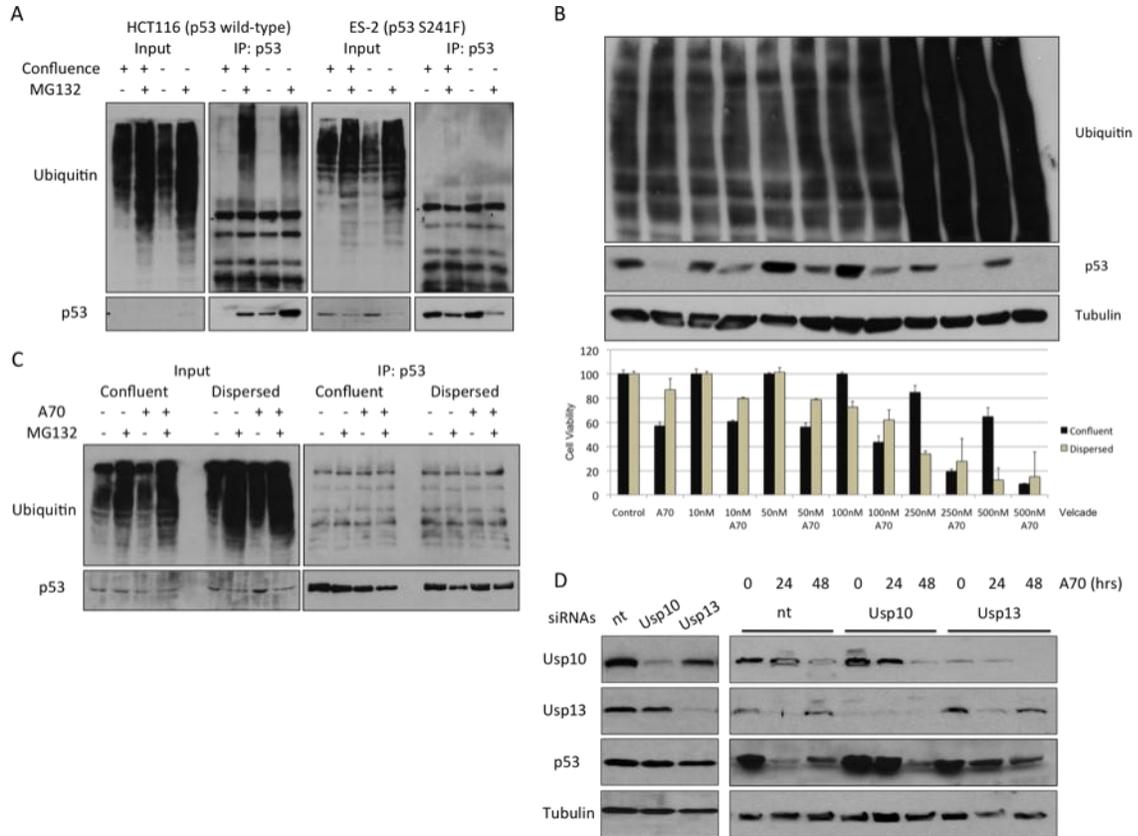


Figure 3.6. Mutant p53 Is Degraded by A70 in Glucose-deprived Condition

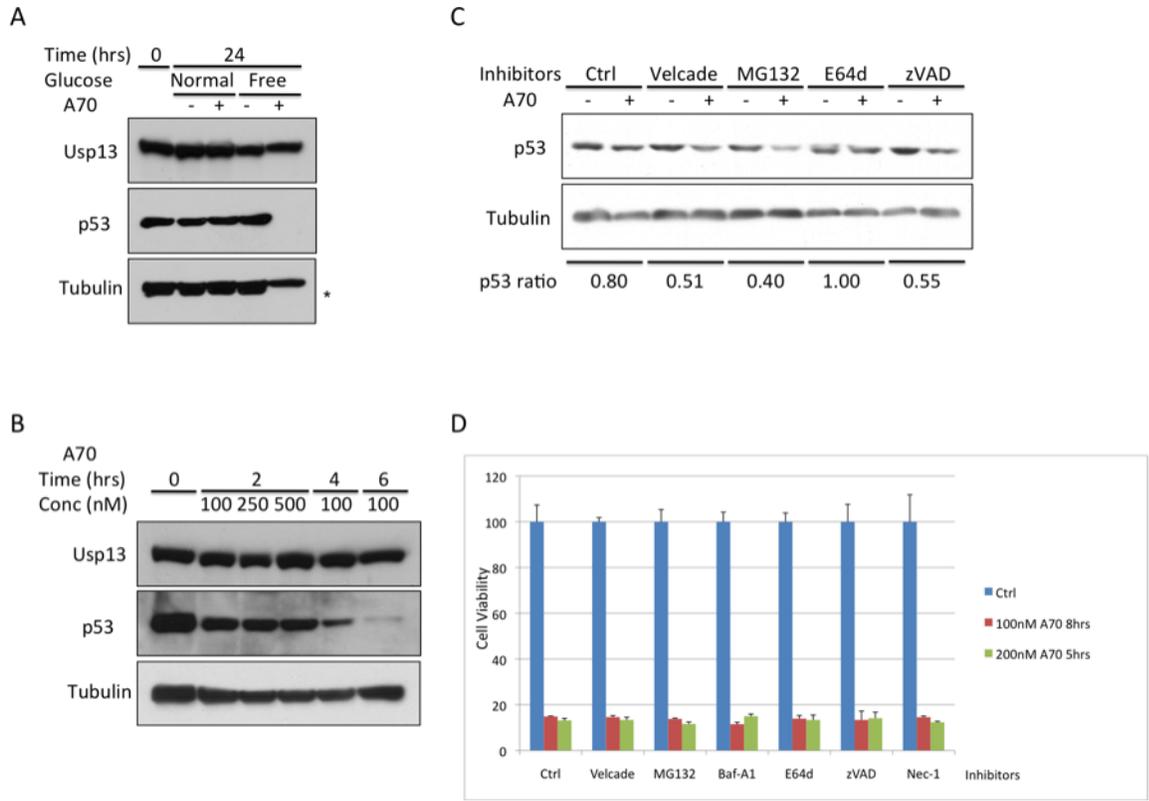
(A) ES-2 cells were treated with 100nM A70 for 24 hours in normal glucose or glucose-free media. Cell lysates were harvested and analyzed by western blotting. * non-specific band.

(B) ES-2 cells were treated with different concentration of A70 in glucose-free media and harvested at different time points. Protein levels were analyzed by western blotting.

(C) ES-2 cells were treated with different inhibitors with or without A70 for 2 hours in glucose-free media. Cell lysates were harvested and analyzed by western blotting. The bands were quantified by densitometry. The relative ratios of p53 are indicated.

(D) ES-2 cells were treated with different inhibitors with or without the treatment of A70 as indicated and the cellular ATP levels were measured. Error bars indicate standard deviation.

Figure 3.6 (Continued).



inhibitors blocks cell death induced by A70, possibly due to the toxicity of the inhibitors because in glucose-free conditions the cells heavily depend on recycling (Figure 3.6D). Therefore, A70 as a potent inhibitor of autophagy will be toxic to the cells under nutritional deprivation condition. This cell death is not caspase-dependent apoptotic cell death or RIPK1 kinase-dependent necrotic cell death because neither zVAD nor Nec-1 has any protective effect. Interestingly, degradation of p53 is not as much inhibited by another lysosomal inhibitor, Bafilomycin A1 (data not shown). Since Bafilomycin A1 inhibits lysosomal degradation of autophagic cargoes by blocking the fusion between autophagosome and lysosome, this suggests that mutant p53 is degraded by other lysosomal pathway than autophagy. This is not surprising because the degradation of mutant p53 is triggered by A70, which is an inhibitor of autophagy.

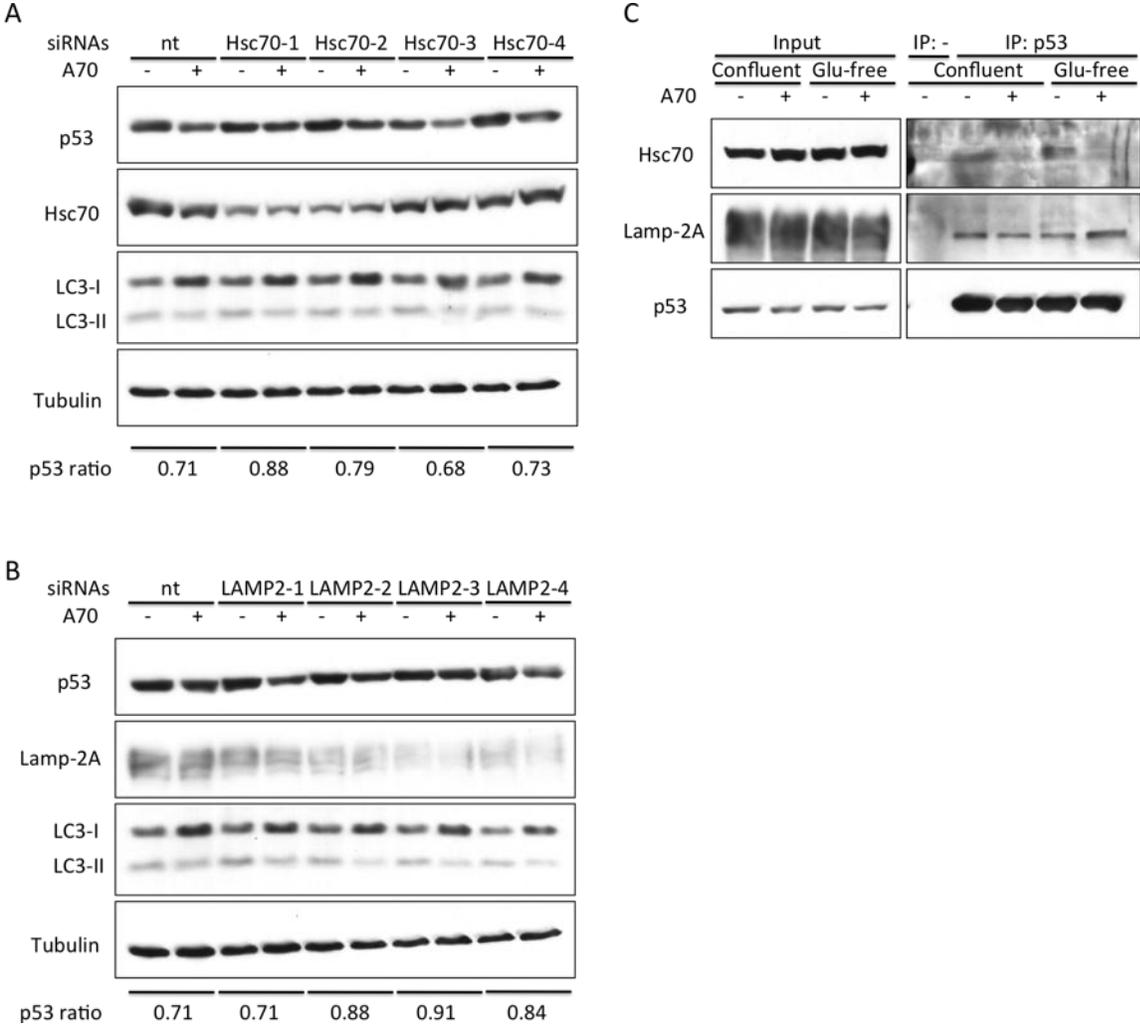
To further study the mechanism of mutant p53 degradation, we hypothesize that p53 is degraded by chaperone-mediated autophagy (CMA) because it is known to be activated when macroautophagy is inhibited. Moreover, p53 contains two consensus pentapeptide sequences, (₂₀₀NLRVE₂₀₄) and (₃₄₀FRELN₃₄₄), to be substrates for CMA (Orenstein and Cuervo, 2010). We show that p53 degradation is inhibited by knockdown of Hsc70 or Lamp-2A in the CMA pathway (Figure 3.7A, B). Interestingly, the inhibition has a strong correlation with the knockdown efficiency. We also show that mutant p53 binds to Hsc70 and Lamp-2A, further confirming that p53 is degraded by CMA pathway upon A70 treatment (Figure 3.7C). It is also possible that mutant p53 is one of many substrates that are degraded by CMA upon A70 treatment. However, the possibility of massive non-specific degradation was excluded by comparing the total protein expression patterns by Ponceau S staining.

Figure 3.7. Mutant p53 Is Degraded by CMA upon A70 Treatment

(A, B) ES-2 cells were transfected with indicated siRNAs. After 48 hours, they were treated with 100nM A70 for 24 hours in confluent condition. Cell lysates were harvested and analyzed by western blotting. The bands were quantified by densitometry. The relative ratios of p53 are indicated.

(C) ES-2 cells were treated as indicated with 100nM A70 for 2 hours, and p53 was immunoprecipitated. Coimmunoprecipitated proteins were analyzed by western blotting. Input samples are 5% of the lysates used for immunoprecipitation.

Figure 3.7 (Continued).



Discussion

Autophagy has been proposed to be involved in tumor suppression. However, most of the known tumor suppressors in the autophagy pathway are the subunits of the class III PI3 kinase complex, rather than downstream effectors, suggesting that the kinase complex has autophagy-independent anti-tumor function. Here, we propose a model in which loss of the subunits of the class III PI3 kinase complex can lead to reduced levels of p53. This adds another layer of explanation as to how the class III PI3 kinase complex suppresses tumor formation. A deficiency in any subunit of the class III PI3 kinase complex may reduce the effectiveness in cellular quality control, and thus subject to higher level of stress. Moreover, inability to mitigate cellular stress when cells have reduced levels of p53 may further promote tumorigenesis.

As opposed to the earlier stage of tumorigenesis, autophagy functions to promote the tumor survival at the later stage (White and DePaola, 2009). For this reason, autophagy inhibitors could be useful in cancer treatment when used together with the chemotherapy. Although this combination therapy can lead to increased cytotoxicity against the tumors, other proliferating cells in the body such as hematopoietic stem cells will be seriously affected. A70, however, specifically targets mutant p53 in confluent cells. Given that most cells in solid tumor are surrounded by neighboring cells and have difficulty in the access of nutrients, tumors harboring mutant p53 may be susceptible to A70. Furthermore, mutant p53 tend to be more accumulated in tumor cells compared with the normal cells, which may render the tumor cells more sensitive to the loss of mutant p53. All together, further studies with A70 on various *in vivo* cancer models with different p53 background will help to develop a mechanism-based therapeutic method for cancers.

Experimental Procedures

Chemicals and Antibodies

The sources of the antibodies used were as follows: mouse monoclonal antibodies against tubulin (Sigma), p53 (Cell Signaling Technology), and Lamp-2A (Santa Cruz Biotechnology), rabbit polyclonal antibodies against LC3 (Novus Biologicals), Hsc70, Vps34, Usp13 (Proteintech Group), Usp10, p150 (Abcam), Beclin 1 (Santa Cruz Biotechnology), UVRAG (Sigma), p53, Atg14L (Cell Signaling Technology), and ubiquitin (Dako).

Immunoprecipitation

For interaction studies, cells were harvested with lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1% NP-40 and protease inhibitor cocktail), and incubated overnight with p53 antibody (Cell Signaling Technology). After incubating with protein A/G beads (Thermo Scientific) for two hours, the beads were washed four times with the lysis buffer. Protein sample buffer was added to the beads to denature the immunoprecipitated proteins. After harvesting the cells, all experiments were performed at 4°C or on ice. For ubiquitination studies, RIPA buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1% Triton-X, 0.1% SDS, 0.5% sodium deoxycholate, and protease inhibitor cocktail) was used. The rest of the procedure is the same as described above.

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Chapter 4

Conclusions

Autophagy is a stress-responsive mechanism of the cells, and thus is tightly regulated by environmental cues. The class III PI3 kinase complex is in the center of the regulation because most of the signals regulating autophagy in nutrient-rich conditions also regulate the class III PI3 kinase complex (Lipinski *et al.*, 2010). Nevertheless, the mechanisms by which the class III PI3 kinase complex is regulated are not well understood.

In Chapter 2, we demonstrate that Vps34, the catalytic subunit of the class III PI3 kinase complex is negatively regulated by Cdk1 and Cdk5. Phosphorylation of Vps34 by those Cdk5 results in the dissociation of Vps34 from its regulatory subunit Beclin 1, leading to decreased lipid kinase activity of Vps34 and inhibition of autophagy. Therefore, autophagy is inhibited in mitotic cells, and post-mitotic cells with elevated Cdk5 activity.

Since cell division only occurs when the cells are surrounded with ample nutrients and growth factors, there is less need for the cells to activate autophagy. Moreover, in dividing cells, inhibition of autophagy may contribute to the preservation of important cellular components. Although growing evidence supports the selective autophagic degradation of cellular cargoes, cells will not be able to block non-selective degradation of unintended cellular components unless cells also counter-select autophagic cargoes. Especially during mitosis, when the nuclear membrane is fragmented and the genetic material is exposed to cytoplasm, the possibility of chromosome loss by autophagy must be avoided. This hypothesis may be tested by over-activating autophagy in dividing cells with the induction of DNA double strand break.

Another plausible explanation is that inhibition of autophagy in mitosis prevents the premature degradation of midbody ring. Midbody ring is a physical barrier between two daughter cells after cell division, and it has been shown to be degraded by autophagy (Pohl and

Jentsch, 2009). Midbody ring formation is important to guarantee the completion of cytokinesis and the separation of two daughter cells. Thus, autophagy may be reactivated after the cell division is completed to degrade the midbody ring. In this case, forced activation of autophagy in mitosis may result in the polynucleated cells due to abnormal cytokinesis.

In post-mitotic neurons, elevated Cdk5 activity has been known to be associated with neurodegeneration (Cruz *et al.*, 2003). Since neurons are not able to dilute intracellular toxic materials by cell division, cellular quality control by autophagy is extremely important. This is supported by the observations that brain-specific inhibition of basal autophagy in mice leads to the neurodegenerative features (Komatsu *et al.*, 2006; Hara *et al.*, 2006). Therefore, inhibition of autophagy by Cdk5 is thought to contribute, at least in part, to the Cdk5-mediated neurodegeneration.

Activated Cdk1 and Cdk5 phosphorylate T159 site on Vps34. T159A mutant of Vps34 interacts with Beclin 1 more strongly than the wild-type Vps34, and the interaction between mutant Vps34 and Beclin 1 is not disrupted by the expression of Cdk5, suggesting that this phosphorylation site is critical for the interaction. Interestingly, we found that the T159D and T159E mutants give the same results as the T159A mutant. This raises a possibility of a putative mediator that only binds to pT159, but not to the mutants. Cdk1 and Cdk5 are proline-directed serine/threonine protein kinases that phosphorylate SP/TP sites to generate pSP/pTP, which are recognized by a peptidyl-prolyl isomerase Pin1 (Ranganathan *et al.*, 1997). This implies that any substrate of Cdk1 and Cdk5 can be a putative substrate of Pin1 when phosphorylated. However, whether Pin1 is involved in the regulation of Vps34 remains to be investigated.

In Chapter 3, we report another mechanism by which the class III PI3 kinase complex is regulated. Upon starvation, the activity of the class III PI3 kinase complex is increased in a couple of hours, suggesting that it is regulated by post-translational modifications. Using a small molecule inhibitor of autophagy, we report ubiquitination and deubiquitination as important regulatory mechanisms in the pathway. Interestingly, the class III PI3 kinase complex and p53 share the deubiquitinases. This provides a regulatory interaction between the class III PI3 kinase complex and p53, supporting the tumor-suppressive role of the kinase complex.

Beclin 1 heterozygous mice spontaneously develop tumors in different tissues (Qu *et al.*, 2003; Yue *et al.*, 2003). It has been thought for a long time that this tumorigenesis is attributed to reduced autophagy. However, knockout of essential autophagy gene Atg7 only results in benign tumors, which is different from the aggressive tumor phenotype of Beclin 1^{+/-} mice (Takamura *et al.*, 2011; Qu *et al.*, 2003). Instead, the tumor patterns of Beclin 1^{+/-} mice is similar to that of p53^{+/-} mice, further supporting the idea that the tumorigenicity of monoallelic loss of Beclin 1 is due to compromised p53 (Jacks *et al.*, 1994).

A plausible explanation for concerted regulation of the class III PI3 kinase complex and p53 may be related to p53 induced cell death triggered by cellular stress. Although prolonged inhibition of autophagy is detrimental to the cells, temporary inhibition of autophagy may only cause some damage in the cells that can be overcome and repaired later by induction of autophagy and other stress-responsive pathways. Therefore, cells need to down-regulate the stress threshold for cell death when autophagy is compromised, which is achieved by transient decrease of p53. Alternatively, decreased activity of p53 may be a negative feedback mechanism of the cells to recover the levels of autophagy. Since p53 of physiological expression

level is shown to inhibit autophagy, degradation of p53 induces autophagy (Tasdemir *et al.*, 2008). Although the activation of p53 also leads to increased autophagy, this is a long term and sustained effect because it involves gene transcription. Thus, any perturbation of the class III PI3 kinase complex may down-regulate p53 to rescue the levels of autophagy to maintain homeostasis.

In contrast to that of the wild-type p53, mutant p53 is regulated in a distinctive mechanism by the autophagy inhibitor. Misfolded mutant p53 can interact with various chaperones including some of which are involved in chaperone-mediated autophagy (CMA). Therefore, mutant p53 is likely to be degraded by CMA that is up-regulated by inhibition of autophagy. Since mutant p53 is implicated in a wide range of cancers, selective degradation of mutant p53 can be used for therapeutic purposes, for the following reasons. First, removal of mutant p53 has been reported to ameliorate the tumor phenotype in mice, suggesting that the expression level of mutant p53 correlates with the aggressiveness of the tumor (Bossi *et al.*, 2008). Moreover, mutant p53 tends to be accumulated only in the cancer cells, instead of normal cells. Thus, cancer cells can be selectively targeted over normal cells by degrading mutant p53. From the experiments, we also claim that mutant p53 is degraded by A70 only in confluent or glucose-deprived cells, which is a hallmark of cancer cells. This may allow us to develop a therapeutic method to specifically induce cell death in cancer cells, which has been an issue for a long time, due to the cytotoxicity of many chemotherapeutic agents towards normal cells. However, *in vivo* studies using mutant p53 mouse models have yet to be performed to further demonstrate the effect of A70 on cancers.

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Appendix I

Interaction of the Class III PI3 Kinase Complex and RIPK1 Complex

Abstract

Necroptosis, also known as programmed necrotic cell death, is a cell death pathway dependent of RIPK1 and its interacting partners. In L929 cells, inhibition of caspase-8 by zVAD induces RIPK1-dependent necrotic cell death. In this model, autophagy has been shown to play a crucial role, and thus inhibition of autophagy protects the cells from cell death, hence referred to as autophagic cell death. We report that spautin-1, a novel autophagy inhibitor we identified in a screen, protects zVAD induced cell death in L929. Spautin-1 also inhibits necroptosis induced by $\text{TNF}\alpha$, suggesting that it is involved in the pathway downstream of $\text{TNF}\alpha$. We also show that the class III PI3 kinase complex interacts with RIPK1/EDD complex, and UVRAG is ubiquitinated by EDD. From the observation, we suppose that autophagy is directly regulated at earlier time point by RIPK1/EDD complex rather than induced by cell damage in the course of cell death.

Introduction

Cells undergo programmed cell death, when triggered by death signals. Depending on the morphology and key players of cell death, it can be divided into subcategories. Necroptosis is a subset of necrotic cell death that is tightly regulated by signaling pathways (Christofferson and Yuan, 2010). It was defined by the identification of a small molecule Nec-1, which specifically inhibits necrotic cell death triggered by death signals (Degterev *et al.*, 2005). Necroptosis is characterized by necrotic cell morphology including substantial increase of autophagy. In this pathway, receptor interacting protein kinase 1 (RIPK1) plays a crucial role, and its kinase activity is indispensable (Holler *et al.*, 2000; Degterev *et al.*, 2008). RIPK1 forms a complex to mediate down-stream signaling including NF- κ B activation, apoptosis, and necroptosis. Depending on the environment of the cell, RIPK1 binds to different proteins to determine which pathway to turn on.

In mouse fibroblast L929 cells, pan-caspase inhibitor zVAD or knockdown of caspase-8 induces necroptosis. Interestingly, inhibition of autophagy by using chemical inhibitors targeting Vps34, or by knockdown of autophagy genes increases the cell survival upon zVAD treatment (Yu *et al.*, 2004). In a genome-wide siRNA screen to identify a regulator of necroptosis, knockdown of EDD has been shown to inhibit zVAD induced cell death in L929 cells (Hitomi *et al.*, 2008). EDD is an E3 ligase involved in DNA damage checkpoint, and often affected in cancer cells (Henderson *et al.*, 2006; Clancy *et al.*, 2003). In the necroptosis pathway, EDD has been shown to interact with RIPK1 and mediate TNF α secretion upon zVAD treatment (Christofferson *et al.*, 2012). The TNF α signaling cascade is crucial in zVAD induced necroptosis

pathway because knockdown of TNF receptor or neutralizing TNF α with antibodies protects L929 cells from zVAD induced cell death.

Here, we show that a small molecule inhibitor of autophagy, spautin-1, that we identified in a screen also protects L929 cells from necroptosis. We also demonstrate the interaction between RIPK1/EDD complex and the class III PI3 kinase complex, suggesting the regulation of autophagy by RIPK1/EDD complex.

Results

Spautin-1 Protects L929 Cells from Autophagic Cell Death

Since spautin-1 specifically inhibits autophagy, we tested whether spautin-1 protects the cells from necrotic cell death in L929 cells. Similar to what has been shown with catalytic inhibitors of Vps34, spautin-1 inhibits cell death triggered by zVAD (Figure A.1A, B). Interestingly, it also protects L929 cells from TNF α induced cell death, whereas knockdown of autophagy genes only rescues zVAD-induced necroptosis. Those two different signals require TNF receptor in common, and spautin-1 reduces the expression levels of TNF receptor, which explains why spautin-1 blocks both pathways (Figure A.1C). However, spautin-1 does not block the necrotic pathway in FADD^{-/-} Jurkat cells which does not require autophagy (Figure A.1D).

The Class III PI3 Kinase Complex Interacts with RIPK1/EDD Complex

To further study the relationship between autophagy and necrotic cell death, we identified interaction partners of PI3 kinase complex in the *Drosophila* system. Among these, we identified Hyd, a fly homologue of EDD, as an interaction partner of UVRAG. In 293T cells,

Figure A.1. Inhibition of zVAD and TNF α Induced Cell Death in L929 Cells by Spautin-1

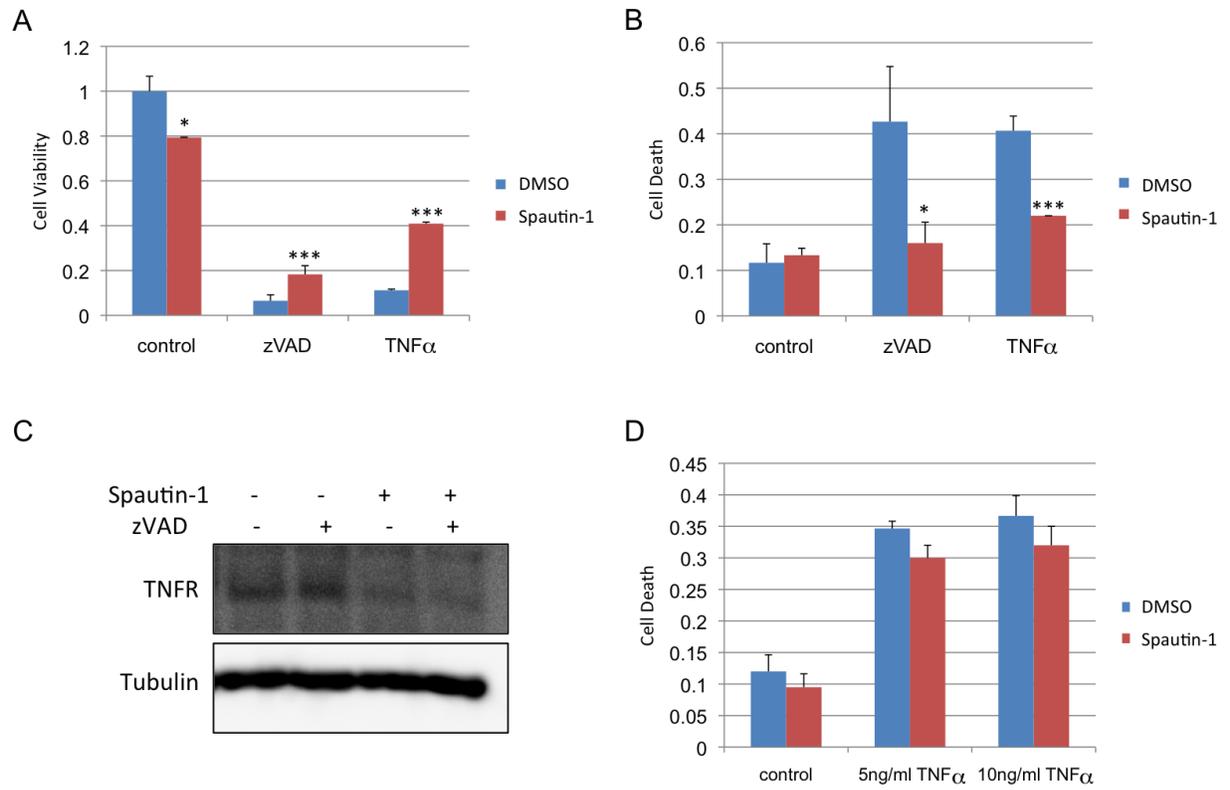
(A) L929 cells were treated with 20 μ M zVAD or 20ng/ml TNF α with or without 20 μ M spautin-1. 24 hours after treatment, cellular ATP levels were measured.

(B) L929 cells were treated as in (A) and the cells were stained with Sytox Green labeling membrane permeabilized cells and Hoechst labeling all cells. The ratio of Sytox Green positive cells to Hoechst positive cells was measured using high-throughput microscope. Error bars indicate standard deviation. * $p < 0.05$, *** $p < 0.01$.

(C) L929 cells were treated with spautin-1 and/or zVAD as indicated for 8 hours and harvested. Cell lysates were analyzed by western blotting.

(D) FADD $^{-/-}$ Jurkat cells were treated with different concentrations of TNF α with or without spautin-1 for 16 hours and the cells were stained with Sytox Green labeling membrane permeabilized cells and Hoechst labeling all cells. The ratio of Sytox Green positive cells to Hoechst positive cells was measured using high-throughput microscope. Error bars indicate standard deviation.

Figure A.1 (Continued).



EDD coimmunoprecipitates with UVRAG and the interaction is reduced under various insults (Figure A.2A). EDD also interacts with Vps34 and Beclin 1, which are the core subunits in the PI3 kinase complex (Figure A.2B). However, EDD does not coimmunoprecipitate with Atg14L, suggesting that EDD specifically interacts with UVRAG containing complex instead of Atg14L containing complex. Since EDD has been shown to interact with RIPK1, the interaction between RIPK1 and the class III PI3 kinase complex was also tested. As opposed to EDD, RIPK1 interacts both with Atg14L and UVRAG as well as Vps34 and Beclin 1, suggesting that RIPK1 and EDD might regulate the class III PI3 kinase complex by more than one mechanism (Figure A.2C). To determine the mechanism by which RIPK1/EDD complex regulates the class III PI3 kinase complex, we tested if EDD has an E3 ligase activity on UVRAG. Overexpression of EDD leads to UVRAG ubiquitination, which is increased with the treatment of MG132 suggesting that ubiquitinated UVRAG is subject to the proteasomal degradation (Figure A.2D). Since both EDD and the class III PI3 kinase complex play important roles in zVAD induced cell death, we suppose that EDD mediates L929 cell death, at least in part by degrading UVRAG. Consistent with this hypothesis, UVRAG is degraded after zVAD treatment (Figure A.2E). Moreover, knockdown of UVRAG sensitizes L929 cells to zVAD (Figure A.2F). However, further studies will be necessary to delineate the mechanism by which RIP1 and EDD coordinately regulate the class III PI3 kinase complex to induce autophagic cell death in L929 cells.

Discussion

Autophagy has been reported to be significantly increased in necroptosis (Degterev *et al.*, 2005). However, whether autophagy contributes to necroptosis depends on the cell types

Figure A.2. Interaction of the Class III PI3 Kinase Complex with RIP1/EDD

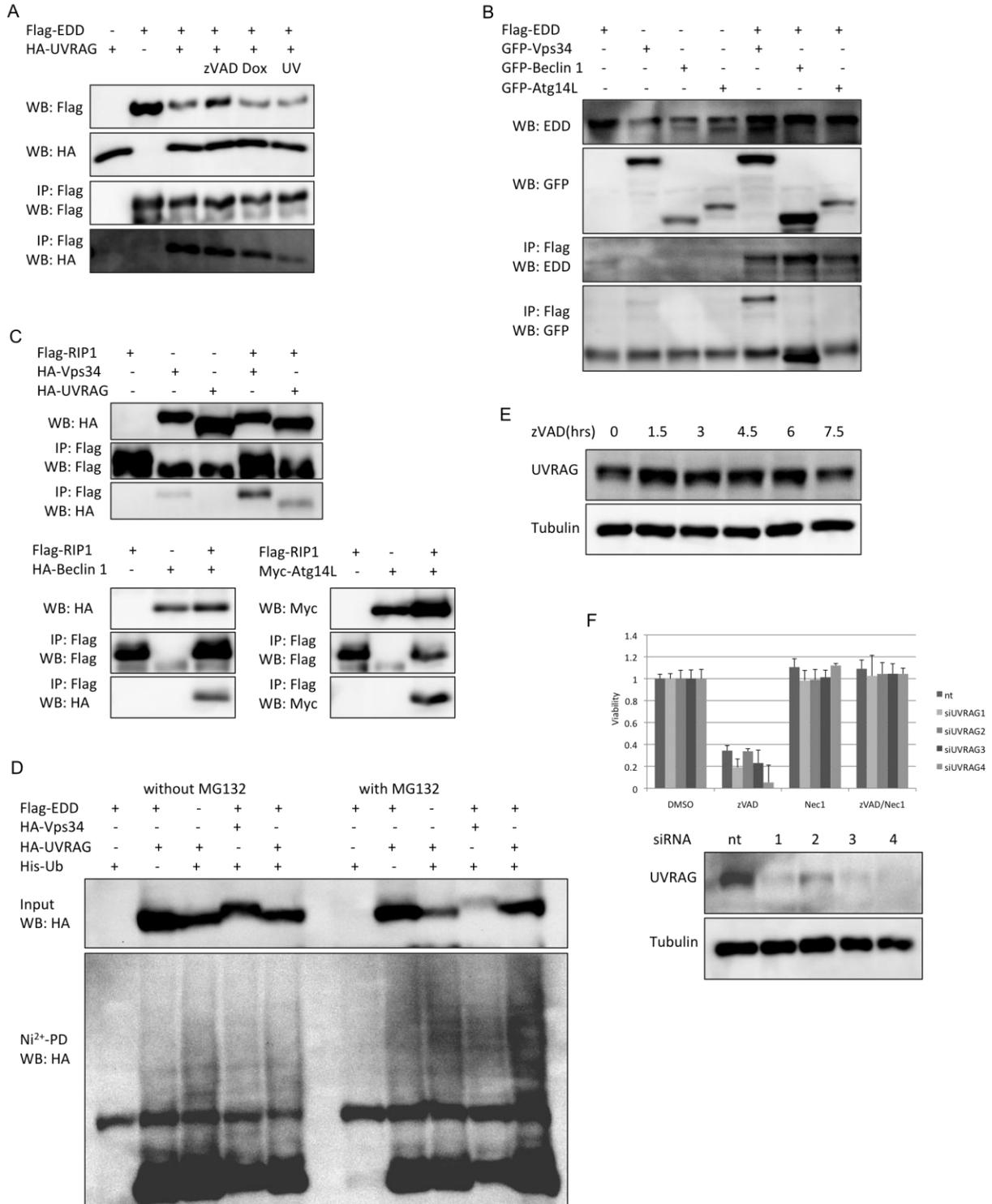
(A - C) 293T cells were transfected with indicated expression vectors. After 24 hrs of transfection, cell lysates were harvested and immunoprecipitated using agarose beads coated with Flag antibody, and coprecipitated proteins were analyzed by western blotting.

(D) 293T cells were transfected with indicated expression vectors. After 24 hrs of transfection, cells were harvested using denaturing lysis buffer containing 8M urea. 6x His-tagged ubiquitin was pulled down by Ni²⁺ beads, and the ubiquitinated proteins were analyzed by western blotting.

(E) L929 cells were treated with zVAD for indicated time. Cell lysates were harvested and analyzed by western blotting.

(F) L929 cells were treated with four different UVRAG siRNAs. After 48 hours, they were treated with zVAD for 24 hours. Cell lysates were harvested and analyzed by western blotting. For the viability assay, cellular ATP levels were measured.

Figure A.2 (Continued).



and death signals. Although the production of ROS and mitochondrial damage occurring during necroptosis may lead to the activation of autophagy, the detailed mechanism of autophagy induction in necroptosis is still elusive (Nakagawa *et al.*, 2005). Moreover, in autophagic cell death, autophagy must be activated before the cell is severely damaged because autophagy is a driving force for cell death. In zVAD induced autophagic cell death model, catalase is degraded by autophagy, resulting in increased ROS (Yu *et al.*, 2006). Therefore, in this case, autophagy is activated before ROS production. In our studies, we show that RIPK1 and EDD that play important roles in the early step of necroptosis interact with the class III PI3 kinase complex. This suggests the possibility that autophagy may be regulated by RIPK1 directly in the early step of necroptosis in general, considering that the activity of RIPK1 is indispensable for most of the necroptotic models.

Experimental Procedures

Chemicals and Antibodies

The sources of the antibodies used were as follows: mouse monoclonal antibodies against tubulin and Flag (Sigma), rabbit polyclonal antibodies against EDD (Novus Biologicals), GFP, HA (Santa Cruz Biotechnology), and UVRAG (Sigma). zVAD and TNF α were from Cell Sciences (Canton, MA).

Immunoprecipitation

293T cells were transfected with indicated overexpression constructs for 24hrs. Cells were harvested with lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1% NP-40

and protease inhibitor cocktail), and incubated overnight with Flag beads (Sigma). After washing four times with the lysis buffer, protein sample buffer was added to the beads to denature the immunoprecipitated proteins. After harvesting the cells, all experiments were performed at 4°C or on ice.

Ni²⁺ Pulldown assay

293T cells were transfected with indicated constructs including 6X His-tagged ubiquitin for 24hrs. Cells were harvested with denaturing lysis buffer (10mM Tris-HCl pH 8.0, 100mM NaH₂PO₄, 500mM NaCl, 10% glycerol, 0.1% Triton X-100, 10mM b-mercaptoethanol, 10mM imidazole), and incubated for 4hrs with Ni²⁺-NTA beads (Qiagen). After washing four times with the lysis buffer with 30mM imidazole, the beads were eluted with elution buffer (0.15M Tris-HCl pH 6.7, 5% SDS, 30% glycerol, 200mM imidazole, 0.72M b-mercaptoethanol). All experiments were performed at room temperature.

Cell Viability Assay

ATP assay was done with CellTiter-Glo Luminescent Cell Viability Assay (Promega). L929 cells were plated in 96-well plate. After 18-24hrs of treatment, the assay reagent was added to each well and the luminescence was measured with the plate reader.

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Appendix II

Supplemental Figures for Chapter 2

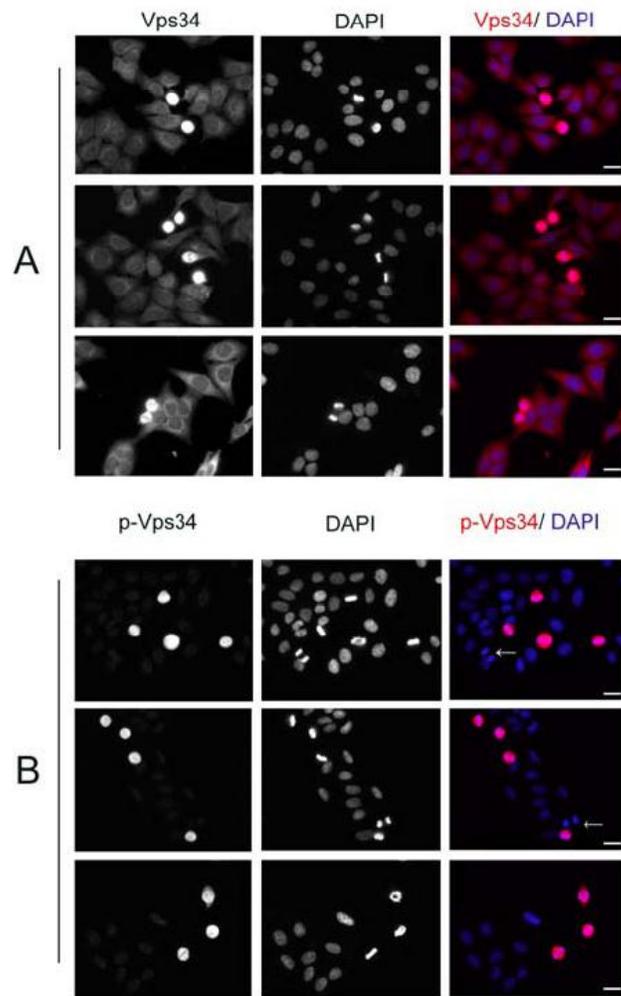


Figure A2.1. Immunofluorescence localization of Vps34 (A) and phosphorylated Vps34 (B) in asynchronous HeLa cells

HeLa cells were fixed and then subjected to immunostaining. Cells were double-labeled with anti-Vps34 or affinity-purified phosphorylated Vps34 antibodies and DAPI as nuclear staining. In cells of the telophase/cytokinesis phosphorylated Vps34 expression was dramatically reduced (Arrows). Bar, 20 μm .

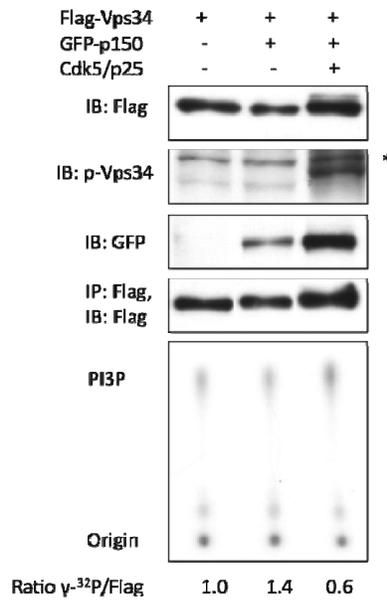


Figure A2.2. The effects of p150 and Cdk5 expression on T159 phosphorylation and lipid kinase activity of Vps34

293T cells were transfected with indicated expression vectors. The cell lysates were subject to western blotting using indicated antibodies. The Vps34 lipid kinase activity in the immune complex isolated using anti-Flag antibody was measured in the presence of γ -³²P-ATP as described in the Materials and Methods. Relative ratios of the γ -³²P signal divided by the amount of Flag-Vps34 protein as measured by densitometry are indicated.

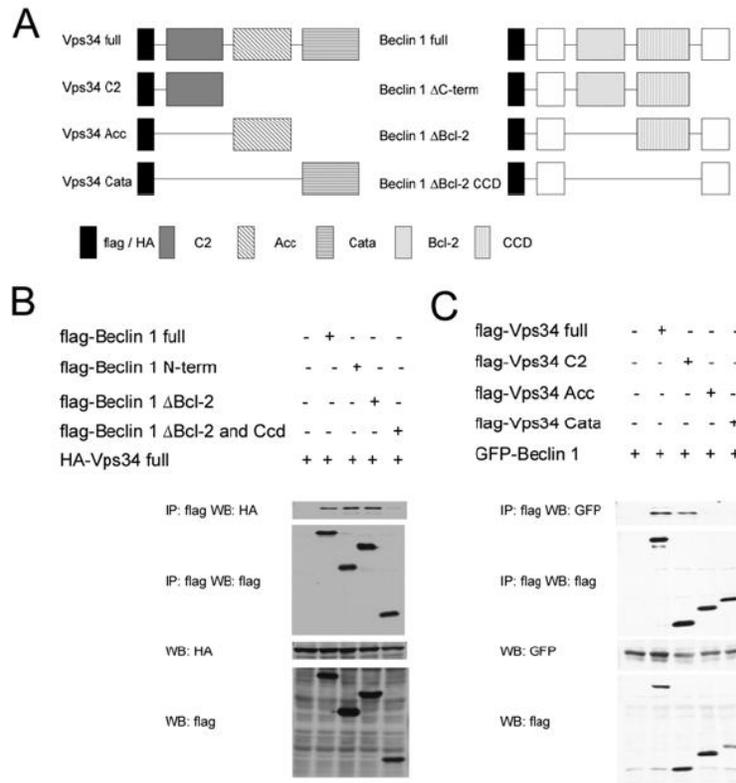


Figure A2.3. Interaction of Beclin1 and Vps34

(A) A schematic representation of Vps34 and Beclin 1 protein structure. Acc, accessory domain; Cata, catalytic domain; CCD, coiled-coil domain. (B) The N-terminal C2 domain of Vps34 interacts with Beclin 1. The cells were transfected with expression vectors of GFP-Beclin 1 and full length or truncated fragments of flag-tagged Vps34. Twenty-two hrs after the transfection, the cell lysates were immunoprecipitated using anti-flag followed by western blotting with anti-GFP and flag antibodies. (C) The CCD of Beclin 1 is required for binding Vps34. The cells were transfected with expression vectors of flag-tagged full length or truncated fragments of Beclin 1 together with that of HA-Vps34; 22 hrs after the transfection, the cell lysates were immunoprecipitated using anti-flag followed by western blotting with anti-HA and anti-flag antibodies.