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# Negative Regulation of Vps34 by Cdk Mediated Phosphorylation

Tsuyoshi Furuya,[1,7,8] Minsu Kim,[1,7] Marta Lipinski,[1] Juying Li,[1] Dohoon Kim,[3,4] Tao Lu,[2] Yong Shen,[5] Lucia Rameh,[6] Bruce Yankner,[2] Li-Huei Tsai,[3,4] and Junying Yuan[1,*]
[1]Department of Cell Biology
[2]Department of Pathology
Harvard Medical School, Boston, MA 02115, USA
[3]Department of Brain and Cognitive Sciences
[4]Howard Hughes Medical Institute
Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[5]Haldeman Laboratory of Molecular and Cellular Neurobiology, Banner Sun Health Research Institute, Sun City, AZ 85351, USA
[6]Boston Biomedical Research Institute, Watertown, MA 02472, USA
[7]These authors contributed equally to this work
[8]Present address: Department of Neurology, Juntendo University Shizuoka Hospital, Izunokuni, Shizuoka 410-2295, Japan
*Correspondence: jyuan@hms.harvard.edu
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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 Cdks. We show that Thr159 of Vps34 can be phosphorylated by Cdk1 and Cdk5, which inhibits 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

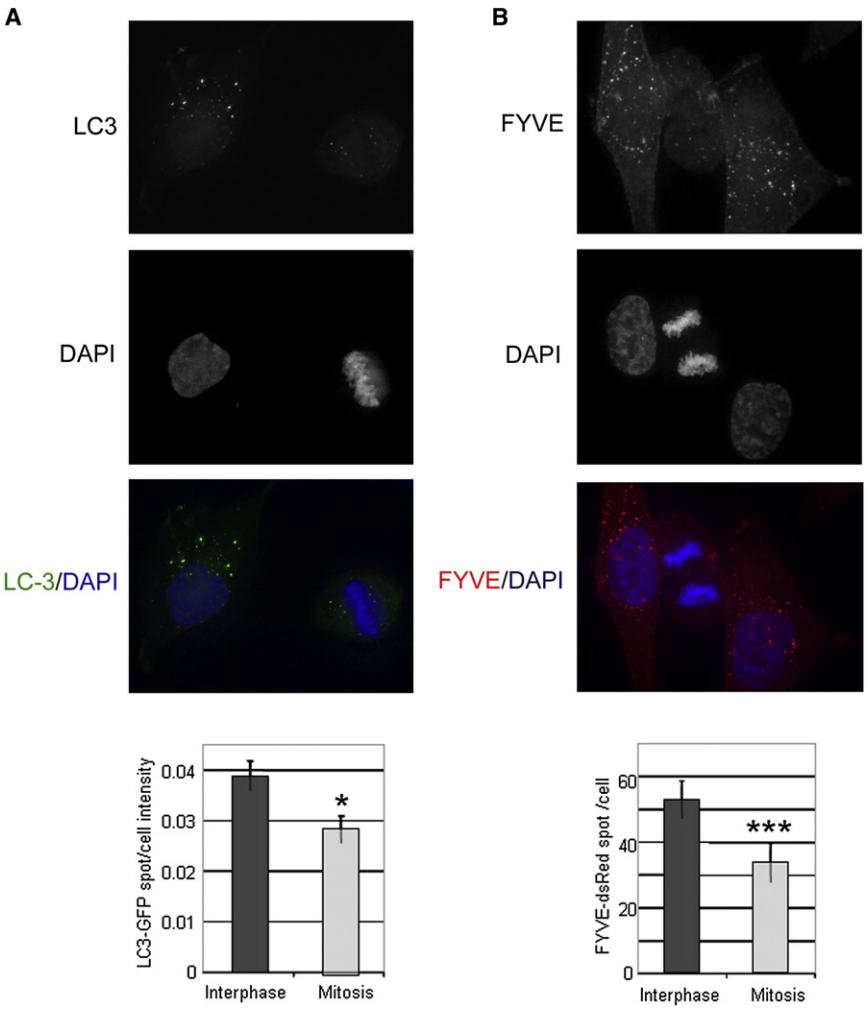


**Figure 1. The Levels of Autophagy and PtdIns3P Are Decreased during Mitosis**

(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 60× 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 60× 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.

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 1A). We quantified the intensity of LC3-GFP present on the autophagosomes versus the total intensity of LC3-GFP 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 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 phosphatidylinositol-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 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,