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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 D-3 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...
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) (Gaulier 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,
we incubated immunoprecipitated Vps34 protein with or without recombinant Cdk1/cyclin B complex in the presence of [γ-32P]-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 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 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 2C). From these results, we conclude that Cdk1/cyclin B can phosphorylate Thr159 of Vps34.

**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 3A, phosphorylation of Thr159 in Vps34 was increased in a time-dependent manner upon nocodazole treatment. Treatment with Cdk1 inhibitors roscovitine (10 μM) or alsterpaullon (1 μM) dramatically reduced nocodazole-induced Thr159 phosphorylation on Vps34 (Figure 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 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 hypothesized 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 3A, phosphorylation of Thr159 in Vps34 was increased in a time-dependent manner upon nocodazole treatment. Treatment with Cdk1 inhibitors roscovitine (10 μM) or alsterpaullon (1 μM) dramatically reduced nocodazole-induced Thr159 phosphorylation on Vps34 (Figure 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 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 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 3E, the levels of phosphorylated Vps34 were
significantly elevated at 10 hr after the release when cells were in the mitotic phase. 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 3F and see Figure S1A available online). 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 3G and Figure S1B). This result is consistent with the western blotting analysis as shown in Figure 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-32P]-ATP. As shown in Figure 4A, the levels of 32P-labeled Vps34 were increased after the incubation 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 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 vectors or without 10 μM roscovitine (Ros). After 20 hr, the whole-cell lysates were analyzed by western blotting using 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) Lysates from H4 cells expressing Cdk5/p25 were either untreated or treated with λPP prior to western blotting with 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).

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 4D). Mutation of Thr159 to Ala (T159A) resulted in a complete loss of recognition by anti-pThr159 Vps34 antibody or the levels of phosphorylation in the presence of cdk5/p25 (lanes 3 and 4, Figure 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 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 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 S2). Thus, it is most likely that Cdk5s 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, we coexpressed individual Vps34 domains (Figure S3A) 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 S3B). Conversely, the coiled-coil domain (CCD) and the ECD of Beclin 1 were required for binding of Vps34 (Figure S3C). 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 5A). However, the interaction of Vps34 and Beclin 1 was partially rescued in the Cdk5/p25-expressing cells in the presence of roscovitine (Figure 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 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 endogenous Beclin 1 in the absence of p25 expression, but this interaction was disrupted by p25 expression (Figure 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 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 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 p25 expression, while the interaction of flagged-Beclin 1 with T159A Vps34 was not affected by Cdk5/p25 expression (Figure 5F). Interestingly, in both experiments the phosphomutant Vps34 (T159A) showed an increased interaction with Beclin 1 as compared with WT Vps34 (Figures 5E and 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 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 in vitro, 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-32P]-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 6B). As a control, treatment with wortmannin, a general PtdIns3 kinase inhibitor, completely inhibited the PtdIns3 kinase activity (Figure 6B).

As discussed above, the phosphomutant Vps34 (T159A) has shown an increased interaction with Beclin 1 as compared with that of WT Vps34 (Figures 5D and 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 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 6D).

To further determine whether autophagy is regulated consistently with the lipid kinase activity of Vps34 by Cdk5 and p25 expression vectors.
Figure 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 μM) 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 32P 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 were quantified. Error bars indicate standard error. *p < 0.01.
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 PtdIns3P formation by p25 (Figure 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 7B).

Finally, since we found that T668 can also be phosphorylated by Cdk5 (Figure 4B), we examined the requirement of T668 for the lipid kinase activity of Vps34. As shown in Figure 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 7D); furthermore, overexpression of p25 in H4 cells resulted in inhibition of autophagy even in the presence of T159A Vps34 overexpression (Figure 7E). Thus, Cdk5 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 Cdk5. 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 Barbadic, 2009) and abnormal regulation of Cdk5 has also been implicated in tumorigenesis (Malumbres and Barbacid, 2009), our study has implications for understanding 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 Cdk5s 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 Cdk5s 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 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 to 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 Cdk5s, 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

Figure 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 6.

(D) 293T cells were transfected with indicated expression vectors, and lipid kinase assays were conducted as in Figure 6. Relative ratios of the 32P 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.
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.

**Cdk5, 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 Cdns 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 phase entry and DNA replication have been well documented 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 Cdns 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 JPP 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 60× 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 v.7.0 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 μg sonicated phosphatidylinositol (Sigma). Finally we added 10 μCi [γ-32P]ATP and 1 mM cold ATP for 15 min at room temperature. The kinase reactions were stopped by the addition of 20 μl 8 M HCl and extracted with 160 μl 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 with this article online at doi:10.1016/j.molcel.2010.05.009.

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