

# hVps15, but not Ca<sup>2+</sup>/CaM, is required for the activity and regulation of hVps34 in mammalian cells

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The mammalian Class III PI3K (phosphoinositide 3-kinase), hVps34 [mammalian Vps (vacuolar protein sorting) 34 homologue], is an important regulator of vesicular trafficking, autophagy and nutrient sensing. In yeast, Vps34 is associated with a putative serine/threonine protein kinase, Vps15, which is required for Vps34p activity. The mammalian homologue of Vps15p, hVps15 (formerly called p150), also binds to hVps34, but its role in hVps34 signalling has not been evaluated. In the present study we have therefore compared the activity and regulation of hVps34 expressed without or with hVps15. We find that hVps34 has low specific activity when expressed alone; co-expression with hVps15 leads to a marked increase in activity. Notably, beclin-1/UVRAG (UV radiation resistance-associated gene) activation of hVps34 requires co-expression with hVps15; this may be explained by the observation that beclin-1/UVRAG expression increases hVps34/hVps15 binding. Regulation of hVps34 activity by nutrients also requires co-expression with

hVps15. Finally, given a recent report that hVps34 activity requires Ca<sup>2+</sup>/CaM (calmodulin), we considered whether hVps15 might be involved in this regulation. Although hVps34 does bind CaM, we find its activity is not affected by treatment of cells with BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] or W7. Removal of CaM by EDTA or EGTA washes has no effect on hVps34 activity, and hVps34 activity *in vitro* is unaffected by Ca<sup>2+</sup> chelation. The results of the present study show that, in mammalian cells, hVps34 activity is regulated through its interactions with hVps15, but is independent of Ca<sup>2+</sup>/CaM.

**Key words:** autophagy, beclin-1, vacuolar protein sorting 15 (Vps15), mammalian target of rapamycin (mTOR), mammalian vacuolar protein sorting 34 (Vps34), UV radiation resistance-associated gene (UVRAG).

## INTRODUCTION

Class III PI3Ks (phosphoinositide 3-kinases) (reviewed in [1]) specifically produce the lipid PtdIns3P [2,3], which acts by recruiting downstream effectors containing FYVE or PX (Phox homology) domains [4,5]. This activity is distinct from that of Class I PI3Ks, which produce PtdIns(3,4,5)P<sub>3</sub> in intact cells, and Class II PI3Ks, which produce PtdIns3P and PtdIns(3,4)P<sub>2</sub> [6].

hVps34 [mammalian Vps (vacuolar protein sorting) 34 homologue] is required for Rab5-mediated fusion in early endosomes [7,8], and is also required for normal maturation of Rab7-positive late endosomes and multivesicular bodies [9,10]. Vps34p and hVps34 are also required for starvation-induced autophagy in yeast and mammalian cells [11,12]. In mammalian cells, this is in part mediated by the association of hVps34 with the autophagy-related proteins beclin-1, UVRAG (UV radiation resistance-associated gene) and Bif-1 (Bax-interacting factor 1) [12–14]. Finally, we have shown previously that hVps34 is required for activation of the mTOR [mammalian TOR (target of rapamycin)]/S6K1 (S6 kinase 1) pathway by insulin in cultured mammalian cells, but is not itself regulated by insulin [15]. Instead, hVps34 activity is regulated by nutrients (glucose and amino acids), consistent with it contributing to the nutrient-regulated inputs to mTOR. Similarly, hVps34 was shown to be required for stimulation of mTOR/S6K1 by amino acids [16]. A recent study has proposed that in cultured mammalian cells, hVps34 activity requires its binding to Ca<sup>2+</sup>/CaM (calmodulin),

and that amino-acid regulation of hVps34 occurs via changes in intracellular Ca<sup>2+</sup> levels [17]. Of note, Vps34 is not required for activation of the TOR pathway in *Drosophila* [18], and the role of hVps34 in mTOR signalling in mammalian animal models has not yet been investigated.

In yeast, Vps34 is associated with a putative serine/threonine protein kinase, Vps15 [19]; the mammalian homologue, hVps15 (formerly called p150) also binds to mammalian hVps34 [20]. Yeast Vps15p is required for the activity of Vps34p, as deletion of *VPS15* leads to a loss of PtdIns3P production and a disruption of vesicular trafficking [21]. Although Vps34p does not appear to be a substrate of Vps15p [22], the mutations in the kinase domain of Vps15p abolish its binding to Vps34p and lead to a loss of PtdIns3P production [21]. Interestingly, increases in PtdIns3P can be detected in *VPS15*-null strains that overexpress Vps34p, but this does not lead to a restoration of Vps34p-dependent vesicular trafficking [21]. Thus, while Vps34p activity may not be strictly dependent on Vps15p, its function in vesicular trafficking is Vps15p dependent.

To date, the role of hVps15 in the regulation of hVps34 in mammalian cells has not been extensively studied. *In vitro*, hVps15 leads to a 2-fold activation of hVps34 lipid kinase activity [20]. We previously showed that activated Rab5 binds to the WD40 domains of hVps15, and that Rab5 targets hVps34 and hVps15 to early endosomes [7,8]. Similarly, in late endosomes, Rab7 and the PtdIns3P phosphatases MTM1 and MTM2 (myotubularin 1 and 2) bind to the hVps15 WD40

Abbreviations used: BAPTA, 1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; BAPTA/AM, BAPTA(acetoxymethyl ester); Bif-1, Bax-interacting factor 1; CaM, calmodulin; D-PBS, Dulbecco's PBS; FBS, foetal bovine serum; HA, haemagglutinin; HEK, human embryonic kidney; hVps15, mammalian vacuolar protein sorting 15 homologue; hVps34, mammalian vacuolar protein sorting 34 homologue; MEM, minimal essential medium; MLCK, myosin light-chain kinase; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; S6K1, S6 kinase 1; TOR, target of rapamycin; UVRAG, UV radiation resistance-associated gene; Vps, vacuolar protein sorting; YFP, yellow fluorescent protein.

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domains, in a mutually exclusive manner [23,23a]. These studies suggest an important role for hVps15 in the targeting of hVps34 to distinct endosomal locations. Based on results from yeast and flies [11,24], hVps15 would be expected to play a role in hVps34-dependent autophagy, but this has not been examined in mammalian cells. However, several groups have reported that the mammalian autophagy proteins beclin-1, UVRAG and Bif-1 bind to overexpressed hVps34 and/or regulate its activity in the absence of overexpressed hVps15 [12–14].

In the present study, we have examined the role of hVps15 in the regulation of hVps34. We find that the specific activity of hVps34 is significantly increased by co-expression with hVps15. Moreover, regulation of hVps34 activity by autophagy-related proteins and by nutrients requires the presence of hVps15. In contrast, we find no evidence to support the hypothesis that hVps34 activity is regulated by  $\text{Ca}^{2+}$ /CaM. These experiments provide new information on the role of hVps15–hVps34 interactions in mammalian cells, and provide strong evidence against the putative role of  $\text{Ca}^{2+}$ /CaM in the regulation of hVps34 activity.

## EXPERIMENTAL

### Cell lines and materials

The insulin-responsive CHO (Chinese-hamster ovary)-derived cell line GRC+LR-73 (referred to as LR73 cells) has been previously described [25]. Anti-hVps34 and anti-hVps15 antibodies have been previously described [8,15]. Anti-FLAG antibodies and anti-V5 antibodies were from Sigma and Invitrogen respectively. The FLAG–beclin cDNA was a gift from Dr Beth Levine (The University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.). The HA (haemagglutinin)–UVRAG construct was a gift from Dr J.U. Jung (Harvard Medical School, Boston, MA, U.S.A.). YFP (yellow fluorescent protein)–CaM was a gift from Dr F. Bukauskas (Albert Einstein College of Medicine, Bronx, NY, U.S.A.). BAPTA [1,2-bis-(*o*-aminophenoxy)ethane-*N,N,N,N*-tetra-acetic acid], BAPTA/AM [BAPTA(acetoxymethyl ester)] and W7 were from Calbiochem; BAPTA/AM and W7 were dissolved in DMSO. Recombinant CaM and anti-CaM antibodies were purchased from Millipore.

### Expression of hVps34 and hVps15

The human hVps34 and hVps15 cDNAs [3,20] were subcloned into the pVITRO2-mcs (Invivogen), which uses the ferritin light- and heavy-chain promoters to drive co-ordinated expression. Transient transfections of HEK (human embryonic kidney)-293T cells were performed using FuGENE™ 6 (Roche). Stable transfectants were selected with hygromycin B. Unless specified, mixed populations of transfected cells were used.

### hVps34 activity assays

hVps34 activity was measured as previously described [15], in anti-Myc, anti-V5 or anti-hVps34 immunoprecipitates. Control experiments showed that immunoprecipitations were highly specific (results not shown). Standard assay conditions for hVps34 involved cell lysis in 20 mM Tris/HCl (pH 7.5), 137 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 10% glycerol and protease and phosphatase inhibitors. Immunoprecipitated hVps34 was washed sequentially in PBS containing 1% Nonidet P40 (three times), 100 mM Tris (pH 7.4) containing 500 mM LiCl (three times) and TBS [Tris-buffered saline; 10 mM Tris (pH 7.4) and 100 mM NaCl] containing 1 mM EDTA (twice) [15,26]. Activity was normalized to the amount of hVps34 in the immunoprecipitates by blotting with a distinct anti-hVps34 antibody [15],

followed by quantification using a LICOR Odyssey system. For nutrient-deprivation experiments, cells were incubated in medium containing 1% (v/v) FBS (foetal bovine serum) for 24 h prior to the experiment, then acutely transferred to D-PBS (Dulbecco's PBS) without or with  $1 \times$  MEM (minimal essential medium) amino acids and 50 mM glucose.

When indicated, the following variations on the standard hVps34 assays were performed: (i) pretreatment of cells with 50  $\mu\text{M}$  BAPTA/AM in  $\text{Ca}^{2+}$ -free buffer, or with 100  $\mu\text{M}$  W7 in  $\text{Ca}^{2+}$ -containing buffer; (ii) lysis of cells in TBS containing 0.3% CHAPS, 1 mM  $\text{MgCl}_2$ , and 1 mM  $\text{CaCl}_2$ ; (iii) washing of immunoprecipitates in PBS/1% Nonidet P40 containing 2 mM EGTA [three times for 20 min at room temperature (22°C)], without or with additional washes in TBS containing 1 mM EDTA, 1 mM BAPTA or 2 mM  $\text{CaCl}_2$ /1  $\mu\text{M}$  CaM; (iv) addition of 1 mM EGTA or 1 mM BAPTA, without or with 2 mM  $\text{CaCl}_2$ , to the final assay.

### Statistical methods

Errors bars indicate S.D. or S.E.M., depending on the experiment, as indicated in the Figure legends. Statistical significance was calculated using a two-tailed Student's *t* test.

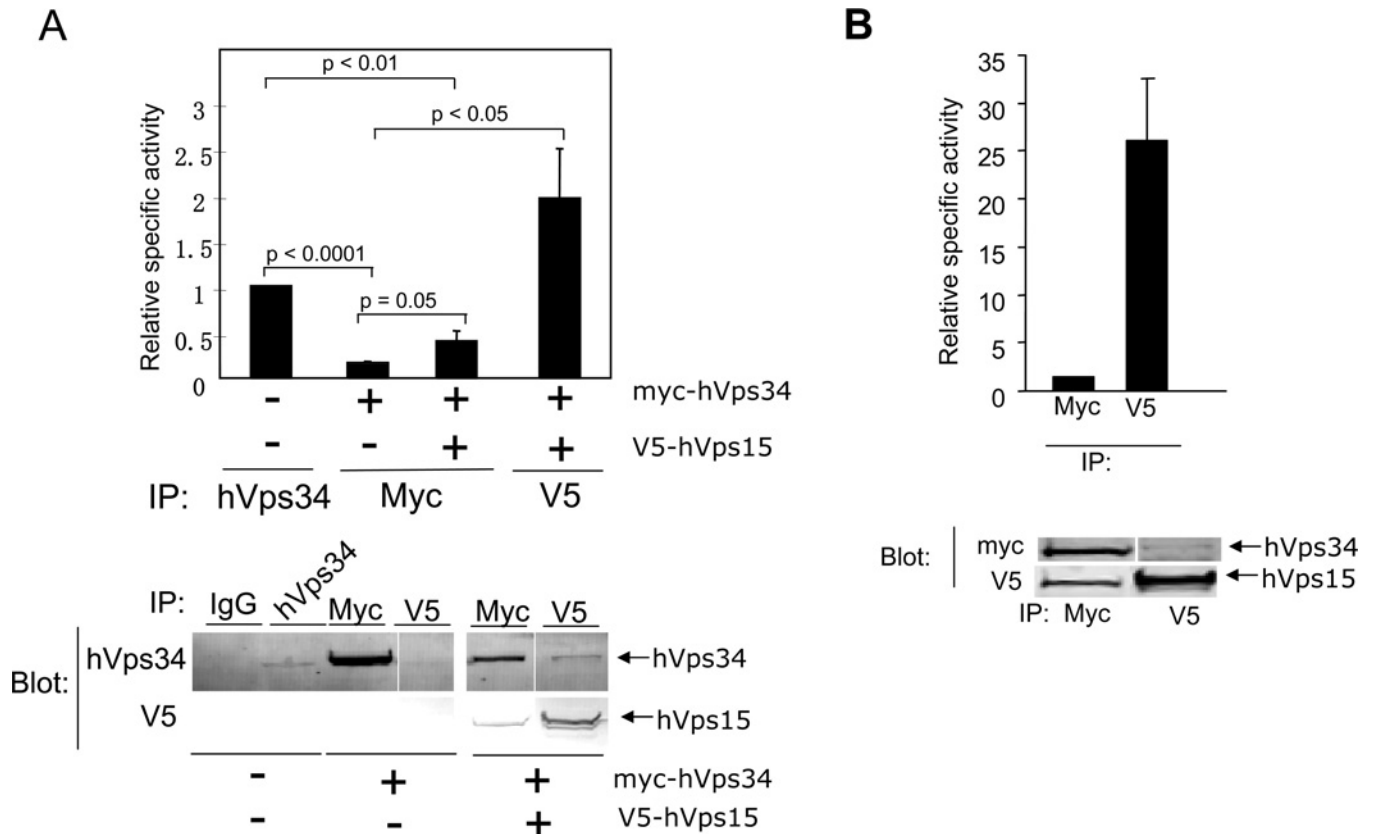
## RESULTS

### Optimal hVps34 activity requires hVps15

hVps34 is active when expressed as a monomer in baculovirus [3], and transfection of hVps34 in HEK-293T cells leads to robust expression and activity (results not shown). However, when the activity of overexpressed hVps34 was normalized for protein expression (determined by Western blotting with anti-hVps34 antibodies, which allows us to directly compare the amount of hVps34 in immunoprecipitates from untransfected compared with transfected cells), the specific activity of overexpressed hVps34 was in fact only 15% of endogenous hVps34 (Figure 1). However, upon co-expression of hVps34 and hVps15, net hVps34 expression was lower, but its specific activity was increased by 2.5-fold. These results suggest that hVps34 is activated when associated with hVps15. To directly test this conclusion, we compared the specific activity of hVps34 in anti-Myc compared with anti-V5 immunoprecipitates from cells transiently expressing Myc–hVps34 and V5–hVps15. Although the amount of hVps34 in anti-V5 immunoprecipitates was less than in anti-Myc immunoprecipitates, the specific activity of hVps34 in anti-V5 immunoprecipitates was 5-fold higher than in anti-Myc immunoprecipitates. The higher activity seen in anti-V5 immunoprecipitates, as compared with anti-Myc immunoprecipitates, presumably reflects the fact that not all of the Myc–hVps34 is bound to V5–hVps15. Thus in cells transfected with both Myc–hVps34 and V5–hVps15, there was less hVps34 in anti-V5 compared with anti-Myc immunoprecipitates, and less hVps15 in anti-Myc compared with anti-V5 immunoprecipitates (Figure 1A, lower panel). Similar results were obtained using cells stably transfected with Myc–hVps34 and V5–hVps15 (Figure 1B). Taken together, these results suggest that hVps15-associated hVps34 is highly active relative to monomeric hVps34.

### Regulation of hVps34 by beclin-1/UVRAG requires hVps15

Studies from yeast suggest that Vps34p and Vps15p interact with Vps30p (the yeast beclin-1 homologue) through the intermediary proteins Atg14 or Vps38 [11]. In contrast, mammalian hVps34 interacts directly with beclin-1 [12], which in turn binds to UVRAG [13]. We therefore examined how the presence of



**Figure 1** hVps34 activity requires hVps15

(A) Control HEK-293T cells or cells transfected with Myc-hVps34 alone or with V5-hVps15 were lysed, and anti-hVps34, anti-Myc or anti-V5 immunoprecipitates were prepared and assayed under standard conditions as described in the Experimental section. Specific activity was determined by measuring hVps34 protein expression by Western blot analysis and analysis using a LICOR Odyssey imaging system. All lanes for each blotting antibody are from the same membrane. The values were normalized to the specific activity of endogenous hVps34, and are the means  $\pm$  S.E.M. (from three experiments). (B) Anti-Myc or anti-V5 immunoprecipitates from LR73 cells stably transfected with Myc-hVps34 and V5-hVps15 were assayed for hVps34 activity, and blotted with anti-Myc and anti-V5 antibodies. The values were normalized to the specific activity of Myc-hVps34, and are the means  $\pm$  S.E.M. (from three experiments). IP, immunoprecipitation.

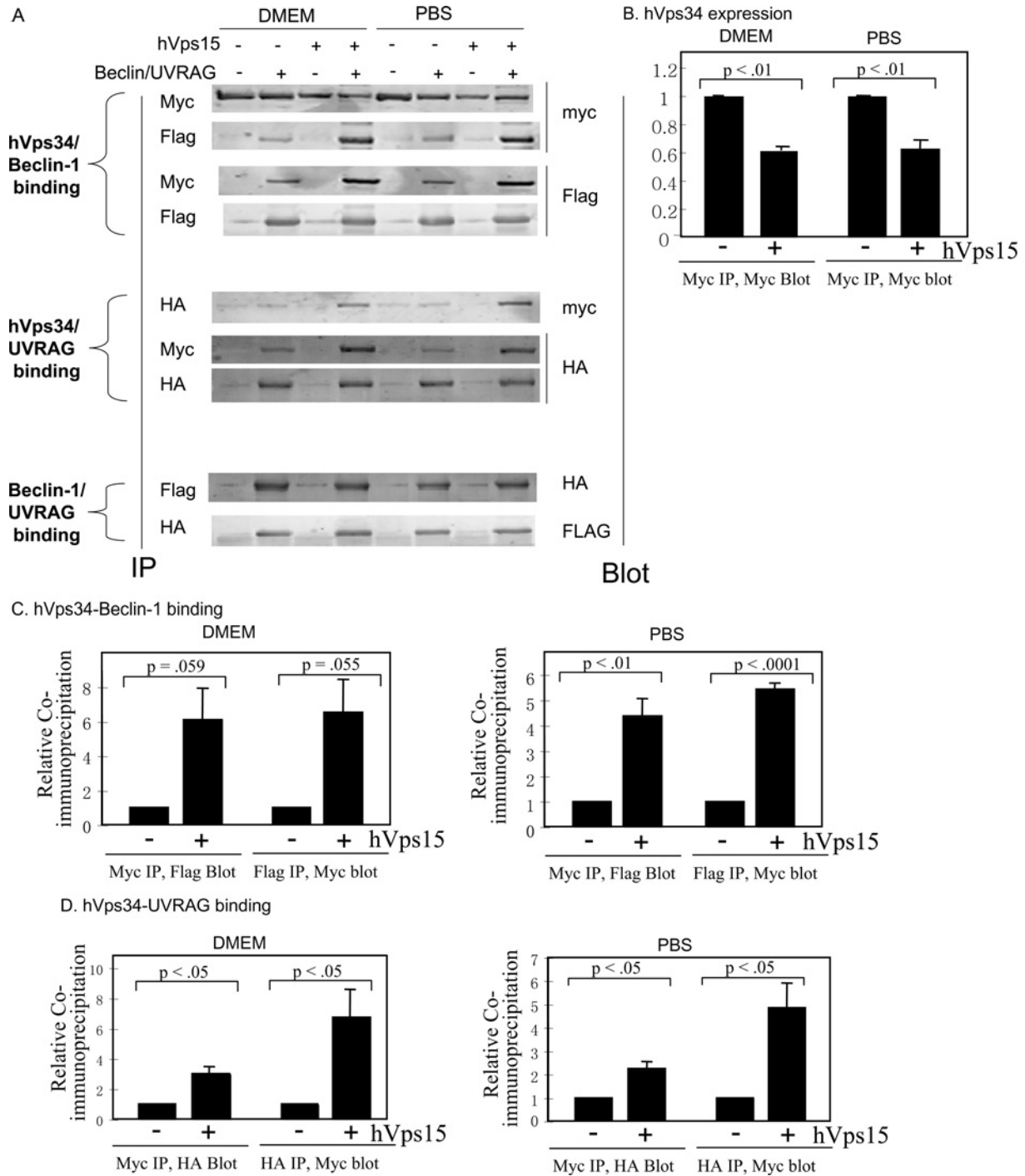
hVps15 affects interactions between hVps34, beclin-1 and UVRAG. HEK-293T cells were transfected with a bicistronic vector containing hVps34 alone or with hVps15, without and with plasmids for FLAG-beclin-1 and HA-UVRAG. Co-expression of hVps15 caused a decrease in the expression of hVps34 (Figures 2A and 2B); this finding is consistent with preliminary experiments showing that hVps15 decreases the half-life of hVps34 in cultured cells (results not shown). hVps34 could be detected in both anti-FLAG-beclin-1 and anti-HA-UVRAG immunoprecipitates (Figure 2A). However, co-expression with hVps15 caused a marked increase in the co-immunoprecipitation of hVps34 with either FLAG-beclin-1 or HA-UVRAG (Figure 2A, left-hand panel, and Figures 2C and 2D); this was seen in anti-Myc-hVps34 immunoprecipitates blotted with anti-FLAG or anti-HA, and anti-HA-UVRAG or anti-FLAG-beclin-1 immunoprecipitates blotted with anti-Myc. Co-expression of hVps15 had similar effects on the association of beclin-1/UVRAG with hVps34 in nutrient-starved cells (Figure 2A, right-hand panel, and Figures 2C and 2D). Expression of hVps15 had no effect on beclin-1/UVRAG co-immunoprecipitation (as seen in anti-FLAG and anti-HA immunoprecipitates blotted with anti-HA or anti-FLAG antibodies respectively; Figure 2A). These results show that hVps15 enhances the ability of beclin-1 and UVRAG to interact with hVps34.

We next examined the effect of beclin-1/UVRAG expression on hVps34 activity. Although previous studies have suggested that

these proteins increase the activity of hVps34 in co-expression assays [13], we saw minimal changes in hVps34 activity upon co-transfection of beclin-1/UVRAG (Figure 3A). However, beclin-1/UVRAG caused a 2-fold activation of hVps34 in cells co-expressing hVps15. This activation by beclin-1/UVRAG was unaffected by nutrient starvation (Figure 3B).

#### Beclin-1/UVRAG enhances hVps34-hVps15 binding

Given that maximal hVps34 activity requires hVps15, and given that the ability of beclin-1/UVRAG to activate hVps34 also requires hVps15, we tested the possibility that beclin-1/UVRAG might regulate hVps34-hVps15 binding. We measured the co-immunoprecipitation of Myc-hVps34 with V5-hVps15 in the absence or presence of beclin-1/UVRAG (Figure 4A). Expression of beclin-1/UVRAG caused a marked increase in hVps34-hVps15 co-immunoprecipitation (Figure 4A). This effect was not dependent on the nutritional status of the cells, as it was seen in both fed and starved cells (Figure 4B). Quantitative analysis of the Western blots showed minimal changes in total hVps34 and hVps15, but a clear increase in their association upon expression of beclin-1/UVRAG (Figure 4C). The increase in hVps15-hVps34 binding provides a plausible explanation for the increased hVps34 activity seen in cells co-expressing beclin-1/UVRAG.



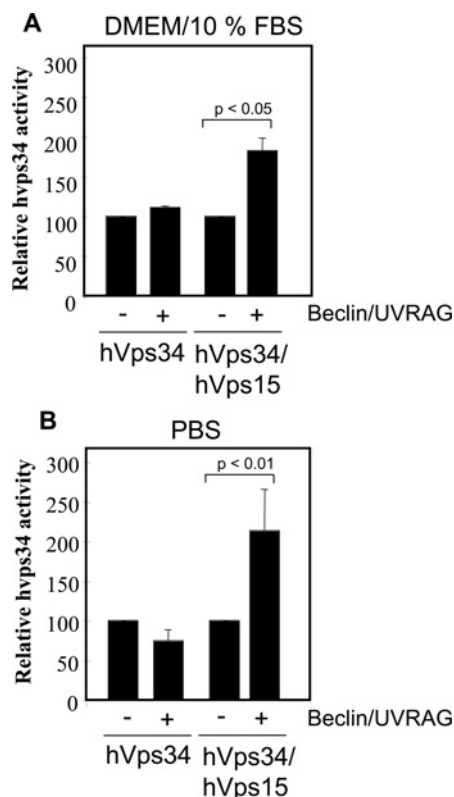
**Figure 2** hVps15 enhances beclin-1/UVRAG binding to hVps34

(A) HEK-293T cells were transfected with Myc-hVps34 alone or with V5-hVps15, without or with plasmids for HA-UVRAG and FLAG-beclin-1. Cells were incubated in medium containing 1% (v/v) FBS overnight, and then transferred to fresh serum-free medium or D-PBS for 2 h. Immunoprecipitation and Western blots with each antibody were performed to assess expression and co-immunoprecipitation. (B) Quantification of hVps34 expression in cells transfected as above. The DMEM values show the means  $\pm$  S.D. (from two experiments). The PBS values show the means  $\pm$  S.E.M. (from three experiments). (C) Quantification of hVps34 binding to beclin-1 and UVRAG in cells transfected as above. The DMEM values show the means  $\pm$  S.D. (from two experiments). The PBS values show the means  $\pm$  S.E.M. (from three experiments). IP, immunoprecipitation.

### Nutrient regulation of hVps34 requires hVps15

We have previously shown that endogenous hVps34 activity is decreased in nutrient-starved cells [15]. Given the role of hVps15 in autophagy-related regulation of hVps34, we

compared the effects of nutrient deprivation in stable cell lines overexpressing Myc-hVps34 without or with V5-hVps15. The relative expression of hVps34 and hVps15 was demonstrated by blotting with anti-Myc, anti-V5, anti-hVps34 and anti-hVps15 antibodies (Figure 5A). Interestingly, the activity of overexpressed



**Figure 3** Beclin-1/UVRAG activation of hVps34 requires hVps15

HEK-293T cells were transfected with Myc-hVps34 alone or with V5-hVps15, without or with beclin-1/UVRAG. (A) Anti-Myc immunoprecipitates were assayed for hVps34 activity. Activity was normalized for hVps34 protein expression, as determined by Western blotting and analysis using a LICOR Odyssey imaging system. The values are the means  $\pm$  S.E.M. (from three experiments). (B) Cells were incubated in medium containing 1% (v/v) FBS overnight, and then transferred to D-PBS for 2 h. hVps34 specific activity was assayed as above. The values are the means  $\pm$  S.E.M. (from four experiments).

hVps34 was unaffected by nutrient starvation in Myc-hVps34 cells, whereas it was inhibited in Myc-hVps34/V5-hVps15 cells (Figure 5B). The magnitude of the decrease was less than we have previously reported [15], presumably owing to the fact that not all of the overexpressed hVps34 is present in complexes with hVps15 (Figure 1). These results show that nutrient regulation of hVps34 requires its presence in a complex with hVps15.

### hVps34 activity is unaffected by inhibitors of $Ca^{2+}$ signalling *in vivo* and *in vitro*

Deretic and co-workers [27] showed that both hVps34 and hVps15 could be pulled down with CaM-agarose beads, and it has recently been suggested that hVps34 binding to  $Ca^{2+}$ /CaM is required for hVps34 activity and for nutrient-regulated hVps34 signalling to the mTOR pathway [17]. In order to evaluate the role of hVps15 in this process, we first sought to confirm the regulation of endogenous hVps34 by  $Ca^{2+}$ /CaM. Treatment of LR73 cells with the  $Ca^{2+}$  chelator BAPTA/AM completely blocked insulin-stimulated activation of S6K1, similar to treatment with rapamycin (Figure 6A, upper panel). However, BAPTA/AM had no effect on hVps34 activity as measured in immune complex assays (Figure 6A, lower panel). Similarly, treatment of cells with the CaM inhibitor W7 (100  $\mu$ M) did not inhibit hVps34.

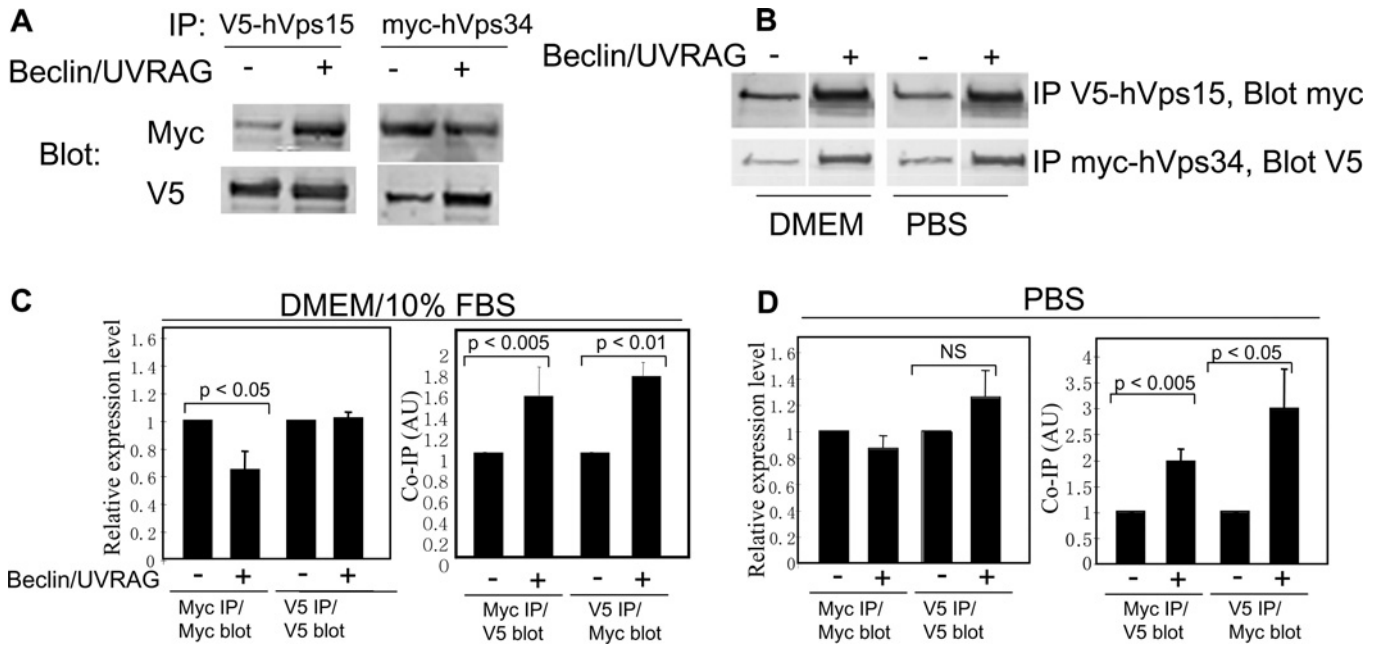
We next tested whether  $Ca^{2+}$ /CaM was required for hVps34 activity *in vitro*. Gulati et al. [17] suggested that hVps34/CaM

binding can be disrupted by lengthy washes in EGTA, which leads to a loss of hVps34 activity. Using cells transfected with YFP-CaM to enhance detection by immunoblotting, we confirmed that CaM did co-immunoprecipitate with hVps34 when washes were performed in the presence of  $Ca^{2+}$ ; however, we found that standard assay conditions for hVps34, which include washes in EDTA, lead to a complete loss of CaM binding (Figure 6B); similar results were seen with immunoprecipitates of MLCK (myosin light-chain kinase), a known CaM-dependent enzyme (Figure 6C). Calmodulin was also removed by washes in EGTA (Figures 6B and 6C). Nonetheless, activity measurements of hVps34 immunoprecipitates prepared under conditions that preserve or disrupt CaM binding showed no differences, suggesting that hVps34 activity does not correlate with the preservation of CaM binding (Figure 6D).

To determine the reason for the disparity between our results and the recently published data [17], we assayed hVps34 activity after washes in EGTA as described by Gulati et al. [17]. Consistent with their findings, hVps34 activity in EGTA-washed samples was nearly abolished (Figure 7A). However, if the EGTA was removed from the samples by washes in  $Ca^{2+}$ -free buffer containing either EDTA or BAPTA, hVps34 activity was completely restored (Figure 7A). These results suggest that the loss of activity is due to a  $Ca^{2+}$ /CaM-independent effect of EGTA. To test this directly, we added  $Ca^{2+}$  chelators to hVps34 immunoprecipitates that were first washed in EDTA, which removes bound CaM (Figures 6B and 6C). Addition of 1 mM EGTA abolished hVps34 activity in these immunoprecipitates, but this effect was still seen in the presence of 2 mM  $Ca^{2+}$  (Figure 7B). Given that the assay conditions for hVps34 include 10 mM  $MnCl_2$ , the free  $Ca^{2+}$  under these conditions is estimated to be 1.99 mM (WEBMAXC, <http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm>), suggesting that the effects of EGTA are unrelated to  $Ca^{2+}$  chelation. Consistent with these results, hVps34 activity was unaffected by the addition of 1 mM BAPTA, without or with 2 mM  $Ca^{2+}$ . Gulati et al. [17] also showed that addition of  $Ca^{2+}$ /CaM to EGTA-washed immunoprecipitates restores hVps34 activity. However, when we performed this experiment on EGTA-washed immunoprecipitates that were first washed with TBS to remove the EGTA, addition of  $Ca^{2+}$ /CaM had no significant effect on hVps34 activity (Figure 7C). Thus the reported *in vitro* activation of hVps34 by  $Ca^{2+}$ /CaM is likely to be due to the removal of EGTA. These results clearly show that the inhibitory effect of EGTA is unrelated to its effect on  $Ca^{2+}$ /CaM, and that hVps34 activity is CaM-independent.

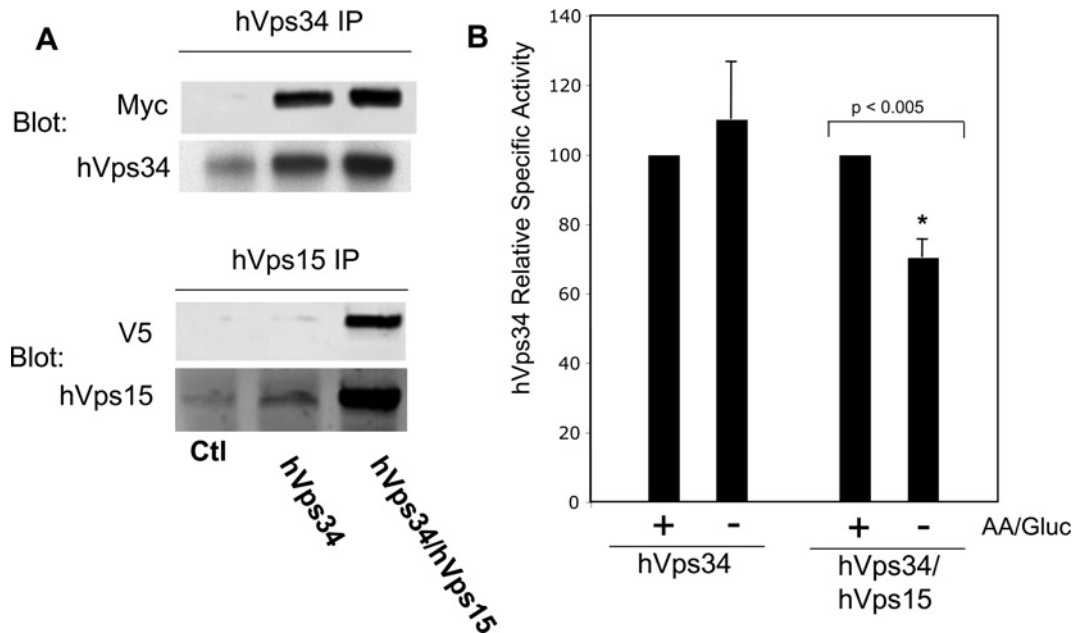
### DISCUSSION

Despite its discovery over 15 years ago [28], the function of Vps15 in Vps34 signalling has been somewhat mysterious. In *Saccharomyces cerevisiae*, hVps34-dependent vesicular trafficking is abolished in *VPS15*-null strains [21]. However, overexpression of Vps34p in *VPS15*-null strains leads to increased production of PtdIns3P, suggesting that Vps34p activity does not strictly require Vps15p [21]. This also appears to be the case in mammalian cells; hVps34 has activity when expressed alone, although the specific activity of the enzyme is only 15% of endogenous hVps34. Co-expression of hVps15 with hVps34 leads to an increase in hVps34 specific activity, and the specific activity of hVps34 in anti-hVps15 immunoprecipitates is 5-fold higher than in anti-hVps34 immunoprecipitates. This difference is much more pronounced than the 2-fold change in recombinant hVps34 activity caused by addition of hVps15 *in vitro* [20]. This



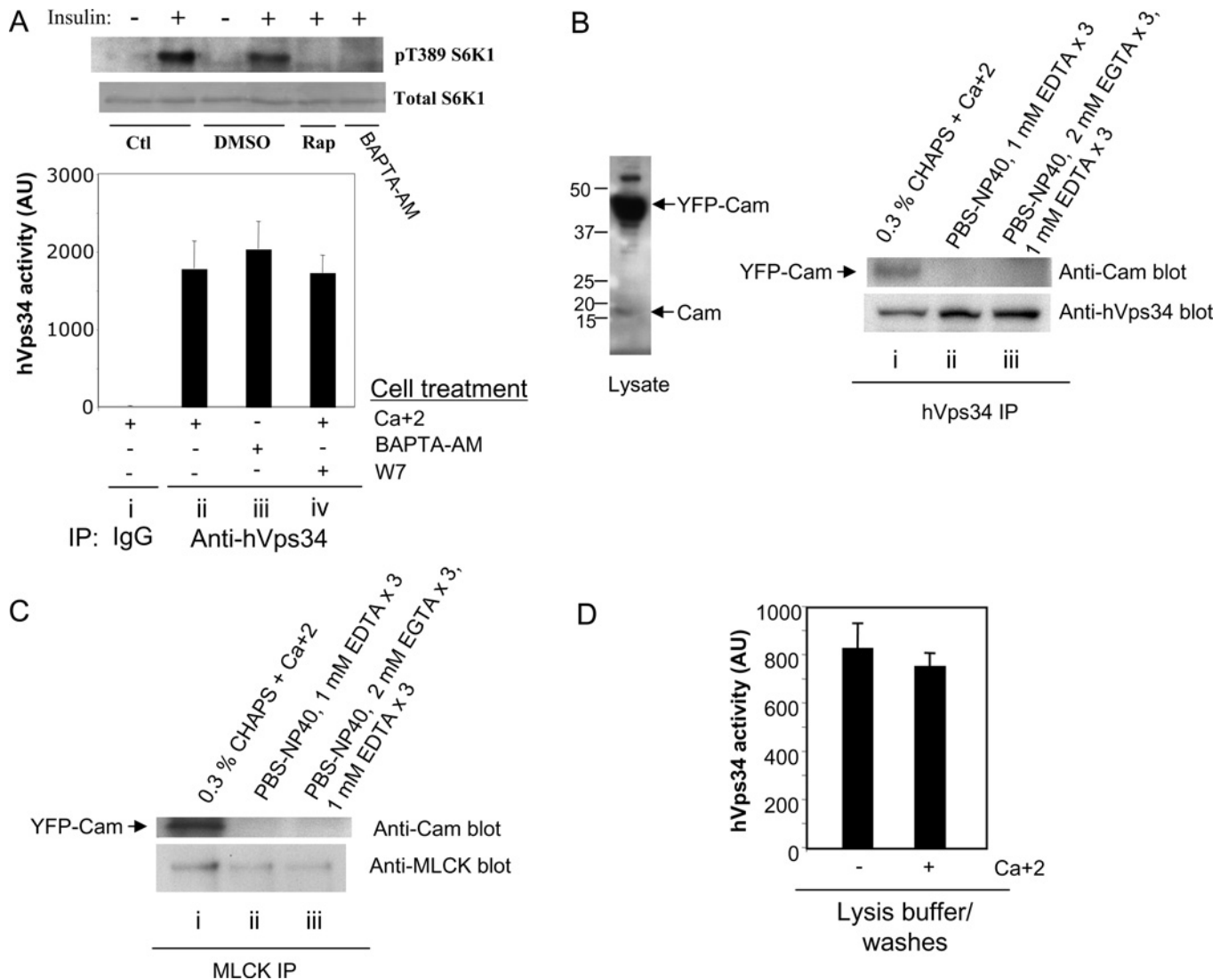
**Figure 4** Beclin-1/UVRAG enhances hVps34 binding to hVps15

(A) HEK-293T cells were transfected with Myc-hVps34 and V5-hVps15, without or with Beclin-1/UVRAG. hVps34-hVps15 association was measured by immunoprecipitating and Western blotting with anti-Myc and anti-V5 antibodies. (B) Cells were transfected as above and incubated overnight in medium containing 1% (v/v) FBS. The cells were then incubated in serum-free medium or buffer for 2 h prior to immunoprecipitation and blotting with anti-V5 and anti-Myc antibodies. All lanes for each blotting antibody are from the same membrane. (C) Cells were transfected with hVps34 and hVps15 without or with beclin-1/UVRAG. Expression and co-immunoprecipitation of hVps34 and hVps15 was measured by Western blotting and analysis using a LICOR Odyssey imaging system. The values are the means  $\pm$  S.E.M. (from three experiments). (D) Cells were transfected as in (C). Prior to immunoprecipitation and analysis by Western blotting, Serum-deprived cells [1% (v/v) FBS] were incubated in D-PBS for 2 h. Expression and co-immunoprecipitation of hVps34 and hVps15 was measured as above. The values are the means  $\pm$  S.E.M. (from four experiments). AU, arbitrary units; Co-IP, co-immunoprecipitation; IP, immunoprecipitation.



**Figure 5** Nutrient regulation of hVps34 requires hVps15

(A) Stable LR73 cell lines expressing Myc-hVps34 alone or with V5-hVps15 were analysed by immunoprecipitation with anti-hVps34 or anti-hVps15 antibodies, followed by blotting with anti-hVps34, anti-hVps15, anti-Myc or anti-V5 antibodies. (B) hVps34- or hVps34/hVps15-expressing cells were incubated in medium containing 1% (v/v) FBS overnight. The cells were then incubated in D-PBS without or with nutrients (1  $\times$  MEM amino acids plus 50 mM glucose) for 2 h. Anti-Myc immunoprecipitates were assayed for activity; activity was normalized to hVps34 protein expression as determined by Western blotting and analysis using a LICOR Odyssey imaging system. The values are the means  $\pm$  S.E.M. (from three experiments).



**Figure 6**  $\text{Ca}^{2+}$ /CaM is not required for hVps34 activity in intact cells or *in vitro*

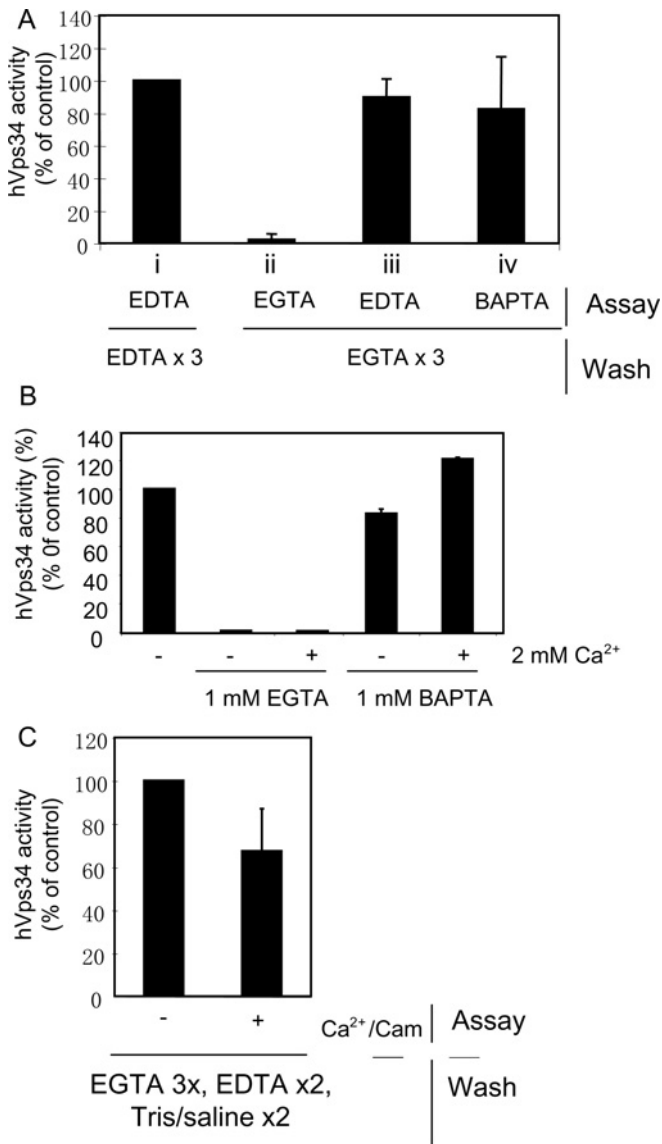
(A) Top panel: LR73 cells transfected with HA-tagged S6K1 were incubated for 15 min in 155 mM NaCl, 10 mM Hepes (pH 7.4), 3 mM KCl, 5 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub> and 3 mM NaH<sub>2</sub>PO<sub>4</sub>, in the presence of DMSO carrier, 100 nM rapamycin or 50  $\mu\text{M}$  BAPTA/AM (without CaCl<sub>2</sub>). The cells were stimulated without or with insulin for 30 min and lysed. Anti-HA immunoprecipitates were blotted with anti-phospho-S6K1 or anti-S6K1 antibodies. The results are representative of three separate experiments. Lower panel: LR73 cells were incubated as above in the presence of (i,ii) 2 mM CaCl<sub>2</sub> plus DMSO carrier, (iii) 50  $\mu\text{M}$  BAPTA/AM or (iv) 2 mM CaCl<sub>2</sub> plus 100  $\mu\text{M}$  W7. Cells were lysed using standard lysis buffer (see the Experimental section) containing (i,ii) 1 mM CaCl<sub>2</sub>, (iii) no Ca<sup>2+</sup> or (iv) 1 mM CaCl<sub>2</sub> plus 100  $\mu\text{M}$  W7. Control IgG (i) or anti-hVps34 (ii–iv) immunoprecipitates were then washed under standard conditions, except that washes for samples i, ii and iv contained 1 mM CaCl<sub>2</sub> and no EDTA. Samples were assayed in TBS without (iii) or with (i,ii,iv) 1 mM CaCl<sub>2</sub>. The values are the means  $\pm$  S.E.M. from triplicates and are representative of three experiments. (B) HEK-293T cells were transfected with YFP–CaM. Cells were lysed in Ca<sup>2+</sup> lysis buffer [20 mM Tris/HCl (pH 7.5), 0.3% CHAPS, 120 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub> and 5% glycerol] with protease/phosphatase inhibitors, followed by washes in (i) Ca<sup>2+</sup> lysis buffer followed by TBS with 1 mM CaCl<sub>2</sub>, (ii) PBS/1% Nonidet P40 followed by TBS with 1 mM EDTA, or (iii) PBS/1% Nonidet P40 with 2 mM EGTA (20 min at 22 °C, three times) followed by TBS/1 mM EDTA. The presence of YFP–CaM and hVps34 in anti-hVps34 immunoprecipitates was determined by Western blot analysis. The results are representative of three experiments. The left-hand panel is an anti-CaM blot of cell lysates, showing the expression of YFP and endogenous CaM. (C) HEK-293T cells transfected with YFP–CaM were lysed as above and anti-MLCK immunoprecipitates were washed and analysed as in (B). The results are representative of three experiments. (D) HeLa cells were lysed either as in (B), with subsequent washes in Ca<sup>2+</sup> lysis buffer followed by TBS with 1 mM CaCl<sub>2</sub>, or in PBS/1% Nonidet P40 followed by TBS with 1 mM EDTA. hVps34 activity in immunoprecipitates was determined. The values are the means  $\pm$  S.E.M. from triplicates, and are representative of three experiments. IP, immunoprecipitation.

could reflect either the ability of hVps15 to recruit other activating factors in cells, or the effect of hVps15 on hVps34 folding and/or post-translational modification.

Experiments to determine the effects of hVps15 knockdown or overexpression on the activity of endogenous hVps34 activity are difficult to interpret. siRNA (small interfering RNA) knockdown of hVps15 leads to a significant loss of hVps34 expression, presumably due to altered stability (results not shown). In contrast, hVps15 overexpression does not lead to increased hVps34 activity. This is probably due to the fact that hVps15

is in excess over hVps34, as was previously shown by exhaustive immunodepletion experiments [8].

The mechanism of hVps15-mediated activation of hVps34 is unknown, and it is not even clear that hVps15 actually possesses kinase activity [1]. The major evidence for the kinase activity of yeast Vps15p is that the immunopurified protein undergoes autophosphorylation *in vitro*, and mutations in its kinase domain abolish this autophosphorylation [22,28]; however, these same mutations abolish Vps34p binding to Vps15p. Thus it remains possible that the autophosphorylation activity observed in Vps15p



**Figure 7** hVps34 is unaffected by Ca<sup>2+</sup> chelation

(A) hVps34 immunoprecipitates from HEK-293T cells were washed in (i) PBS/1% Nonidet P40 followed by TBS/1 mM EDTA, (ii) PBS/1% Nonidet P40/2 mM EGTA followed by TBS/1 mM EDTA/1 mM EGTA, (iii) PBS/1% Nonidet P40/2 mM EGTA followed by TBS/1 mM EDTA, or (iv) PBS/1% Nonidet P40/2 mM EGTA followed by TBS/1 mM EDTA/1 mM BAPTA. hVps34 activity was measured in the final wash buffer. The values are the means  $\pm$  S.D. (from two experiments). (B) hVps34 immunoprecipitates were prepared under standard conditions (as described in the Experimental section). The final assay was performed in the absence or presence of 1 mM EGTA or 1 mM BAPTA, without or with 2 mM CaCl<sub>2</sub> as indicated. The values are the means  $\pm$  S.D. (from two experiments). (C) hVps34 immunoprecipitates were washed in PBS/1% Nonidet P40/2 mM EGTA, followed by TBS/1 mM EDTA, followed by TBS without EDTA. Immune complex kinase assays were performed without or with 2 mM CaCl<sub>2</sub> and 1  $\mu$ M CaM (Cam). The values are the means  $\pm$  S.D. (from two experiments).

immunoprecipitates actually reflects the protein kinase activity of Vps34p [22].

Previous studies have shown that hVps34 is activated by association with beclin-1 and UVRAG [13]. We find that the hVps34 binding and activation by beclin-1/UVRAG requires the presence of hVps15. Beclin-1 binds directly to hVps34 [12], and the requirement for hVps15 could reflect a conformational change in hVps34 that enhances its ability to interact with beclin-1/UVRAG. Interestingly, beclin-1/UVRAG also increases the association of

hVps34 with hVps15. Given that hVps15 binding increases the specific activity of hVps34, the ability of beclin-1/UVRAG to enhance hVps34-hVps15 binding may provide a mechanism for the effects of these proteins on hVps34 activity [13]. The finding that hVps34 binding to hVps15 is regulated by autophagy-related proteins could have important implications for PtdIns3P-dependent steps in autophagy. Studies are in progress to determine whether the increase in hVps34 activity caused by expression of Bif-1 [14] also correlates with an increase in hVps34-hVps15 binding.

A recent study has suggested that hVps34 is regulated by Ca<sup>2+</sup>/CaM [17]. However, in our hands, we see no effect on hVps34 activity in cells treated with Ca<sup>2+</sup> chelators or CaM inhibitors, and no requirement of Ca<sup>2+</sup> or CaM for hVps34 activity *in vitro*. We suspect that the apparent Ca<sup>2+</sup> dependence reported by Gulati et al. [17] reflects the presence of residual EGTA in the assays, which inhibits hVps34 even in the presence of saturating levels of Ca<sup>2+</sup> and manganese. Gulati et al. [17] did show that mutation of a predicted CaM-binding site in hVps34 causes a loss of activity [17]; however, loss-of-function mutants are by nature difficult to interpret. The mutated sites are in the helical domain of hVps34, which contains a cluster of multiple short helices. The mutations were all hydrophobic-to-charged mutations, which might disrupt the folding of the putative CaM-binding helix or its interactions with other helices within this domain, leading to a loss of activity.

In summary, we have shown that mammalian hVps15 is critical for hVps34 activity as well as for regulation of hVps34 by autophagy-related proteins and by nutritional stress. In contrast, our results contradict the hypothesis that hVps34 activity requires the binding of hVps34 to Ca<sup>2+</sup>/CaM. Future experiments will need to address the mechanism by which hVps15 regulates hVps34, as well as the function of the putative kinase activity of hVps15.

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