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(54) Title: REGULATORS OF RAB5 ACTIVITY

(57) Abstract: The present disclosure provides for small molecules that modulate Rab5 activity including novel peptides derived from or corresponding to at least a portion of the p110 β α -helical domain. These peptides possess the ability to block, interfere with or prevent the conversion of Rab5-GTP into Rab5-GDP and enhance Rab5 mediated activity. The disclosure also provides a method of identifying an agent for modulating the level of Rab5 activity in a subject or cell. The present disclosure also provides methods and compositions for modulating Rab5 activity in a subject in need thereof, comprising administering an effective amount of an agent that modulates the activity of Rab5.



REGULATORS OF RAB5 ACTIVITY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims benefit of U.S. Provisional Application No. 61/767,006 filed on February 20, 2013, the entire contents of which are incorporated herein by reference.

GOVERNMENT INTEREST

[0002] The present disclosure was made with Government support under grant numbers GM97355 and CA129536 awarded by the National Institutes of Health. The Government has certain rights in the disclosure.

FIELD OF THE DISCLOSURE

[0003] The present disclosure relates to protein and peptide chemistry. In particular, the present disclosure relates to the discovery, production and isolation of novel peptides whose sequences coincide with regions of the p110 β catalytic subunit (p110 β) of the Class IA phosphoinositide 3-kinases (PI3Ks), more specifically the α -helical domain of the p110 β protein. The disclosure is also directed to the use of these novel peptides to modulate Rab5 GTPase (Rab5)-mediated biological activity. Moreover, the present disclosure relates to the therapeutic effects of treating a subject with novel modulators of Rab5 activity.

BACKGROUND OF THE DISCLOSURE

[0004] Autophagy is a membrane trafficking process that delivers intracellular contents destined for degradation into a double membrane structure termed an autophagosome that then fuses with the lysosome. See Levine, B. and Kroemer, G., *Cell* 132, 27-42 (2008); and Mizushima, N. et al., *Nature* 451, 1069-1075 (2008)). The initiation of autophagy is regulated by a group of phospholipids, phosphoinositides produced by PI3Ks. PI3Ks are lipid kinases central to numerous signaling pathways. See Cantley, L.C., *Science* 296, 1655-1657 (2002); and Engelman, J.A., et al., *Nature Reviews Genetics* 7, 606-619 (2006). PI3Ks are grouped into three classes: Class I, Class II, and Class III. See Domin, J. and Waterfield, M.D., *Febs Letters* 410, 91-95 (1997). Class IA PI3Ks are composed of a p85 regulatory

subunit and a p110 catalytic subunit that produces phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃], which activates the Akt/mTOR signaling pathway. See Franke, et al., *Science* 275, pp. 665-668 (1997); and Sarbassov, D.D., et al., *Current Opinion in Cell Biology* 17, pp. 596-603 (2005). The p110 β subunit has a molecular mass of approximately 110 kDa and plays a role in trafficking small molecules to the plasma membrane. It is believed that Class IA PI3Ks inhibit autophagy by promoting nutrient uptake and metabolic activities through Akt/mTOR. See Levine and Kroemer, (2008); and Petiot, A., et al., *Journal of Biological Chemistry* 275, pp. 992-998 (2000). Conversely, the Class III PI3K catalytic subunit Vps34 is bound to the regulatory subunit Vps15 and converts phosphatidylinositol (PI) to phosphatidylinositol 3-phosphate [PI(3)P], which is essential for autophagy initiation. See Jaber, N., et al., *Proc Natl Acad Sci* 109, pp. 2003-2008 (2012); and Kihara, A., et al., *J Cell Biol* 152, pp. 519-530 (2001). Hence, it is generally recognized that, in metazoans, Class III PI3K Vps34 activates autophagy while Class IA PI3Ks inhibit autophagy.

[0005] Further, it has been reported that the small GTPase Rab5, in its GTP-bound form is known to regulate membrane trafficking. See Barbieri, M.A., et al., *J Biol Chem* 269, pp. 18720-18722 (1994); and Stenmark, H., et al., *EMBO J.* 13, 1287-1296 (1994). However, while, Rab5 has known involvement in endocytic trafficking, much remains unknown regarding Rab5's participation in autophagosome formation. For example, to date, it remains unknown whether p110 β modulates Rab5 activity directly.

SUMMARY OF THE DISCLOSURE

[0006] The present disclosure is directed to small molecules that modulate Rab5 activity. In certain embodiments, novel peptides derived from or corresponding to the p110 β Rab5-binding domain have been synthesized and isolated. The novel peptides of the present disclosure possess the ability to block, interfere with or prevent the conversion of Rab5-GTP (active) into Rab5-GDP (inactive) and enhance Rab5 mediated autophagy or endocytosis.

[0007] In one aspect, the peptides of the present disclosure have an amino acid sequence that corresponds with the p110 β Rab5-binding domain of human, rat, mouse, or rabbit p110 β protein (residues 1 to 1070).

[0008] In a specific embodiment, the peptides of the present disclosure have an amino acid sequence that corresponds with the alpha helical domain of human p110 β (residues 509-780), including variations thereof.

[0009] In another embodiment, the present disclosure has further identified a Rab5 protein binding domain located within the α -helical domain, of the p110 β protein, as X_nLQIX_n as set forth in SEQ ID NO: 8.

[0010] In another aspect of the present disclosure Rab5 activity is governed by binding of an agent, small molecule, antibody or fragment thereof, which binds to Rab5 in the same fashion as p110 β or variations thereof. For example, in certain embodiments of the present disclosure antibodies raised against a Rab5 protein or against the Rab5 modulating peptides whose sequences coincide with the corresponding sequences of a vertebrate Rab5 protein. Both, polyclonal antibodies and monoclonal antibodies are contemplated by the present disclosure.

[0011] The present disclosure also provides methods and compositions for modulating Rab5 activity (e.g., biological activity) in a subject i.e., mammal or humans, in need thereof, which includes administering an effective amount of an agent, small molecule, peptide antibody or fragment thereof that modulates the activity of Rab5. In certain embodiments the Rab5 activity being modulated is the interaction of Rab5 with Rab5 effectors. In a specific embodiment of the present disclosure, the Rab5 effector is the Vps34-Vps15-Beclin 1-Atg14L complex. In yet another embodiment of the disclosure, the Rab5 effector is the Vps34-Vps15 complex. In one embodiment of the present disclosure, the Rab5 effector is Vps34. In another embodiment of the present disclosure, the Rab5 effector is Atg14L. In a embodiment of the present disclosure, the Rab5 effector is EEA1.

[0012] The present disclosure further provides methods of identifying an agent that modulates Rab5 activity. The methods disclosed herein include contacting a test agent with Rab5, and detecting autophagy of a cell or endocytosis, wherein the agent is identified by its ability to modulate Rab5 activity. In one embodiment of the disclosure, the agent is a peptide. In another embodiment of the disclosure the agent is an antibody. In yet another

embodiment, the agent is a small molecule. In yet another embodiment, the agent is a nucleic acid.

[0013] These and other embodiments of the disclosure will be readily apparent to those of ordinary skill in view of the disclosure herein.

BRIEF DESCRIPTION OF DRAWINGS

[0014] **Figure 1. Rab5 plays a critical role in p110 β -mediated autophagy. p110 $\beta^{+/+}$ and p110 $\beta^{-/-}$ MEFs were transfected with GFP-LC3 together with indicated expression constructs.** (A) Western blots of cell lysates were probed with the indicated antibodies. Ponceau S staining shows equal protein loading. (B) Cells were imaged under a deconvolution microscope. Cells with more than 10 cytosolic puncta and diminished nuclear GFP were considered as autophagic. Data are averages of at least 4 blind countings with over 200 cells. Error bars: SD. * $p < 0.005$. (C) Representative images of GFP-LC3 fluorescence in MEFs. Scale bar: 20 μ m. (D) p110 $\beta^{+/+}$ and p110 $\beta^{-/-}$ MEFs were transfected with vector (control), GFP-Rab5-WT, or GFP-Rab5-CA. 48 hours later, cells were left untreated or treated with 50 nM bafilomycin A1 for 6 hours. Relative levels of LC3-II against β -tubulin from three independent experiments are shown on the right. Data were normalized against that of p110 $\beta^{+/+}$. Error bars: SEM; * $p < 0.05$; N.S., non-significant.

[0015] **Figure 2. p110 β promotes Rab5 activation.** (A) HEK293T cells were transfected with GFP-tagged Rab5-WT, Rab5-DN, or Rab5-CA. 48 hours post transfection, cell lysates were subjected to pull-down with GST (control) or GST-R5BD beads. Western blots of precipitates and total cell lysates were probed with GFP antibody. (B) Lysates of MEFs with the indicated genotypes were subjected to GST or GST-R5BD pull-down, and western blotting was used to analyze endogenous Rab5-GTP levels. Relative levels of Rab5-GTP (expressed as normalized Rab5-GTP/total Rab5 ratios) are shown on the right. Data are means of three independent experiments $\pm$ SEM; * $p < 0.05$; N.S., non-significant. (C) p110 $\beta^{-/-}$ MEFs and those stably reconstituted with wild-type p110 β or the kinase-dead K805R mutant were analyzed for Rab5-GTP by the GST-R5BD pull-down assay. Relative levels of Rab5-GTP are shown on the right. Data are average values of three independent experiments $\pm$ SEM; * $p < 0.05$; N.S., non-significant. (D) MEFs

with the indicated genotypes were subjected to immunoprecipitation with control IgG or Rab5 antibody and analyzed for endogenous Vps34 and EEA1. Immunoblots of the precipitates and the input were probed with the indicated antibodies.

[0016] Figure 3. p110 β suppresses the Rab5 GAP activity of p85 α . (A) $\beta^{+/+}$ and $\beta^{-/-}$ MEFs were stably infected with lentivirus encoding a non-targeting control shRNA or shRNA against p85 α . (B) MEFs generated as in (A) were subjected to GST-R5BD pull-down assays to detect Rab5-GTP. Note that p85 α silencing leads to increased levels of Rab5-GTP. Relative levels of Rab5-GTP (expressed as normalized ratios of Rab5-GTP/total Rab5) from three independent experiments are shown on the right. Error bars: SEM; * $p < 0.05$; ** $p < 0.01$. (C) MEFs generated as in (A) were transfected with GFP-LC3 and autophagic cells were quantified. The data are average of at least four blind countings with over 200 cells. Error bars: SD. * $p < 0.05$; ** $p < 0.001$. (D) MEFs generated as in (A) were transfected with mCherry-GFP-LC3. 48 h later, images were taken, and yellow and red puncta were counted. Data are mean values of over 20 cells $\pm$ SEM. * $p < 0.01$; ** $p < 0.001$. (E) Purified Rab5 (200 nM) was loaded with GDP or GTP and subjected to pull-down with GST or GST-R5BD beads. The precipitates were analyzed for Rab5-GTP. (F) For *in vitro* Rab5 GAP assays, 200 nM Rab5-GTP was incubated in the absence of (-), or in the presence of 1 μ M (+) or 2 μ M (++) of purified p85 α , p110 β /p85 α , or p110 α /p85 α . Rab5-GTP was then pulled down with GST-R5BD beads and quantified by immunoblotting. The relative amounts of Rab5-GTP are shown. The quantification of Rab5-GTP levels in control and 1 μ M of purified p85 α , p110 β /p85 α is shown. Data presented are the mean values from 3 independent experiments. Error bars: SEM. * $p < 0.05$. (G) 200 nM Rab5-GTP was incubated with 2 μ M p85 α in the absence or presence of 1 μ M (+) or 2 μ M (++) of p110 β /p85 α or p110 α /p85 α . Rab5 GAP activity was assessed as in (F). The relative amounts of Rab5-GTP are shown. (H) HEK293T cells were transfected with Flag-p85 α expressing construct. 48 h later, cell lysates were divided into 4 aliquots and mixed with HEK293T cell lysates that over expressed untagged-p85 α alone or together with p110 β wild-type or p110 β Q596C. The mixed lysates were subjected to pull-down with GST (control) or GST-Rab5 loaded with GTP γ S. The precipitates and the input were analyzed as indicated. The relative amount of Flag-p85 α against Rab5 is shown.

[0017] Figure 4. Association of p110 β with Rab5 is required for Rab5 activity and autophagy.

(A) HEK293T cells were transfected with Myc-tagged p110 α or p110 β constructs together with Flag-p85 α . 48 hours later, cell lysates were subjected to pull-down with GST or GST-Rab5 loaded with GTP γ S. (B) p110 β -Q596C and p110 β -I597S possess intact kinase activity. Data presented are the means of three independent experiments, normalized against that of p110 β protein levels. Error bars: SEM. (C) HEK293T cells were transfected with indicated constructs. 48 hours later, cell lysates were subjected to immunoprecipitation with Myc-antibody conjugated to agarose. Blots of the precipitates and the input were probed with Myc and Flag antibodies. (D) p110 β ^{-/-} MEFs were stably reconstituted with indicated constructs and were analyzed for indicated proteins. (E) Lysates of stably reconstituted MEFs were subjected to GST-R5BD pull-down assays to determine the amount of Rab5-GTP. The relative amount of Rab5-GTP against total Rab5 is shown. Error bars: SEM; n=3; * p<0.05. (F) MEFs were analyzed for p62. The relative amount of p62 is shown. Error bars: SEM; n=3; * p<0.05. (G) MEFs as indicated were incubated with [¹⁴C] valine for 24 hours. The cells were left untreated or serum-starved for 4 hours. Degradation of long-lived proteins was measured. Error bars: SEM; n=3; * p<0.05. (H) MEFs generated as in (D) were cultured in complete or serum-free medium for 6 hours and treated with or without bafilomycin A1 (50 nM). Western blots of cell lysates were probed for LC3 and β -tubulin. Quantification of LC3-II against β -tubulin is shown. Error bars: SEM; n=3; * p<0.05. (I) MEFs generated as in (D) were transfected with GFP-LC3. 48 hours later, cells were cultured in complete or serum-free medium for 4 hours, in the absence or presence of bafilomycin A1 (50 nM). Representative images of cells without bafilomycin A1 treatment are shown. Scale bar: 20 μ m. Quantification of autophagic cells is shown on the right. Data are average values of at least 4 blind countings with over 200 cells. Error bars: SD; * p<0.05; ** p<0.01.

[0018] Figure 5. Withdrawal of growth factors, but not nutrients, induces p110 β -Rab5 binding and p110 β -dependent Rab5 activation.

(A) p110 β ^{-/-} MEFs stably reconstituted with human p110 β (hp p110 β) were left untreated, deprived of serum, glucose, or amino acids for 6 hours, or treated with the Akt inhibitor (Akti, 10 μ M) or rapamycin (Ra, 50 nM) overnight. (B) Lysates of cells treated as in (A) were subjected to immunoprecipitation with

control IgG or Rab5 antibody. The precipitates were analyzed for Rab5, hp110 β , and Vps34. (C) p110 β ^{+/+} and p110 β ^{-/-} MEFs were left untreated or serum-deprived for 6 hours. GST-R5BD pull-down assays were done to determine the amount of Rab5-GTP in cell lysates. The mean values of relative Rab5-GTP against that of total Rab5 from three independent experiments with SEM is shown. * $p < 0.05$; N.S., non-significant. (D) p110 β ^{+/+}, p110 β ^{-/-}, and the human p110 β -reconstituted p110 β ^{-/-} MEFs were cultured in complete or serum-free medium for 6 h. Cell lysates were subjected to immunoprecipitation with IgG or Rab5 antibody and analyzed for human p110 β and endogenous Vps34 and EEA1. (E) MEFs as indicated expressing GFP-FYVE were untreated or serum-starved, and stained for endogenous Rab5. Around 20 cells were randomly selected and imaged. The numbers of GFP-FYVE puncta and co-localized GFP-FYVE and Rab5 puncta per cell were quantified. Error bars: SEM; * $p < 0.005$; ** $p < 0.0005$. The representative images are shown in Figure 12B.

[0019] Figure 6. p110 β dissociates from growth factor receptor complexes and

interacts with Rab5 upon growth factor deprivation. (A and B) MCF10A cells were grown in complete or basal (without growth factors) medium for 24 h. Cell lysates were subjected to immunoprecipitation with the indicated antibodies or with phosphotyrosine antibody conjugated to agarose. Western blotting of the precipitates and the input is shown. Quantification of each association is shown (A). (B) Immunofluorescence confocal microscopy was used to visualize endogenous p110 β and Rab5 in fixed cells.

Representative images are shown. Scale bar: 10 μ m. The percentage of Rab5 co-localizing with p110 β was quantified in over 50 cells and is shown on the right. Error bars: SEM.

* $p < 0.01$. (C) MCF10A cells were stably infected with lentiviruses encoding a tetracycline-inducible non-targeting control shRNA or shRNA against p110 β . The two cell lines were treated in complete medium with doxycycline (Dox, 1 μ g/ml) for the indicated times and then harvested for western blotting. (D) MCF10A cells generated as in (C) were treated with Dox for 3 days to silence p110 β , and then cultured in complete or basal medium for 24 hours in the presence of Dox. Cell lysates were analyzed for the amount of Rab5-GTP using GST-R5BD pull-down assays. The input of Rab5 and p110 β are shown. n.s., non-specific band. (E) Cells generated as in (C) stably expressing GFP-LC3 were left untreated or

starved in basal medium for 24 or 48 hours in the absence or presence of bafilomycin A1 (20 nM). Representative images of cells not treated with bafilomycin A1 are shown. Scale bar: 20 μ m. Autophagic cells from the indicated culture conditions were quantified and shown in (F). Data are averages of at least 4 blind countings with over 500 cells. Error bars: SD. (G) MCF10A cells were stably infected with tetracycline-inducible control shRNA or shRNA against p110 β . Cells were treated with Dox for 3 days to allow knock-down of p110 β . Cells were then left untreated or cultured in basal medium for 36 hours in the absence or presence of bafilomycin A1 (20 nM). Short and long exposures of the LC3 immunoblots are shown. Relative amount of LC3-II normalized against β -tubulin was quantified from three independent experiments. Error bars: SEM; * $p < 0.05$; N.S., non-significant.

[0020] Figure 7. Cells with membrane-targeted p110 β -CAAX display impaired Rab5 association and autophagy. (A) HEK293T cells were transfected with wild-type Flag-p110 β or Flag-p110 β -CAAX. 48 hours later, cells were left untreated or serum-deprived for 24 hours. Cell lysates were subjected to immunoprecipitation with IgG or Rab5 antibody. The normalized relative binding of Flag-p110 β to Rab5 from three independent experiments with SEM is shown. * $p < 0.05$; N.S., non-significant. (B) $\beta^{-/-}$ MEFs expressing indicated constructs were left untreated or serum-deprived in the absence or presence of bafilomycin A1 (50 nM) for 6 hours. The relative amount of LC3-II normalized against that of vector control from three independent experiments with SEM is shown. * $p < 0.05$, comparing wild-type p110 β versus vector and p110 β -CAAX mutant. (C) p110 $\beta^{-/-}$ MEFs were transfected with the indicated constructs together with GFP-LC3. 48 hours later, cells were left untreated or serum-deprived for 6 hours, in the absence or presence of bafilomycin A1 (50 nM). The pictures shown are of cells in the absence of bafilomycin A1. Scale bar: 20 μ m. Expression of the constructs and quantification of autophagic cells are shown on the right. Data are average of 4 blind countings with over 200 cells. Error bars: SD; * $p < 0.05$; ** $p < 0.005$. (D) Schematic representation of the role of p110 β in regulating autophagy.

[0021] Figure 8. Overexpression of Vps34-Vps15 or Atg14L failed to rescue autophagy deficiency of the p110 $\beta^{-/-}$ MEFs. (A) Wild-type MEFs were transfected with construct expressing bicistronic Vps34-Vps15. 48 hours post transfection, cells were left untreated or cultured in the presence of bafilomycin A1 (50 nM) for 6 hours. The lysates were probed

for indicated antibodies. The relative amount of LC3-II is shown. Note that overexpressing Vps34-Vps15 increases autophagy flux. (B) p110 $\beta^{+/+}$ and p110 $\beta^{-/-}$ MEFs were transfected with vector control or expression constructs encoding the bicistronic Vps34-Vps15, or Flag-Atg14L. 48 hours post transfection; cells were left untreated or treated with 50 nM rapamycin for 6 hours, in the presence of 50 nM bafilomycin A1. The cell lysates were subjected to immunoblotting with indicated antibodies. Ponceau staining is shown for equal loading. The relative amount of LC3-II in untreated plus bafilomycin A1 conditions is quantified and shown at the bottom. Data presented are the average values from three independent experiments. Error bars: SEM; n.s., non-significant.

[0022] Figure 9. p110 β -mediated Rab5 activation is independent of the catalytic activity of p110 β . (A) p110 $\beta^{-/-}$ MEFs were transfected with vector control, expression construct of p110 β -WT or the kinase dead p110 β -K805R mutant. 48 hours post transfection, cell lysates were subjected to GST-R5BD pull-down for the amount Rab5-GTP. Relative Rab5-GTP against total Rab5 is shown. (B) p110 β is required for the activity of ectopically expressed GFP-Rab5. HEK293T were stably infected with the tet-inducible shRNA control or that against p110 β . Cells were treated with doxycycline (Dox, 1 μ g/ml) for 3 days to reach a significant silencing of p110 β . Cells were transfected with GFP-Rab5 after 1 day treatment of Dox. Two days post transfection, cells were untreated or serum-deprived for 24 hours (with Dox). Cell lysates were analyzed for the amount of GFP-Rab5-GTP using the R5BD pull-down assay. The average values of GFP-Rab5-GTP against that of total GFP-Rab5 from three independent experiments with SEM is shown. *p<0.05; n.s., non-significant.

[0023] Figure 11. p110 β Q596 and I597 residues are critical for Rab5 binding and autophagy induction. (A) Schematic domain organization of human p110 β , highlighting residues Q596 and I597 in the helical domain. (B) Sequence alignment of amino acids 593 to 611 of the alpha helical domain in p110 α and p110 β . 596 and 597 residues are highlighted in red. (C) Full view and side view (90° rotation) of mouse p110 β complex with p85 β iSH2 and cSH2 domains. p85 β is labeled in dark gray. The Q590 and I591 residues corresponding to human Q596 and I597 are highlighted. (D) Detailed views of the two key residues in the helical domain. (E) MEFs with indicated genotypes were serum-starved

overnight, and stimulated with 10 μ M LPA for 5 min. The lysates were probed for indicated antibodies. (F and G) Rab5 association, but not the kinase activity of p110 β , is required for autophagy. (F) p110 $\beta^{-/-}$ MEFs expressing vector control, p110 β -K805R mutant, or p110 β -K805R/I597S double mutant were subjected to GST-Rab5-GTP γ S pull-down. The precipitates together with input lysates were analyzed as indicated. Note that while the K805R mutant possesses intact binding to Rab5, the K805R/I597S double mutant fails to do so. (G) MEFs as in (F) were transfected with GFP-LC3. 48 h post transfection, cells were treated as indicated, fixed, and imaged under a deconvolution microscope. Representative images are shown. Scale bar: 20 μ m. Quantification of autophagic cells is shown on the right. Data presented are the mean values of four countings with over 200 cells. Error bars: SD; * $p < 0.05$; ** $p < 0.01$.

[0024] Figure 12. Rab5 binding-deficient p110 β mutants are unable to promote Rab5-Vps34 association. (A) MEFs with indicated genotypes were untreated or serum-starved for 6 hours. The lysates were subjected to immunoprecipitation with IgG control or Rab5 antibodies, and analyzed for endogenous Vps34 and EEA1. The input is shown on the right. (B) Representative images of GFP-FYVE (green) and Rab5 staining (red) are shown. Inset shows higher magnifications of the two colors and merged channels. Note that the wild-type p110 β reconstituted cells displayed more GFP-FYVE puncta and GFP-FYVE Rab5 colocalizing puncta than the control and the mutant-expressing cells. Quantification of this result is shown in Fig. 5E. (C) Wild-type MEFs were transfected with GFP-LC3 together with control vector or Rab5-DN expressing plasmid. 48 hours later, cells were untreated or serum-starved, in the absence or presence of bafilomycin A1. Autophagic cells were quantified. Data are average values of 4 countings with over 200 cells. Error bars: SD; * $p < 0.05$; ** $p < 0.001$. (D) Wild-type MEFs were transfected with GFP or GFP-Rab5-DN construct. 48 hours later, cells were left untreated or cultured in serum-free media for 6 h, in the absence or presence of bafilomycin A1 (50 nM). The relative amount of LC3-II with SEM is shown. Data presented are average values from three independent experiments normalized against that of GFP untreated control. * $p < 0.05$; ** $p < 0.01$.

[0025] Figure 13. Association of p110 β with Rab5 is critical for autophagy induced by trophic factor limitation. (A) MCF10A cells stably infected with lentivirus encoding

tetracycline-inducible shRNA against p110 β (as in Figure 6C) were reconstituted with vector control or shRNA-resistant p110 β wild-type or the I597S mutant. Cells were treated with Dox for 3 days to allow knock-down of endogenous p110 β . The cells were cultured in basal medium without trophic factors for 24 hours, and then subjected to immunoprecipitations with IgG control or Rab5 antibodies. The precipitates and the input lysates were analyzed for p110 β . Note that while wild-type p110 β showed positive binding to Rab5, the I597S mutant did not. (B) Validation of p110 β antibody for immunofluorescence. Control or p110 β knock-down MCF10A cells were fixed and stained with p110 β antibody. The slides were observed and imaged under a deconvolution microscope. DAPI was used to show nucleus. Scale bar: 10 μ m. (C) MCF10A cells as generated in (A) were cultured in trophic factor deprived medium for 24 hours, and stained for p110 β and Rab5 antibodies. Representative images were taken under a deconvolution microscope. Scale bar: 10 μ m. Note that while p110 β wild-type reconstitution showed colocalization with Rab5, the I597 mutant failed to do so. (D) p110 β knock-down MCF10A cells reconstituted with indicated shRNA-resistant constructs were cultured in complete or trophic factor deprived medium for 24 hours. Cell lysates were subjected to GST-R5BD analysis for Rab5-GTP. The relative Rab5-GTP against total Rab5 is shown. (E) Cells as in (A) stably expressing GFP-LC3 was left untreated or deprived of trophic factors for 24 hours. The cells were fixed and imaged under a deconvolution microscope. Representative images are shown. Scale bar: 20 μ m. Quantification of autophagic cells is shown on the right. Data presented are the average values of 4 countings with over 200 cells. Error bars: SD; * $p < 0.05$. (F) Cells were left untreated or deprived of trophic factors for 24 hours, in the presence of 20 nM of bafilomycin A1. The lysates were probed for indicated antibodies. The relative amount of LC3-II is shown at the bottom. (G) MCF10A cells were stably infected with tetracycline-inducible control shRNA or shRNA against p110 β . Cells were treated with Dox for 3 d to allow knock-down of p110 β . Cells were then left untreated or treated with rapamycin (Ra, 50 nM), tunicamycin (0.1 μ g/ml), or MG132 (0.25 η M), in the absence or presence of 20 nM bafilomycin A1 for 36 hours. Relative amounts of LC3-II (expressed as normalized LC3-II/ β -tubulin ratios) and fold change are shown at the bottom. Note that the trophic factor deprivation-induced increase in LC3-II was abolished in sh-

p110 β cells (as shown in Figure 6G). While the basal level of LC3-II was lower in sh-p110 β cells, the inducibility of LC3-II upon treatment with rapamycin, tunicamycin, or MG132 was intact.

[0026] Figure 14. Plasma membrane targeted p110 β does not activate autophagy upon trophic factor limitation.

(A) Flag-p110 β -WT or CAAX mutant were transfected into Hs578T cells. 48 hours post transfection, cells were cultured in complete or serum-free medium for 6 hours. Cells were then fixed and stained with Flag antibody, and observed under a deconvolution microscope. Representative images were taken. Scale bar: 20 μ m. Note that serum deprivation led to decreased plasma membrane localization of p110 β -WT but not p110 β -CAAX. Quantification of cells with plasma membrane p110 β was performed by counting a total of over 100 cells. Data presented are the mean values with SD. * $p < 0.005$; N.S., non-significant. (B-D) The failure of p110 β -CAAX mutant to rescue autophagy is not due to its elevated kinase activity. (B) p110 $\beta^{-/-}$ MEFs were transfected with vector control or constructs encoding p110 β -WT or CAAX mutant, together with GFP-LC3. 48 hours post transfection, p110 β -CAAX cells were treated with the p110 β -specific kinase inhibitor TGX-221 (500 nM) for overnight. The cells were then serum-starved for 6 hours, in the absence or presence of 50 nM bafilomycin A1. TGX-221 was present during the treatment. Cells were fixed and quantified for autophagy induction indicated by GFP-LC3 puncta. Data shown are the mean values of at least 4 blind countings with over 200 cells. Error bars: SD; N.S., non-significant. (C) Wild-type MEFs were serum-starved overnight then stimulated with 10 μ M LPA for 5 minutes in the absence or presence of 500 nM TGX-221. Cell lysates were analyzed as indicated. Note that TGX-221 blocked LPA-induced Akt phosphorylation. (D) MEFs with indicated reconstitutions were left untreated or serum-starved for 6 h, in the absence or presence of 500 nM TGX-221. The lysates were probed for indicated antibodies. Note that while the p110 β -CAAX expressing cells showed slight increase of pAkt and pS6K levels, addition of TGX-221 abrogated this effect.

DETAILED DESCRIPTION OF THE DISCLOSURE

[0027] Autophagy is the process, by which cytosolic components and organelles are engulfed and degraded by a double-membrane structure during a specialized, multistep

membrane transport process. Autophagy begins when double-membrane autophagosomes engulf portions of the cytoplasm. These vesicles are then fused with lysosomes and the contents of such vesicles are degraded. In addition to the vital homeostatic role and the processes role in modulating cell survival of Autophagy, this degradation process is involved in various human disorders, including metabolic conditions, neurodegenerative diseases, cancers and infectious diseases.

[0028] Autophagy is induced under conditions of nutrient or growth factor deprivation in the cell and during various conditions of stress. Several molecules are known to be involved in the initiation and process of autophagy, including but not limited to PI3P, double FYVE-domain containing protein (DFCP1), AMP-dependent kinase (AMPK), Class III PI3 kinases such as, the Vps34-Vps15-Bec1-Atg14L complex. See Dou et al., (2010). Additionally, a number of Rab GTPases which regulate secretory and endocytic membrane traffic have been shown to play either critical or accessory roles in autophagy including, but not limited to, Rab1, Rab5, Rab7, Rab9, Rab24, and Rab33.

[0029] Endocytosis refers to a process by which mammalian cells take up extracellular materials. Endocytosis serves many important cellular functions including the uptake of extracellular nutrients, regulation of cell-surface receptor expression, maintenance of cell polarity, and antigen presentation. Endocytic pathways are also utilized by viruses, toxins, and symbiotic microorganisms to gain entry into cells. There are several types of endocytosis pathways, namely receptor-mediated endocytosis via clathrin-coated pits, caveolae or membrane buds, macropinocytosis, and phagocytosis. The roles of endocytosis in physiological and pathological processes have been elucidated including, the maintenance of cell polarization, antigen presentation, glucose transport, atherosclerosis, Alzheimer's disease, and the endocytosis of toxins and viruses. See Mukherjee, S., et al. *Physiol Rev.* 77(3), 759-803 (1997).

[0030] Generally, p110 β is a PI3K that phosphorylates Phosphatidylinositol, Phosphatidylinositol 4-phosphate and Phosphatidylinositol 4, 5-bisphosphate to generate phosphatidylinositol 3, 4, 5-trisphosphate (PIP3). PIP3 plays a key role by recruiting PH domain (Pleckstrin homology domain)-containing proteins to the membrane, including

AKT1 and PDPK1, activating signaling cascades involved in cell growth, survival, proliferation, motility and morphology. Moreover, p110 β is involved in the activation of AKT1 upon stimulation by G-protein coupled receptors (GPCRs) ligands such as CXCL12, sphingosine 1-phosphate, and lysophosphatidic acid. p110 β also modulates the intracellular level of Phosphatidylinositol 3-phosphate and activates PIK3C3 kinase activity and can act as a scaffold, independently of its lipid kinase activity to positively regulate autophagy.

[0031] Rab5 is a small GTPase protein that is generated from the expression of the RAB5A gene. It is found in two forms; 1) GTP-bound, otherwise known as its active state (herein “Rab5-GTP”, “active Rab5” or “Rab5”), and 2) GDP bound, or its inactive form (hereinafter “GDP-Rab”). The present disclosure provides that Rab GTPases regulate secretory and endocytic membrane traffic and play a critical role in autophagy. The present disclosure further describes the interaction between Rab5 and Rab5 effector molecules including, but not limited to, the Rabaptin protein family such as Rabaptin 5, enzymes such as Rab GTPase-binding effector protein 1 (RABEP1) and Rab proteins geranylgeranyltransferase component A 2, Syntenin-1, Rep-2, Rabenosyn-5, EEA1 complex, Vps34-Vps15 (see Murray, JT et al., *Traffic* **3(6)** 416-27(2002)), Vps34-Vps15-Beclin 1-Atg14L, (see Ravikumar et al., (2008)) and the angiotensin II type 1A receptor.

COMPOSITIONS

[0032] In one aspect of the present disclosure, small molecules, agents, peptides and antibodies are provided, which modulate Rab5 activity. In certain embodiments of the present disclosure novel peptides have been synthesized, which are derived from or correspond to the α -helical domain of the p110 β protein, i.e., LEDVAQLQALLQI, as set forth in SEQ ID NO: 3. The present disclosure has further identified a Rab5 protein binding domain located within the α -helical domain, of the p110 β protein, X_nLQIX_n (SEQ ID NO: 8). The peptides disclosed herein positively regulate Rab5 activity and possess the ability to block, interfere with, and prevent the conversion of Rab5-GTP into Rab5-GDP, and thus enhance Rab5 mediated autophagy and endocytosis. For example, modulation of Rab5 activity includes increasing the amount of Rab5-GTP protein in a cell or subject, the

ability of active Rab5 to bind a Rab5 effector, and increasing the amount of Rab5 mediated autophagy or endocytosis.

[0033] The term “agent” as used herein refers to any kind of compound or combination of compounds. In one embodiment of the invention the agent is a small molecule. In another embodiment of the disclosure, the agent is a biological molecule, including, but not limited to, a protein or a peptide or a nucleic acid. In one embodiment, the nucleic acid is an interfering RNA. The term “interfering RNA” is employed herein to refer to small interfering RNAs (siRNAs), short hairpin RNAs (shRNAs), microRNAs (miRNAs), antisense oligonucleotides, ribozymes, or any RNA-based molecule that interferes with the expression of a protein from its corresponding gene.

[0034] In the context of this disclosure, the term “small molecule” refers to small organic compounds, such as heterocycles, peptides, saccharides, steroids, and the like. The small molecule modulators preferably have a molecular weight of less than about 1500 Daltons, and more preferably less than 500 Daltons. The compounds can be modified to enhance efficacy, stability, pharmaceutical compatibility, and the like. Candidate modulator compounds from libraries of synthetic or natural compounds can be screened. Synthetic compound libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, N. J.), Brandon Associates (Merrimack, N.H.), and Microsource (New Milford, Conn.). Combinatorial libraries are available or can be prepared according to known synthetic techniques. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from e.g., Pan Laboratories (Bothell, Wash.) or MycoSearch (NC), or are readily producible by methods well known in the art. Additionally, natural and synthetically produced libraries and compounds can be further modified through conventional chemical and biochemical techniques.

[0035] The term “peptide”, “polypeptide” or “protein” refers to a linear series of amino acid residues linked to one another by peptide bonds between the alpha-amino and carboxy groups of adjacent amino acid residues.

[0036] The term “isolated” and “purified”, when used in reference to a molecule (such as a peptide, protein or polypeptide), means that the molecule has been removed from its naturally occurring environment and is substantially free of other molecules (such as other proteins). By “substantially free” of other proteins, it is meant that a protein of interest accounts for at least 60%, 70%, 80%, 90%, or 95% (by dry weight) of total proteins in a composition. When an isolated protein is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20% of the volume of the protein preparation, less than about 10% of the volume of the protein preparation or less than about 5% of the volume of the protein preparation. For example, the proteins of the present disclosure can be purified to homogeneity or other varying degrees of purity. The level of purification can be based on the intended use. In certain non-limiting examples, isolated peptides of the present disclosure can be purified from cells that express such protein, as further described below, or can be synthetically made using known protein synthesis methods. The term “synthetic peptide” is intended to refer to a chemically derived chain of amino acid residues linked together by peptide bonds that are isolated or substantially isolated from other materials or elements. Specifically, the term “synthetic peptide” is intended to refer to recombinantly produced peptides in accordance with the present disclosure.

[0037] In one aspect, the peptides of the present disclosure have an amino acid sequence that corresponds with the p110 β catalytic domain of human, rat, mouse, or rabbit p110 β (residues residues 1 to 1070), including variations thereof.

[0038] The term “p110 β protein” or “p110 β peptide” used herein shall mean phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta protein regardless of the organism from which said p110 β protein originates, which includes a conserved α -helical domain, LEDVAQLQALLQI (SEQ ID NO: 3), which interacts with the Rab5 protein. Non-limiting examples of p110 β proteins include NP_006210.1 (human), NP_083370.2 (murine), NP_445933.1 (rat) and homologs thereof.

[0039] The term “Rab5” as used in the present disclosure shall mean a member of the ras oncogene family of small GTPases also known as RAB5A that is generated from the

expression of the RAB5A gene located on human chromosome 3 (3p24-p22). The term “Rab5 gene” as used herein includes the nucleic acid molecule represented by AAB08927.1 (human). The Rab5 gene is highly conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, *C.elegans*, and *S.cerevisiae*, and thus the Rab5 gene also includes homologs of the human Rab 5 gene in these other species.

[0040] The Rab5 gene produces an mRNA transcript, including NM_004162.4, which translate into a “Rab5 protein” or “wild-type Rab5 protein” or “Rab5 peptide”, which is represented by NP_004153.2.

[0041] Homologs, analogs and fragments of these peptides are also contemplated by the present invention as modulators of Rab5 activity.

[0042] By “homologs” it is meant that the corresponding p110 β proteins of other vertebrate species are substantially homologous at the overall protein (i.e., mature protein) level to human, p110 β . In certain embodiments, homologs of a human p110 β protein have an amino acid sequence substantially identical to the human wild-type p110 β protein, i.e., at least 80-85%, at least 90-95% or more sequence identity. Further, homologs of p110 β proteins retain the ability to positively regulate Rab5 activity. Certain non-limiting examples of homologs of the human p110 β protein include, mouse, turtle or rat p110 β .

[0043] The term “fragment” as used herein shall mean any portion of a molecule (e.g., peptide or antibody) that is, by some measure smaller than the whole including, but not limited to, a peptide that contains fewer amino acids than the protein or domain of said protein as a whole.

[0044] The term “analogs” shall mean peptides that differ by one or more amino acids alterations, which alterations, e.g., substitutions, additions or deletions of amino acid residues, do not abolish the ability to positively regulate Rab5 activity. Thus, an analog can comprise a peptide having a substantially identical amino acid sequence to a peptide provided herein and in which one or more amino acid residues have been conservatively or non-conservatively substituted. Examples of a conservative substitution include the substitution of a hydrophobic residue such as isoleucine, valine, leucine or methionine for

another. Likewise, the present disclosure contemplates the substitution of one aromatic residues such as phenylalanine, tryptophan or tyrosine for another. The substitution of a polar residue such as lysine, arginine, glutamine or asparagine for another or the substitution of a polar residue such as aspartate, glutamate, glutamine or asparagine for another. Additionally, the present disclosure contemplates the substitution of a non-polar aliphatic residue, such as between glycine and alanine, or a polar aliphatic residue such as between serine and threonine. Examples of non-conservative substitutions include the substitution of a non-polar residue e.g., isoleucine, valine, leucine, alanine or methionine for a polar residue e.g., glutamine, glutamate, lysine, and/or a polar residue for a non-polar residue.

[0045] The phrase “conservative substitution” also includes the use of chemically derivatized residues in place of non-derivatized residues, so long as the modified peptide retains the ability to modulate Rab5 activity. Analogs also include the presence of additional amino acids or the deletion of one or more amino acids which do not affect the peptides ability to modulate Rab5 activity. For example, analogs of the subject peptides can be covalently attached to a carrier protein, e.g., albumin. Such attachment can minimize clearing of the peptide from the blood and also prevent proteolysis of the peptides. Additionally, for purposes of the present disclosure, peptides containing D-amino acids in place of L-amino acids are also included in the term “conservative substitution.” The presence of such D-isomers can help minimize proteolytic activity and clearing of the peptide. Moreover, a peptide of the present disclosure can have domains attached that modulate or facilitate trafficking of said peptide, including but not limited to, a trans-activating transcriptional activator domain (Tat), commonly identified by the amino acid sequence: GRKKRRQRRR, as set forth in SEQ ID. NO. 9.

[0046] According to the present disclosure, the isolated peptide is preferably at least about two hundred and seventy two (272) amino acids in length and corresponds at least a portion of the alpha helical domain of human p110 β , including variations thereof. In another embodiment, the peptides of the present disclosure are preferably at least about fifty seven (57) amino acids in length corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In yet another embodiment, the peptides of the present disclosure are preferably at least about fifty five (55) amino acids in length corresponding

with a portion of the alpha helical domain of p110 β , including variations thereof. In still another embodiment, the peptides of the present invention are preferably at least about twenty (20) amino acids in length, corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In yet another embodiment, the peptides of the present invention are preferably at least about fourteen (14) amino acids in length, corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In another embodiment, the peptides of the present disclosure are preferably at least about thirteen (13) amino acids in length corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In still another embodiment, the peptides of the present disclosure are preferably at least about five (5) amino acids in length corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In still another embodiment, the peptides of the present disclosure are preferably at least about four (4) amino acids in length corresponding with a portion of the alpha helical domain of p110 β , including variations thereof. In another embodiment, the peptides of the present disclosure are preferably at least about three (3) amino acids in length, corresponding with a portion of the alpha helical domain of p110b.

[0047] More specifically, the present disclosure provides peptides with the ability to modulate Rab5 activity which substantially correspond to sequences found in the alpha helical domain of Rab5.

[0048] A preferred modulator of Rab5 activity of the present disclosure is a one thousand and seventy (1070)-residue length peptide having the sequence X_nLQIX_n (SEQ ID NO: 8), wherein X_n can be any amino acid which includes:

A=Ala=Alanine

R=Arg=Arginine

N=Asn=Asparagine

D=Asp=Aspartate

B=Asx=Asparagine or Aspartate

C=Cys=Cysteine

Q=Gln=Glutamine

E=Glu=Glutamate

Z=Glx=Glutamine or Glutamate

G=Gly=Glycine

H=His=Histidine

I=Ile=Isoleucine

L=Leu=Leucine

K=Lys=Lysine

F=Phe=Phenylalanine

P=Pro=Proline

S=Ser=Serine

T=Thr=Threonine

W=Trp=Tryptophan

Y=Tyr=Tyrosine

V=Val=Valine

[0049] Preferably, X_n are those amino acids that are present in homologs of the native human residues found in the alpha helical domain of p110 β .

[0050] More preferably, X_n are those amino acids found in the native sequence of a vertebrate p110 β .

[0051] Even more preferably, X_9 -L-Q-I- X_8 is a twenty (20)-residue length peptide identical to the amino acid residues 586-605 of the native human p110 β protein. An example of such

twenty-residue length peptide includes the sequence EDVAQLQALLQIWPKLPPRE (SEQ ID NO: 4). Homologs and analogs of this twenty-residue length peptide are also contemplated by the present disclosure, as long as such homologs and analogs maintain the ability to modulate Rab5 activity.

[0052] Even more preferably, X_6 -L-Q-I- X_5 is a fourteen (14)-residue length peptide identical to the amino acid residues 589-602 of the native human p110 β alpha helical domain. An example of such twenty-residue length peptide includes the sequence AQLQALLQIWPKLP (SEQ ID NO: 5). Homologs and analogs of this fourteen-residue length peptide are also contemplated by the present disclosure, as long as such homologs and analogs maintain the ability to modulate Rab5 activity.

[0053] In a preferred embodiment, the peptides of the present disclosure are at least about thirteen (13) amino acids in length corresponding with a portion of the alpha helical domain of p110 β (i.e., residues 585-597), including variations thereof. An example of such thirteen-residue length peptide includes the sequence LEDVAQLQALLQI (SEQ ID NO: 3). Homologs and analogs of this thirteen-residue length peptide are also contemplated by the present disclosure, as long as such homologs and analogs maintain the ability to modulate Rab5 activity.

[0054] Preferably, X_1 -L-Q-I- X_1 is a five (5)-residue length peptide corresponding to the amino acid residues 594-598 of the native human p110 β alpha helical domain. An example of such five-residue length peptide includes the sequence LLQIW (SEQ ID NO: 7). Homologs and analogs of this four-residue length peptide are also contemplated by the present disclosure, as long as such homologs and analogs maintain the ability to modulate Rab5 activity.

[0055] Another embodiment of the present disclosure, X_0 -L-Q-I- X_0 is a three (2)-residue length peptide identical to the amino acid residues 596-597 of the native human p110 β alpha helical domain. An example of such two-residue length peptide includes the amino acid sequence LQI. Homologs and analogs of this two-residue length peptide are also contemplated by the present invention, as long as such homologs and analogs maintain the ability to modulate Rab5 activity.

The sequences of peptides of the present disclosure are derived from and/or correspond to the amino acid sequence of the p110 β alpha helical domain. Non-limiting examples of peptides useful for modulating the activity of Rab5 include, but are not limited to, DKIIIEKAAEIASSDSANVSSRGGKKFLPVLKEILDRDPLSQLCENEMDLIWTLRQDCR EIFPQSLPKLLLSIKWNKLEDVAQLQALLQIWPKLPPREALELLDFNYPDQYVREYA VGCLRQMSDEELSQYLLQLVQVLKYEPFLDCALSRFLLERALGNRRIGQFLFWHLR SEVHIPAVSVQFGVILEAYCRGSVGHMKVLSKQVEALNKLKTLNSLIKLNNAVKLNR AKGKEAMHTCLKQSAYREALSDLQSPLNPCVILSELYVEKCKYMD (SEQ ID NO: 1), ILDRDPLSQLCENEMDLIWTLRQDCREIFPQSLPKLLLSIKWNKLEDVAQLQALLQI (SEQ ID NO: 2), LEDVAQLQALLQI (SEQ ID NO: 3), EDVAQLQALLQIWPKLPPRE (SEQ ID NO: 4), AQLQALLQIWPKLP (SEQ ID NO: 5), LQALLQIWPKLP (SEQ ID NO: 6), LLQIW (SEQ ID NO: 7), and LQI.

[0056] In another aspect of the present disclosure, Rab5 activity is governed by binding of a small molecule that binds to Rab5 in the same fashion as p110 β .

[0057] Accordingly, as used herein the term “increasing the activity” or “decreasing the activity” can mean increasing the concentration or decreasing the concentration or amount of a molecule in a subject or cell. In a specific, non-limiting example, the amount of Rab5 is increased in a cell, thereby resulting in an increase in Rab5 mediated activity. The term, “Rab5 activity” or “Rab5 mediated activity” refers to the amount of Rab5 bound to GTP, otherwise known as its active form, as well as the Rab5 protein’s ability to interact with its effectors and modulate cellular processes such as, autophagy and endocytosis.

[0058] In the context of this disclosure, the term “interaction” refers to an action that occurs between two or more molecules or entities that has an effect. A non-limiting example is a protein that binds to another protein, such as p110 β binding to Rab5, whereby such interaction causes Rab5 deactivation (e.g., Rab5-GTP to Rab5-GDP) to be inhibited.

[0059] Rab5 activity or levels thereof, in a subject or elsewhere, can be detected and their amount and concentration measured by any method commonly known in the art. This includes, for example, methods involving mass spectrometry, high pressure liquid chromatography (HPLC), combined gas chromatography-mass spectrometry, and liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. See, *for example*, De Marchi et al., *Lipids Health Dis.* 2:5, (2003). The modulation of the level of a

compound, for example the increase or decrease of the concentration of Rab5-GTP in a subject, can be measured by detecting the compound in samples taken at different times. Rab5 can be measured in samples taken from, for example, tissue samples or biopsy.

[0060] The phrase “modulating the activity” as employed herein refers to increasing the level or decreasing the activity of a molecule or agent. For example, the present disclosure provides agents that modulate the ability of Rab5 to interact with Rab5 effectors.

[0061] The phrase “effector” or “effectors” refers to any small molecule, protein, ligand, or complex thereof that binds to, or interacts with the Rab5 protein. The result of this interaction can modulate a biological activity including but not limited to, autophagy, endocytosis, cell signaling, enzymatic activity, protein-protein interaction. Certain non-limiting examples of Rab5 protein effectors include a Vps34-Vps15-Becn1-Atg14L complex, a Vps34-Vps15 complex or individual proteins thereof. In an embodiment of the present disclosure, the Rab5 effector is Vps34. In yet another embodiment of the present disclosure, the Rab5 effector is Atg14L. In an alternative embodiment of the present disclosure, the Rab5 effector is EEA1.

[0062] The peptides of the present disclosure, homologs, analogs and fragments thereof can be synthesized by a number of known techniques. For example, the peptides can be prepared using the solid-phase synthetic technique initially described by Merrifield, in *J. Am. Chem. Soc.* 85, pp. 2149-2154 (1963). Other peptide synthesis techniques can be found in M. Bodanszky, et al. *Peptide Synthesis*, John Wiley & Sons, 2d Ed., (1976) and other references readily available to those skilled in the art. A summary of polypeptide synthesis techniques can be found in J. Stuart and J. D. Young, *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, Ill., (1984). Peptides can also be synthesized by solution methods as described in *The Proteins*, Vol. II. 3d Ed., Neurath, H. et al., Eds., p. 105-237, Academic Press, New York, N.Y. (1976). Appropriate protective groups for use in different peptide syntheses are described in the above-mentioned texts as well as in J. F. W. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, N.Y. (1973). The peptides of the present disclosure can also be prepared by chemical or enzymatic cleavage from larger portions of the p110 β protein or from the entire p110 β protein.

[0063] Specific examples of conventional techniques include methods such as the Merrifield solid phase technique. In general, the Merrifield solid phase method comprises the sequential addition of one or more amino acid residues to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid residue is protected by a suitable, selectively removable protecting group. A different, selectively removable protecting group is utilized for amino acids containing a reactive side group such as lysine.

[0064] A preferred method of solid phase synthesis the protected or derivatized amino acid is attached to an inert solid support through its unprotected carboxyl or amino group. The protecting group of the amino or carboxyl group is then selectively removed and the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected is admixed and reacted under conditions suitable for forming the amide linkage with the residue already attached to the solid support. The protecting group of the amino carboxyl group is then removed from this newly added amino acid residue, and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining terminal and side group protecting groups including the solid support are removed sequentially or concurrently to yield the final peptide. The lyophilized oligopeptides are resuspended in double distilled H₂O at 2 mg/ml as stock solutions and subsequently diluted in M199-HPS for experiments.

[0065] Additionally, the peptides of the present disclosure can also be prepared by recombinant DNA techniques known by one of ordinary skill in the art. See, e.g., *Current Protocols in Molecular Cloning* Ausubel et al., 1995, John Wiley & Sons, New York); Sambrook et al., (1989), *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, New York; Coligan et al. *Current Protocols in Immunology*, John Wiley & Sons Inc., New York, N.Y. (1994). The skilled artisan understands that any of a wide variety of expression systems can be used to provide the recombinant peptides of the present invention. The precise host cell used is not critical to the present methods. The peptides of the present disclosure can be produced in a prokaryotic host (e.g. *E. coli*), or in a eukaryotic host (e.g., *S. cerevisiae* or mammalian cells, e.g. COS1, CHO, NIH3T3, and JEG3 cells, or in the cells of an arthropod, e.g. *S. frugiperda*). Such cells are available from, for example, the American Type Culture

Collection, Manassas, Va. It is appreciated by the skilled artisan that the method of transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g. in Sambrook et al., (1989); expression vehicles can be chosen from those provided. See, e.g., P. H. Powels et al., Cloning Vectors: A Laboratory Manual. (1985).

[0066] For most of the amino acids used to build proteins, more than one coding nucleotide triplet (codon) can code for a particular amino acid residue. This property of the genetic code is known as redundancy. Therefore, a number of different nucleotide sequences can code for a particular peptide corresponding to the p110 β catalytic domain. The present disclosure also contemplates a deoxyribonucleic acid (DNA) molecule or segment that defines a gene coding for, i.e., capable of expressing, a subject peptide or a subject chimeric peptide from which a peptide of the present disclosure can be enzymatically or chemically cleaved.

[0067] DNA molecules that encode peptides of the present disclosure can be synthesized by chemical techniques, for example, the phosphotriester method of Matteucci, et al., *J. Am. Chem. Soc.* 103:3185 (1981), which is incorporated herein by reference. Using a chemical DNA synthesis technique, desired modifications in the peptide sequence can be made by making substitutions for bases which code for the native amino acid sequence. Ribonucleic acid equivalents of the above described DNA molecules can also be used.

[0068] A nucleic acid molecule comprising a vector capable of replication and expression of a DNA molecule defining coding sequence for a subject polypeptide or subject chimeric polypeptide is also contemplated.

[0069] Another aspect of the present disclosure is directed to antibodies raised against the Rab5 activity modulating peptides or homologs, analogs or fragments of the present disclosure. The antibodies of the present disclosure are raised against the Rab5 modulating peptides whose sequences coincide with the corresponding sequences of a vertebrate Rab5 protein.

[0070] The peptides can be coupled to a carrier protein such as KLH as described in Ausubel et al. (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York. The KLH-antagonist peptide is mixed with Freund's adjuvant and injected into guinea pigs, rats, donkeys and the like or preferably into rabbits. Antibodies can be purified by peptide antigen affinity chromatography.

[0071] Said antibodies can be prepared using Rab5 activity modulating peptides and standard hybridoma technology. See, e.g., Kohler et al., *Nature* 256:495 (1975)); Hammerling et al., (1981); and Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y.. For example, monoclonal antibodies to Rab5 activity modulating peptides homologs, analogs or fragments thereof can be raised in Balb/C or other similar strains of mice by immunization with purified or partially purified preparations of Rab5 activity modulating peptides. The spleens of the mice can be removed, and their lymphocytes fused to a mouse myeloma cell line. After screening of hybrids by known techniques, a stable hybrid will be isolated that produces antibodies against Rab5 activity modulating peptides. Such activity can be demonstrated by the ability of the antibody to prevent the radio labeled Rab5 activity modulating peptides from interfering or blocking the Rab5 activity or by measuring the level of active Rab5 protein. The monoclonal antibody can then be examined for its ability to inhibit the biological activity of Rab5, e.g., autophagy or endocytosis. Once produced, monoclonal antibodies are tested for specific Rab5 recognition by Western blot or immunoprecipitation analysis. The antibodies of the present disclosure can be used in diagnostic assays or to further characterize Rab5, fragments thereof or Rab5 activity modulating peptides. Both, polyclonal antibodies and monoclonal antibodies are contemplated by the present disclosure. Further, any of these approaches may be used in connection with an *in vivo*, *ex vivo*, or *in vitro* experimental setup.

THERAPEUTIC METHODS

[0072] The present disclosure further provides methods and compositions for modulating Rab5 activity in a subject in need thereof, comprising administering an effective amount of an agent that modulates the activity of Rab5. The present disclosure provides specific compositions that regulate Rab5 activity. For example, a composition that regulates Rab5

activity by modulating at least one Rab5-mediated biological activity in a mammal, including humans. In another embodiment of the present disclosure, the composition is a peptide that binds Rab5. In yet another embodiment of the present disclosure, the composition is a small molecule that functions in the same manner as p110 β to modulate Rab5 activity. In yet another embodiment, the agent is a nucleic acid.

[0073] The phrase “modulating the level” is employed herein to refer to increasing the level or decreasing the level of a molecule. For example, modulating the level includes altering the amount of GTP bound Rab5 in a subject.

[0074] According to the disclosure, modulating Rab5 activity is accomplished by modulating the amount of GTP bound (active) Rab5 available to interact with Rab5 effectors, or by modulating the binding interaction between Rab5 and its effectors directly. The amount of active Rab5, or the binding of Rab5 to an effector can be detected and quantified according to methods disclosed herein as well as method otherwise commonly known in the art. The mechanism by which the agent modulates the interaction is not limited. For example, the agent can bind to Rab5 or the Rab5 effector. In an embodiment of the disclosure, the agent binds to Rab5 with an affinity of at least about 1000 nM, or at least about 500 nM, or at least about 250 nM, or at least about 100 nM, or at least about 50 nM, or at least about 10 nM, or at least about 5 nM, or at least about 2 nM. In an embodiment of the disclosure, the agent has a higher affinity for Rab5 than a Rab5 effector. In an embodiment of the disclosure, the agent selectively inhibits binding of the Rab5 to a Rab5 effector. For example, the agent inhibits binding of the Rab5 to a Rab5 effector such as VPS34 with an IC₅₀ that is lower than the IC₅₀ of inhibition of binding of endogenous Rab5 to a Rab5 effector.

[0075] Furthermore, active Rab5, in a subject or elsewhere, can be detected and their amount and concentration measured by any method commonly known in the art. This includes, for example, methods involving mass spectrometry, high pressure liquid chromatography (HPLC), combined gas chromatography-mass spectrometry, and liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. See, *for example*, De Marchi et al., (2003). The modulation of the level of a compound, for example

the increase or decrease of the concentration of Rab5 in a subject, can be measured by detecting the compound in samples taken at different times. Active Rab5 can be measured in samples taken from, for example, blood or tissue biopsies.

[0076] In one embodiment of the present disclosure, the agent used for modulating the activity of Rab5 modulates binding of the Rab5 to a Rab5 effector, for example VPS34. The binding of Rab5 to Rab5 effectors, for example, can be detected and quantified according to methods commonly known in the art. See, *for example*, Glatz et al., *J. Biol. Chem.*, 259 4295-4300, (1984); and Morrow & Martin, *J. Lipid Res.*, 24 pp. 324-331, (1983).

Accordingly, examples of such methods can include or involve incubation of GTP bound Rab5 with radio-labeled effectors in the presence or absence of agents to be tested with respect to their effect on the binding or interaction between Rab5 to a Rab5 effector.

Examples of such methods can further include the subsequent separation of Rab5-bound and unbound effectors and quantification of Rab5 effectors bound to Rab5 in the presence or in the absence of said agents.

[0077] The phrase “subject in need thereof” as used herein refers to any organism in need of treatment, or requiring preventative therapy to prevent a condition resulting from lower or higher than normal levels of Rab5 activity in the organism, with the methods of the disclosure. A subject animal can include: fish, birds, or mammals. The subject can also be livestock, such as cattle, canines, swine, sheep, poultry, and horses. In a specific embodiment of the present disclosure, the subject is a human.

[0078] The term “effective amount” is employed herein to refer to the amount of an agent that is effective in modulating the activity of Rab5 in a subject.

[0079] The dosage of an agent that is administered to a subject in need thereof can vary, depending on the reason for use and the individual subject. The dosage can be adjusted based on the subject's weight, the age and health of the subject, and tolerance for the compound or composition.

[0080] The amount of therapeutic agent to be used depends on many factors. Dosages can include about 2 mg/kg of bodyweight/day, about 5 mg/kg of bodyweight/day, about 10

mg/kg of bodyweight/day, about 15 mg/kg of bodyweight/day, about 20 mg/kg of bodyweight/day, about 25 mg/kg of bodyweight/day, about 30 mg/kg of bodyweight/day, about 40 mg/kg of bodyweight/day, about 50 mg/kg of bodyweight/day, about 60 mg/kg of bodyweight/day, about 70 mg/kg of bodyweight/day, about 80 mg/kg of bodyweight/day, about 90 mg/kg of bodyweight/day, about 100 mg/kg of bodyweight/day, about 125 mg/kg of bodyweight/day, about 150 mg/kg of bodyweight/day, about 175 mg/kg of bodyweight/day, about 200 mg/kg of bodyweight/day, about 250 mg/kg of bodyweight/day, about 300 mg/kg of bodyweight/day, about 350 mg/kg of bodyweight/day, about 400 mg/kg of bodyweight/day, about 500 mg/kg of bodyweight/day, about 600 mg/kg of bodyweight/day, about 700 mg/kg of bodyweight/day, about 800 mg/kg of bodyweight/day, and about 900 mg/kg of bodyweight/day. Routine experimentation will determine the appropriate value for each patient by monitoring the agent's effect on Rab5 activity levels, cell autophagy or endocytosis, which can be frequently and easily monitored by one of ordinary skill in the art. The agent can be administered once or multiple times per day. The frequency of administration can vary from a single dose per day to multiple doses per day. Preferred routes of administration include oral, intravenous and intraperitoneal, but other forms of administration can be chosen as well.

[0081] The effective amount of an agent according to the present disclosure can be administered along any of the routes commonly known in the art. This includes, for example, (1) oral administration; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection; (3) topical administration; or (4) intravaginal or intrarectal administration; (5) sublingual or buccal administration; (6) ocular administration; (7) transdermal administration; (8) nasal administration; and (9) administration directly to the organ or cells in need thereof.

[0082] The term "effective amount" further includes the amount of a Rab5 modulating agent that will elicit the biological or medical response of a tissue, system, animal or human that is being sought by the researcher, veterinarian, medical doctor or other clinician. It will be understood, however, that the specific amount of such agent and frequency of administration for any particular subject may be varied and will depend upon a variety of factors including the activity of the specific cells employed, the stability of the agent and, the age, body

weight, general health, sex, diet, mode and time of administration, rate of engraftment, drug combination, the severity of the particular condition, and the host undergoing therapy.

[0083] The effective amount of an agent according to the present disclosure can be formulated together with one or more pharmaceutically acceptable excipients. The active ingredient and excipient(s) can be formulated into compositions and dosage forms according to methods known in the art. These compositions and dosage forms can be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, tablets, capsules, powders, granules, pastes for application to the tongue, aqueous or non-aqueous solutions or suspensions, drenches, or syrups; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin, lungs, or mucous membranes; or (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; (5) sublingually or buccally; (6) ocularly; (7) transdermally; or (8) nasally.

[0084] The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject with toxicity, irritation, allergic response, or other problems or complications, commensurate with a reasonable benefit/risk ratio.

[0085] The phrase “pharmaceutically-acceptable excipient” as used herein refers to a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, carrier, manufacturing aid (e.g., lubricant, talc magnesium, calcium or zinc stearate, or stearic acid), solvent or encapsulating material, involved in carrying or transporting the therapeutic compound for administration to the subject. Each excipient should be “acceptable” in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable excipients include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; gelatin; talc; waxes;

oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as ethylene glycol and propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents; water; isotonic saline; pH buffered solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. If desired, certain sweetening and/or flavoring and/or coloring agents can be added. Other suitable excipients can be found in standard pharmaceutical texts, e.g. in “Remington's Pharmaceutical Sciences”, The Science and Practice of Pharmacy, 19th Ed. Mack Publishing Company, Easton, Pa., (1995).

[0086] Excipients are added to the composition for a variety of purposes. Diluents increase the bulk of a solid pharmaceutical composition, and can make a pharmaceutical dosage form containing the composition easier for the patient and caregiver to handle. Diluents for solid compositions include, for example, microcrystalline cellulose (*e.g.* Avicel[®]), microfine cellulose, lactose, starch, pregelatinized starch, calcium carbonate, calcium sulfate, sugar, dextrates, dextrin, dextrose, dibasic calcium phosphate dihydrate, tribasic calcium phosphate, kaolin, magnesium carbonate, magnesium oxide, maltodextrin, mannitol, polymethacrylates (*e.g.* Eudragit[®]), potassium chloride, powdered cellulose, sodium chloride, sorbitol and talc.

[0087] Solid pharmaceutical compositions that are compacted into a dosage form, such as a tablet, can include excipients whose functions include helping to bind the active ingredient and other excipients together after compression. Binders for solid pharmaceutical compositions include acacia, alginic acid, carbomer (*e.g.* carbopol), carboxymethylcellulose sodium, dextrin, ethyl cellulose, gelatin, guar gum, hydrogenated vegetable oil, hydroxyethyl cellulose, hydroxypropyl cellulose (*e.g.* Klucel[®]), hydroxypropyl methyl cellulose (*e.g.* Methocel[®]), liquid glucose, magnesium aluminum silicate, maltodextrin, methylcellulose, polymethacrylates, povidone (*e.g.* Kollidon[®], Plasdone[®]), pregelatinized starch, sodium alginate and starch.

[0088] The dissolution rate of a compacted solid pharmaceutical composition in the subject's stomach can be increased by the addition of a disintegrant to the composition.

Disintegrants include alginic acid, carboxymethylcellulose calcium, carboxymethylcellulose sodium (*e.g.* Ac-Di-Sol[®], Primellose[®]), colloidal silicon dioxide, croscarmellose sodium, crospovidone (*e.g.* Kollidon[®], Polyplasdone[®]), guar gum, magnesium aluminum silicate, methyl cellulose, microcrystalline cellulose, polacrillin potassium, powdered cellulose, pregelatinized starch, sodium alginate, sodium starch glycolate (*e.g.* Explotab[®]) and starch.

[0089] Glidants can be added to improve the flowability of a non-compacted solid composition and to improve the accuracy of dosing. Excipients that can function as glidants include colloidal silicon dioxide, magnesium trisilicate, powdered cellulose, starch, talc and tribasic calcium phosphate.

[0090] When a dosage form such as a tablet is made by the compaction of a powdered composition, the composition is subjected to pressure from a punch and dye. Some excipients and active ingredients have a tendency to adhere to the surfaces of the punch and dye, which can cause the product to have pitting and other surface irregularities. A lubricant can be added to the composition to reduce adhesion and ease the release of the product from the dye. Lubricants include magnesium stearate, calcium stearate, glyceryl monostearate, glyceryl palmitostearate, hydrogenated castor oil, hydrogenated vegetable oil, mineral oil, polyethylene glycol, sodium benzoate, sodium lauryl sulfate, sodium stearyl fumarate, stearic acid, talc and zinc stearate.

[0091] In liquid pharmaceutical compositions of the present disclosure, the modulator of a eukaryotic pathogen's adenylyl cyclase and any other solid excipients are dissolved or suspended in a liquid carrier such as water, water-for-injection, vegetable oil, alcohol, polyethylene glycol, propylene glycol or glycerin.

[0092] Liquid pharmaceutical compositions can contain emulsifying agents to disperse uniformly throughout the composition an active ingredient or other excipient that is not soluble in the liquid carrier. Emulsifying agents that can be useful in liquid compositions of the present invention include, for example, gelatin, egg yolk, casein, cholesterol, acacia, tragacanth, chondrus, pectin, methyl cellulose, carbomer, cetostearyl alcohol and cetyl alcohol.

[0093] Liquid pharmaceutical compositions of the present invention can also contain a viscosity enhancing agent to improve the mouth-feel of the product and/or coat the lining of the gastrointestinal tract. Such agents include acacia, alginic acid bentonite, carbomer, carboxymethylcellulose calcium or sodium, cetostearyl alcohol, methyl cellulose, ethylcellulose, gelatin guar gum, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, maltodextrin, polyvinyl alcohol, povidone, propylene carbonate, propylene glycol alginate, sodium alginate, sodium starch glycolate, starch tragacanth and xanthan gum.

[0094] Sweetening agents such as sorbitol, saccharin, sodium saccharin, sucrose, aspartame, fructose, mannitol and invert sugar can be added to improve the taste. Flavoring agents and flavor enhancers can make the dosage form more palatable to the patient. Common flavoring agents and flavor enhancers for pharmaceutical products that can be included in the composition of the present invention include maltol, vanillin, ethyl vanillin, menthol, citric acid, fumaric acid, ethyl maltol and tartaric acid.

[0095] Preservatives and chelating agents such as alcohol, sodium benzoate, butylated hydroxy toluene, butylated hydroxyanisole and ethylenediamine tetraacetic acid can be added at levels safe for ingestion to improve storage stability.

[0096] According to the present disclosure, a liquid composition can also contain a buffer such as gluconic acid, lactic acid, citric acid or acetic acid, sodium gluconate, sodium lactate, sodium citrate or sodium acetate. Selection of excipients and the amounts used can be readily determined by the formulation scientist based upon experience and consideration of standard procedures and reference works in the field.

[0097] Solid and liquid compositions can also be dyed using any pharmaceutically acceptable colorant to improve their appearance and/or facilitate patient identification of the product and unit dosage level.

[0098] The dosage form of the present disclosure can be a capsule containing the composition, for example, a powdered or granulated solid composition of the invention,

within either a hard or soft shell. The shell can be made from gelatin and optionally contain a plasticizer such as glycerin and sorbitol, and an opacifying agent or colorant.

[0099] A composition for tableting or capsule filling can be prepared by wet granulation. In wet granulation, some or all of the active ingredients and excipients in powder form are blended and then further mixed in the presence of a liquid, typically water, that causes the powders to clump into granules. The granulate is screened and/or milled, dried and then screened and/or milled to the desired particle size. The granulate can then be tableted, or other excipients can be added prior to tableting, such as a glidant and/or a lubricant

[0100] A tableting composition can be prepared conventionally by dry blending. For example, the blended composition of the actives and excipients can be compacted into a slug or a sheet and then comminuted into compacted granules. The compacted granules can subsequently be compressed into a tablet.

[0101] As an alternative to dry granulation, a blended composition can be compressed directly into a compacted dosage form using direct compression techniques. Direct compression produces a more uniform tablet without granules. Excipients that are particularly well suited for direct compression tableting include microcrystalline cellulose, spray dried lactose, dicalcium phosphate dihydrate and colloidal silica. The proper use of these and other excipients in direct compression tableting is known to those in the art with experience and skill in particular formulation challenges of direct compression tableting.

[0102] A capsule filling can include any of the aforementioned blends and granulates that were described with reference to tableting; however, they are not subjected to a final tableting step.

[0103] In the context of the present disclosure, the effective amount of the agent modulating the activity of Rab5 can be administered alone or in combination with one or more of other therapeutic agents. In a combination therapy, the effective amount of the agent modulating the activity of Rab5 can be administered before, during, or after commencing therapy with another agent, as well as any combination thereof, *i.e.*, before and during, before and after, during and after, or before, during and after commencing the additional therapy.

[0104] Consistent with the observed properties of the peptides of the disclosure, the present peptides can be used to inhibit, suppress or cause the cessation of at least one Rab5 mediated disease pathology including but not limited to cancer, Parkinson's disease, Alzheimer's disease and Huntington's disease, pancreatitis, obesity and diabetes, infectious disease such as, bacterial infection, HIV, hepatitis, and tularemia or atherosclerosis, Alzheimer's disease, and the endocytosis of toxins and viruses.

ASSAY FOR IDENTIFYING AN AGENT THAT MODULATES Rab5 ACTIVITY

[0105] The present disclosure also provides methods of identifying an agent that modulates Rab5 activity. Said disclosure comprises contacting a test agent with Rab5, and detecting autophagy of a cell or endocytosis, wherein the agent is identified by its ability to modulate Rab5 activity. In an embodiment of the disclosure, the agent is a peptide. In another embodiment of the disclosure the agent is an antibody. In yet another embodiment, the agent is a small molecule. In yet another embodiment, the agent is a nucleic acid.

[0106] Another aspect of the present disclosure provides methods of identifying an agent for modulating the activity of Rab5 in a subject comprising contacting the agent with Rab5, and detecting the interaction of Rab5 with Rab5 effectors. In an embodiment of the present disclosure, the Rab5 effector is the Vps34-Vps15-Becn1-Atg14L complex. In yet another embodiment of the disclosure, the Rab5 effector is the Vps34-Vps15 complex. In an embodiment of the present disclosure, the Rab5 effector is Vps34. In an embodiment of the present disclosure, the Rab5 effector is Atg14L. In an embodiment of the present disclosure, the Rab5 effector is EEA1.

[0107] In a non-limiting example of the disclosure, a modulator of Rab5 activity can be prepared and subsequently used by an embodiment of the disclosure by a known method. For example, a "split-pool" strategy can be implemented in the following way: beads of a functionalized polymeric support are placed in a plurality of reaction vessels; a variety of polymeric supports suitable for solid-phase peptide synthesis are known, and some are commercially available (for examples, see, e.g., M. Bodansky, Principles of Peptide Synthesis, 2nd ed., Springer-Verlag, Berlin (1993)). To each aliquot of beads is added a solution of a different activated amino acid, and the reactions are allowed to proceed to yield

a plurality of immobilized amino acids, one in each reaction vessel. The aliquots of derivatized beads are then washed, "pooled" (i.e., recombined), and the pool of beads is again divided, with each aliquot being placed in a separate reaction vessel. Another activated amino acid is then added to each aliquot of beads. The cycle of synthesis is repeated until a desired peptide length is obtained. The amino acid residues added at each synthesis cycle can be randomly selected; alternatively, amino acids can be selected to provide a "biased" library, e.g., a library in which certain portions of the inhibitor are selected non-randomly, e.g., to provide an inhibitor having known structural similarity or homology to a known peptide capable of interacting with an antibody, e.g., the an anti-idiotypic antibody antigen binding site. It will be appreciated that a wide variety of peptidic, peptidomimetic, or non-peptidic compounds can be readily generated in this way.

[0108] The "split-pool" strategy can result in a library of peptides, e.g., modulators, which can be used to prepare a library of test compounds of the disclosure. In another illustrative synthesis, a "diversomer library" is created by the method of Hobbs DeWitt et al., *Proc. Natl. Acad. Sci. USA*, 90:6909 (1993). Other synthesis methods, including the "tea-bag" technique of Houghten (see, e.g., Houghten et al., *Nature*, 354, 84-86 (1991)) can also be used to synthesize libraries of compounds according to the subject invention.

[0109] Libraries of compounds can be screened to determine whether any members of the library have a desired activity and, if so, to identify the active species. Methods of screening combinatorial libraries have been described. See, e.g., Gordon, et al., *J Med Chem.* 13;37(10) pp. 1385-401 (1994). After screening, compounds that have a desired activity can be identified by any number of techniques (e.g., mass spectrometry (MS), nuclear magnetic resonance (NMR), matrix-assisted laser desorption ionisation/time of flight (MALDI-TOF) analysis, and the like). Exemplary assays useful for screening libraries of test compounds are described herein.

[0110] The disclosure is further illustrated by the following specific examples which are not intended in any way to limit the scope of the disclosure.

EXAMPLES

[0111] The following examples further illustrate the disclosure, but should not be construed to limit the scope of the disclosure in any way.

[0112] **Example 1. *Active Rab5 rescues the autophagy deficiency in p110 β -/- cells.*** It has been reported that p110 β associates with the Vps34-Vps15-Beclin 1-Atg14L complex and stimulates Vps34 kinase activity to promote PI(3)P production. See Dou et al., (2010). A further examination using purified or in vitro-translated proteins revealed a lack of direct interaction of p110 β with Vps34, Vps15, or Beclin 1, raising the possibility that p110 β interacts with other proteins to modulate Vps34 catalytic activity. One candidate is the small GTPase Rab5. If p110 β promotes autophagy by activating Rab5, one would predict that a constitutively active form of Rab5 will restore autophagy in p110 β -deficient cells. To test this hypothesis the following proteins were expressed: wild-type Rab5 (Rab5-WT), the Rab5-Q79L constitutively active mutant (Rab5-CA), Vps34-Vps15, or Atg14L in p110 β control (p110 β +/+) and p110 β knockout (p110 β -/-) mouse embryonic fibroblasts (MEFs) (Fig. 1A) and autophagy was measured. As expected, expression of Vps34-Vps15 or Atg14L induced autophagy in p110 β +/+ cells, as indicated by an increase in autophagic GFP-LC3 puncta and LC3-II (Fig. 1B, 1C, and Fig. 8). However, in p110 β -/- cells, Vps34-Vps15 or Atg14L failed to induce autophagy under basal conditions (Fig. 1B and 1C) or in cells treated with the mTOR inhibitor rapamycin (Fig. 8B). This result is consistent with the finding that p110 β induces autophagy via the regulation of Vps34-Vps15-Beclin 1-Atg14L complex activity. Moreover, Rab5-CA but not Rab5-WT enhanced the accumulation of LC3-II in cells treated with the lysosomal inhibitor bafilomycin A1 (Fig. 1D). Taken together, these data suggest that Rab5-CA can bypass the requirement for p110 β in autophagy induction, and that Rab5-WT can be activated in a p110 β -dependent manner.

[0113] **Example 2. *p110 β is required for Rab5 activity.*** The differential effects of Rab5-WT and Rab5-CA in p110 β -/- MEFs suggest that p110 β can act upstream of Rab5 to increase its autophagy-promoting activity. Like other G proteins, Rab5 cycles between an inactive GDP-bound state and an active GTP-bound form that binds and activates its effectors. To determine whether p110 β can promote Rab5 activation, pull-down assays were

performed using the GST-tagged Rab5-binding domain of Rabaptin5 (residues 739-862, R5BD), which specifically binds to Rab5-GTP. The specificity of GST-R5BD binding to active Rab5 was verified by its more efficient binding to Rab5-CA compared to Rab5-WT and lack of binding to a dominant-negative Rab5 S43N mutant (Rab5-DN) (Fig. 2A). Importantly, GST-R5BD pulled down less active endogenous Rab5 from p110 β ^{-/-} cells than from p110 β ^{+/+} cells, while no difference was detected between p110 α control (p110 β ^{+/+}) and p110 α knockout MEFs (Fig. 2B). Stable (Fig. 2C) or transient (Fig. 9A) expression of either p110 β -WT or the kinase-dead mutant p110 β . K805R in p110 β ^{-/-} cells increased the amount of Rab5-GTP. Consistent with the lower amount of activated Rab5 in p110 β ^{-/-} versus p110 β MEFs, co-immunoprecipitation of Rab5 with two of its effectors related to autophagy and endocytosis, Vps34 and EEA1, was reduced in p110 β ^{-/-} cells (Fig. 2D). Re-expression of p110 β restored Rab5 binding to Vps34 and EEA1 (Fig. 2D). Consistent with the effect seen in MEFs, shRNA silencing of p110 β in HEK293T cells resulted in a large decrease in Rab5-GTP (Fig. 9B), compare lanes 1 and 3). Taken together, these data suggest that p110 β plays a critical role in promoting the activation of Rab5.

[0114] Example 3. *p110 β antagonizes the Rab5 GTPase-activating protein (GAP) activity of p85 α .* The mechanisms by which p110 β regulates Rab5 activity were analyzed.

Sequence and structure analysis of p110 β did not implicate p110 β as a putative guanine nucleotide exchange factor (GEF) that activates monomeric GTPases by stimulating the release of GDP to allow binding of GTP. In addition, as p110 β binds specifically to Rab5-GTP, p110 β does not act as a Rab5 GEF or guanine nucleotide dissociation inhibitor (GDI) because these functions require binding to Rab5-GDP. A working model is, that while p110-free p85 α can function as a Rab5 GAP, the presence of p110 β in the Rab5 complex can sequester Rab5 from the p85 α GAP function. Meaning that the loss of p85 α will lead to an increase in Rab5-GTP levels and enhanced autophagy. Indeed, stable silencing of p85 α (Fig. 3A) led to increased amounts of Rab5-GTP (Fig. 3B) and increased GFP-LC3 puncta formation (Fig. 3C) in both p110 β ^{+/+} and p110 β ^{-/-} cells. To test the effect of p85 α silencing on autophagosome maturation, LC3 with the tandem conjugation of mCherry and GFP was expressed in MEFs. Due to the more stable nature of mCherry fluorescence in the acidic environment, the appearance of red puncta indicates autolysosomes while the

formation of yellow puncta (owing to merged green and red signals) represents autophagosomes. While the number of both red and yellow puncta was higher in p110 β ^{+/+} than in p110 β ^{-/-} cells. Furthermore, consistent with defective autophagy in β ^{-/-} cells, silencing of p85 α led to a marked increase in the number of autophagosomes and autolysosomes in both cell lines (Fig. 3D). These results show that downregulation of p85 α relieves the inhibition of Rab5 and thus bypasses the requirement of p110 β for autophagy induction. Furthermore, p85 α knockdown led to a slight decrease in the steady-state level of p110 α , loss of p85 α also increased phosphorylation of Akt and S6K in both p110 β ^{+/+} and p110 β ^{-/-} cells (Fig. 3A), which is consistent with the finding that p85 α plays a role in activating PTEN. Therefore, downregulation of p85 α in these MEFs leads to an increase in autophagy, which is not mediated by Akt/mTOR inhibition, but rather by relief of Rab5 inhibition as shown above.

[0115] To test the effect of p85 α and p110 β on Rab5 activation in a more direct fashion, Rab5 GAP activity was analyzed in vitro using purified Rab5, p85 α , and the p110 β /p85 α and p110 α /p85 α PI3K complexes. Control experiments showed that GST-R5BD specifically pulled down Rab5 loaded with GTP but not GDP (Fig. 3E). Incubation of Rab5-GTP with free p85 α led to a decrease in the amount of Rab5-GTP in a dose-dependent manner (Fig. 3F), consistent with the ability of p85 α to act as a Rab5 GAP in vitro. p85 α complexed with p110 β showed no Rab5 GAP activity, whereas p85 α complexed with p110 α possessed GAP activity toward Rab5 (Fig. 3F). These results show that p110 β , but not p110 α , has a negative effect on the Rab5 GAP activity of p85 α . The addition of the p110 β /p85 α complex antagonized the Rab5 GAP activity of free p85 α , whereas the p110 β /p85 α complex failed to do so (Fig. 3G). In line with this, the p110 β /p85 α complex competed with Flag-tagged p85 α for binding to Rab5-GTP, while the Rab5-binding deficient p110 β mutant (Q596C) showed impaired ability to do so (Fig. 3H). Taken together, these data show that p110 β /p85 α sequesters Rab5 from p85 GAP activity to maintain a higher level of Rab5-GTP. In addition to p85 α , p110 β can also protect Rab5-GTP from other Rab5 GAPs, as the p110 β /p85 α complex was able to reduce RabGAP5 binding to Rab5 (Fig. 10).

[0116] Example 4. *The p110 β -Rab5 interaction plays an essential role in Rab5 activation and autophagy.* Using a structure based scanning mutagenesis strategy; mutations in p110 β that have no effect on p110 β activity but block interactions with Rab5 were identified. In a non-limiting example, two point mutants of p110 β , namely Q596C and I597S, showed loss of interaction with Rab5 (Fig. 4A). Examination of the crystal structure of p110 β (see Zhang et al., *Mol Cell* 41, 567-578. (2011)) revealed that Q596 and I597 are exposed on the outer surface of the alpha helical domain of p110 β (Fig. 11A-D). The two mutants possessed intact catalytic activity (Fig. 4B) and p85 α binding (Fig. 4C) and induced normal phosphorylation of Akt and S6K in cells treated with lysophosphatidic acid (LPA) (Fig. 11E).

[0117] To test the ability of these mutants to activate Rab5 and autophagy, the p110 β -Q596C and p110 β -I597S proteins were stably expressed in p110 β β -MEFs to levels similar to those seen in cells reconstituted with p110 β -WT (Fig. 4D). The steady-state levels of p110 α , p85 α , and phosphorylated Akt and S6K showed no change (Fig. 4D). GST-R5BD pull-down assays revealed that p110 β -WT, but not the Q596C or I597S mutants, increased the level of Rab5-GTP (Fig. 4E). Consistent with the autophagy-promoting role of p110 β -mediated Rab5 activation, cells expressing the mutants showed impaired ability to degrade the autophagy substrate p62 (Fig. 4F) and long-lived proteins (Fig. 4G). In addition, cells expressing the two mutants showed reduced accumulation of LC3-II (Fig. 4H) and GFP-LC3 autophagic puncta (Fig. 4I) in both untreated and serum-deprived conditions. Taken together, the data also show that the p110 β -Rab5 association, but not the kinase activity of p110 β , is important for autophagy. While the kinase-deficient p110 β K805R mutant was able to rescue Rab5 activation (Fig. 2C and Fig. 9A) and autophagy, the K805R/I597S double mutant failed to interact with Rab5 and to induce autophagy (Fig. 11F and Fig. 11G). Taken together, these results demonstrate that residues Q596 and I597 in the p110 β alpha helical domain are essential for the p110 β -Rab5 interaction and that the physical interaction of p110 β with Rab5 is required for Rab5 activity and its autophagy-promoting function.

[0118] Example 5. *p110 β -mediated Rab5 activation is selectively regulated by growth factor availability, but not by nutrient signaling.* The results herein show that p110 β can function to activate Rab5 and autophagy (Fig. 1-4). On the other hand, Class IA PI3Ks are

well known to be recruited to growth factor receptor signaling complexes where they produce PI(3,4,5)P₃ to stimulate downstream signaling cascades, such as the Akt/mTOR pathway (see Engelman et al., (2006)). To examine whether metazoan cells are able to respond to growth factor limitation, and thus activate autophagy in a manner that can be directly transduced from growth factor receptor, immunoprecipitation of Rab5 under various conditions was performed in genetically modified MEFs. The effect of such modifications on cell signaling was verified by monitoring the phosphorylation of Akt, S6 (a downstream effector of mTOR), and AMPK (Fig. 5A). Co-immunoprecipitation experiments showed that only serum deprivation enhanced the p110 β -Rab5 association (Fig. 5B). Moreover, Vps34 binding to Rab5 was also selectively enhanced by serum deprivation (Fig. 5B).

[0119] To determine whether Rab5 activation, induced by serum deprivation is dependent on p110 β , GST-R5BD pull-down assays were performed on lysates of p110 β ^{+/+} and p110 β ^{-/-} MEFs. Indeed, serum withdrawal increased the level of Rab5-GTP in p110 β ^{+/+} but not in p110 β ^{-/-} cells (Fig. 5C). A similar effect was also seen in HEK293T cells with shRNA silencing of p110 β (Fig. 9B). As a correlative measure of Rab5 activation, enhanced coimmunoprecipitation of Rab5 with its effectors Vps34 and EEA1 was observed in response to serum deprivation only in p110 β ^{+/+} MEFs (Fig. 5D). Reconstitution of β ^{-/-} MEFs with wild-type p110 β restored Vps34 and EEA1 association with Rab5 that are induced by serum deprivation (Fig. 5D), whereas the Rab5-binding deficient p110 β mutants failed to rescue the defects (Fig. 12A). Vps34 activity leads to production of PI(3)P that can be visualized by the GFP-conjugated FYVE domain. Expression of GFP-FYVE revealed an increase in puncta formation upon serum deprivation in p110 β ^{-/-} MEFs reconstituted with wild-type p110 β (Fig. 5E and Fig. 12B). In addition, an enhanced co-localization between GFP-FYVE and Rab5 was seen upon serum deprivation in p110 β -reconstituted cells (Fig. 5E and Fig. 12B), consistent with a role of Rab5 in stimulating Vps34 activity. By contrast, the increase in GFP-FYVE puncta and the co-localization with Rab5 were drastically impaired in p110 β ^{-/-} MEFs and in p110 β ^{-/-} MEFs reconstituted with p110 β I597S (Fig. 5E and Fig. 12B), which is correlated with the lack of Vps34 association to Rab5 in these cells (Fig. 5D and Fig. 12A). In addition, expression of the dominant-negative Rab5 S43N mutant (Rab5-DN) led to impaired GFP-LC3 puncta and LC3-II formation (Fig. 12C and

Fig. 12D), supporting the role of Rab5 in mediating autophagy induced by serum deprivation. Taken together, these results indicate that growth factor limitation selectively enhances the p110 β -Rab5 interaction, which plays an essential role in Rab5 activation and autophagy.

[0120] Example 6. p110 β dissociates from growth factor signaling complexes and increases its interaction with Rab5 upon growth factor limitation. The results show that serum withdrawal selectively induces p110 β -mediated Rab5 activation, which is responsible for autophagy induction. Although serum is well regarded as a source of growth factors, it is a relatively undefined mixture that mediates complex changes in signaling and contains various nutrients. Therefore, to further pursue the possible selective role of p110 β in Rab5 activation and autophagy in response to growth factor limitation, the immortalized breast epithelial MCF10A cell line, whose growth is dependent on the presence of defined growth factors (insulin, EGF, cholera toxin, and hydrocortisone) was used. Removal of the growth factors has been shown to induce autophagy in MCF10A cells. To detect changes in p110 β partitioning between subcellular pools, insulin receptor signaling complexes were immunoprecipitated. The results show that the association of p110 β with insulin receptor substrate (IRS)-1 and IRS-2 was diminished upon deprivation of the growth factors (Fig. 6A, left panel). Similarly, pull-down with phosphotyrosine antibodies showed reduced binding of p110 β to tyrosine-phosphorylated proteins (Fig. 6A, left panel). By contrast, p110 β and Rab5 co-precipitation increased upon withdrawal of the growth factors (Fig. 6A, middle panel). The Rab5-binding deficient mutant p110 β I597S showed abrogated binding to Rab5 even upon growth factor deprivation (Fig. 13A). To visualize the change in subcellular localization of p110 β immunofluorescence microscopy imaging of MCF10A cells was performed. The specificity of the p110 β antibody used for immunofluorescence was verified using cells treated with shRNA to knock down p110 β (Fig. 13B). Staining of endogenous p110 β and Rab5 in cells cultured in complete medium showed a portion of p110 β on the plasma membrane and Rab5 mostly in the cytoplasm (Fig. 6B). In the absence of the hormonal factors, the plasma membrane p110 β signal was greatly reduced, and enhanced co-localization of p110 β and Rab5 was observed (Fig. 6B). The p110 β I597S mutant showed loss of co-localization with Rab5 (Fig. 12C). These data show that growth

factor limitation leads to p110 β dissociation from growth factor receptor signaling complexes and increased p110 β -Rab5 interaction.

[0121] To study the requirement for p110 β in growth factor limitation-induced autophagy in MCF10A cells, a shRNA against p110 β or a non-targeting control shRNA under the control of a tetracycline-inducible system was stably expressed in MCF10A cells. Addition of doxycycline led to a progressive reduction in p110 β protein levels (Fig. 6C), with no substantial effect on the steady-state level of p110 α , p85, or phosphorylated Akt and S6K (Fig. 6C). Growth factor deprivation led to an increased amount of endogenous Rab5-GTP in the control but not in p110 β knock-down MCF10A cells with endogenous Rab5 (Fig. 6D). Reconstitution with shRNA-resistant wild-type p110 β , but not the I597S mutant, rescued Rab5-GTP levels (Fig. 13D). Upon removal of the growth factors, there was a marked increase in GFP-LC3 puncta in control MCF10A cells, whereas this response was greatly impaired in the p110 β knock-down cells (Fig. 6E and 6F). In MCF10A cells, the LC3 signal was not readily detectable by immunoblotting (Fig. 6G). In the presence of the lysosomal inhibitor bafilomycin A1 a significant increase in LC3 was observed. p110 β knock-down cells displayed a lower level of LC3-II than the control cells (Fig. 6G), consistent with the positive role of p110 β in basal level Rab5 activity and autophagy. Noticeably, the amount of LC3-II induced by growth factor withdrawal was greatly reduced in p110 β knock-down cells. Reconstitution with shRNA-resistant wild-type p110 β rescued the GFP-LC3 puncta and LC3-II formation, whereas the I597S mutant failed to do so (Fig. 13E and Fig. 13F). In contrast to growth factor deprivation, other autophagic stimuli including rapamycin, the ER stressor tunicamycin, or the proteasome inhibitor MG132 did induce significant LC3-II production in the p110 β knock-down cells (Fig. 13G). The data herein show that p110 β plays an indispensable and selective role in growth factor limitation-induced autophagy.

[0122] **Example 7. Plasma membrane targeted p110 β does not activate autophagy upon growth factor limitation.** To further investigate the role of p110 β translocation in mediating growth factor limitation-induced autophagy, ectopically expressed p110 β was targeted to the plasma membrane by tagging it with the carboxy-terminal CAAX sequence of K-Ras. Immunofluorescence imaging of the Flag-p110 β -CAAX mutant expressed in Hs578T cells

confirmed that it did not dissociate from the plasma membrane upon serum deprivation (Fig. 14A). Co-immunoprecipitation experiments showed a reduced interaction between Rab5 and the p110 β -CAAX mutant under basal conditions and a lack of enhanced binding upon serum starvation (Fig. 7A). Importantly, while reconstitution of wild-type p110 β in p110 β $-/-$ MEFs restored the serum deprivation-induced increases in LC3-II level (Fig. 7B) and GFP-LC3 puncta accumulation (Fig. 7C), expression of the p110 β -CAAX mutant failed to do so. The failure of p110 β -CAAX to restore autophagy is not due to its possible elevated kinase activity because the p110 β -specific kinase inhibitor TGX-221 did not rescue the GFP-LC3 puncta in the p110 β $-/-$ cells expressing p110 β -CAAX (Fig. 14B). The same concentration of TGX-221 inhibited LPA-stimulated Akt phosphorylation (Fig. 14C) and abrogated the slightly elevated phosphorylation of Akt and S6K imposed by p110 β -CAAX (Fig. 14D). Therefore, these results show that cytosolic p110 β , through its interaction with Rab5, plays a critical role in promoting autophagy induced by growth factor limitation.

[0123] Example 8. *Materials and Methods. Cell lines, culture, transfection, and*

treatment. MEFs, HEK293T, and Hs578T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen), and transfected using Lipofectamine 2000 (Invitrogen). Glucose-free DMEM, Hank's buffer (with calcium and glucose), and dialyzed FBS were also obtained from Invitrogen. For short-term amino acid starvation, cells were incubated in Hank's buffer supplemented with 10% dialyzed FBS and 1% HEPES (Invitrogen). MCF10A cells were cultured in DMEM/F12 (Invitrogen) and supplemented with 5% horse serum (Invitrogen), 20 ng/ml EGF (Sigma-Aldrich), 0.5 μ g/ml hydrocortisone (Sigma-Aldrich), 100 ng/ml cholera toxin (Sigma-Aldrich), 10 μ g/ml insulin (Sigma-Aldrich), 100 units/ml penicillin, and 100 μ g/ml streptomycin. For growth factor removal experiments, MCF10A cells were cultured in basal medium (DMEM/F12 plus 5% horse serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin) for the indicated times. The SV40-immortalized p110 $\beta^{+/+}$, p110 $\beta^{-/-}$, p110 $\alpha^{+/+}$, and p110 $\alpha^{-/-}$ MEFs from p110 $\alpha^{\text{flox/+}}$ or p110 $\beta^{\text{flox/+}}$ matings were described previously in Dou et al., (2010); and Lu et al., (2009), which are incorporated herein by reference.

[0124] *Plasmids, antibodies, and other reagents.* LPC-GFP-LC3, GFP-FYVE, pCMV-3xFlag-p110 α , p110 β -WT, p110 β -K805R, and p85 α were previously described in Dou et al., (2010). GFP-Rab5a-WT, GFP-Rab5a-CA and GFP-Rab5a-DN made according to Volpicelli et al., 2001, and the Rab5a sequences were cloned into pDsRed vector. Myc-Vps34-V5-Vps15 (see Yan et al., (2009)), Flag-Atg14L (see Zhong et al., 2009), GFP-mCherry-LC3 (see Pankiv et al., *J Biol Chem* 282, 24131-24145 (2007)), Myc-RabGAP5 and GST-R5BD (see Liu et al., 2007) were used as described in the references cited herein, which are incorporated herein by reference. GST-Rab5a was generated by cloning the Rab5a fragment into pGEX-2T. Myc-p110 β , Myc-p110 β -Q596C and Myc-p110 β -I597S were subcloned into the retroviral LPC vector. The p110 β -CAAX construct was made by using PCR to add sequences encoding the carboxy-terminal 17 amino acids of K-Ras (KDGKKKKKKSKTKCVIM) (SEQ ID NO: 10) to the 3' end of the p110 β cDNA and cloning the fragment into pCMV-3xFlag. shRNA resistant p110 β cDNA was generated by mutagenesis from the shRNA target sequence 5' gattgtgccctctctagattccta 3' (SEQ ID NO: 13) to shRNA resistant sequence 5' gactgcgcattatcaaggtttcta 3' (SEQ ID NO: 14).

[0125] Bafilomycin A1 was from Enzo; Ponceau S, GTP, GDP, GTP γ S, and doxycycline were from Sigma-Aldrich. Akt inhibitor, rapamycin, tunicamycin, MG132, TGX-221, and LPA were from sources described previously, which are incorporated herein by reference. See Dou et al., 2010; and Pan et al., *Mol Cell Biol.* 31, pp. 3158-3170 (2011). The following antibodies were used: Rab5 (Santa Cruz Biotechnology, BioVision, and Cell Signaling Technology), Vps34 (Cell Signaling Technology), Atg14L (see Zhong et al., *Nature Cell Biology* 11, pp. 468-U262(2009)), LC3 (MBL and Cell Signaling Technology), GFP (Santa Cruz Biotechnology), β -tubulin (Sigma-Aldrich), EEA1 (BD Biosciences and Cell Signaling Technology), p85 α (Millipore), Myc (9E10, Iowa University Hybridoma Bank), Flag (Sigma-Aldrich), p110 β (Cell Signaling Technology), IRS-1 and IRS-2 (Santa Cruz Biotechnology), p-Akt T308 (Cell Signaling Technology), p-S6 S240/S244 (Cell Signaling Technology), p-S6K T389 (Cell Signaling Technology), p-AMPK T172 (Cell Signaling Technology), and Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen).

[0126] *Retroviral and lentiviral infection.* Retrovirus infection using LPC-based viral constructs was performed as previously described in Dou et al., (2010). The stable cell lines

were generated by, for example, retroviral infection. Lentiviral infection for shRNA knock-down was done as previously described Dou et al., (2010). To generate the tetracycline-inducible shRNA virus against p110 β , we followed the procedure of the “all-in-one” system for the inducible expression of shRNA. See Wiederschain et al., *Cell Cycle* 8, pp. 498-504 (2009). The Tet-pLKO-puro vector was purchased from Addgene. The oligo corresponding to the sh-p110 β sequence was synthesized by Operon and inserted into the Tet-pLKO-puro vector. After lentiviral infection, cells were selected with puromycin, and the expression of shRNA was induced by addition of 1 μ g/ml doxycycline.

[0127] *GFP-LC3 puncta observation and quantification.* Quantification of GFP-LC3 was performed as described previously in Dou et al., 2010. In brief, cells expressing GFP-LC3 were treated and fixed in 4% paraformaldehyde (PFA) in PBS and observed under an inverted deconvolution microscope (Axiovert 200M; Carl Zeiss, Inc.) using the 63 $\times$ oil objective. Over 200 cells were randomly selected and counted for autophagy induction in multiple fields. Cells with more than 10 green puncta and diminished nuclear GFP signal were considered autophagic.

[0128] *Immunofluorescence.* Cells were fixed in 4% PFA in PBS for 20 min at room temperature. Cells were washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. After washing two times, cells were blocked in 10% BSA in PBS for 2 hours at room temperature. Cells were incubated with primary antibodies in 5% BSA in PBS overnight at 4°C. The next day, cells were washed four times with PBS containing 0.1% Tween 20 (PBST) each for 10 minutes, followed by incubation with fluorophore-conjugated secondary antibody in 5% BSA in PBST for 1 hour at room temperature. Cells were then washed four times in PBST, twice with PBS, and mounted with Immu-Mount (Thermo Fisher Scientific). The slides were analyzed and imaged using an inverted deconvolution microscope (Axiovert 200M; Carl Zeiss, Inc.), or a two-photon laser scanning confocal microscope (LSM 510 META NLO; Carl Zeiss, Inc.) and the co-localization was analyzed by the LSM software.

[0129] *Immunoprecipitation.* Cells were lysed with lysis buffer (20 mM Tris, pH 7.5, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% NP-40, 10% glycerol, and 100 μ M PMSF)

supplemented with EDTA-free protease inhibitor cocktail (Roche). For cell lysates used for detecting phosphoproteins, 10 mM sodium pyrophosphate, 0.1 mM Na_3VO_4 , 20 mM β -glycerolphosphate disodium, and 10 mM NaF were added to the lysis buffer. The presence of MgCl_2 is essential for Rab5-related immunoprecipitations. Cell lysates were centrifuged at 17,000 g for 5 minutes; 500 μg to 1 mg of soluble protein was incubated with primary antibodies overnight at 4°C . Protein A- or protein G-agarose (Roche) was added the next day, and samples were incubated at 4°C for 2 hours. Myc and phosphotyrosine immunoprecipitations were performed using anti-Myc (Sigma-Aldrich) or anti-phosphotyrosine (4G10) antibody conjugated to agarose (Millipore) following the manufacturer's protocols. The precipitates were then washed 4–5 times with lysis buffer, boiled with $1\times$ SDS sample buffer, and subjected to polyacrylamide gel electrophoresis and immunoblotting.

[0130] *GST-Rab5 pull-down assay.* GST-Rab5-GTP γ S pull-downs were performed according to a published protocol in Christoforidis et al., (1999) with the following modifications: pGEX-2T-Rab5a was transformed into BL21-CodonPlus *E. coli* and induced with 0.2 mM IPTG for 4 hours at 28°C . Bacterial lysates were loaded onto glutathione agarose beads (Invitrogen), washed, and incubated with buffer containing 20 mM HEPES, 100 mM NaCl, 10 mM EDTA, 5 mM MgCl_2 , 1 mM DTT, and 1 mM GTP γ S (freshly made), pH 7.5, for 90 minutes at room temperature. The product of the lysis was stabilized by incubating the beads with the above buffer without EDTA for 20 minutes at room temperature. The HEK293T cells were lysed in buffer containing 25 mM HEPES, 100 mM NaCl, 5 mM MgCl_2 , 1 mM DTT, 1% NP-40, 10% glycerol, 100 μM PMSF, and EDTA-free protease inhibitor cocktail, pH 7.5. After centrifugation, supernatants of cell lysates were pre-cleared with GST beads, and then incubated with GST beads or GST-Rab5-GTP γ S beads in the presence of 1 mM GTP γ S for 2 hours at 4°C . The beads were then washed 4 times with lysis buffer, boiled with $1\times$ SDS sample buffer and subjected to immunoblotting.

[0131] *GST-R5BD pull-down assay.* The GST-R5BD construct and pull-down assays were described previously in Liu et al., 2007 with the following modifications: GST-R5BD was expressed in BL21-CodonPlus *E. coli* and purified with glutathione agarose beads. For pull-down of cell lysates, cells in 10-cm plates were lysed in buffer containing 25 mM HEPES,

pH 7.4, 100 mM NaCl, 1 mM CaCl₂, 5 mM MgCl₂, 1% NP-40, 10% glycerol, 1 mM DTT, 100 μM PMSF, and EDTA-free protease inhibitor cocktail. After centrifugation, supernatants were incubated with GST-R5BD beads at 4°C, washed with lysis buffer, boiled in 1× SDS sample buffer and subjected to immunoblotting.

[0132] *In vitro* Rab5 GAP assays. Purified proteins for *in vitro* Rab5 GAP assays were purchased or prepared as follows. Rab5a was made by removing the protein from GST-Rab5a beads with thrombin (GE Healthcare). Purified p110β/p85α expressed in Sf21 cells were purchased from Millipore, and p110α/p85α was obtained from Invitrogen. Free p85α was purified from Sf9 cells infected with baculovirus. Two days after infection, cells were lysed and low-speed supernatants were loaded onto a HiTrap Ni²⁺ column (Amersham Pharmacia Biotech). Free p85α was eluted by washing the column with 50 mM imidazole. Fractions containing p85α were loaded onto a HiTrap QFF column (Amersham Pharmacia Biotech) and the proteins were eluted with a gradient of NaCl. p85α emerging at ~150-230 mM NaCl was pooled and concentrated, then purified by gel filtration on a Superdex 200 column (Amersham Pharmacia Biotech). Fractions containing highly purified p85α with little or no p110 were pooled, concentrated and stored at -20°C. The concentrations of p110β/p85α and p110α/p85α were normalized against that of p85α on immunoblots probed with p85α antibody. Signals were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences). For *in vitro* Rab5 GAP assays, 200 nM Rab5 was incubated with 200 nM GDP or GTP in loading buffer (20 mM Tris-HCl, pH 8, 2 mM EDTA, and 1 mM DTT) at 25°C for 30 minutes. Rab5-GTP hydrolysis was initiated by addition of p85α or the p110 complex in the presence of MgCl₂ at a final concentration of 10 mM. After 10 minutes at 25°C, the reactions were stopped by addition of ice cold buffer containing 25 mM HEPES, 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, and 0.1% NP-40. The samples were pre-cleared with GST beads and incubated with GST-R5BD beads. The beads were washed, boiled in 1× SDS sample buffer, and subjected to immunoblotting.

[0133] *Peptide development.* The peptides of the instant disclosure are designed using the peptide sequence of p110β that flank two critical residues Gln (Q)596 and Ile (I)597. The peptides of the present disclosure are synthesized using Solid Phase Peptide Synthesis by Peptide 2.0, Inc. Generally, Solid Phase Peptide Synthesis comprises a solid support

comprising a synthetic polymer that bears reactive groups including but not limited to -OH. The reactive group reacts easily with the carboxyl group of an N-a-protected amino acid, thereby covalently binding it to the polymer. The amino protecting group can then be removed and a second N-protected amino acid can be coupled to the attached amino acid. These steps are repeated until the desired sequence is obtained. At the end of the synthesis, a different reagent is applied to cleave the bond between the C-terminal amino acid and the polymer support; the peptide then goes into solution and can be obtained from the solution. TATdomain(s) were added to make the peptides cell more cell permeable in cell culture and *in vivo*. See Wagstaff, K.M. and Jans, D. A., *Current Medicinal Chemistry* 13, pp. 1371-1387(2006).

[0134] *Measurement of long-lived protein degradation.* Long-lived protein degradation assay was performed as previously described in Dou et al., 2010, which is incorporated herein by reference.

[0135] *Statistics.* Student's *t* test was used to compare the differences between two groups. One-way ANOVA with Tukey's post-hoc test was used for comparisons between more than two groups. Results were considered significant when *p* was less than 0.05.

[0136] *Image processing and densitometry measurement.* Images captured by deconvolution and confocal microscopes were viewed and processed by AxioVision LE and Zeiss LSM image browser, respectively. Images were processed in Adobe Photoshop to enhance the brightness and contrast. Densitometry of immunoblot bands was determined by ImageJ software or by the Odyssey Infrared Imaging System.

We claim:

1. A peptide comprising the amino acid sequence set forth in SEQ ID NO: 8.
2. The peptide of claim 1, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7.
3. The peptide of claim 1, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 15.
4. The peptide of claim 1, wherein said peptide modulates Rab5 activity.
5. A method of modulating Rab5 activity comprising contacting Rab5 with a fragment of an isolated p110 β protein.
6. The method of claim 5, wherein said fragment comprises the amino acid sequence set forth in SEQ ID NO: 8.
7. The method of claim 5, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7.
8. The method of claim 5, wherein said amino acid sequence is selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 15.
9. A method of modulating the level of Rab5 activity in a subject in need thereof comprising administering an effective amount of an agent that modulates the activity of Rab5.
10. The method of claim 9, wherein said agent increases the level of Rab5-GTP in the subject.
11. The method of claim 9, wherein the agent reduces the level of Rab5-GDP in the subject.

12. The method of claim 9, wherein the agent modulates the interaction between Rab5 and Vps34-Vps15-Becn1-Atg14L complex.
13. The method of claim 9, wherein the agent modulates the interaction between Rab5 and the Vps34-Vps15 complex.
14. The method of claim 9, wherein the agent modulates the interaction between Rab5 and an effector thereof selected from the group consisting of Vps34, Atg14L, and EEA1.
15. The method of claim 9, wherein the agent modulates the level of autophagy or endocytosis in a cell.
16. The method of claim 9, wherein said agent selected from the group consisting of a small molecule, and a peptide.
17. The method of claim 16, wherein said agent is a peptide.
18. The method of claim 17, wherein said peptide comprises the sequence set forth in SEQ ID NO: 8.

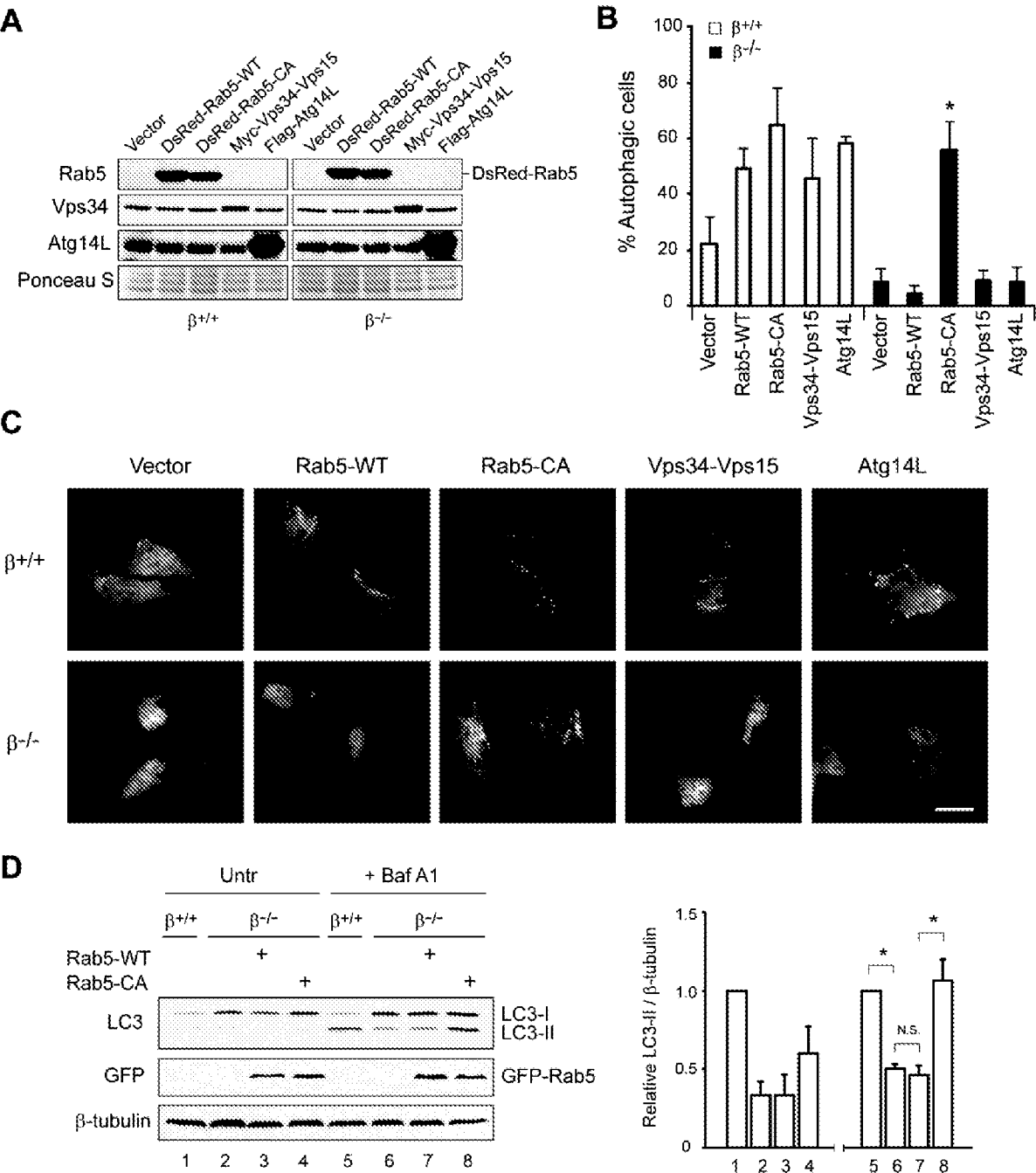


Figure 1

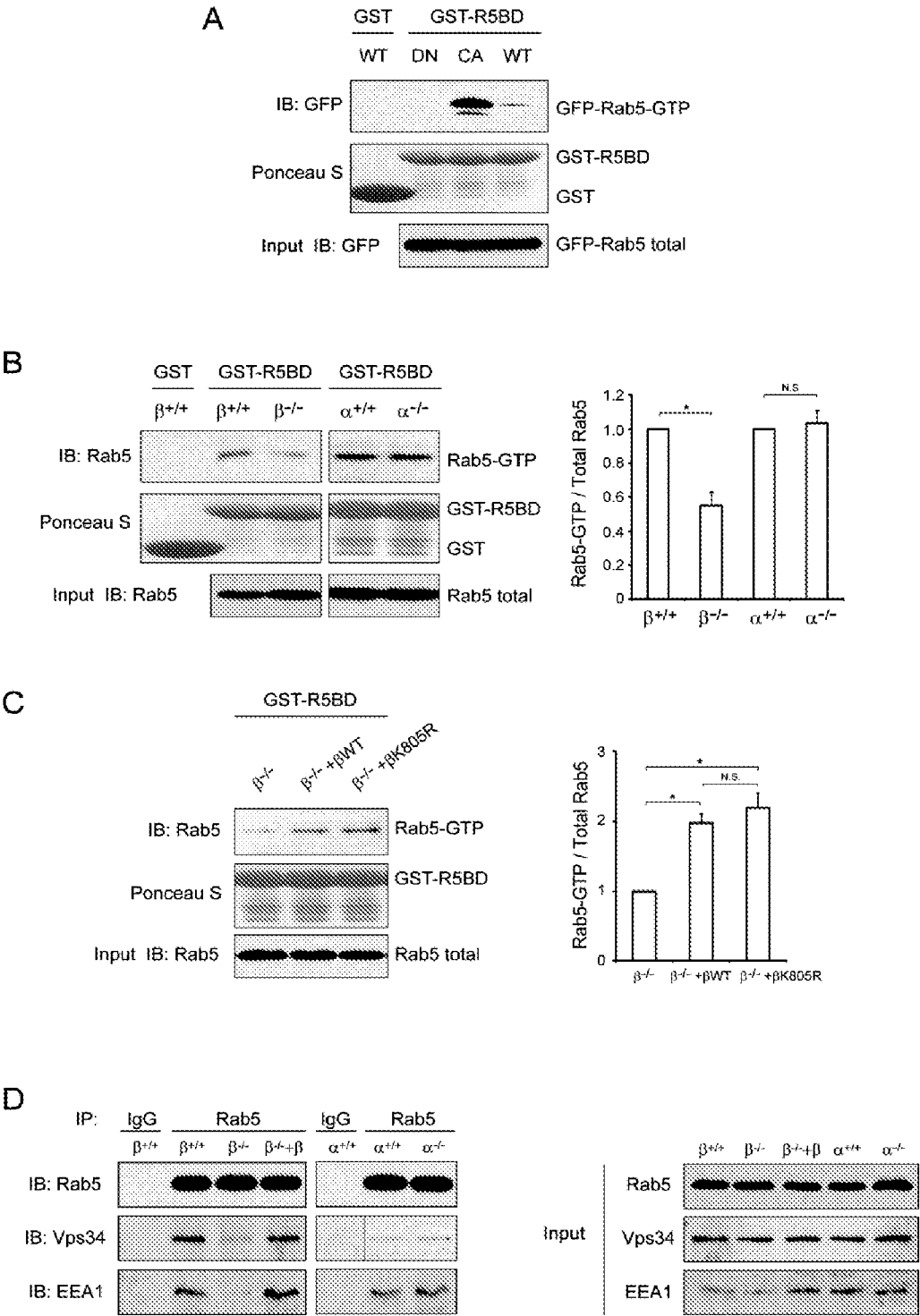
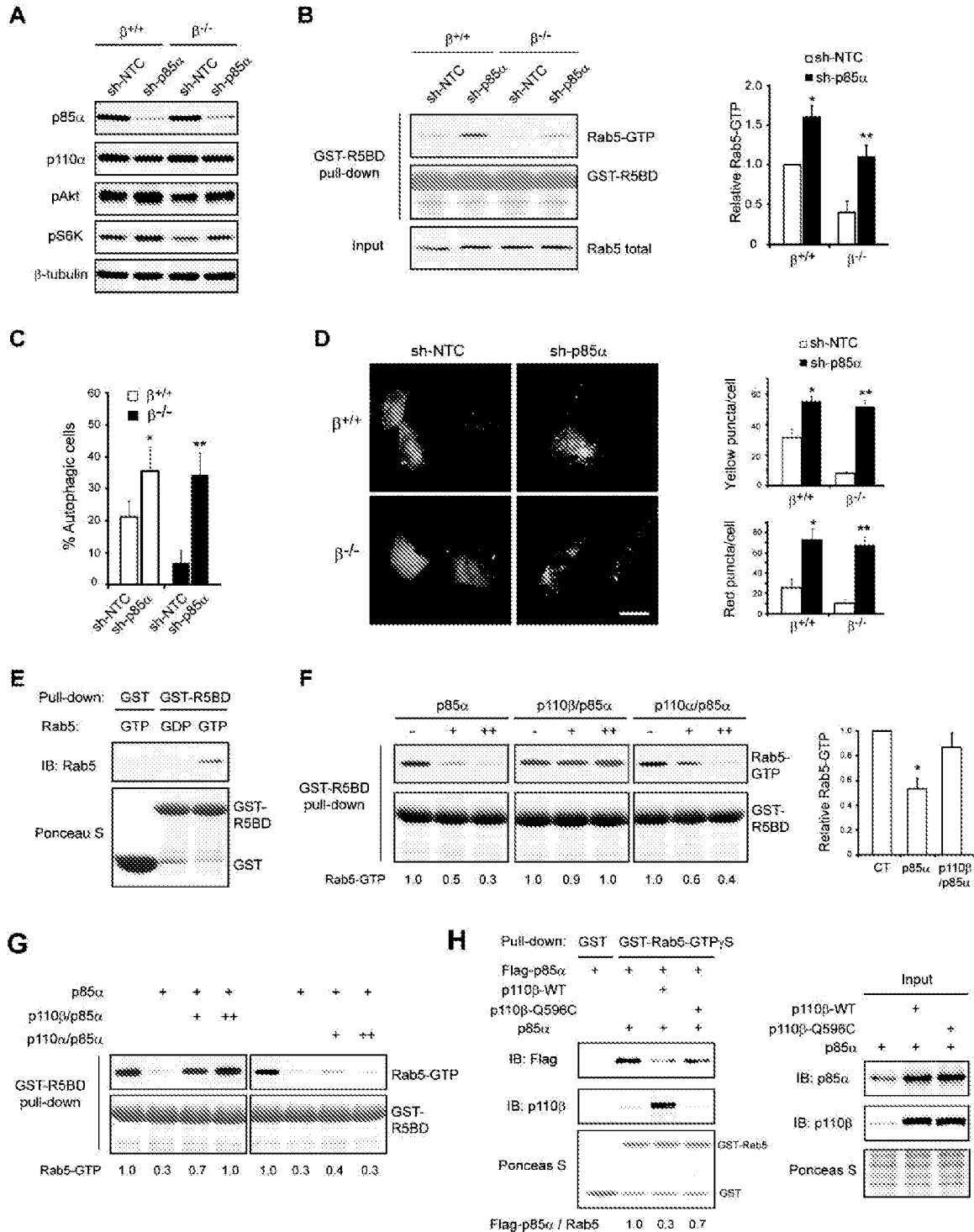


Figure 2



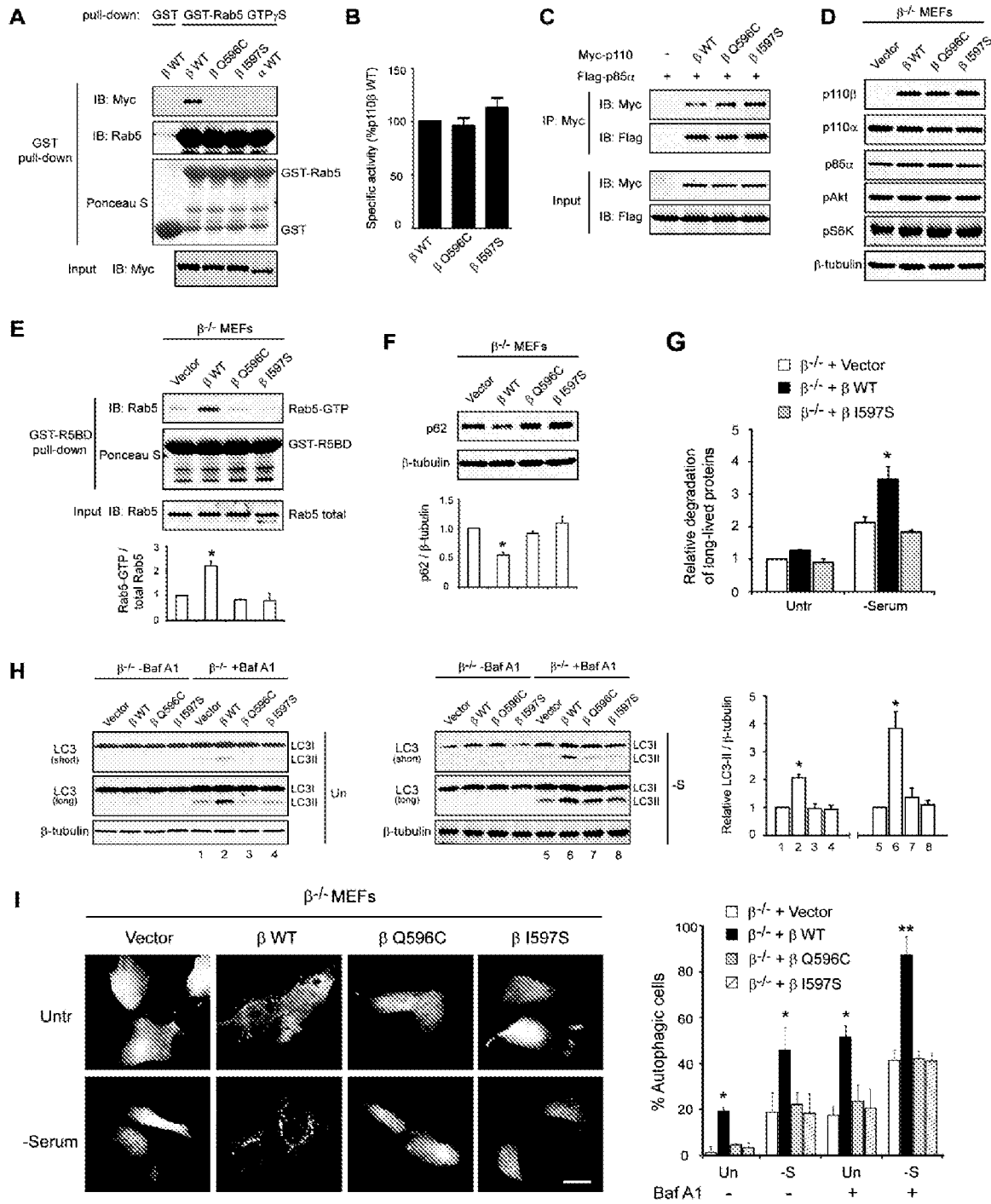


Figure 4

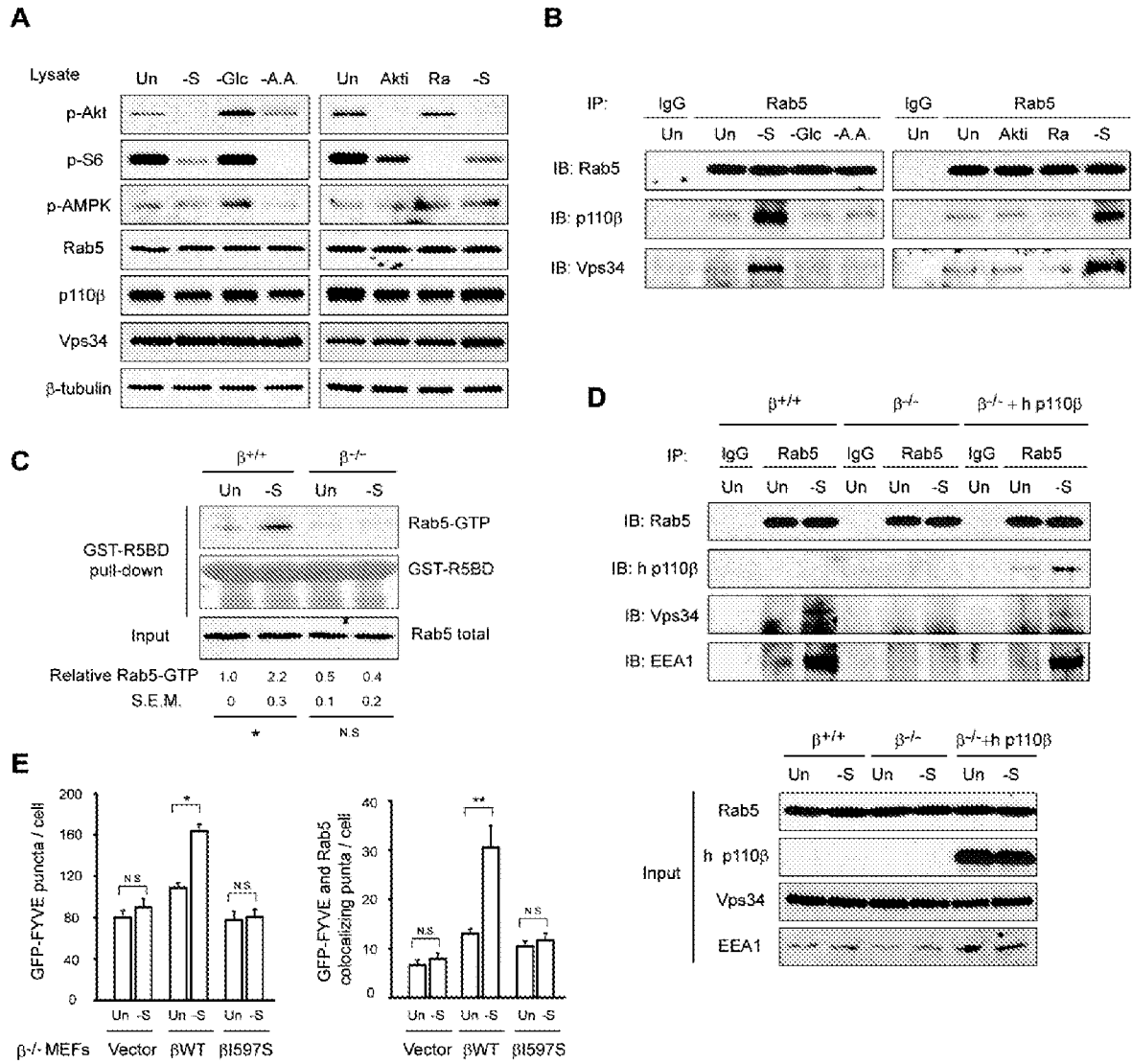


Figure 5

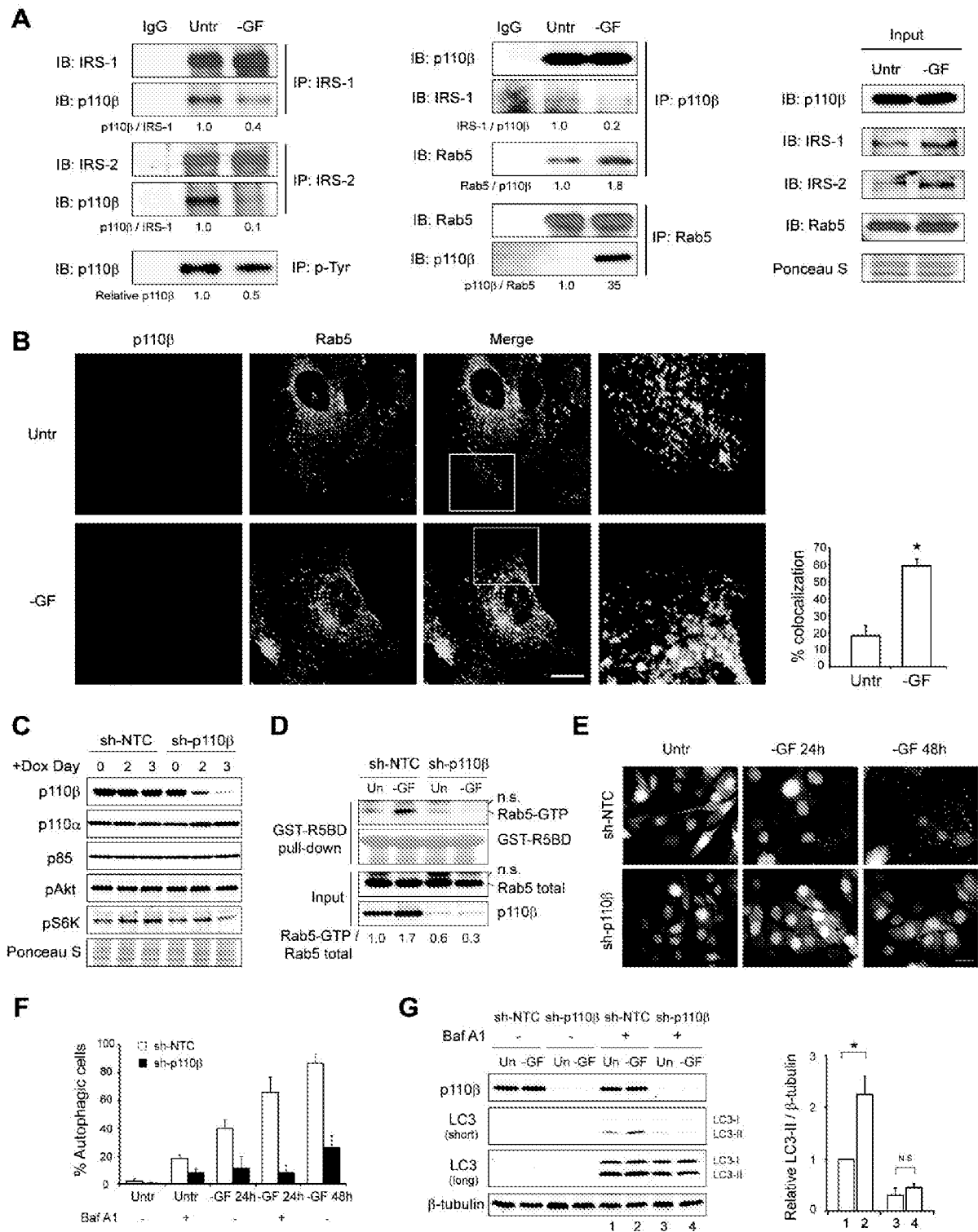


Figure 6

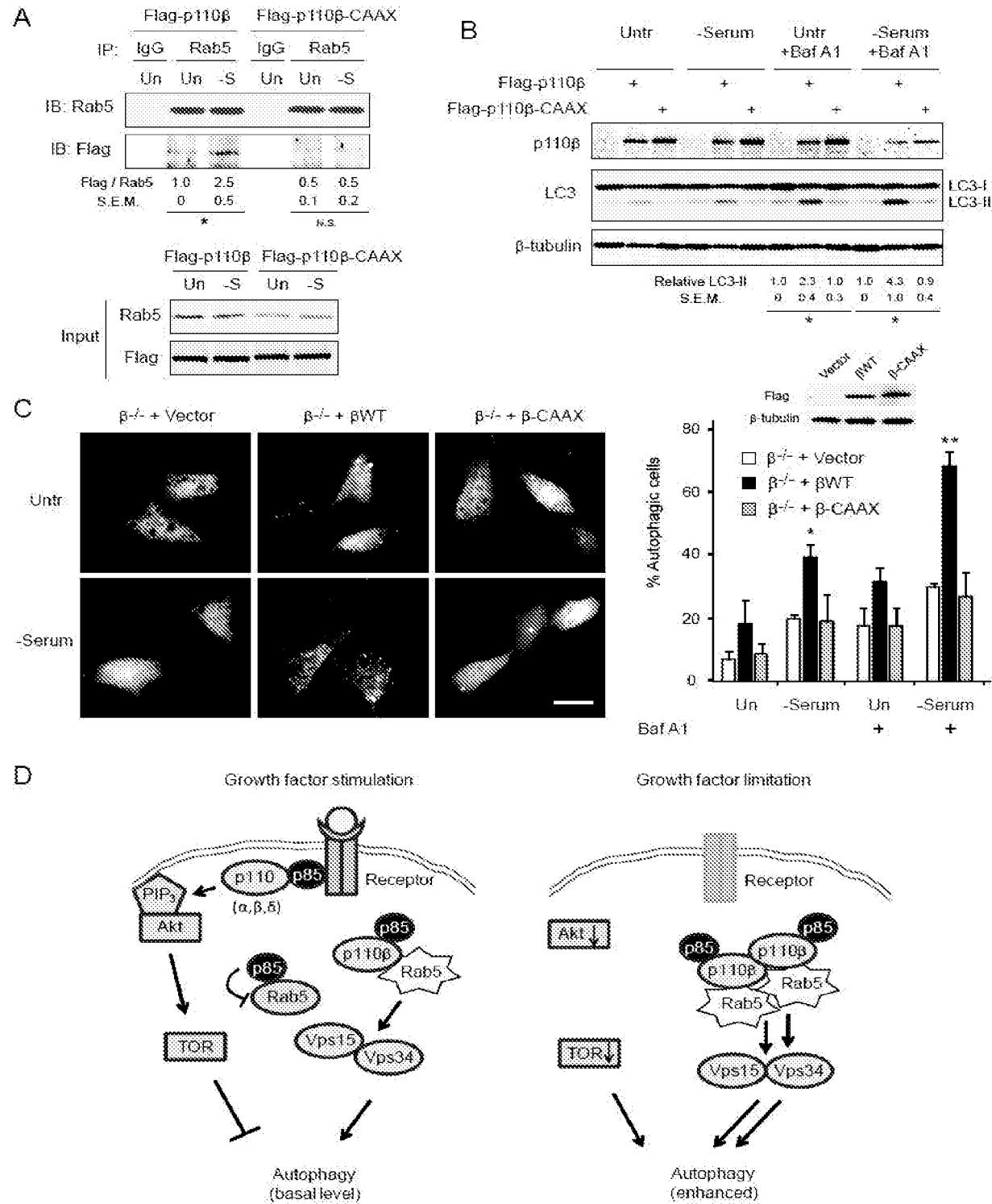


Figure 7

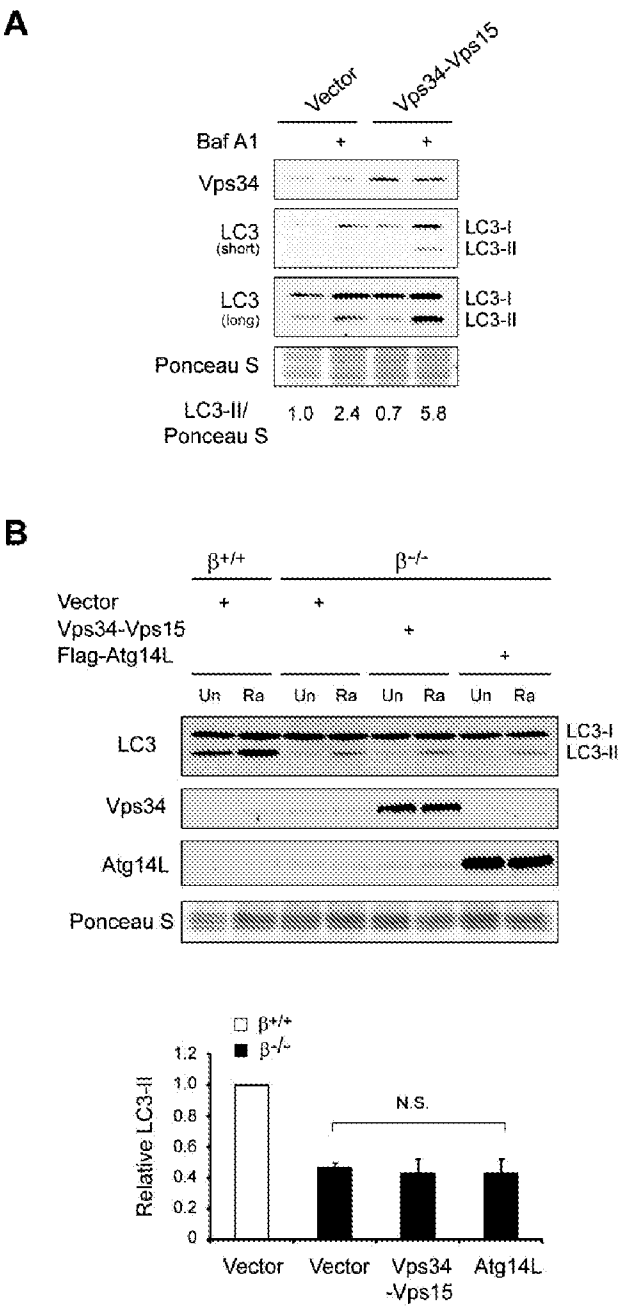


Figure 8

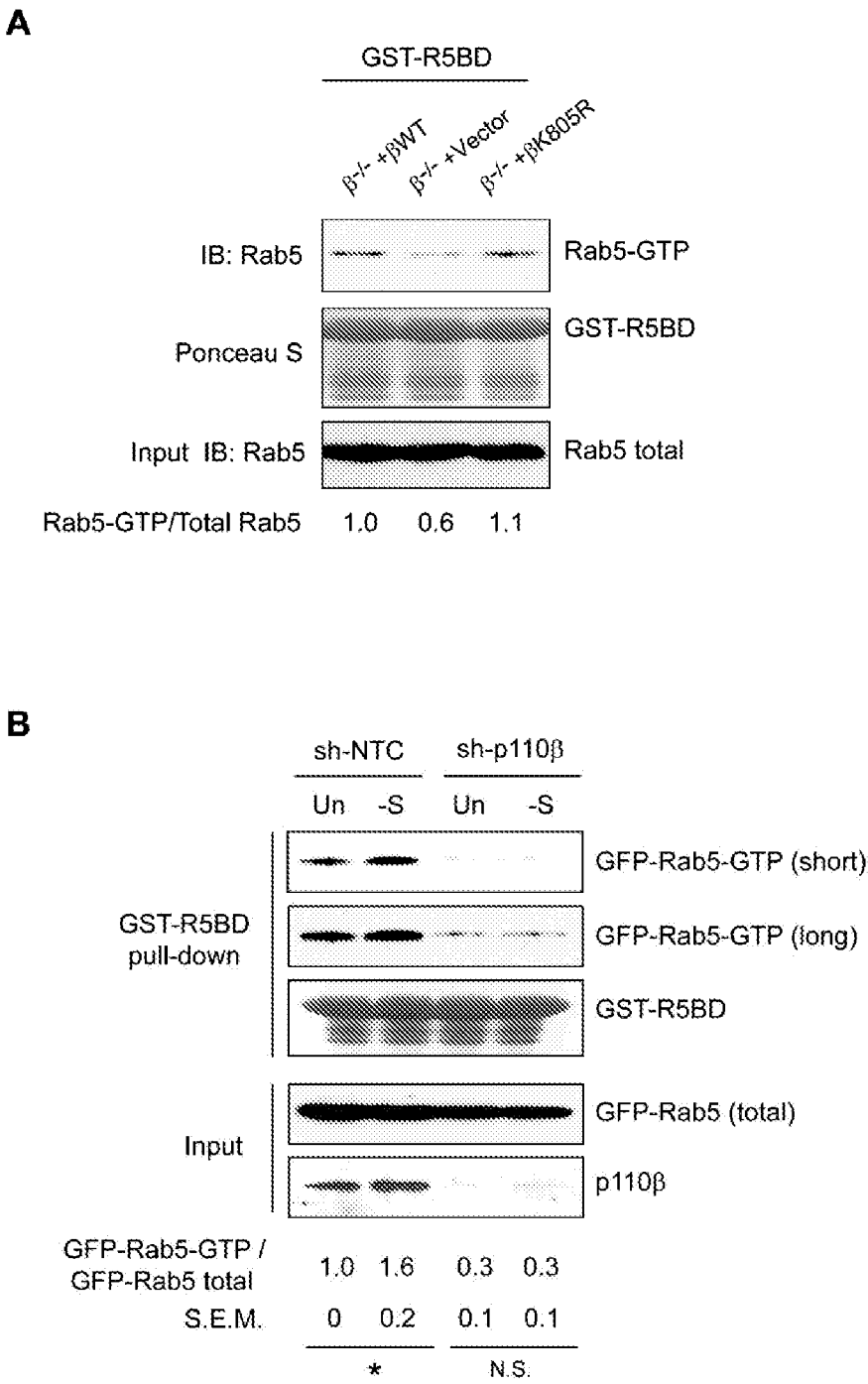


Figure 9

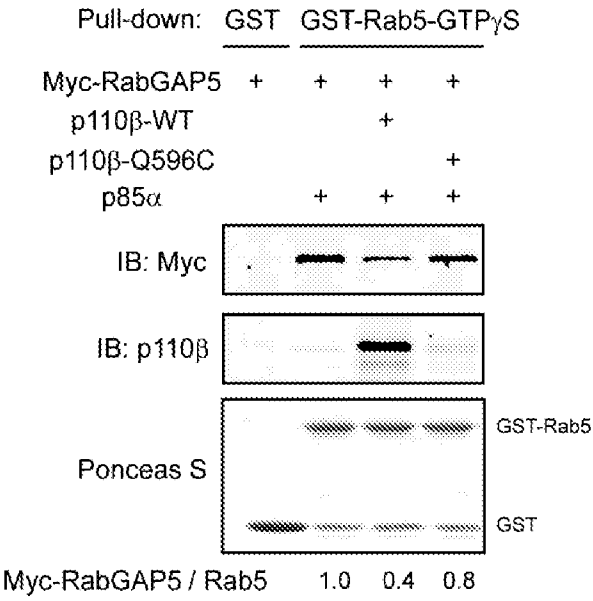


Figure 10

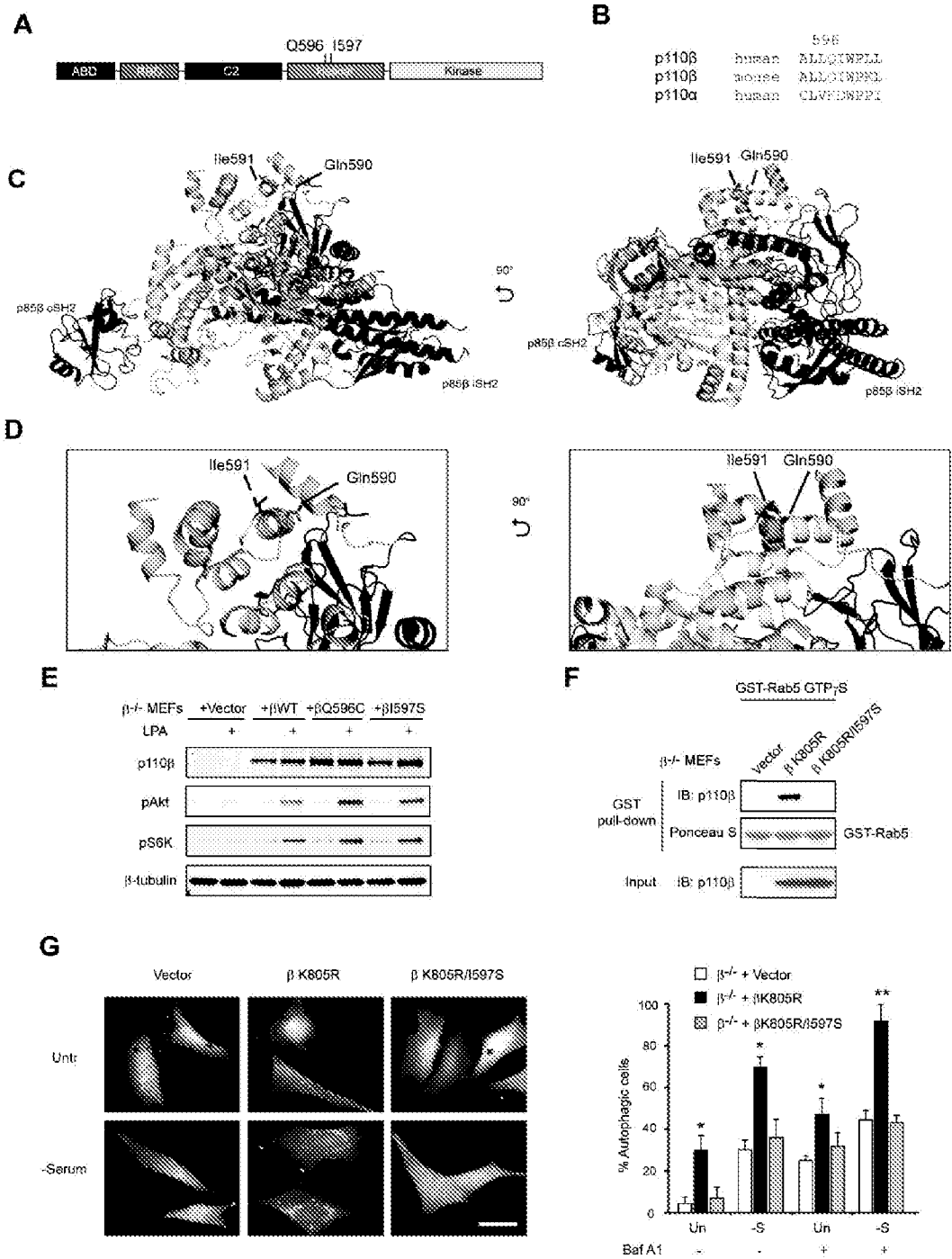


Figure 11

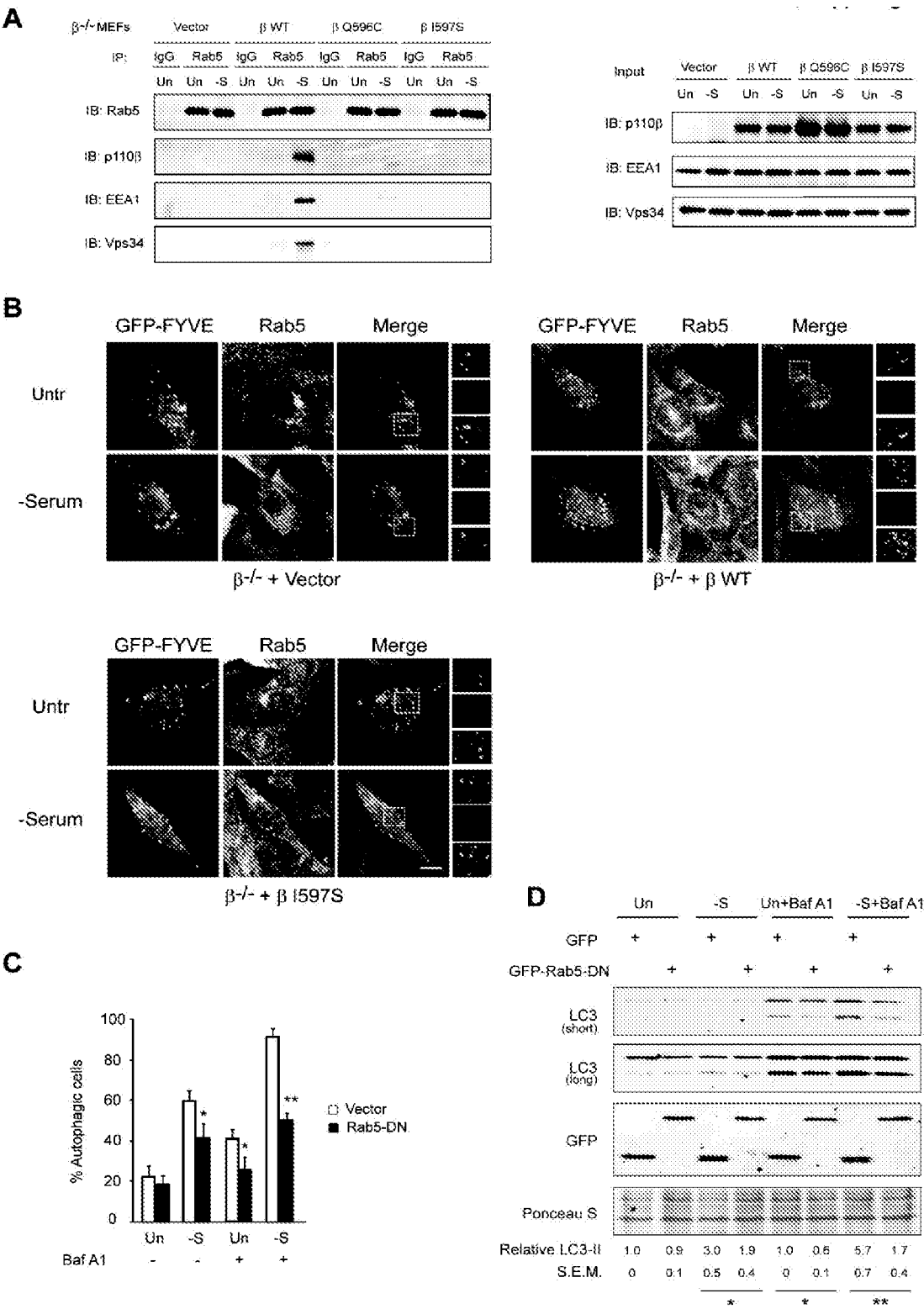


Figure 12

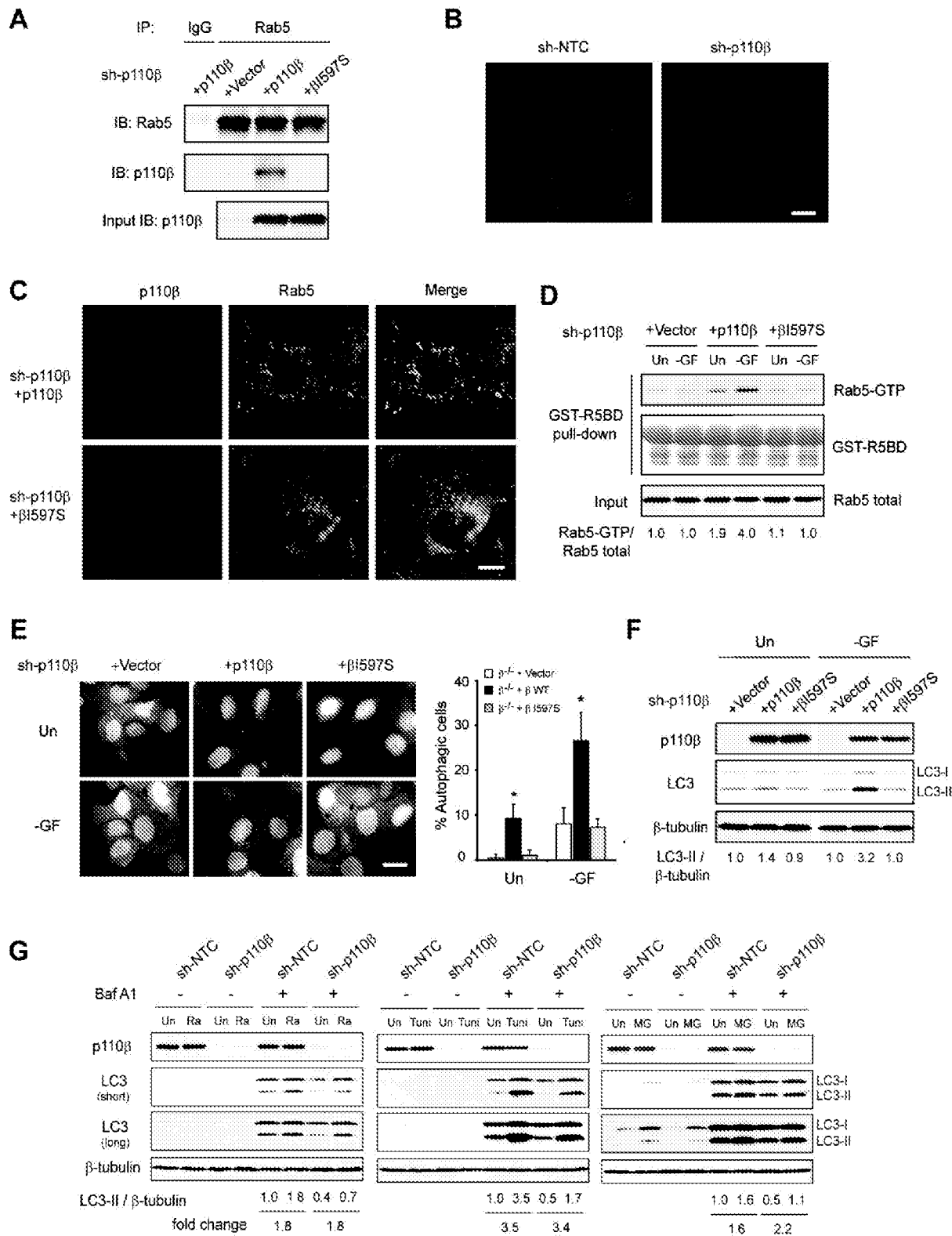


Figure 13

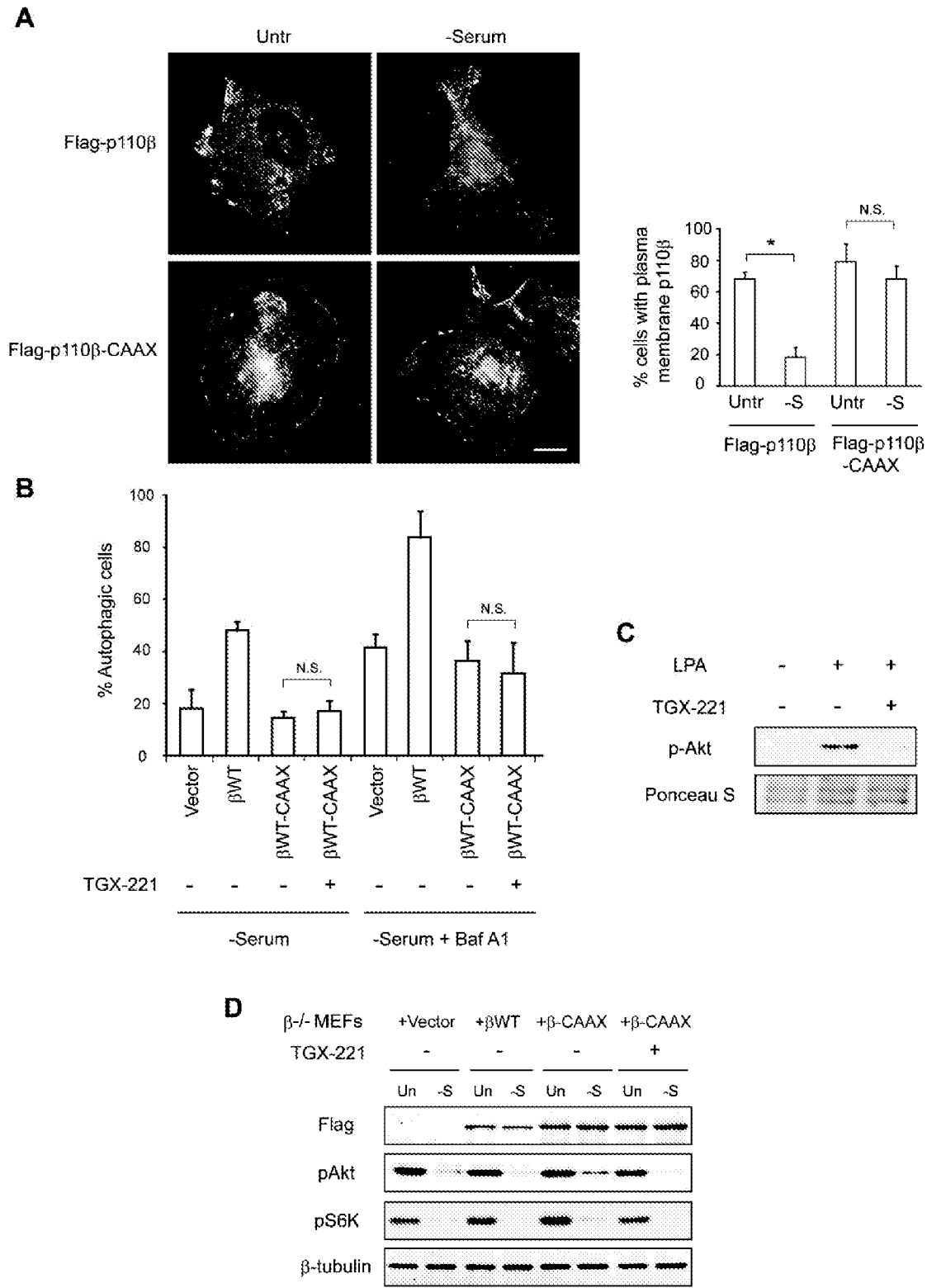


Figure 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2014/016759

A. CLASSIFICATION OF SUBJECT MATTER		<i>C12N 9/12 (2006.01)</i> <i>C07K 7/04 (2006.01)</i> <i>A61K 38/04 (2006.01)</i> <i>A61K 38/43 (2006.01)</i>	
According to International Patent Classification (IPC) or to both national classification and IPC			
B. FIELDS SEARCHED			
Minimum documentation searched (classification system followed by classification symbols)			
C12N 9/12, C07K 7/04, A61K 38/04, 38/43			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)			
CIPO, DEPATISnet, DWPI, EAPATIS, EMBL, EPO-Internal, Espace, Espacenet, Google, KIPRIS, PAJ, PabMed, RUPTO, ScienceDirect, SIPO, USPTO, Patentscope, VINITI			
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category*	Citation of document, with indication, where appropriate, of the relevant passages		Relevant to claim No.
X Y	DATABASE: GenBank: Acc. no AFL58920.1, 06.06.2012		1-2, 4 3, 6-8, 18
X Y	CHRISTOFORIDIS S. et al. Phosphatidylinositol-3-OH kinases are Rab5 effectors. NATURE CELL BIOLOGY, 1999, Vol. 1, pp.249-252, especially p. 250		5 3, 6-8
X Y	US 2008/0145865 A1 (GUANGPU LI) 19.06.2008, paragraphs [0008], [0024], [0029], [0121], fig.12, 17, paragraph [0008]		9-11, 15-17 12-14, 18
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.			
* Special categories of cited documents:		"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family	
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"O"	document referring to an oral disclosure, use, exhibition or other means		
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Date of the actual completion of the international search		Date of mailing of the international search report	
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INTERNATIONAL SEARCH REPORT

International application No.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAVIKUMAR B. et al. Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. Journal of Cell Science, 2008, vol.121 no.10, pp.1649-1660, especially pp. 1651-1652	12-14
Y	WO 2007/115578 A1 (GEORGE-AUGUST-UNIVERSITAT GOTTINGEN STIFTUNG OFFENTLICHEN RECHTS et al) 18.10.2007, pp.8-9	3, 8
A	VIEIRA O.V. et al. Modulation of Rab5 and Rab7 Recruitment to Phagosomes by Phosphatidylinositol 3-Kinase. Molecular and Cellular Biology, 2003, Vol.23, no.7, pp.2501-2514	1-18
A	WO 2005/005475 A2 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V. et al.) 20.01.2005, abstract	1-18