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PI4KIIα phosphorylation by GSK3 directs vesicular trafficking to lysosomes

James W. Robinson*, Iryna Leshchyns'ka†, Hovik Farghaian*, William E. Hughes‡, Vladimir Sytnyk†, Graham G. Neely§[1] and Adam R. Cole*[1]

*Neurosignalling and Mood Disorders Group, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia
†School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW 2052, Australia
‡Diabetes and Obesity Group, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia
§Functional Genomics Research Group, Garvan Institute of Medical Research, Sydney, NSW 2010, Australia

Glycogen synthase kinase 3 (GSK3) is essential for normal development and function of the central nervous system. It is especially important for regulating neurotransmission, although the downstream substrates mediating this function are not yet clear. In the present paper, we report the lipid kinase phosphatidylinositol 4-kinase II α (PI4KIIα) is a novel substrate of GSK3 that regulates trafficking and cell-surface expression of neurotransmitter receptors in neurons. GSK3 phosphorylates two distinct sites in the N-terminus of PI4KIIα (Ser^{5} and Ser^{47}), promoting binding to the adaptor protein 3 (AP-3) complex for trafficking to the lysosome to be degraded. Blocking phosphorylation reduces trafficking to the lysosome, stabilizing PI4KIIα and its cargo proteins for redistribution throughout the cell. Importantly, a reduction in PI4KIIα expression or phosphorylation increases α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression at the surface of hippocampal neurons. These studies implicate signalling between GSK3 and PI4KIIα as a novel regulator of vesicular trafficking and neurotransmission in the brain.

Key words: adaptin, adaptor protein 3 (AP-3) complex, glycogen synthase kinase 3 (GSK3), lysosome, phosphatidylinositol 4-kinase II α (PI4KIIα), phosphorylation, trafficking.

INTRODUCTION

Glycogen synthase kinase 3 (GSK3) is a serine/threonine protein kinase that is critical for normal development and function of the central nervous system. It regulates a variety of brain functions, including neurotransmission, neurite outgrowth, growth cone dynamics, cytoskeletal dynamics, synaptic plasticity, gene transcription, apoptosis and neurogenesis (for reviews, see [1–6]). Hyperactivation of GSK3 is implicated in psychiatric disorders, particularly bipolar disorder and schizophrenia [7,8]. Accordingly, it is a key target of lithium and other psychoactive drugs currently used in the clinic [9–13]. Lithium is also being trialled as a treatment for Alzheimer's disease, age-related cognitive decline, stroke rehabilitation, multiple sclerosis and various types of cancers ([14]; http://ClinicalTrials.gov).

GSK3 is a potent regulator of transmission across synapses. In a pharmacological screen of kinase inhibitors, it was found to be the only kinase among 58 serine/threonine kinases that was required for induction of *N*-methyl-D-aspartate (NMDA)-induced long-term depression (LTD) in hippocampal neurons [15]. Induction of LTD increases GSK3 activity, whereas long-term potentiation (LTP) reduces it [16,17]. At the pre-synapse, high GSK3 activity reduces glutamate release, antagonizing LTP [17–19]. At the post-synapse, GSK3 regulates cell-surface expression of various neurotransmitter receptors, including NMDA receptors [20,21], α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [22], calcium channels [23] and serotonin receptors [24,25]. However, targets of GSK3 mediating these functions are not yet clear.

So far, over 70 substrates of GSK3 have been reported in the literature [1,2,26] with various levels of confidence/evidence [26,27]. These include a wide range of proteins with diverse functions, including transcription factors, cytoskeletal proteins, signalling and metabolic proteins. GSK3 is highly active under basal conditions; however, most (if not all) of its substrates require prior 'priming' phosphorylation by another kinase four residues C-terminal to the GSK3 target site before they can be efficiently phosphorylated by GSK3 [28]. GSK3 is the only kinase in the genome that requires this 'priming' phospho-serine/threonine residue at the +4 position in order to phosphorylate its substrates. Therefore, we used its unique phosphorylation consensus sequence to predict and experimentally validate a novel GSK3 substrate associated with vesicular trafficking and neurotransmission in the brain.

EXPERIMENTAL

Materials

The cDNA encoding full-length mouse PI4KIIα (UniProt code Q2TBE6) was amplified by PCR from Image clone 5346917 using the primers 5′-GAATTCGCCACCATGGACGAGACGAGCCC-3′ and 5′-GGCGAATTCCTAGGATCCCCACCATGAAAAGAAGGGC-3′. Truncation mutants were generated using 5′-GAATTCGCCACCATGGGAGCTCACTTTCCGCAAGTACC-3′ (ΔG24) and 5′-GAATTCGCCACCATGGGCCACGACCGGGAGCG-3′ (ΔG52). PCR products were sub-cloned into pRK5 [cytomegalovirus (CMV) promoter] with a 3′ FLAG tag for mammalian expression or into pEGFP with a 3′ GFP tag. All mutant forms of phosphatidylinositol 4-kinase IIα (PI4KIIα) were synthesized

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP-3, adaptor protein 3; Baf, bafilomycin A; Cdk5, cyclin-dependent kinase 5; CRMP2, collapsin response mediator protein 2; DIV, day(s) *in vitro*; DMEM, Dulbecco's modified Eagle's medium; GluA1, glutamate receptor A1; GSK3, glycogen synthase kinase 3; HEK, human embryonic kidney; Lamp-1, lysosome-associated membrane protein 1; LTD, long-term depression; LTP, long-term potentiation; NMDA, *N*-methyl-D-aspartate; PI, phosphatidylinositol; PI4KIIα, phosphatidylinositol 4-kinase II α; PIP4K, phosphatidylinositol 5-phosphate 4-kinase; Tfn, transferrin.

[1] Correspondence may be addressed to either of these authors (email g.neely@garvan.org.au or a.cole@garvan.org.au).

by DNA2.0 and GeneArt (Life Technologies). Constructs were verified by DNA sequencing. The shRNA sequence targeted to mouse and rat PI4KIIα (5′-GATTTGATTCTTCCAAAGA-3′) was cloned into pSUPER vector.

Antibodies

Phospho-specific antibodies were generated by injection of rabbits with the following peptides: pSer^{5}-CMDETpSPLVS, pSer^{9}-CSPLVpSPERA, pSer^{47}-AGSGPpSPPC, pSer^{51}-SPPCpSPGHD, non-pSer^{5}-CMDETSPLVS and non-pSer^{47}-AGSGPSPPC (where pS is phosphoserine). Peptides were conjugated to keyhole limpet haemocyanin. Antisera were affinity-purified on a phosphopeptide antigen–agarose column. Immunoblotting and immunofluorescence analyses using purified phospho-specific antibodies were routinely performed in the presence of 1 μM non-phosphopeptides to reduce non-specific binding to non-phosphorylated PI4KIIα (vice versa for non-phospho-specific antibodies).

Cell culture and immunofluorescence microscopy

Human embryonic kidney (HEK)-293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium, supplemented with 10 % heat-inactivated FBS, 2 mM GlutaMAX, penicillin and streptomycin. Transfections were performed using DharmaFECT 1 (Dharmacon). Cells were fixed in 4 % paraformaldehyde, permeabilized (0.5 % Triton X-100), blocked (2 % BSA) and incubated with primary antibodies for 2 h at room temperature. Image acquisition was performed on a Zeiss Axiocam mRm microscope using a $\times$63 objective.

Primary cortical neuron cultures from embryonic day 17 (E17) Sprague–Dawley rats were generated as previously described [29]. Neurons were co-transfected with PI4KIIα and GFP constructs at 1 day *in vitro* (DIV) using calcium phosphate precipitation and harvested at 3 DIV. Primary hippocampal neurons from P2 C57BL6 mice were transfected before plating by electroporation using the Neon transfection system (Life Technologies) and maintained in Neurobasal medium containing 2 % B27 serum replacement (Life Technologies) and 2 mM GlutaMAX. Surface expression of glutamate receptor A1 (GluA1) at 12 DIV was detected using a rabbit polyclonal GluA1 antibody (Alomone Labs), as previously described [30]. Images were acquired using a confocal laser-scanning microscope C1si, NIS Elements software and oil Plan Apo VC $\times$60 objective (numerical aperture 1.4; all from Nikon). All image analyses were performed using ImageJ software and statistical analyses were performed using paired Student's *t*-test, with results considered significant when $P < 0.05$. All animal care and experiments were approved by the Garvan/St Vincent's and UNSW Animal Care and Ethics Committees.

Western blotting and co-immunoprecipitation

Cells were lysed in buffer containing 1 % (v/v) Triton X-100, 50 mM Tris/HCl, pH 7.5, 0.27 M sucrose, 1 mM EDTA, 0.1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.1 % 2-mercaptoethanol and Complete™ protease inhibitor tablets (Roche Applied Science) (4 °C). Lysates were subjected to Western blotting and visualized using a Li-COR Odyssey infrared imaging system. Densitometry was performed using Odyssey application software (version 3). Statistical analyses were performed using paired Student's *t*-test and results were considered significant when $P < 0.05$. PI4KIIα was immunoprecipitated from 3 mg of transfected HEK-293 cell lysate using 10 μl of anti-FLAG-agarose beads, washed three times in lysis buffer, resuspended in SDS loading buffer and subjected to Western blotting.

Single-round endocytosis and recycling of transferrin

HeLa cells were incubated in serum-free DMEM to deplete endogenous transferrin (Tfn; 3 h, 37 °C), followed by ice-cold, serum-free DMEM, 1.0 % (w/v) BSA containing 5 μg/ml Tfn conjugated with Alexa Fluor® 488 (Invitrogen) to allow ligand binding to the receptor (4 °C, 45 min). Extracellular Tfn was removed by washing in ice-cold serum-free DMEM; then cells were incubated in pre-warmed serum-free DMEM at 37 °C for the indicated times. Cells were fixed and localization of Tfn was manually scored under blinded conditions ($n = 150$ cells from randomly chosen fields; three independent experiments). Statistical analyses were performed using two-way ANOVA and results were considered significant when $P < 0.05$.

RESULTS

Mapping and characterization of phosphosites in PI4KIIα

PI4KIIα is a lipid kinase that phosphorylates the 4′ position of phosphatidylinositol (PI) lipids. It performs this function at intracellular vesicles, regulating trafficking between the Golgi, endosomes and lysosomes [31–33]. It contains two conserved GSK3 phosphorylation consensus sequences in its N-terminal region (Figure 1A), whereby Ser^{5} and Ser^{47} are the putative GSK3 target sites, whereas Ser^{9} and Ser^{51} are their respective priming sites. To determine which is targeted by GSK3, we subjected full-length and truncated forms of PI4KIIα lacking the N-terminal 24 amino acids (removing the Ser^{5}–Ser^{9} site but not the Ser^{47}–Ser^{51} site) to an assay we previously developed for identifying another novel GSK3 substrate [29]. Briefly, candidates were expressed in HEK-293 cells in the presence or absence of the highly-specific GSK3 inhibitor CT99021 [34], immunoprecipitated and subjected to *in vitro* kinase assays with recombinant GSK3β and radiolabelled ATP. In the present study, recombinant GSK3 should be able to incorporate more radiolabelled phosphate into candidates isolated from inhibitor-treated cells compared with untreated cells, since the GSK3 target sites should be vacant due to inhibition of endogenous GSK3. If so, this indicates the candidate is a good substrate for GSK3 *in vitro* and in cells. Significantly more radiolabelled phosphate was incorporated into full-length PI4KIIα isolated from inhibitor-treated (Figures 1B and 1C; lane 2) than non-treated cells (lane 1), indicating it is a *bona fide* substrate of GSK3. This was blocked in the truncated form of PI4KIIα (lane 3), indicating the majority of phosphorylation occurred at the Ser^{5}–Ser^{9} site, not the Ser^{47}–Ser^{51} site.

Phospho-specific antibodies were generated to all four putative phosphosites (Ser^{5}, Ser^{9}, Ser^{47} and Ser^{51}) and their specificity was validated by Western blotting of wild-type and phospho-mutant forms of PI4KIIα (Figure 1D). Ser^{5} was confirmed as a GSK3 target site, since phosphorylation was reduced by treatment with CT99021 (Figure 1D), lithium (Figure 1E) and mutation of the priming site Ser^{9} to non-phosphorylatable alanine (Figure 1D). Phosphorylation of Ser^{47} was more complicated, since it was inhibited by mutation of Ser^{51} (therefore priming-dependent), but was not affected by CT99021 (Figures 1D and 1F) or lithium (Figure 1E). A possible explanation is that pSer^{47} is relatively resistant to dephosphorylation by phosphatases, as found for other GSK3 substrates {β-adducin [29] and collapsin response mediator protein 2 (CRMP2) [35]}. To test this, a dose–response assay using increasing concentrations of CT99021 on

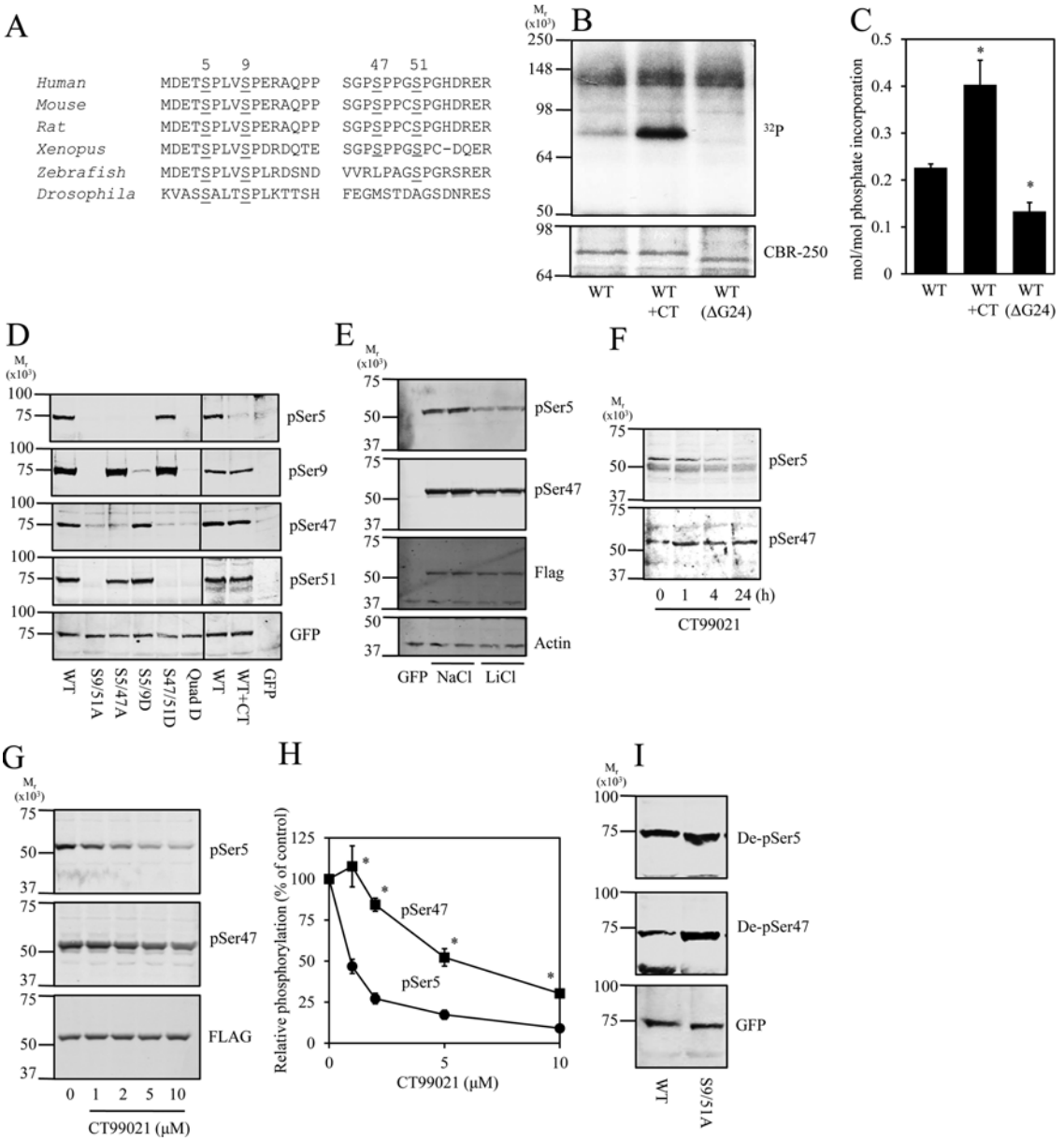

Figure 1 Characterization of GSK3 phosphosites in PI4KIIα

(**A**) Sequence alignment of putative GSK3 target sites in the N-terminal region of PI4KIIα from various species. GSK3 sites and the priming sites at the + 4 position are numbered and underlined. (**B**) PI4KIIα was expressed in HEK-293 cells that were untreated (lane 1), treated with CT99021 (lane 2) or expressed a truncated form of PI4KIIα lacking the N-terminal 24 amino acids (ΔG24; lane 3). Full-length and truncated PI4KIIα was immunoprecipitated via its C-terminal GFP-tag and subjected to an *in vitro* kinase assay with recombinant GSK3 and radiolabelled ATP. (**C**) The mol/mol incorporation of phosphate is shown as a histogram [means ± S.E.M.; $^*P < 0.05$, Student's *t* test, $n = 3$]. (**D**) HEK-293 cells were transfected with PI4KIIα wild-type and phosphosite mutants, immunoprecipitated and subjected to Western blot analysis using the indicated phosphospecific antibodies, as well as an antibody recognizing the C-terminal GFP-tag as a loading control. Also, wild-type PI4KIIα was expressed with/without CT99021 (2 μM). (**E**) HEK-293 cells were transfected with GFP or PI4KIIα wild-type, then treated with 20 mM NaCl (control) or 20 mM LiCl for 4 h. Lysates were subjected to Western blotting using the anti-pSer^5, anti-pSer^{47}, anti-FLAG and anti-actin antibodies. (**F**) Primary rat cortical neurons were treated with 2 μM CT99021 for 0, 1, 4 or 24 h and subjected to Western blotting. Endogenous PI4KIIα was detected using the anti-pSer^5 and anti-pSer^{47} antibodies. (**G**) HEK-293 cells transfected with wild-type PI4KIIα were treated with various concentrations of CT99021 for 4 h. Lysates were subjected to Western blotting using anti-pSer^5, anti-pSer^{47} and anti-FLAG antibodies. (**H**) The relative amount of dephosphorylation induced by CT99021 treatment in (**G**) is presented as a graph (means ± S.E.M.; $^*P < 0.05$, Student's *t* test, $n = 3$). (**I**) HEK-293 cells transfected with PI4KIIα wild-type or S9/51A were subjected to Western blotting using antibodies that recognize Ser^5 and Ser^{47} when non-phosphorylated, as well as an antibody against the C-terminal GFP tag as a loading control. WT, wild-type.

PI4KIIα-transfected HEK-293 cells was performed to compare the relative rates of dephosphorylation at the Ser^5 and Ser^{47} sites. Low concentrations of CT99021 were sufficient to reduce phosphorylation of Ser^5, but not Ser^{47} (Figures 1G and 1H), indicating that Ser^{47} is relatively resistant to dephosphorylation. It would therefore be expected that, under basal conditions, Ser^{47} has a higher stoichiometry of phosphorylation than Ser^5. Antibodies were generated that specifically recognize Ser^5 and Ser^{47} when non-phosphorylated. Mutation of Ser^9 and Ser^{51} priming sites completely blocks phosphorylation of Ser^5 and Ser^{47} respectively (Figure 1D), but causes a much larger increase in staining using the non-pSer^{47} antibody compared with non-pSer^5 (Figure 1I). This indicates the stoichiometry of phosphorylation is higher for Ser^{47} compared with Ser^5 due to its relative resistance to phosphatases.

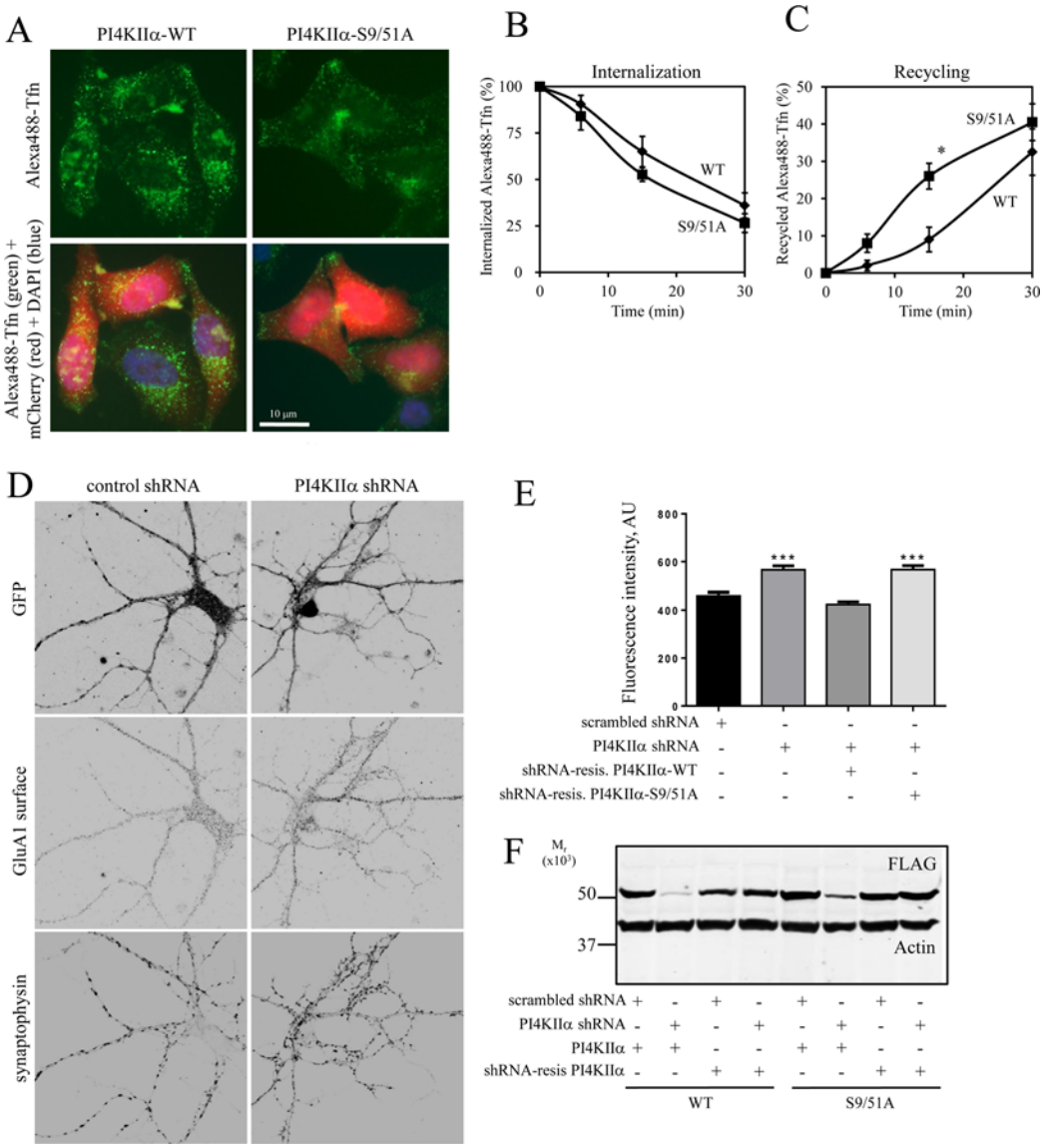

Figure 2 Phosphorylation of PI4KIIα by GSK3 regulates trafficking in cells

(**A**) HeLa cells transfected with wild-type or S9/51A PI4KIIα were pulse-labelled with fluorescent Tfn, then incubated in Tfn-free medium for 0, 6, 15 or 30 min. Cells were analysed using fluorescence microscopy for Tfn (green), transfected cells (mCherry, red) and cell nuclei (blue). Images are representative of the 15 min time-point. (**B**) Relative rates of Tfn internalization were scored and results are presented as a graph (blinded, means ± S.E.M. for three independent experiments, $n = 150$ cells). (**C**) Relative rates of Tfn recycling were scored and results are presented as a graph ($^*P < 0.05$, two-way ANOVA). (**D**) Phosphorylation of PI4KIIα by GSK3 regulates GluA1 trafficking in neurons. Primary mouse hippocampal neurons (12 DIV) co-transfected with scrambled or PI4KIIα shRNA constructs, as well as shRNA-resistant wild-type and mutant PI4KIIα as labelled, were fixed and incubated with antibodies to GluA1 and synaptophysin under non-permeabilizing conditions to label surface-exposed proteins. (**E**) Quantification of GluA1 surface expression from (**D**) is presented as a histogram (means ± S.E.M.; $^{***}P < 0.005$, Student's *t* test). Au, arbitrary units. (**F**) Confirmation of effective knockdown by shRNA. Wild-type and S9/51A forms of PI4KIIα, with or without synonymous mutations rendering them resistant to shRNA-mediated knockdown, were co-transfected into HEK-293 cells with scrambled or PI4KIIα shRNA. Lysates were subjected to Western blotting for FLAG and actin. WT, wild-type.

In summary, GSK3 phosphorylates two sites in the N-terminal region of PI4KIIα (Ser[5] and Ser[47]), which is dependent upon prior priming phosphorylation at Ser[9] and Ser[51] respectively.

Phosphorylation of PI4KIIα by GSK3 regulates vesicular trafficking

To determine whether PI4KIIα phosphorylation by GSK3 regulates vesicular trafficking, HeLa cells were transfected with wild-type or a non-phosphorylatable mutant form of PI4KIIα (S9/51A), then trafficking of fluorescently-labelled Tfn through cells was monitored using fluorescence microscopy (a well-established model of vesicular trafficking; Figure 2A). The rate of Tfn internalization was similar to cells transfected with wild-type and PI4KIIα-S9/51A (Figure 2B); however, recycling back to the cell surface was significantly increased by the presence of PI4KIIα-S9/51A compared with wild-type (Figure 2C). Thus, phosphorylation of PI4KIIα by GSK3 slows recycling of Tfn back to the surface of HeLa cells.

To determine whether PI4KIIα phosphorylation also regulates trafficking in neurons, we measured cell-surface expression levels of the AMPA receptor subunit GluA1 in transfected primary mouse hippocampal neurons. Endogenous PI4KIIα was knocked down using a shRNA construct and the surface expression of GluA1 was determined by immunofluorescence microscopy

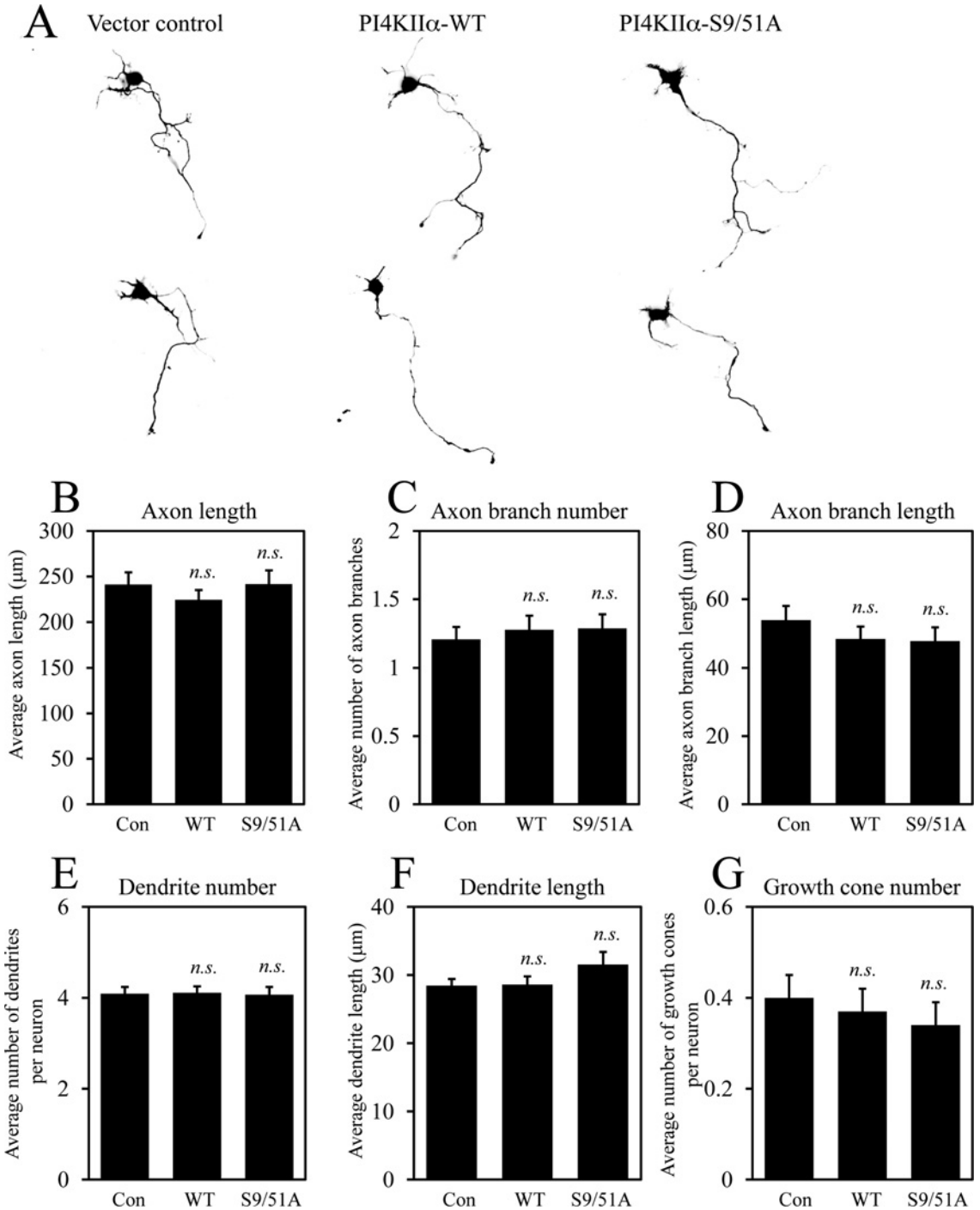

Figure 3 PI4KIIα does not affect neurite outgrowth in cortical neurons

(**A**) Cortical neurons were co-transfected with GFP and either empty vector, PI4KIIα wild-type or PI4KIIα-S9/51A. Neurons were fixed and visualized using fluorescence microscopy. The morphology of transfected neurons was quantified using ImageJ software and is presented as histograms: (**B**) average axon length, (**C**) number of axon branches, (**D**) axon branch length, (**E**) dendrite number, (**F**) dendrite length and (**G**) growth cone number (GFP control $n = 161$, PI4KIIα wild-type $n = 141$, PI4KIIα-S9/51A $n = 111$; means ± S.E.M.; *n.s.*, not significant, Student's *t* test).

using an antibody recognizing the extracellular region of GluA1 under non-permeabilizing conditions [30]. PI4KIIα knockdown significantly increased GluA1 at the surface of hippocampal neurons compared with controls (Figures 2D and 2E). This was rescued back to control levels by co-expression of a shRNA-resistant form of wild-type PI4KIIα, but not the PI4KIIα-S9/51A mutant. Validation of the knockdown and shRNA-resistant constructs is shown in Figure 2(F). These data indicate that GluA1 is a cargo protein of PI4KIIα that is normally directed away from the cell surface by PI4KIIα. Phosphorylation by GSK3 is required for this activity, since the non-phosphorylated form (S9/51A) was unable to restore normal surface expression of GluA1. These observations are consistent with the Tfn trafficking assays in HeLa cells (Figures 2A–2C), in that phosphorylation by GSK3 promotes trafficking of PI4KIIα and its cargo proteins away from the cell surface. In separate experiments, expression

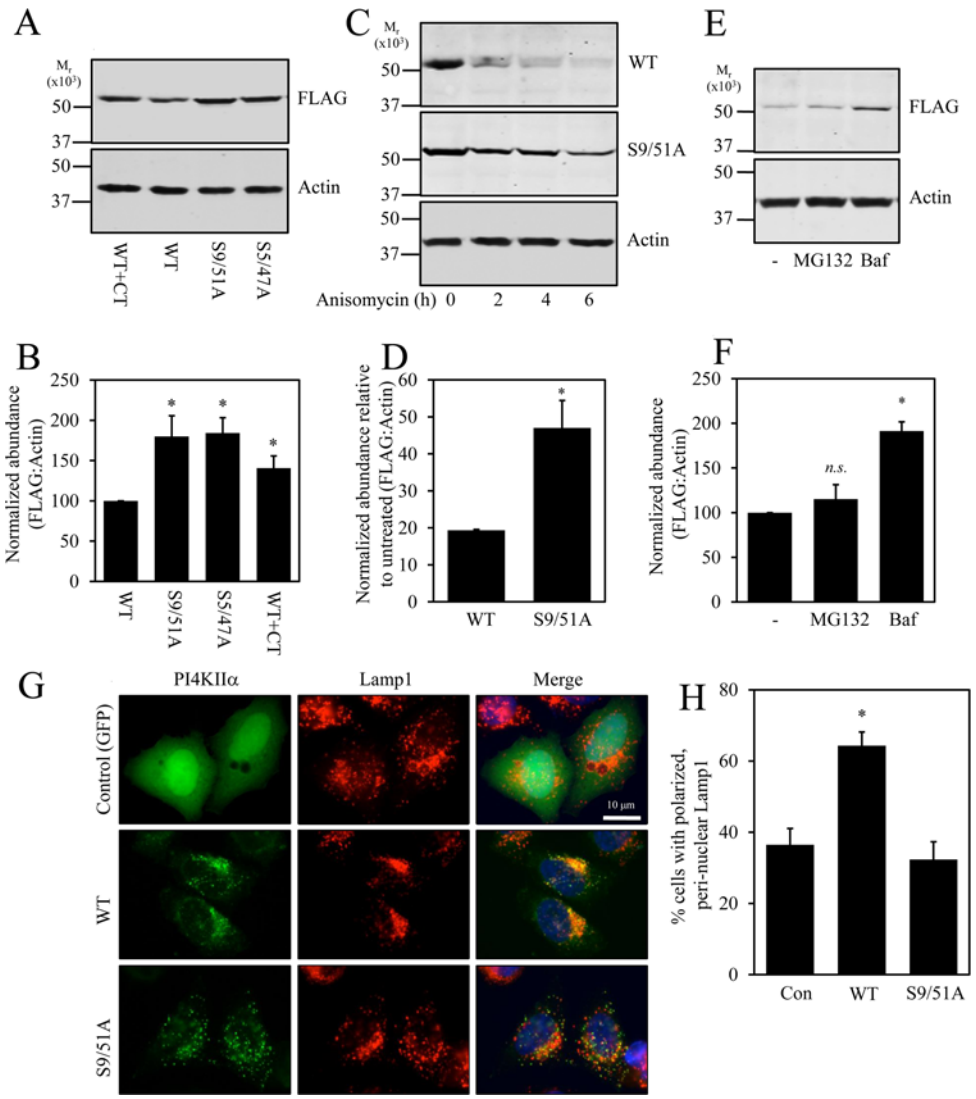

Figure 4 Phosphorylation regulates the abundance of PI4KIIα

(**A**) Lysates from HEK-293 cells transfected with wild-type or phosphomutant PI4KIIα, untreated or treated with the GSK3 inhibitor CT99021 (2 μM, 16 h), were subjected to Western blotting for the C-terminal FLAG tag and actin as a loading control. (**B**) Relative abundance of PI4KIIα was quantified as the ratio between FLAG and actin and is presented as a histogram. (**C**) HEK-293 cells transfected with PI4KIIα wild-type or S9/51A were treated with 10 μg/ml anisomycin for the times indicated. Lysates were subjected to Western blotting for the C-terminal FLAG tag and actin as a loading control. (**D**) The ratio of FLAG to actin after 2 h of anisomycin treatment is presented as a histogram. (**E**) HEK-293 cells transfected with wild-type PI4KIIα were treated with 10 μM MG132 or 50 nM Baf for 2 h. Lysates were subjected to Western blotting for FLAG and actin. (**F**) The ratio of FLAG to actin after treatment with MG132 and Baf is presented as a histogram. (**G**) HeLa cells were transfected with GFP (control), wild-type or S9/51A PI4KIIα (FLAG-tagged, green). Lysosomes were visualized using an antibody to Lamp1 (red), whereas nuclei were visualized using DAPI (blue). (**H**) Localization of Lamp-1 in a focused perinuclear position, polarized to one side of the cell was scored and results are presented as a histogram ($n = 150$ cells, means ± S.E.M., $^*P < 0.05$, *t*-test).

of wild-type and S9/51A forms of PI4KIIα had no affect on the morphology of cultured cortical neurons (Figure 3).

Phosphorylation of PI4KIIα by GSK3 promotes trafficking to the lysosome by the AP-3 complex

We next investigated the molecular mechanisms by which GSK3 regulates PI4KIIα function. Increased abundance levels were consistently observed for non-phosphorylated forms of PI4KIIα compared with wild-type protein (Figures 4A and 4B), suggesting that GSK3 regulates PI4KIIα stability. Indeed, treatment with the translation inhibitor anisomycin demonstrated that wild-type PI4KIIα is more rapidly degraded than PI4KIIα-S9/51A (Figures 4C and 4D). Degradation is mediated by the lysosome and not the proteasome, since PI4KIIα abundance is increased by the lysosome inhibitor bafilomycin A (Baf), but not the proteasome inhibitor MG132 (Figures 4E and 4F). Consistent with this, wild-type and PI4KIIα-S9/51A clustered with lysosome-associated membrane protein 1 (Lamp-1) staining of lysosomes in HeLa cells (Figure 4G). Interestingly, wild-type PI4KIIα and Lamp-1 were more tightly clustered in the perinuclear region compared with PI4KIIα-S9/51A, which was more diffusely spread throughout the cell (Figure 4H). Together, these observations show that phosphorylation by GSK3 promotes degradation of PI4KIIα by lysosomes.

We next investigated binding partners of PI4KIIα that could mediate its trafficking to lysosomes. Wild-type and S9/51A forms of PI4KIIα were expressed in HEK-293 cells, immunoprecipitated and subjected to Western blotting using antibodies against

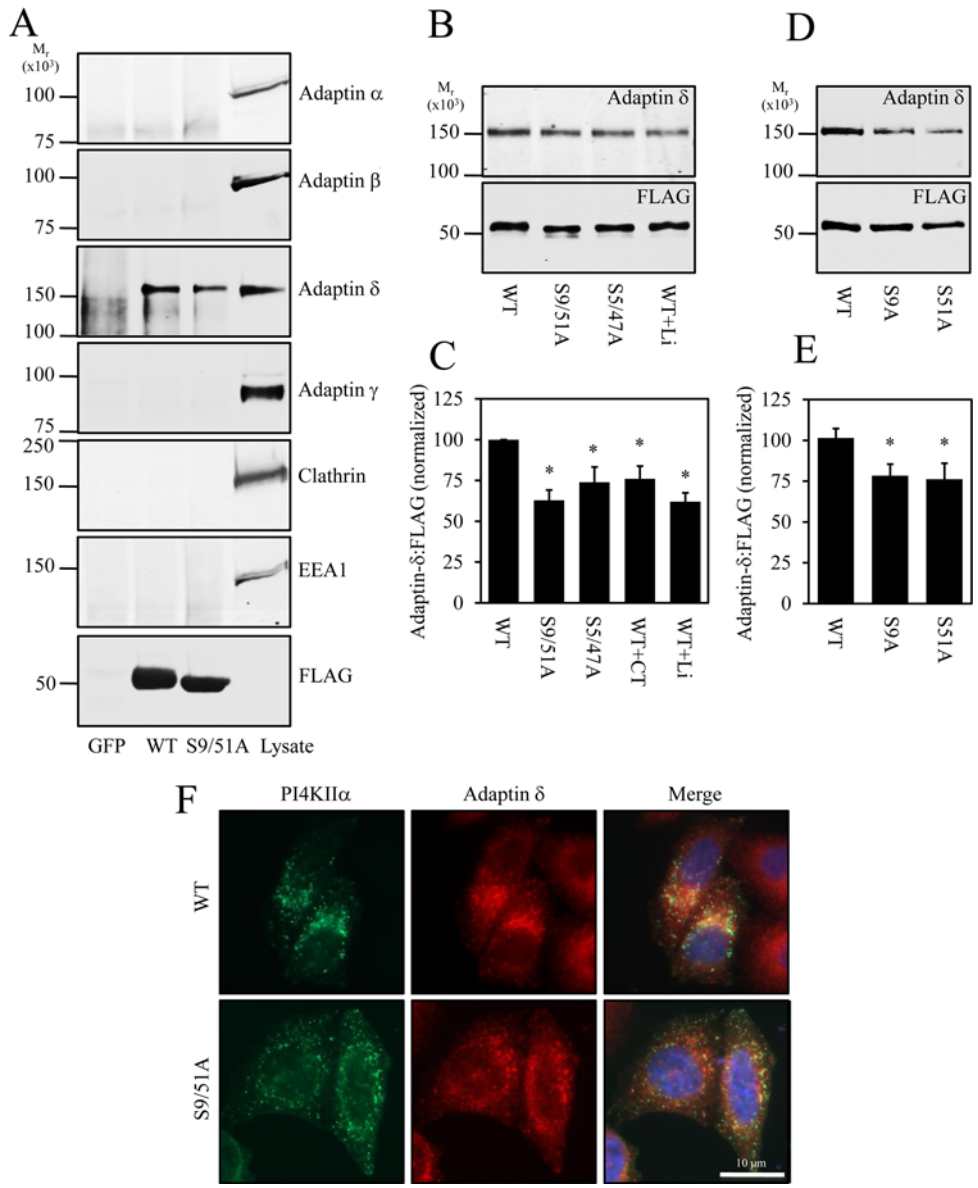

Figure 5 Phosphorylation of regulates binding to adaptin δ

(**A**) HEK-293 cells transfected with PI4KIIα wild-type or S9/51A were immunoprecipitated via their C-terminal FLAG-tag and subjected to Western blotting for various trafficking proteins, as well as FLAG as a loading control. (**B**) Western blots of immunoprecipitates of PI4KIIα wild-type, phosphomutants and GSK3 inhibitor-treated wild-type PI4KIIα for adaptin δ and FLAG. (**C**) Relative amounts of adaptin δ binding to PI4KIIα is shown as a histogram (means ± S.E.M.; $*P < 0.05$, Student's t test; $n = 6$). (**D**) Western blots of immunoprecipitates of PI4KIIα wild-type and phosphomutants for adaptin δ and FLAG. (**E**) Relative amounts of adaptin δ binding to PI4KIIα is shown as a histogram (means ± S.E.M.; $*P < 0.05$, Student's t test). (**F**) HeLa cells were transfected with wild-type or S9/51A PI4KIIα (FLAG-tagged, green) and stained for adaptin δ (red) and nuclei using DAPI (blue).

various trafficking proteins. PI4KIIα bound to adaptin δ of the AP-3 complex, but not adaptins α, β (AP-2 complex), γ (AP-1 complex), clathrin or early endosome antigen 1 (EEA1) (Figure 5A). Binding of adaptin δ was decreased for PI4KIIα-S9/51A compared with wild-type and by treatments with GSK3 inhibitors (Figures 5B and 5C). Mutation of each site individually also reduced the interaction with adaptin δ (i.e. S9A and S51A; Figures 5D and 5E). PI4KIIα co-localized with endogenous adaptin δ in HeLa cells (Figure 5F), with the wild-type form more tightly clustered with adaptin δ in the perinuclear region than PI4KIIα-S9/51A, similar to Lamp-1 (Figure 4G).

We speculated that GSK3 may decrease the stability of PI4KIIα (Figure 4) by promoting binding to the adaptin δ/AP-3 complex (Figure 5) for trafficking to the lysosome to be degraded (Figures 4E and 4F). To test this, mutants of PI4KIIα were generated that could potentially block its interaction with adaptin δ (L60/61A [36] and P16/Y18A [37]). The L60/61A mutant, but not the P16/Y18A mutant, successfully blocked binding to adaptin δ in co-immunoprecipitation assays (Figure 6A). This was accompanied by a dramatic increase in PI4KIIα abundance levels (Figure 6B). Inhibition of lysosomes using bafilomycin increased the abundance of wild-type PI4KIIα, but not the L60/61A or S9/51A mutants (Figures 6C and 6D). Meanwhile, reducing phosphorylation of PI4KIIα using the GSK3 inhibitor CT99021 increased the abundance of wild-type PI4KIIα, but not L60/61A or S9/51A mutants (Figures 6E and 6F). Together, these observations support the hypothesis that phosphorylation by GSK3 promotes

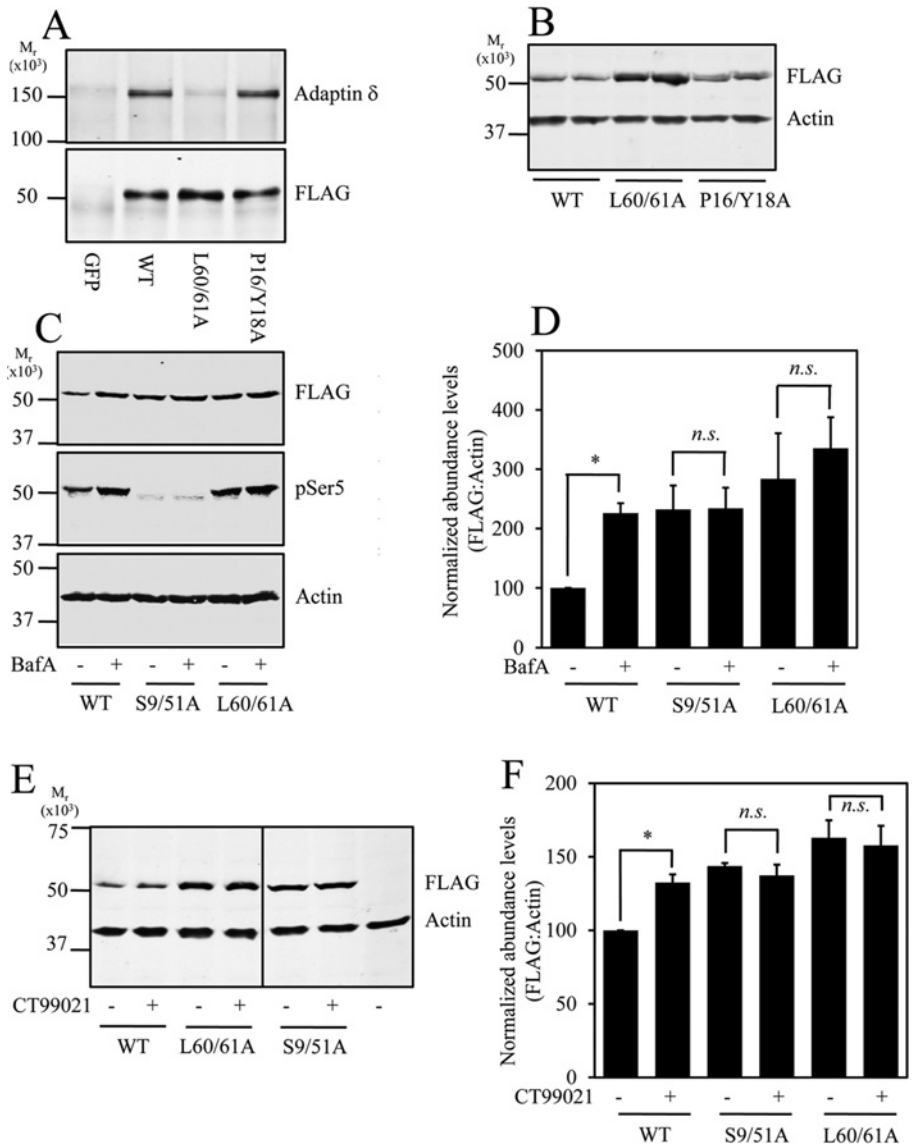

Figure 6 Phosphorylation promotes PI4KIIα binding to adaptin δ and trafficking to the lysosome for degradation

(**A**) HEK-293 cells transfected with GFP control, PI4KIIα wild-type, L60/61A or P16/Y18A mutants were immunoprecipitated via their C-terminal FLAG-tags and subjected to Western blotting for adaptin δ and FLAG. (**B**) HEK-293 cell lysates transfected with PI4KIIα wild-type, L60/61A or P16/Y18A were subjected to Western blotting for FLAG and actin. (**C**) HEK-293 cell lysates transfected with PI4KIIα wild-type, S9/51A or L60/61A and treated without/with Baf were subjected to Western blotting for FLAG. (**D**) The ratio of FLAG to actin after bafilomycin treatment in (**C**) is presented as a histogram (means ± S.E.M.; $*P < 0.05$, Student's *t* test; n.s., not significant). (**E**) HEK-293 cells transfected with PI4KIIα wild-type, L60/61A or S9/51A and treated without/with CT99021 were subjected to Western blotting for FLAG and actin. (**F**) The ratio of FLAG to actin after CT99021 treatment in (**E**) is presented as a histogram (means ± S.E.M.; $*P < 0.05$, Student's *t* test; n.s., not significant).

PI4KIIα binding to adaptin δ/AP-3 for trafficking to the lysosome to be degraded. However, the influence of other unknown binding partners contributing to this process cannot be ruled out.

The N-terminal region regulates binding of the dileucine motif to the AP-3 complex

The GSK3 phosphosites are located adjacent to the Leu^{60}-Leu^{61} dileucine motif on the flexible N-terminal domain of PI4KIIα (Figure 7A). We postulated that the N-terminal region might be able to fold back on itself to restrict access of the AP-3 complex to the dileucine motif. To test this, PI4KIIα truncation mutants were generated lacking the N-terminal 24 (ΔG24) or 52 (ΔG52) amino acids and access to the dileucine motif was measured by relative binding to adaptin δ in co-immunoprecipitation experiments. Figures 7(B) and 7(C) show that binding of the truncation mutants to adaptin δ is dramatically increased compared with full-length PI4KIIα. This is accompanied by decreased abundance levels of PI4KIIα. This supports the idea that the N-terminal region blocks the interaction of the dileucine motif with the AP-3 complex for trafficking to the lysosome.

Analysis of the amino acid sequences surrounding the dileucine motif and Ser^5–Ser^9 phosphosites revealed a series of complementary charged and hydrophobic residues that could constitute an interaction site between these regions (Figure 7D). To test this, four residues were mutated to alanine to reduce this interaction, exposing the dileucine motif for binding to the AP-3 complex (Quad A; Figure 7E). Co-immunoprecipitation experiments revealed increased binding of adaptin δ to PI4KIIα-Quad

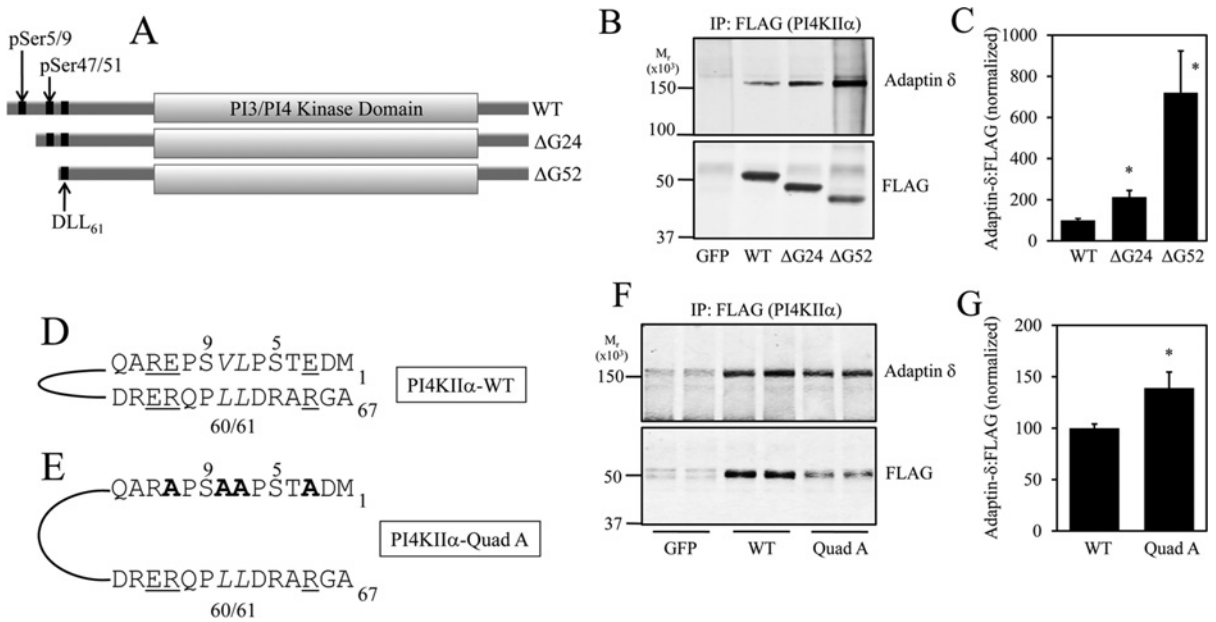

Figure 7 The N-terminal region of PI4KIIα restricts access of the AP-3 complex to the dileucine motif

(**A**) Schematic representation of the domain structure of PI4KIIα, showing the position of the phosphorylation sites, dileucine motif and truncation sites at the N-terminus. (**B**) HEK-293 cells transfected with GFP control, PI4KIIα full-length, ΔG24 or ΔG52 truncation mutants were immunoprecipitated via their C-terminal FLAG-tags and subjected to Western blotting for adaptin δ and FLAG. (**C**) Relative amounts of adaptin δ binding to PI4KIIα full-length and truncation mutants is shown as a histogram (means ± S.E.M.; $*P < 0.05$, Student's *t* test). (**D**) Amino acid sequences surrounding the dileucine motif and Ser^5–Ser^9 phosphosites (numbered) at the N-terminus of PI4KIIα. Charged (underlined) and hydrophobic (italics) residues predicted to form an interaction site are shown. (**E**) Same as (**D**), except the four residues mutated to alanine in PI4KIIα-Quad A to disrupt the potential interaction site are shown in bold. (**F**) HEK-293 cells transfected with GFP control, PI4KIIα wild-type or Quad A mutant were immunoprecipitated via their C-terminal FLAG-tags and subjected to Western blotting for adaptin δ and FLAG. (**G**) Relative amounts of adaptin δ binding to PI4KIIα wild-type and QuadA mutant is shown as a histogram (means ± S.E.M.; $*P < 0.05$, Student's *t* test). IP, immunoprecipitation; WT, wild-type.

A compared with wild-type, together with decreased abundance levels of the mutant form (Figures 7F and 7G). These observations identify a novel binding site between the N-terminus and dileucine regions that restricts access of the AP-3 complex to the dileucine motif. All together, we propose a model whereby the N-terminal region restricts access to the dileucine motif, but phosphorylation by GSK3 exposes this site for binding to AP-3 and subsequent trafficking to the lysosome to be degraded (Figure 8).

DISCUSSION

GSK3 is essential for normal brain development and function and deregulation of GSK3 can lead to psychiatric or neurodegenerative illnesses. It is an important regulator of neurotransmission at both the pre- and post-synapse, although the downstream substrates meditating this are not yet clear. The only trafficking proteins previously reported to be phosphorylated by GSK3 are dynamin-1 [38,39] and phosphatidylinositol 5-phosphate 4-kinase β (PIP4Kβ) [40], although CRMP2 is a cytoskeletal protein that has also been linked to endocytosis [41–45]. In the present study, we identified the trafficking protein PI4KIIα as a novel GSK3 substrate, strengthening the case that GSK3 may be an important regulator of vesicular trafficking in cells. It is interesting to note that two of these substrates are lipid kinases (PI4KIIα and PIP4Kβ). Indeed their PI lipid products [e.g. PtdIns4*P*, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3] are known to be important for endocytosis and trafficking. Therefore it will be interesting to determine whether GSK3 directly regulates PI lipid levels at plasma or intracellular membranes.

PI4KIIα is highly expressed in the brain and synapses, where it accounts for the majority of 4′ PI phosphorylation activity [33]. It localizes to the *trans*-Golgi network (TGN)/endosomal membranes [31,32] via palmitoylation [46,47] and has been shown to bind to the AP-3 complex [48]. GSK3 phosphorylates two sites in the N-terminal region of PI4KIIα (Ser^5 and Ser^{47}), following obligatory priming phosphorylation at Ser^9 and Ser^{51}. The identity of the priming kinase(s) is not yet known, although both sites fit the consensus sequences of cyclin-dependent kinase 5 (Cdk5) and dual-specificity tyrosine-regulated kinase (DYRK) (i.e. proline-directed sites with a basic residue at P + 3). Both of these kinases are known to prime other GSK3 substrates (e.g. CRMPs [49]) and Cdk5 primed for GSK3 phosphorylation at Ser^5 and Ser^{47} *in vitro* (results not shown), although this remains to be proven *in vivo*. The Ser^{47}/Ser^{51} phosphosites appeared later in evolution than the Ser^5/Ser^9 sites (Figure 1A). These sites are relatively resistant to phosphatases (Figures 1D–1H) and are highly phosphorylated in cells (Figure 1I), suggesting they may not be regulatable. Therefore, it is likely that phosphorylation of Ser$^{5/9}$ is the primary mechanism for dynamically regulating PI4KIIα function, whereas constitutive phosphorylation of Ser^{47}/Ser^{51} evolved later to enhance this, although its precise function is not yet clear.

Phosphorylation of PI4KIIα promotes binding to the AP-3 complex for trafficking to the lysosome to be degraded. Since Leu^{60}-Leu^{61} in the primary sequence of PI4KIIα is permanent/non-modifiable, it is likely that the nearby phosphosites evolved to dynamically regulate the interaction of PI4KIIα with the AP-3 complex. Interestingly, conserved adaptin-binding sequences are located adjacent to predicted GSK3 phosphosites in other GSK3 substrates (PIP4Kβ, CRMP2), suggesting that phosphorylation by GSK3 could be a common

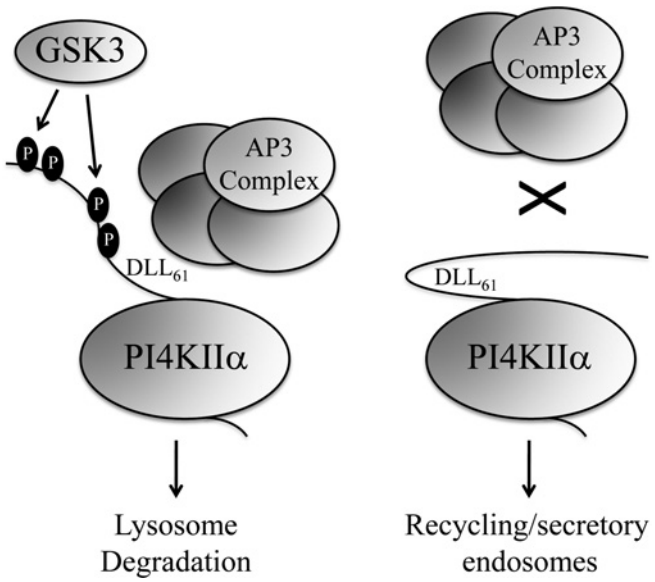

Figure 8 Model of GSK3-mediated regulation of PI4KIIα binding to AP-3 and trafficking to the lysosome

Phosphorylation of PI4KIIα by GSK3 induces structural rearrangement of its N-terminal region, exposing the dileucine (DLL) motif for binding to the AP-3 complex and subsequent trafficking to the lysosome to be degraded. In its non-phosphorylated form, the N-terminal region blocks access to the DLL motif, reducing binding of PI4KIIα to the AP-3 complex and trafficking to the lysosome. Instead, PI4KIIα abundance increases and is trafficked to recycling and secretory endosomes.

mechanism for regulating the function of several trafficking proteins. Elsewhere, it has been suggested that ubiquitination of PI4KIIα promoted by a PPxY motif in its N-terminal region directs it towards the degradative endosomal pathway [37]. However, when we mutated this site, we did not observe any effect on its binding to the AP-3 complex or any change in its abundance (Figure 6), implying that the dileucine motif at Leu^{60}-Leu^{61} is the predominant motif on PI4KIIα for regulating its abundance.

In addition to regulating itself, PI4KIIα regulates trafficking of several cargo-proteins, including Tfn (Figure 2A), GluA1 (Figure 2), epidermal growth factor receptor (EGFR) [50], lysosome membrane protein 2 (LIMP2) and glucocerebrosidase (GBA) [51]. Since PI4KIIα acts as an integral membrane protein due to its palmitoylation [46,47], it probably links the AP-3 complex to lipid bilayer vesicles containing these and other cargo proteins. It is possible that its lipid kinase activity is important for trafficking, since PtdIns4*P* production has been implicated in recruitment of GGAs and the AP-1 complex to the Golgi, promoting Golgi-to-endosomal transport [51,52]. GSK3-mediated phosphorylation may indirectly regulate PtdIns4*P* production and trafficking by controlling the abundance of PI4KIIα. Interestingly, a population of GSK3 located at the Golgi was previously shown to promote cargo transport from the Golgi to pre-lysosomal compartments [53]. Therefore, PI4KIIα may be a major target of GSK3 for maintaining a transport route from Golgi/endosomes to lysosomes for degradation of cargo proteins.

High surface expression of AMPA receptors at synapses promotes LTP, whereas its removal promotes LTD [54–56]. In the present study, PI4KIIα knockdown and the S9/51A mutant form of PI4KIIα increased surface expression of GluA1 in hippocampal neurons, favouring LTP. Therefore, it would be expected that inhibition of GSK3 would stabilize GluA1 levels and increase its surface expression, promoting LTP. Indeed, low GSK3 activity promotes LTP, whereas high GSK3 activity promotes LTD [15–17]. Surprisingly, inhibition of GSK3 was previously shown to reduce surface expression of AMPA [22] and NMDA [20] receptors on cortical neurons. This discrepancy may reflect differences in synaptic transmission between hippocampal and cortical neurons. Alternatively, it may reflect difficulties in interpreting results from global inhibition of a multi-functional kinase, like GSK3. Inhibition of particular pools of GSK3 in different subcellular compartments (especially in neurons) or inhibiting phosphorylation of individual substrates, as performed in the present study, may be a more precise approach for delineating the role of GSK3 and its substrates in neurotransmission.

In summary, we have discovered a novel substrate of GSK3 that regulates cell-surface expression of the AMPA receptor GluA1 and is therefore likely to affect neurotransmission in the brain. Future studies will confirm its functional effect on synaptic transmission in the brain, as well as investigate a potential role for PI4KIIα in mood and neurodegenerative disorders in which GSK3 activity is dysregulated.

AUTHOR CONTRIBUTION

James Robinson, Graham Neely and Adam Cole conceived and designed the project, wrote the paper and assisted with experiments. James Robinson and Hovik Farghaian performed most of the experiments. Iryna Leshchyns'ka and Vladmir Sytnyk performed the experiments shown in Figures 2(D)–2(F). William Hughes helped with the microscopy experiments shown in Figures 2, 4 and 5.

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