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# Phosphorylation of Amyloid Precursor Protein at Threonine 668 Is Essential for Its Copper-responsive Trafficking in SH-SY5Y Neuroblastoma Cells\*

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**Background:** The function, localization, and processing of the amyloid precursor protein (APP) is regulated by phosphorylation.

**Results:** Copper promotes APP trafficking by phosphorylation at threonine 668 in SH-SY5Y cells.

**Conclusion:** By promoting APP phosphorylation, copper regulates its intracellular localization.

**Significance:** Understanding the role copper plays in regulating APP function in normal neuronal cells will provide insight into the interplay between copper and APP in normal and pathological conditions.

Amyloid precursor protein (APP) undergoes post-translational modification, including *O*- and *N*-glycosylation, ubiquitination, and phosphorylation as it traffics through the secretory pathway. We have previously reported that copper promotes a change in the cellular localization of APP. We now report that copper increases the phosphorylation of endogenous APP at threonine 668 (Thr-668) in SH-SY5Y neuronal cells. The level of APPT668-p (detected using a phospho-site-specific antibody) exhibited a copper-dependent increase. Using confocal microscopy imaging we demonstrate that the phospho-deficient mutant, Thr-668 to alanine (T668A), does not exhibit detectable copper-responsive APP trafficking. In contrast, mutating a serine to an alanine at residue 655 does not affect copper-responsive trafficking. We further investigated the importance of the Thr-668 residue in copper-responsive trafficking by treating SH-SY5Y cells with inhibitors for glycogen synthase kinase 3- $\beta$  (GSK3 $\beta$ ) and cyclin-dependent kinases (Cdk), the main kinases that phosphorylate APP at Thr-668 in neurons. Our results show that the GSK3 $\beta$  kinase inhibitors LiCl, SB 216763, and SB 415286 prevent copper-responsive APP trafficking. In contrast, the Cdk inhibitors Purvalanol A and B had no significant effect on copper-responsive trafficking in SH-SY5Y cells. In cultured primary hippocampal neurons, copper promoted APP re-localization to the axon, and this effect was inhibited by the addition of LiCl, indicating that a lithium-sensitive kinase(s) is involved in copper-responsive trafficking in hippocampal neurons. This is consistent with APP axonal transport to the synapse, where APP is involved in a number of functions. We conclude that copper promotes APP trafficking by promoting a GSK3 $\beta$ -dependent phosphorylation in SH-SY5Y cells.

Amyloid precursor protein (APP)<sup>2</sup> is an integral type I transmembrane protein that is synthesized in the endoplasmic reticulum and transported through the Golgi network via the secretory pathway where it undergoes post-translational modifications including *N*- and *O*-glycosylation, ubiquitination, and phosphorylation (1–11). A proportion of APP reaches the plasma membrane where it is rapidly endocytosed and trafficked through endocytic and recycling compartments back to the cell surface or degraded by lysosomes (for review, see Refs. 12 and 13). The trafficking process of APP has been intensively studied as it is closely linked to its processing and the generation of the toxic amyloid  $\beta$  (A $\beta$ ) peptide central to Alzheimer disease pathogenesis. APP can be cleaved by  $\alpha$ ,  $\beta$ , and  $\gamma$ -secretases, which are localized to specific subcellular compartments. For instance, APP is cleaved by the  $\beta$ -secretase BACE1 in acidic compartments (the trans-Golgi, early endosomes) to generate a soluble ectodomain (sAPP $\beta$ ) and a C-terminal fragment ( $\beta$ -CTF). C-terminal fragments can be further processed by the  $\gamma$ -secretase complex, which resides in the endocytic compartment or late endosomes to release the A $\beta$  peptide. Processing by  $\beta$ - and  $\gamma$ -secretases to generate A $\beta$  is referred to as the amyloidogenic processing pathway (for review, see Refs. (12 and 13).  $\alpha$ -Secretase cleavage (non-amyloidogenic) occurs primarily at the plasma membrane (PM). There has been much debate as to the normal function of APP. In neurons APP function has been associated with neurite outgrowth, neuronal migration, and repair via interaction with extracellular matrix proteins (14–16). APP undergoes rapid kinesin-1 dependent anterograde transport and reaches presynaptic terminals (17, 18). At the synapse APP is involved in synapse formation, synaptic transmission, plasticity, and learning and memory (for review, see Ref. 19). Relevant to this study, APP is also involved in copper homeostasis (20, 21). Identifying the cellular signals, which

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<sup>2</sup> The abbreviations used are: APP, amyloid precursor protein; A $\beta$ , amyloid  $\beta$  peptide; PM, plasma membrane; GSK3 $\beta$ , glycogen synthase kinase 3- $\beta$ ; Cdc2, cell division cycle protein 2; Cdk, cyclin-dependent kinase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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mediate APP trafficking, is central to understanding its normal function and processing, including yielding the toxic A $\beta$  peptide of Alzheimer disease.

The subcellular localization of APP is regulated by phosphorylation at a number of sites within the intracellular domain. APP is phosphorylated at eight residues (Tyr-653, Thr-654, Ser-655, Ser-675, Thr-668, Tyr-682, Thr-686, and Tyr-687; APP695 numbering) within the APP intracellular domain. Phosphorylation at these sites has been reported to impact APP processing and cellular localization (for review, see Ref. 22). The phosphorylation of APP at Thr-668 results in a significant conformational change that may affect interactions with binding partners and hence impact its subcellular localization and metabolism (23). Phosphorylation at Thr-668 is a normal process associated with neurite extension, anterograde transport of vesicular cargo, and in signaling to the nucleus (24–28). APP is phosphorylated at Thr-668 *in vitro* and *in vivo* by a number of kinases including glycogen synthase kinase 3 $\beta$ , Jun N-terminal kinase-3 (JNK3), cell division cycle protein (Cdc2), and cyclin-dependent kinase 5 (Cdk5) (7, 29–32). Whether and how phosphorylation at Thr-668 impacts APP processing remains controversial, with studies showing varying results. For instance, one study reported that phosphorylation of APP at Thr-668 increased A $\beta$  production by enhancing  $\beta$ -secretase cleavage (33), whereas a later study showed a decrease in A $\beta$  due to the inhibition of  $\gamma$ -secretase cleavage (34). In contrast, knock-in mice expressing APP with a threonine to alanine substitution showed no change in APP metabolism including brain levels of A $\beta$  (35). A recent study has shown that non-phosphorylated forms (at Thr-668) of C-terminal APP fragments are associated with lipid raft-like microdomains where the  $\gamma$ -secretase complex (amyloidogenic) resides, whereas Thr-668-phosphorylated C-terminal fragments reside predominantly in cytoplasmic fractions (36). Hence phosphorylation regulates the localization of APP and thus affects its processing by  $\gamma$ -secretases (36).

We previously reported that copper promotes the relocation of APP from a predominant Golgi localization to a wider distribution (37) including the PM, which is the predominant site of non-amyloidogenic cleavage by  $\alpha$ -secretase. Copper-responsive APP trafficking was due to both a stimulation of exocytosis and suppression of endocytosis of APP (37). Our earlier studies on the copper transport protein, which is mutated in Menkes disease, ATP7A, demonstrated that copper induces the trafficking of ATP7A via phosphorylation at specific residues in its C terminus (38). This was demonstrated by targeted mutagenesis of phosphorylatable residues. In the current study we investigated whether phosphorylation at Thr-668, a widely studied phosphorylation site, is required for copper-responsive APP trafficking. We investigated this by 1) studying copper-responsive trafficking of a phospho-deficient mutant T668A, 2) studying the level of phosphorylated Thr-668 using a phosphosite-specific antibody after copper treatment, and 3) using kinase inhibitors including lithium chloride (LiCl) to inhibit phosphorylation at Thr-668. Our results from these various approaches strongly suggest that copper promotes a relocation of APP by phosphorylation at Thr-668 in the neuronal cell model SH-SY5Y. This involves GSK3 $\beta$  and importantly identifies a

novel mechanism by which copper can regulate APP function in neuronal cells.

### EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—The following antibodies were used in this study: GM130 (BD Transduction Laboratories),  $\beta$ -catenin (Abcam), Ankyrin-G (NeuroMab, Davis, CA), C20 (C-terminal APP antibody; Calbiochem), phospho-APP (Thr-668 (D90B8); Cell Signaling Technology);  $\beta$ -actin (Sigma), and W0–2. The antibody CT77 was used to detect the copper transport protein, ATP7A, and was a kind gift from Prof. B. Eipper (Neuroscience and Molecular, Microbial, and Structural Biology Division, University of Connecticut). GM130 and Ankyrin-G were used as markers for the cis-Golgi network and as an axonal marker in primary hippocampal neurons, respectively. The C-terminal APP antibody C20 specifically recognizes residues 751–770 and will detect full-length APP and C-terminal fragments. The W0–2 epitope lies within the A $\beta$  domain (1–4 amino acids) and will detect full-length APP as well as the sAPP- $\alpha$  ectodomain and A $\beta$  peptide. Lithium chloride (Sigma) was used as a GSK3 $\beta$  inhibitor. Other kinase inhibitors for GSK3 $\beta$  and cyclin-dependent kinases were obtained from the Tocriscreen Kinase Inhibitor Toolbox (Tocris Bioscience). PhosSTOP Phosphatase inhibitor mixture tablets (Roche Applied Science) were used to inhibit phosphatase activity after cell lysis. Western lysis buffer was also supplemented with Complete EDTA-free protease inhibitor mixture tables (Roche Applied Science).

**Cell Culture and Generation of Stable Cell Lines**—Human neuroblastoma SH-SY5Y cells (American Type Culture Collection catalogue no. CRL-2266) were cultured in DMEM (Invitrogen) containing GLUTAMAX<sup>TM</sup>-I (Invitrogen) supplemented with 10% fetal calf serum and 1 mM sodium pyruvate. Cell lines were cultured at 37 °C and in the presence of 5% CO<sub>2</sub>. To generate SH-SY5Y stable cell lines, cells grown in 6-well plates were transfected with 2.4  $\mu$ g of plasmid DNA using the Lipofectamine 2000<sup>TM</sup> reagent (Invitrogen) according to the manufacturer's instructions. Stable SH-SY5Y cell lines were selected and maintained with Geneticin (0.5 mg/ml; Invitrogen) 48 h after transfections. The SH-SY5Y cell lines generated express APP695 or APP with point mutations at the threonine 668 or the serine 655 residue with a C-terminal mCherry fluorescent tag in the pcDNA3.1 vector (Invitrogen). The generation of the pcDNA3.1-APP-cherry expression vector has been previously described (37). To obtain an enriched population of APP-mCherry expressing cells, cell lines were subjected to flow cytometry using the FACS Aria III cell sorter (BD Biosciences).

**Isolation of Mouse Hippocampal Primary Cultures**—Hippocampal neuronal cultures were prepared from E17 mouse C57BL/6 embryos as described previously (39, 40) in accordance with ethics committee approval of the University of Melbourne. Briefly, hippocampi were removed, dissected free of meninges, and dissociated in 0.025% (w/v) trypsin. Dissociated cells were plated onto poly-L-lysine-coated coverslips in sterile 24-well culture plates in minimal essential medium supplemented with 10% fetal calf serum. Cultures were maintained at 37 °C in 5% CO<sub>2</sub> for 2 h before the plating medium was replaced with Neurobasal growth medium containing B27 supplements

(Invitrogen). Experiments were performed in fresh Neurobasal medium.

**Copper, Copper Chelator, and Kinase Inhibitor Treatment**—SH-SY5Y cell lines were treated with copper ( $\text{CuCl}_2$ ) or copper chelators at a concentration of  $150 \mu\text{M}$  for 3 h in normal growth medium (see above) containing 10% fetal calf serum. The copper chelators used were bathocuproine disulfonate, which chelates Cu(I) and D-penicillamine for Cu(II), both of which were used at equal concentrations (*i.e.*  $150 \mu\text{M}$ ). The copper and copper chelator concentrations used were based on previously published data and take into account the presence of copper binding proteins in the serum such as albumin and  $\alpha$  fetoprotein, which reduce the level of bioavailable copper (37, 38, 41, 42). As hippocampal neurons were cultured in defined Neurobasal medium without serum, a lower concentration of copper was used in these experiments ( $15 \mu\text{M}$ ). LiCl at 10 mM was used to inhibit GSK3 $\beta$  activity (43). The potent selective GSK3 $\beta$  inhibitors, SB 216763 and SB 415286, were obtained from the Tocris inhibitor toolbox and were used at  $10 \mu\text{M}$  as per the manufacturer's instructions. Purvalanol A and Purvalanol B were used to inhibit cyclin-dependent kinases at a concentration of  $10 \mu\text{M}$  (Tocris inhibitor toolbox).

**Immunocytochemistry**—SH-SY5Y stable cells were plated onto 12-mm coverslips at a density of  $0.05 \times 10^6$ /well in 24-well plates. After copper/copper chelator treatment, cells were fixed in 4% paraformaldehyde in PBS (pH 7.2, Sigma) and permeabilized with 0.1% Triton X-100 (Sigma) and nonspecific sites blocked with 1% bovine serum albumin (Sigma) overnight. Antibodies used for immunocytochemistry were used at the following dilutions: GM130 (1:200), AnkG (1:500), W0-2 (1:20), C20 (1:500), and CT77 (1:500). Primary antibodies were detected using secondary IgG antibodies conjugated to AlexaFluor<sup>®</sup> 488, 594, or 647 fluorophores (Invitrogen) at a dilution of 1:400. Rhodamine phalloidin (Invitrogen), a fluorescent probe that labels filamentous actin, was used at a concentration of 1:50 to mark the perimeter of individual cells. Images were captured using the Leica SP8 confocal microscope.

**Image Analysis**—Images captured were deconvoluted using the Huygens essential software Version 4.3 (Scientific Volume Imaging B.B., Hilversum, The Netherlands). For hippocampal neurons, the average intensity of APP fluorescence in an  $\sim 200\text{-}\mu\text{m}$  length axon (as determined by AnkG fluorescence) and a dendrite segment were quantified per z-stack using the Metamorph Meta Imaging Service Software Version 7.1 (Molecular Devices, Sunnyvale, CA). The average intensity of APP fluorescence in axons and two dendrites was measured for 10 randomly selected hippocampal neurons per treatment.

To quantify copper-responsive trafficking of APP in the exocytic pathway, we utilized an analysis we previously used for quantifying the copper-responsive post-Golgi trafficking of the copper-efflux protein ATP7A (44). APP or ATP7A fluorescence intensity was measured from equivalent areas containing the perinuclear regions (defined using nuclear (DAPI) and Golgi (GM130) staining) of at least 10 cells cultured in both copper-chelated and copper-supplemented medium. Fluorescent intensity was measured for the maximum intensity projection per treatment using ImageJ (Wayne Rasband, National Institutes of Health).

**Site-directed Mutagenesis**—The threonine 668 and serine 655 residues (APP695 numbering) were substituted to an alanine to create a phospho-deficient mutant using the QuikChange Site-directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer's instructions. Briefly, an amino acid substitution was introduced by replicating both plasmid strands of the pcDNA3.1-APPcherry expression vector using high fidelity DNA polymerase (*PfuTurbo*) and two complementary primers, which contained the desired mutation. The synthesized plasmid containing the desired substitution was verified by DNA sequencing.

**Western Blot Analysis**—Cell lysates were prepared in 50 mM Tris-HCl, 120 mM NaCl, 1% Triton X-100 containing protease and phosphatase inhibitors (Roche Applied Science). Proteins were separated by SDS-PAGE using a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and MES running buffer (Invitrogen) followed by protein transfer to Amersham Biosciences<sup>™</sup> Hybond<sup>™</sup>-ECL 0.2- $\mu\text{m}$  membrane (GE Healthcare). After transfer, membranes were boiled in PBS for 45 s and blocked in 5% skim milk in Tris-buffered saline (TBS). The membrane was then probed with primary antibodies diluted in TBS containing 0.1% Tween 20. The antibodies and their corresponding dilutions include W0-2 (1:40),  $\beta$ -catenin (1:2000), phospho-APP (Thr-668) (1:1000), and  $\beta$ -actin (1:500). Proteins were visualized by probing with the corresponding secondary IgG horseradish peroxidase-conjugated antibody (DAKO; 1:5000). Membranes were developed using the ECL<sup>™</sup> Western blotting detection system (GE Healthcare) as per the manufacturer's instructions and visualized using the LAS-3000 Imaging system (Fuji).

**Densitometric Analysis**—Protein levels after treatment with copper and copper chelators ( $\pm$ LiCl) were measured from three independent experiments by densitometry. The pixel intensity (arbitrary unit) per given protein band was quantified using the Multi Gauge software (Fuji). The level of phospho-APP<sup>Thr-668</sup> was normalized to total APP detected in the cell lysate.

**Data Analysis**—Statistical analyses were performed using one-way analysis of variance, and Student's *t* test was expressed as the mean  $\pm$  S.E. A level of  $p < 0.05$  was considered statistically significant (\*). To determine whether changes in APP fluorescence in axons and dendrites were statistically significant between treatments, the average APP fluorescence intensity was measured per z-stack for 10 randomly selected axons ( $n = 10$ ) and 20 dendrites ( $n = 20$ ; 2 per hippocampal neuron) using the Metamorph imaging software. These values were averaged per axon/dendrite and expressed in relation to axon area ( $\mu\text{m}^2$ ).

## RESULTS

**The Phospho-deficient Mutant, APP<sub>T668A</sub>, Does Not Traffic in Response to Copper**—We previously reported that copper promotes the trafficking of the copper-transporting P-type ATPase, ATP7A (38), and of APP (37), and the exocytic trafficking pathways appear to be distinct (37). The copper-responsive APP trafficking involved both a stimulation of exocytosis and a suppression of endocytosis. Increased intracellular copper enhanced post-Golgi trafficking of ATP7A to the PM for copper efflux and transport across epithelial cell barriers (44), and this is mediated by phosphorylation at specific sites in the C

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terminus of ATP7A (38). Serine 1469, a copper-responsive phosphorylation site, was identified as being required for copper-responsive trafficking of ATP7A from the trans-Golgi Network and the PM. In the current study we investigated whether, similar to ATP7A, copper-responsive APP trafficking (37) is also regulated by phosphorylation at specific sites. Previous studies have reported that APP is phosphorylated at several sites within its intracellular domain, including Thr-668, which is phosphorylated both *in vivo* and *in vitro* and has been widely studied (22, 32, 36, 45–47).

To investigate whether phosphorylation of Thr-668 is required for copper-responsive APP trafficking, the Thr-668 residue was substituted to an alanine to create a phospho-deficient mutant at this site. Previous studies have shown that a threonine to alanine substitution at APP<sup>Thr-668</sup> effectively mimics the non-phosphorylated state with respect to the helical structure of the cytoplasmic domain (35, 36). To determine whether the APP<sup>T668A</sup> mutant traffics in response to copper in comparison to wild type APP (APP<sup>WT</sup>), SH-SY5Y stable cell lines were generated that expressed APP<sup>T668A</sup> and APP<sup>WT</sup> with a C-terminal cherry fluorescent tag. Both of these cell lines were treated with either copper (150  $\mu$ M CuCl<sub>2</sub>) or with the copper chelators bathocuproine disulfonate and D-penicillamine (150  $\mu$ M bathocuproine disulfonate/D-penicillamine) for 3 h. Cells were immunolabeled with the Golgi marker GM130 and the nuclear stain DAPI (Fig. 1). As previously reported, APP<sup>WTcherry</sup> relocates from a predominant Golgi localization to a wider distribution after copper treatment (Fig. 1A). By Western blot analysis we found that copper-responsive APP relocation is not accompanied by a change in the protein levels of both endogenous APP protein or the APP<sup>WTcherry</sup> (data not shown), which we have also demonstrated previously (37).

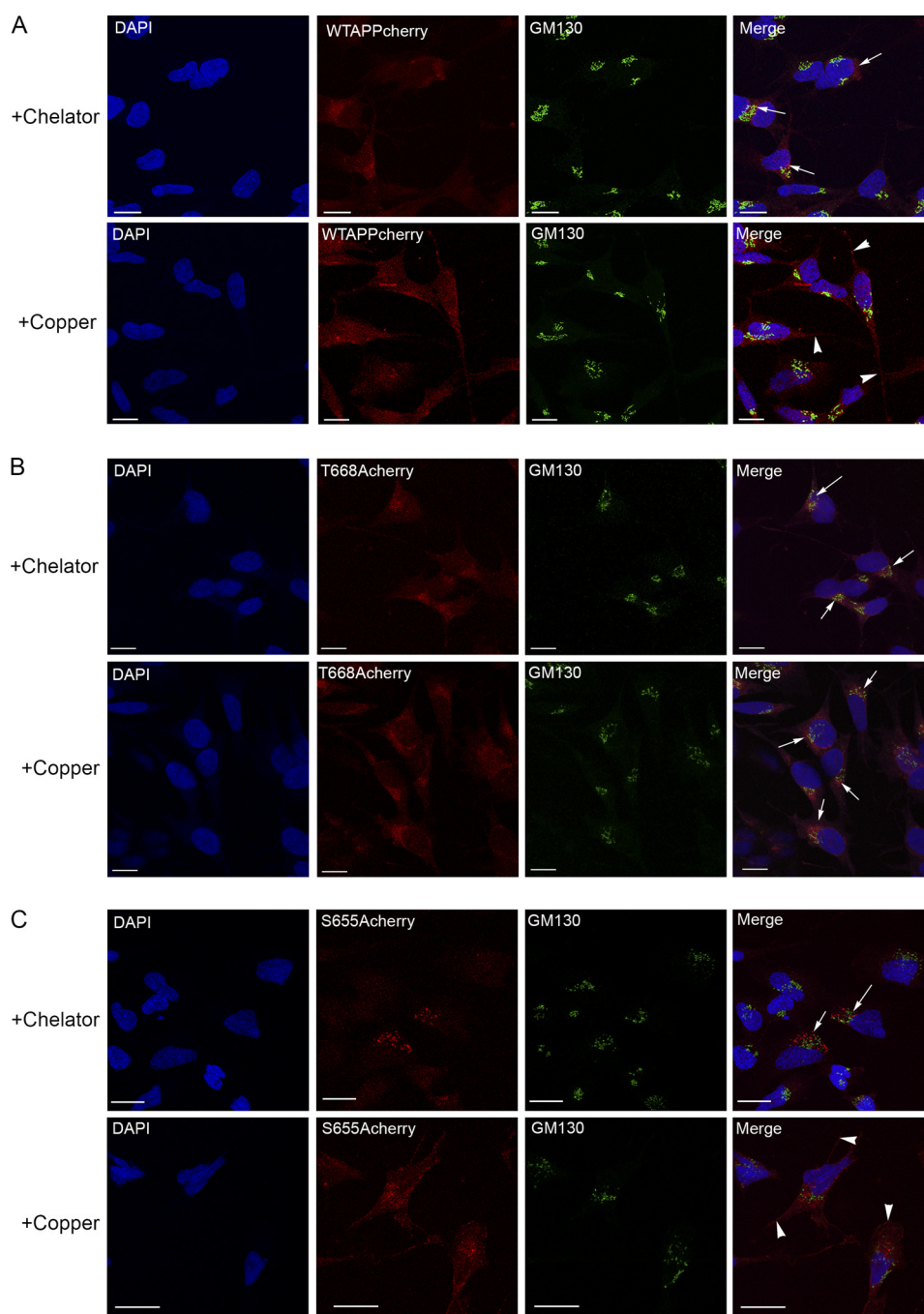
In contrast to APP<sup>WTcherry</sup>, APP<sup>T668Acherry</sup> was localized to a perinuclear localization with partial co-localization with the Golgi marker GM130 after both copper and copper chelator incubation (Fig. 1B). These results provide strong evidence that copper-responsive trafficking of APP requires phosphorylation at Thr-668, as the phospho-deficient mutant T668A does not exhibit copper-responsive trafficking. We also investigated whether substituting Ser-655 to an alanine affects copper-responsive APP trafficking. Fig. 1C shows that the phospho-deficient mutant APP<sup>S655Acherry</sup> exhibits relocation similar to wild type APP after copper treatment, suggesting Ser-655 is not required for copper-responsive trafficking of APP. Under chelator conditions, APP<sup>S655Acherry</sup> did not appear to exhibit co-localization with GM130, unlike APP<sup>T668Acherry</sup> and APP<sup>WTcherry</sup>.

**Copper Promotes APP Phosphorylation at Thr-668**—The above results are consistent with copper promoting the phosphorylation of APP at Thr-668. To investigate this using an independent approach, SH-SY5Y cells were incubated with copper or copper chelators  $\pm$  10 mM LiCl for 3 h. Thr-668 phosphorylation is mediated by the kinases GSK3 $\beta$  and Cdk5 (29, 32). LiCl is an inhibitor of GSK3 $\beta$  (43) and also indirectly (via the p35/p25 system) an inhibitor of Cdk5 (48, 49). Cell lysates were prepared, and protein levels were analyzed by Western blotting. An antibody to phospho-APP (Thr-668) was used to detect endogenous levels of phospho-APP (Thr-668) (7,

25, 32, 50). The membrane was then stripped and re-probed with W0-2 to determine the total level of APP. APP is detected as three bands by both the phospho-specific antibody and W0-2, which represent glycosylated forms of APP (5). Actin was used as a loading control. Our results show that copper promotes an increase in the level of phospho-APP at Thr-668 in comparison to copper chelator treatment (Fig. 2A, *top panel*). This effect was abolished when cells were treated with both copper and LiCl ( $n = 3$ ;  $p = 0.025$ ). Densitometry analysis was conducted in all three independent experiments comparing the level of pAPP-<sup>Thr-668</sup> with that of total APP, which in turn was normalized to actin levels. Results show that copper increased the level of p-APP<sup>Thr-668</sup> by 1.65-fold in comparison to copper chelator treatment ( $n = 3$ ;  $p = 0.018$ ; Fig. 2B).

As substantiation that LiCl was inhibiting glycogen synthase kinase 3 $\beta$  activity, we analyzed the protein levels of  $\beta$ -catenin, an essential protein of the Wnt signaling pathway, by Western blot. Phosphorylation of  $\beta$ -catenin by glycogen synthase kinase 3 $\beta$  leads to its degradation via the ubiquitin-proteasome pathway, thus reducing its total levels. Conversely, inhibition of GSK3 $\beta$  leads to an increase in total protein levels of  $\beta$ -catenin (for review, see Ref. 51). Thus, measuring  $\beta$ -catenin protein levels is a robust readout of glycogen synthase kinase 3 $\beta$  activity. We found that treating SH-SY5Y cells with LiCl for 3 h led to a 1.65-fold increase in the total protein level of  $\beta$ -catenin (data not shown;  $n = 3$ ,  $p = 0.047$ ). These results provide strong evidence that treating with LiCl effectively inhibits GSK3 $\beta$  activity.

**Treatment with LiCl Inhibits Copper-responsive APP Trafficking**—As glycogen synthase kinase 3 $\beta$  and Cdk5 phosphorylate APP at Thr-668 in neurons (29), we tested whether 10 mM LiCl decreases the level of phosphorylation at this site. LiCl is a commonly used and efficient inhibitor of GSK3 $\beta$  and has been shown to down-regulate the expression of Cdk5 (48, 52). SH-SY5Y cells grown on coverslips were treated with either copper or copper chelators and LiCl for 3 h in normal growth medium containing 10% fetal calf serum (see Fig. 3). As a control, cells were treated with either copper chelators or copper alone (Fig. 3A). After the 3-h incubation period, cells were fixed and permeabilized and immunolabeled with the APP C-terminal antibody C20, the Golgi- marker GM130, and rhodamine phalloidin as well as the nuclear DAPI stain. A change in the cellular localization of APP was quantified by measuring APP fluorescence intensity at a defined region of interest at the Golgi as determined by GM130 staining for at least 10 cells after copper chelator and copper treatment (see “Experimental Procedures”). APP is predominantly localized to a Golgi/perinuclear localization and thus a change in its subcellular localization would lead to a decrease in fluorescence intensity in this region. As previously reported, after incubation with copper, endogenous APP relocates from a predominant Golgi localization (see *arrows*, Fig. 3A, *top panel*) to a wider distribution including cell extensions (as shown by *arrowheads* in Fig. 3A). After copper treatment, a proportion of APP reaches the PM, as we demonstrated previously (37). Copper treatment led to a 25% decrease in APP fluorescence intensity at a defined Golgi region in comparison to chelator treatment. These data further verify that APP exits the Golgi after copper treatment ( $n = 13$ ,  $p =$



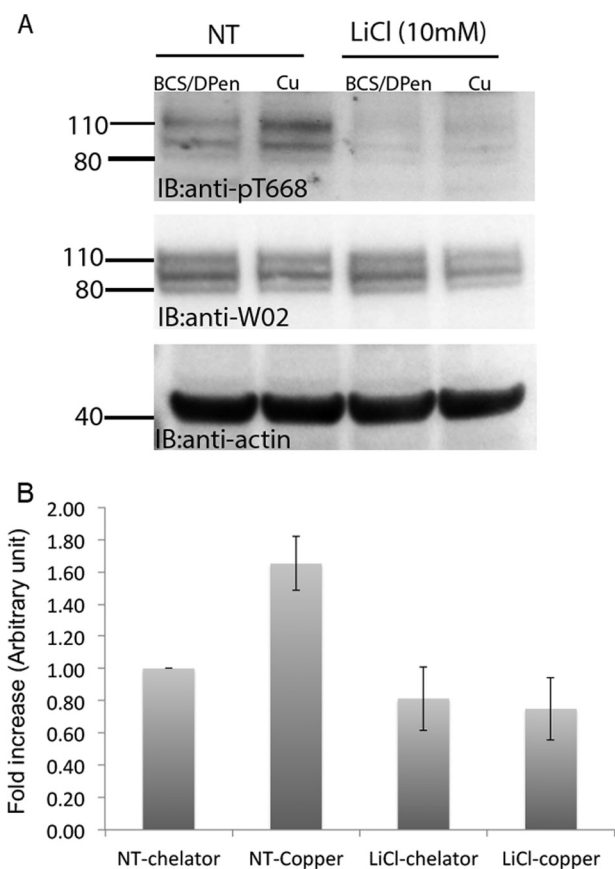
**FIGURE 1. The phospho-deficient mutant T668A does not exhibit copper-responsive trafficking.** SH-SY5Y cell lines stably expressing either APP<sub>WT</sub> (A) APP<sub>T668A</sub> (B), or APP<sub>S655A</sub> (C) with a fluorescent cherry tag were incubated with either 150  $\mu$ M copper or 150  $\mu$ M copper chelators bathocuproine disulfonate and D-penicillamine (bathocuproine disulfonate/*dPEN*) for 3 h. A, after copper treatment APP<sub>WT-cherry</sub> traffics from the Golgi to a wider distribution throughout the cell (see *arrowheads*). B, APP<sub>T668A</sub> is localized to a perinuclear localization, with partial co-localization with GM130 under both copper chelator and copper conditions. C, under chelator conditions, the phospho-mutant APP<sub>S655A</sub> localizes to the perinuclear region (*arrows*) with no co-localization with the GM130 marker. After copper treatment APP<sub>S655A</sub> re-distributes throughout the cell. Scale bar = 20  $\mu$ m.

0.003). In contrast, cells that were treated with both copper and LiCl did not exhibit APP trafficking with APP remaining in a Golgi/perinuclear localization similar to chelator LiCl conditions (see the *arrows* in Fig. 3, B and C). Moreover, there was no statistically significant difference in APP fluorescence intensity at the Golgi when cells were treated with copper chelator + LiCl and copper + LiCl, suggesting that there was no change in APP cellular localization between treatments ( $n = 10$ ,  $p = 0.28$ ). Treating SH-SY5Y cells with a range of LiCl concentra-

tions (1–50 mM) for 3 h had no effect on cell viability as measured by the PrestoBlue<sup>®</sup> cell viability assay (data not shown).

**Treatment with LiCl Does Not Inhibit the Trafficking of the Copper Transporter ATP7A**—We have previously reported copper-responsive trafficking of the copper-transporter ATP7A from the Golgi to the PM (42, 44, 53). To determine whether, similar to APP, copper-responsive ATP7A trafficking is inhibited by LiCl, SH-SY5Y cells were treated with copper chelators or copper together with 10 mM LiCl. After treatment, cells were immunola-

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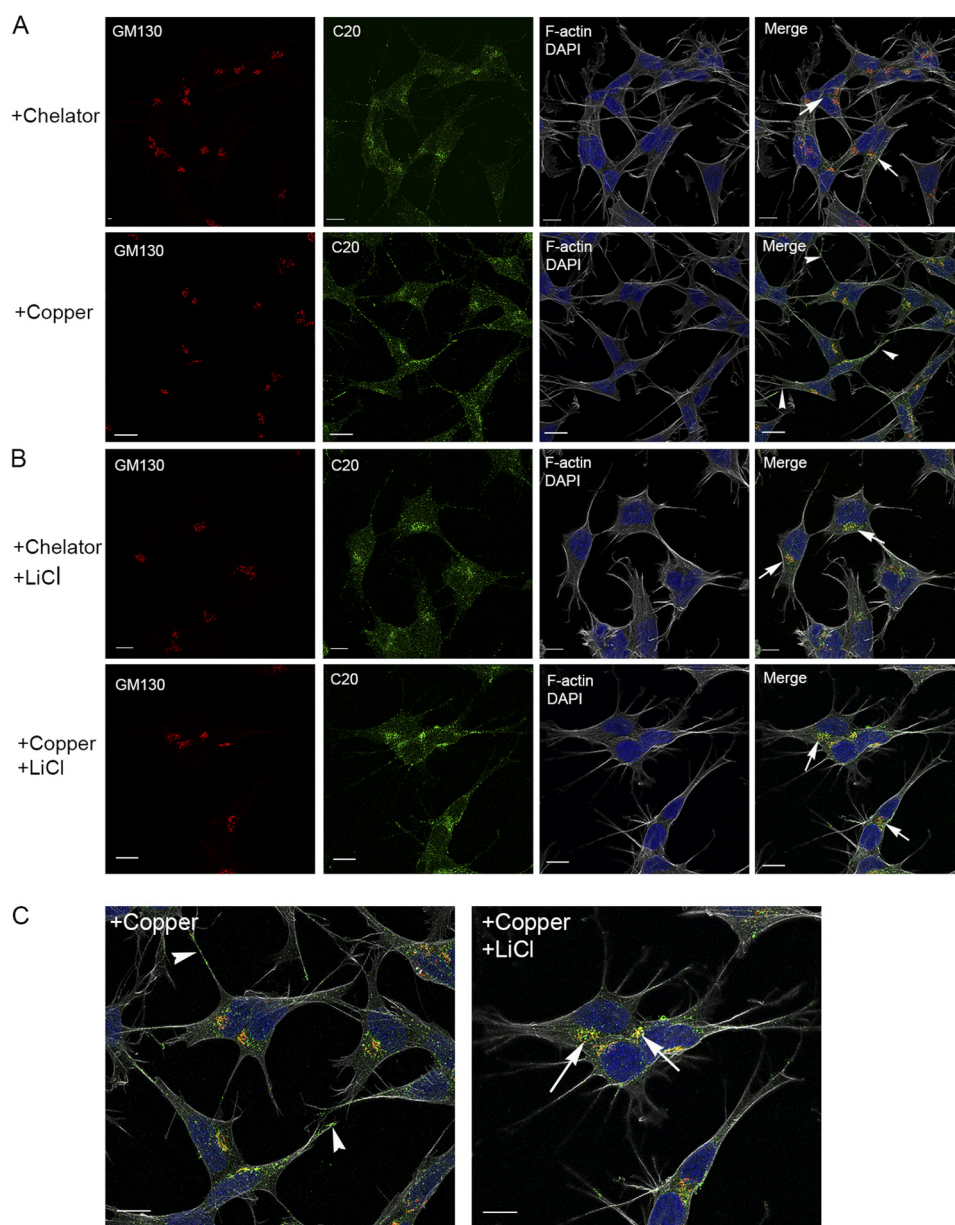


**FIGURE 2. Copper increases the level of phosphorylation at Thr-668.** SH-SY5Y cells were cultured in 6-well plates and treated with copper (150  $\mu\text{M}$ ) or copper chelators (150  $\mu\text{M}$ )  $\pm$  10 mM LiCl for 3 h. *A*, protein levels were analyzed by Western immunoblotting (IB), whereby the membrane was probed with the antibody anti-phospho-APP (Thr-668). The membrane was stripped and then re-probed with W0-2 to detect total APP. The three bands detected in the top two panels represent different glycosylated APP isoforms. Actin was used as the loading control. BCS/DPen, bathocuproine disulfonate/D-penicillamine. NT, not treated. *B*, Western blot results were analyzed from three independent experiments by densitometry. The analysis shows copper increased the level of APP phosphorylation by an average of 1.65-fold in comparison to chelator treatment ( $n = 3$ ;  $p = 0.018$ ). Incubating cells with both copper and LiCl abolished this effect ( $n = 3$ ;  $p = 0.025$ ). NT, not treated.

beled with the antibody W0-2 and CT77 to visualize endogenous APP and ATP7A, respectively. Results show that ATP7A traffics in response to copper in the presence of LiCl, unlike APP (Fig. 4). In chelator (+LiCl) conditions ATP7A was tightly localized to a perinuclear localization and dispersed throughout the cell after copper (+LiCl) incubation (Merge panels, see arrowheads). This was quantified by measuring ATP7A fluorescence intensity at a defined perinuclear region for 10 cells per treatment. Results showed a 35% decrease in fluorescence intensity after copper + LiCl treatment, suggesting that ATP7A is exiting the perinuclear region in response to copper despite the presence of LiCl ( $n = 10$ ;  $p = 0.039$ ). In contrast, APP was localized to a perinuclear region after both chelator + LiCl and copper + LiCl conditions, and there was no statistical difference in APP fluorescent intensity in this region between treatments ( $n = 10$ ;  $p = 0.22$ ). These data provide further evidence that copper promotes APP trafficking via a distinct pathway to that of ATP7A (initial evidence in Acevedo *et al.* (37)). The data are also consistent with LiCl treatment not having a generalized effect on protein trafficking.

*GSK3 $\beta$ -selective Inhibitors, but Not Cdk Inhibitors, Prevent Copper-responsive APP Trafficking in SH-SY5Y Cells*—To determine whether copper promotes APP trafficking via GSK3 $\beta$  or Cdk5, selective inhibitors were used from the Tocris kinase inhibitor toolbox (Tocris Bioscience). The GSK3 $\beta$  inhibitors used include SB 216763 (Fig. 5C) and SB 415286 (data not shown), which have been shown to be selective and potent inhibitors of GSK3 $\beta$  (54–56). Purvalanol A (data not shown) and B (Fig. 5D) were used to inhibit the activity of cyclin-dependent kinases including Cdk5-p35, Cdc2/cyclin B, Cdk2/cyclin E, and cdk4/cyclin D1 (57, 58). Kinase inhibitors were used at a final concentration of 10  $\mu\text{M}$  in the presence of copper chelators or copper (150  $\mu\text{M}$ ) in normal growth media for 3 h. As the above inhibitors were dissolved in DMSO, control cells were treated with copper chelators and copper in the presence of 10  $\mu\text{M}$  DMSO (Fig. 5, A and B). After treatment, cells were immunolabeled with W0-2 and CT77 to visualize the localization of endogenous APP and ATP7A, respectively. We co-labeled with ATP7A to investigate whether copper promoted protein trafficking of APP and ATP7A via the same putative signaling pathway. Results show that cells incubated with copper and GSK3 $\beta$  inhibitors (SB 216763, panel C) did not exhibit copper-responsive trafficking with APP remaining in the Golgi region in the presence of copper as quantified by measuring APP fluorescence intensity at the perinuclear region. Results show no statistical difference in APP fluorescence between copper chelator and copper + SB 216763 treatment ( $n = 10$ ;  $p = 0.13$ ), demonstrating no change in APP cellular localization. In the presence of Purvalanol A (data not shown) and B (Fig. 5D), copper-responsive trafficking was observed with APP relocating throughout the cell including extensions, as indicated by arrowheads. Quantitation showed a 27% decrease in APP fluorescence intensity in the perinuclear region when treated with copper + Purovalanol B in comparison to chelator treatment ( $n = 10$ ;  $p = 0.0023$ ), which is consistent with a relocalization of APP. Importantly, ATP7A traffics from a tight Golgi localization under copper chelator conditions (Fig. 5A) to a wider distribution in the presence of copper and either GSK3 $\beta$  or Cdk inhibitors (Fig. 5, B–D). Taken together these results strongly suggest that copper promotes trafficking of APP and ATP7A via separate pathways.

*Copper Promotes an Increase in APP Trafficking to Axons in Primary Hippocampal Neurons, and This Is Inhibited by LiCl Treatment*—To investigate copper-responsive trafficking of APP in primary neurons and the effect of added LiCl, we repeated the above experiments utilizing cultured mouse primary hippocampal cells. Hippocampal neurons isolated from E17 embryos were cultured for 18 days on coverslips to obtain mature neurons (39, 40). Neurons were then treated with copper or copper chelator  $\pm$  10 mM LiCl for 3 h in Neurobasal medium. Copper was used at 15  $\mu\text{M}$  due to the absence of serum in Neurobasal media as previously described (37, 41, 59). After treatment, cells were immunolabeled with the antibodies C20 and anti-Ankyrin-G, which visualize, respectively, endogenous APP and Ankyrin-G, a protein localized at the initial axon segment (60). After copper treatment there was an increase in APP at the axon in comparison to copper chelator conditions (Fig. 6, A–C). To quantify the level of APP at the axon after treatments,



**FIGURE 3. Treatment with LiCl prevents copper-responsive APP trafficking.** *A*, SH-SY5Y cells were treated with either copper or copper chelators for 3 h. Endogenous APP was visualized by staining with the C-terminal APP antibody C20. Cells were co-stained with the nuclear marker DAPI, Golgi marker GM130, and the actin cytoskeleton stain rhodamine phalloidin to visualize the cell surface. Merged images show a partial co-localization of APP and the Golgi marker. Under copper chelator conditions APP has a predominant Golgi-localization (see the *arrows*). After copper treatment, APP distributes throughout the cell, including extension as shown by *arrowheads*. *B*, cells were treated with 10 mM LiCl and copper chelators or copper for 3 h. LiCl did not affect the localization of endogenous APP after chelator conditions (compare *top panels A and B*). However, in the presence of LiCl copper did not induce a change in APP localization from the Golgi to a wider distribution. *C*, magnification of merged images show that copper treatment induces a re-localization of APP to extensions, and this is inhibited by LiCl (refer to *arrows*). Scale bar = 20  $\mu$ m.

the average fluorescence intensity of APP was measured for 10 hippocampal neurons per treatment using the Metamorph imaging software (see "Experimental Procedures"). The addition of LiCl to the copper treatment abolished this effect (Fig. 6, *A–C*). Fig. 6, *B* and *C*, shows the maximum z-projections of two representative axons per treatment and further demonstrates that copper increases the level of APP at the axons in comparison to chelator  $\pm$  LiCl and copper + LiCl axons. Fig. 6*D* shows that after copper treatment there was a 1.91-fold increase in the level of APP at the axon in comparison to copper chelator treatment ( $n = 10$ ,  $p = 0.016$ ). There was no increase in APP immunolabeling at the axon when neurons were treated with both

copper and LiCl ( $n = 10$ ; copper *versus* copper + LiCl;  $p = 0.0009$ ).

To determine whether copper promoted significant relocalization of APP to dendrites as well as the axon, the APP fluorescence intensity was measured for two dendrites per hippocampal neuron ( $n = 20$ ). Fig. 6*E* summarizes our findings that copper promotes trafficking of APP to axons but not dendrites (*i.e.* there was a significantly greater level of APP at the axons *versus* dendrites after copper treatment). On the other hand, during chelator  $\pm$  LiCl or copper + LiCl treatment there was no statistically significant difference between the levels of APP in axon *versus* dendrites, suggesting APP is evenly

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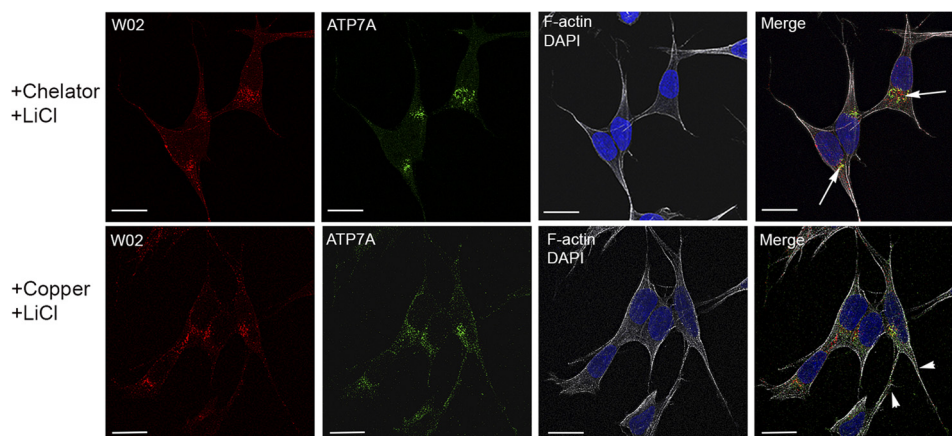


FIGURE 4. **LiCl treatment does not inhibit the copper-responsive trafficking of the copper efflux protein ATP7A.** SH-SY5Y cells were treated with either 150  $\mu\text{M}$  copper chelators or 150  $\mu\text{M}$  copper and LiCl (10 mM) for 3 h and endogenous APP and ATP7A immunolabeled with the antibodies W0-2 and CT77, respectively. Unlike APP, which does not exhibit copper-responsive trafficking in the presence of LiCl, ATP7A trafficked from a tight Golgi-localization to a wider distribution (as shown by arrowheads). Scale bar = 20  $\mu\text{m}$ .

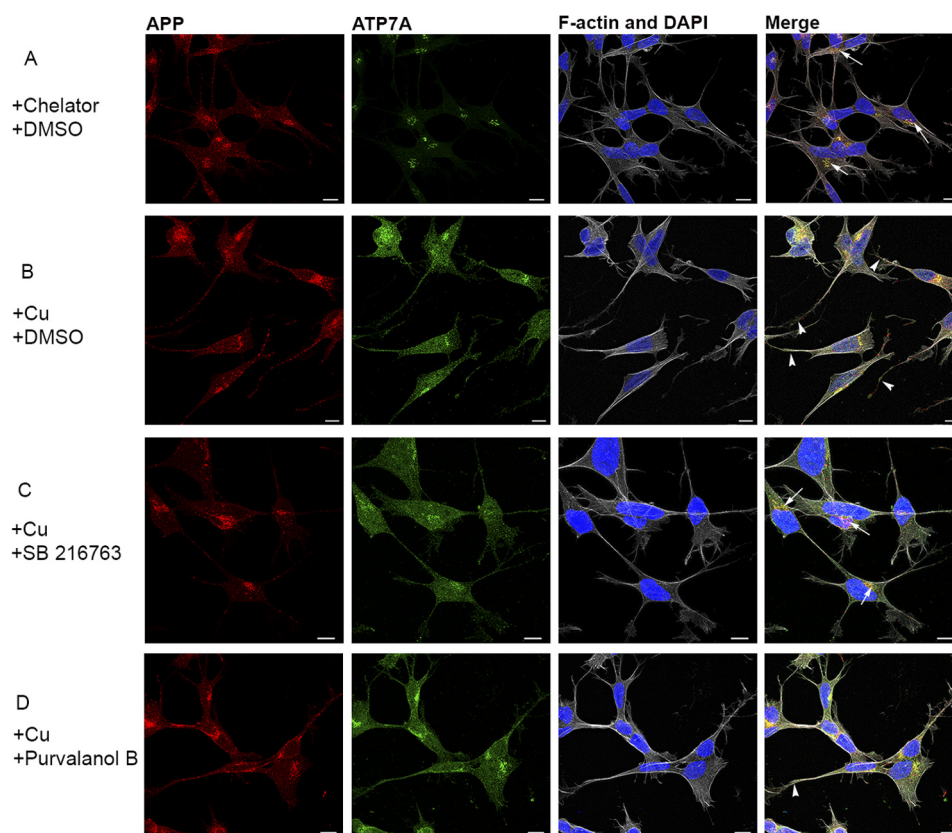


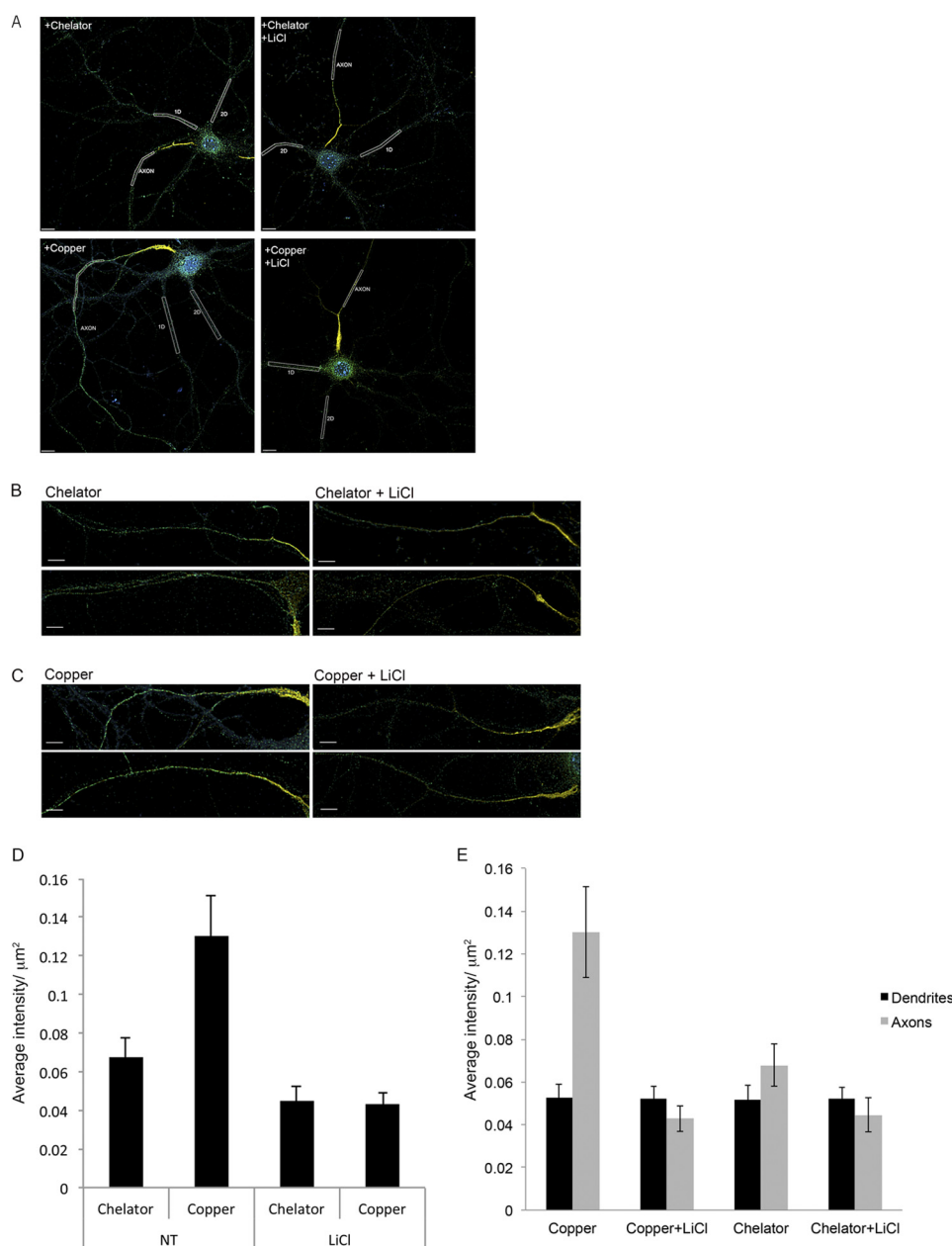
FIGURE 5. **SB 216763, a potent and selective GSK3 $\beta$  inhibitor, prevents copper-responsive APP trafficking but not Purvalanol B, a Cdk inhibitor.** To determine whether GSK3 $\beta$  or Cdk5 activity is required for copper-responsive trafficking, SH-SY5Y cells were treated with copper (150  $\mu\text{M}$ ) in the presence of 10  $\mu\text{M}$  SB 216763 (C) or Purvalanol B (D), a GSK3 $\beta$  and Cdk inhibitor, respectively. A, control panel showing that APP localizes to a Golgi region with partial co-localization to ATP7A in chelator and DMSO conditions. B, both APP and ATP7A traffic to a wider cellular distribution after the addition of copper and DMSO. C, in the presence of copper and SB 216763, APP fails to traffic in response to copper. D, Purvalanol B does not inhibit APP redistribution in the presence of copper. ATP7A traffics in response to copper in the presence of both SB 216763 and Purvalanol B. Scale = 10  $\mu\text{m}$ .

distributed throughout neurites. These findings identify a novel mechanism by which copper may regulate APP function in primary neurons by promoting its trafficking to axons.

### DISCUSSION

Threonine 668 is a major phosphorylation site within APP that can be phosphorylated by a number of kinases. Cdk5 and

GSK3 $\beta$  phosphorylate APP at Thr-668 in neurons, whereas Cdk1/Cdc2 phosphorylate APP in dividing cells (7, 10, 29, 32, 49). After a cellular stress signal, JNK also phosphorylates APP at Thr-668 (27, 30, 61). Interestingly, in human and mouse brains it is the mature form (*O*-glycosylated APP695) that is phosphorylated but not immature APP, suggesting that a fixed pool of APP is constitutively phosphorylated in neurons (32). In



**FIGURE 6. Copper promotes the trafficking of APP to axons, and this is inhibited by treatment with LiCl.** *A*, hippocampal neurons were isolated from E17 embryos and cultured for 18 days before incubation with either 15  $\mu\text{M}$  copper or 15  $\mu\text{M}$  copper chelators  $\pm$  LiCl (10 mM) for 3 h in serum-free Neurobasal medium. Neurons were immunolabeled with the C20 and anti-Ankyrin-G antibody to visualize APP (green) and Ankyrin-G (yellow), a protein localized to axon initial segments, respectively. Ankyrin-G was used as an axonal marker. An  $\sim 200\text{-}\mu\text{m}$  section of the axon and two dendrites (1D and 2D) is outlined by boxes. Shown are maximum intensity z-projections of axonal regions after chelator/chelator + LiCl (*B*) and copper/copper + LiCl treatment (*C*). Images were taken of 10 hippocampal neurons per treatment, and the average intensity of APP fluorescent labeling along an  $\sim 200\text{-}\mu\text{m}$  axonal segment ( $n = 10$ ) and two dendrites per neuron ( $n = 20$ ) was measured using the Metamorph imaging software. Panel *D* shows the average APP fluorescence intensity of 10 axonal regions per  $\mu\text{m}^2$  per treatment. NT, not treated. Copper promotes a 1.91-fold increase in the level of APP at the axon in comparison to chelator condition ( $n = 10$ ;  $p = 0.016$ ), and this effect was abolished in the presence of LiCl ( $n = 10$ ;  $p = 0.0009$ ). Panel *E* shows a comparison between APP fluorescent intensity in the axon and dendrites after various treatments. Copper does not promote an increase in APP fluorescence at the dendrites ( $n = 20$ ;  $p = 0.0026$ ). Scale bar = 10  $\mu\text{m}$ .

this study we used independent approaches to investigate whether copper-responsive trafficking of APP is dependent on phosphorylation, as is the case for ATP7A. The human neuroblastoma cell line, SH-SY5Y, was chosen for this study as it is widely utilized and accepted as a good model for neuronal cells including hippocampal neurons (62, 63). In this study we constructed a phospho-deficient mutant at the Thr-668 site (T668A) to determine whether this residue was important for copper-responsive trafficking of APP. Unlike APP<sub>WT</sub>, the

“phospho-deficient” APP<sub>T668A</sub> protein did not exhibit trafficking after copper treatment in SH-SY5Y cells. Western blot analysis utilizing an antibody to APP phosphorylated at Thr-668 showed that copper promotes an increase in endogenous APP phosphorylation at this site. In addition, we used LiCl as well as selective potent inhibitors of GSK3 $\beta$  to determine whether this kinase is involved in copper-responsive phosphorylation of APP at the Thr-668 residue. Our results clearly show that inhibiting the activity of GSK3 $\beta$  prevents the trafficking of APP from

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the Golgi to a wider distribution (Figs. 3, 4, and 5). Importantly, inhibiting cyclin-dependent kinases did not inhibit copper-responsive trafficking (Fig. 5). In these studies we have identified a novel mechanism by which copper regulates the localization of APP. In SH-SY5Y cells, this may involve copper signaling upstream of GSK3 $\beta$ . The activity of GSK3 $\beta$  is regulated by phosphorylation, modulating its subcellular localization or by protein-protein interactions (for review, see Ref. (79)). Copper may mediate its effect via a signaling pathway or by directly affecting GSK3 $\beta$  activity, for example by regulating the activity of a phosphatase that dephosphorylates GSK3 $\beta$  at its inhibitory residue (Ser-9). It has been previously reported that copper is involved in regulating signal transduction pathways (64, 65).

In neurons APP is phosphorylated at Thr-668 by GSK3 $\beta$  and Cdk5, and phospho-APP<sub>Thr-668</sub> has been observed in neurites and mostly in growth cones of differentiated neuronal cells (25, 29, 32, 66). Phosphorylation of APP at Thr-668 is associated with neurite extension, anterograde transport of vesicular cargo into neurites, and axonal transport (25). Studies have shown that APP is localized to synaptic vesicles in the pre- and post-synaptic compartments (for review, see Ref. 12). At the synapse full-length APP can be transported to the cell surface where  $\alpha$ -secretase cleavage occurs to produce sAPP $\alpha$ , which plays a crucial role in synapse formation and maintenance (67). Only a small fraction of APP is cleaved at the cell surface with the remainder internalized to an early endosome compartment where  $\beta$ -secretase and  $\gamma$ -secretase cleavage occurs to produce sAPP- $\beta$  and A $\beta$  peptides (68, 69). Processing products can then be trafficked back to the plasma membrane for secretion (70). Functions for sAPP $\beta$  and A $\beta$  include promoting axonal pruning and inhibition of long term potentiation, which impacts synaptic plasticity, respectively (71). In this study we found that in hippocampal neurons copper promotes the trafficking of APP to axons and not dendrites and that this is inhibited by LiCl treatment. APP undergoes rapid kinesin and calsyntenin 1-dependent anterograde transport in axons with subsequent retrograde transport to dendrites (72–78). We propose that our data showing a copper-responsive increase at 3 h of APP at the axon is consistent with axonal anterograde trafficking associated with an early step in post-Golgi exocytic trafficking. Indeed, we previously reported that in SH-SY5Y cells (non-polarized) copper promoted APP exocytosis as shown by live cell imaging (37). On the other hand, as we do not observe a significant increase of APP at dendrites or soma, we do not believe the data is consistent with copper stimulating APP retrograde transport.

GSK3 $\beta$  plays a role in a wide range of cellular processes and has numerous targets including proteins involved in metabolism such as glycogen synthase, cytoskeletal proteins, and transcription and transcription factors (for review, see Refs. 79). In neuronal development, GSK3 $\beta$  is associated with the regulation of neuronal outgrowth, motility, and synaptic plasticity as well as axonal polarity and survival (80–84). Our studies demonstrate that copper plays an important role in the trafficking of APP along the axon, thus potentially regulating the function of APP at the synapse. This process was sensitive to LiCl treatment suggesting that copper-responsive APP trafficking to the axon is dependent on a lithium-sensitive kinase(s). This is consistent with the findings in SH-SY5Y cells. Further studies are

required to establish the precise kinase(s) responsible for this effect.

As discussed earlier, whether phosphorylation of APP at Thr-668 regulates its processing remains controversial with various studies yielding contradictory outcomes. The discrepancy between the various studies could be due to the nature of the study. Some studies based their finding on the phosphomimetic APP protein, whereby the threonine is mutated to an aspartate or glutamate. It has been reported that replacing threonine with either of these amino acids does not accurately mimic the phosphorylated state of Thr-668 (36). In contrast, NMR studies have shown that mutating Thr-668 to an alanine accurately simulates the non-phosphorylated state (36). In the current study we investigated the processing products of APP<sub>T668A</sub> and APP<sub>WT</sub> after copper and copper chelator treatments after a 3-h incubation period. We investigated the level of C-terminal fragments, sAPP $\alpha/\beta$  and A $\beta$ , by Western blot and ELISA analysis, respectively. Within the 3-h time frame when we observed copper-responsive APP trafficking we did not observe a detectable change in the level of APP processing products in APP<sub>T668A</sub> and APP<sub>WT</sub> and between treatments (data not shown). This result is consistent with our previous findings, where we reported that copper does not influence the processing of wild type APP within a 3-h incubation period (37).

In this study we used independent approaches to conclude that copper-responsive trafficking of APP requires phosphorylation at Thr-668 in SH-SY5Y cells. Although we have yet to fully elucidate the significance of copper-responsive APP trafficking in neurons, our results using cultured primary hippocampal neurons suggest that copper promoted APP axonal transport, which may impact APP function at the synapse. Elucidation of the cellular function(s) associated with copper-responsive APP anterograde trafficking will be the subject of future investigations.

The physiological and pathological roles of GSK3 $\beta$  in the central nervous system are diverse and complex (for review, see Refs. 85). Understanding the relationship between copper, APP, and GSK3 $\beta$  in normal neuronal cells will provide important insight into the role these play in both normal processes and in pathological conditions like Alzheimer disease.

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