NUB1 modulation of GSK3β reduces tau aggregation

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Abnormal phosphorylation of the microtubule-associated protein tau in neurodegenerative disorders, including Alzheimer’s disease (AD) and frontotemporal lobar degeneration, is associated with disrupted axonal transport and synaptic dysfunction ultimately manifesting as histopathological lesions of protein aggregates. Glycogen synthase kinase 3β (GSK3β) may be critical for the pathological hyperphosphorylation of tau. Here, we examined the role of the proteasome-associated protein Nedd8 ultimate buster 1 (NUB1) in the neuro-pathogenic phosphorylation and aggregation of tau. We reveal that NUB1 interacted with both tau and GSK3β to disrupt their interaction, and abolished recruitment of GSK3β to tau inclusions. Moreover, NUB1 reduced GSK3β-mediated phosphorylation of tau and aggregation of tau in intracellular inclusions. Strikingly, NUB1 induced GSK3β degradation. Deletion of the NUB1 ubiquitin-like (UBL) domain did not impair the interaction with tau and GSK3β, and the ability to suppress the phosphorylation and aggregation of tau was not affected. However, the UBL motif was necessary for GSK3β degradation. Deletion of the NUB1 ubiquitin-associated (UBA) domain abrogated the ability of NUB1 to interact with and degrade GSK3β. Moreover, the UBA domain was required to suppress the aggregation of tau. Silencing of NUB1 in cells stabilized endogenous GSK3β and exacerbated tau phosphorylation. Thus, we propose that NUB1, by regulating GSK3β levels, modulates tau phosphorylation and aggregation, and is a key player in neurodegeneration associated with tau pathology. Moreover, NUB1 regulation of GSK3β could modulate numerous signalling pathways in which GSK3β is a centrally important effector.

INTRODUCTION

Tau is a microtubule-associated protein abundantly expressed in the nervous system (1). Tau is mainly located in neuronal axons, and has been implicated in microtubule stabilization through its microtubule-binding domain. Thus, tau has been reported to be involved in neurite outgrowth, cell signalling and axonal transport (2–4). Strikingly, tau is prone to aggregate in numerous pathologies that lead to neurodegeneration. Tau accumulation in filamentous structures such as paired helical filaments, which form neurofibrillary tangles (NFTs), is the principal feature of tauopathies including corticobasal dementia, frontotemporal dementia and parkinsonism linked to chromosome 17, progressive supranuclear palsy and Pick’s disease, and is also a primary feature of Alzheimer’s disease (AD) (5,6). In tauopathies, tau aggregation is associated with neuronal loss, suggesting a fundamental role for tau in the survival of neurons. Although pathological tau mutations have been identified in inherited tauopathies, aggregated tau in AD is composed of wild-type tau that has been subjected to post-translational modifications including anomalous hyper-phosphorylation and cleavage.

The phosphorylation state of tau is tightly regulated by kinases and phosphatases which target at least 45 sites, some of which modify the ability of tau to bind microtubules (3,7). Among the numerous kinases that phosphorylate tau, glycogen synthase kinase 3β (GSK3β), a proline-directed serine/threonine kinase, is highly expressed in the brain and an increase
in GSK3β levels has been observed in AD (8). Indeed, the dysregulation of GSK3β has been identified as playing a key role in the development of AD and other neurodegenerative disorders (8–10). GSK3β activity is negatively regulated by insulin and Wnt signalling. Exposure of neurons to Amyloid-β peptide (Aβ), which accumulates as Aβ plaques in AD, increases GSK3β activity by antagonizing insulin and blocking the canonical Wnt signalling pathway (11–13). In the hippocampus, GSK3β has been described to be involved in long-term potentiation and depression, two molecular mechanisms essential for learning and memory processes (14). GSK3β is involved in a wide range of cellular activities through the phosphorylation of numerous substrates. In neurons, GSK3β is implicated in apoptosis, synaptic plasticity, axon formation and neurogenesis (10,14,15). GSK3β overexpression in cells and upregulation in transgenic mice enhance tau phosphorylation (16,17). Furthermore, GSK3β has been shown to promote tau aggregation in tangle-like structures in vitro (18,19). On the contrary, lithium treatment, which inhibits GSK3 activity, reduces tau phosphorylation in cells and a mouse model of AD (20). Hence, the development of GSK3 inhibitors is an important strategy for treating AD and other neurodegenerative disorders (15).

Misfolded and non-functional proteins are primarily targeted for degradation by the ubiquitin proteasome system (UPS). In AD, the presence of aggregated tau has been reported to inhibit UPS activity, and thus impair clearance of tau aggregates and exacerbate tau pathology (21). NFTs are often labeled with ubiquitin, and the ubiquitin-like modifier NEDD8 (22). NEDD8 is conjugated to proteins in a similar manner to ubiquitin, but through an independent mechanism. A widely characterized target of NEDD8 is the cullin subunit of the cullin-RING E3 ubiquitin ligases, neddylation of which is necessary for the correct activity of the ligase (23). NEDD8 has been reported to be regulated by the NEDD8 ultimate buster 1 (NUB1) (24). NUB1 possesses an ubiquitin-like (UBL) domain at its N-terminus and two ubiquitin-associated (UAB) domains at its C-terminus and thus belongs to the UBL/UBA family of proteins (24,25). The UBL/UBA proteins are mostly known as proteasome shuttles since the UBL motif is thought to bind the proteasome, while the UBA domains recognize proteasome-targeted substrates (25). Indeed, NUB1L, a longer isoform with an additional UBA domain, interacts directly with the Rpn1 and Rpn10 subunits of the 20S proteasome via its UBL domain (26). NUB1 and NUB1L facilitate the proteasomal degradation of the ubiquitin-like modifiers NEDD8 and FAT10, and protein conjugates thereof, through non-covalent interaction with these ubiquitin-like modifiers (24,27,28).

A number of studies have identified a role for NUB1 in various cellular mechanisms. NUB1 binds AIPL1, mutations in which cause severe neurodegeneration of retinal photoreceptors resulting in early-onset blindness (29). The interaction of NUB1 with AIPL1 abrogates the NUB1-mediated degradation of FAT10 substrates (29). NUB1 can also bind and target the proteasomal degradation of synphilin-1 to suppress the formation of Lewy body-like inclusions, and NUB1 has been shown to co-localize with α-synuclein in presynapses in Lewy body disease (30,31). Finally, NUB1 promotes the cytoplasmic localization and inhibits the transcriptional activity of p53 by reducing the modification of p53 with NEDD8 and conversely stimulating p53 ubiquitination (32). Therefore, NUB1 regulation of substrate proteins is implicated in pathologies including cancer and neurodegeneration.

In the present study, we have tested the hypothesis that NUB1 affects tau degradation and aggregation. We highlight a new role for NUB1 in regulating GSK3β stability and consequently tau phosphorylation and aggregation. Therefore, NUB1 may have an important role in the regulation of GSK3β and tau homeostasis in numerous neurodegenerative disorders.

RESULTS
Wild-type tau forms cellular inclusions when phosphorylated by GSK3β

Aggregation of tau was first optimized in human SK-N-SH neuroblastoma cells by ectopic expression of the largest isoform of wild-type human tau fused to the C-terminus of enhanced green fluorescent protein (EGFP) (33). Western blot analysis demonstrated that SK-N-SH cells do not express endogenous tau protein in detectable amounts, and EGFP-tau was detected with antibodies to tau and GFP (Fig. 1A). Immunocytochemistry revealed that EGFP-tau co-localized with β-tubulin and induced the formation of microtubule bundles (Fig. 1B, arrows). In the absence of proteasome inhibition or co-expression of GSK3β, the formation of cellular inclusions of EGFP-tau over time was rarely observed (<2%) (Supplementary Material, Fig. S1A). Interestingly, inhibition of the proteasome alone was sufficient to induce formation of EGFP-tau inclusions in a time-dependent manner. EGFP-tau inclusions were observed in ~18% of EGFP-tau transfected cells following 4 h of MG132 treatment (Supplementary Material, Fig. S1A). The inclusions were β-tubulin negative, suggesting that aggregated tau was not associated with microtubules (Fig. 1B, arrowheads).

The effect of co-expression of haemagglutinin (HA)-tagged GSK3β on the formation of EGFP-tau inclusions was assessed in SK-N-SH cells, which express very low levels of endogenous GSK3β (data not shown). The phosphorylation of EGFP-tau by GSK3β was detected by western blotting at the AT8 epitope (S199/S202/T205) and serine 396 (S396), both of which are targets of GSK3β phosphorylation (Fig. 2A). A basal level of EGFP-tau phosphorylation on S396 (pS396-tau) was observed, which increased with GSK3β co-expression. In contrast, phosphorylation of tau on the AT8 epitope was only detected in the presence of GSK3β. An upward shift in the electrophoretic mobility of EGFP-tau was observed with GSK3β co-expression, indicative of increased phosphorylation of EGFP-tau. The interaction between GSK3β and EGFP-tau in SK-N-SH cells was analysed by co-immunoprecipitation. GSK3β was specifically co-immunoprecipitated only when EGFP-tau was also present (Fig. 2B). Reciprocally, EGFP-tau was co-immunoprecipitated with GSK3β (Fig. 2C). Therefore, GSK3β interacts with EGFP-tau in SK-N-SH cells to mediate its phosphorylation.

In addition, co-expression of GSK3β led to increased cytoplasmic EGFP-tau, and induced the formation of EGFP-tau inclusions to which GSK3β was recruited (Fig. 2D, arrowheads).
Indeed, the co-expression of GSK3β in the absence of proteasome inhibition was sufficient to induce the formation of inclusions in ∼6% of EGFP-tau transfected cells, representing a basal incidence of EGFP-tau inclusions that remained unchanged over time (Supplementary Material, Fig. S1B). The formation of EGFP-tau inclusions in the presence of GSK3β was further exacerbated by inhibition of the proteasome with the number of inclusions increasing in a time-dependent manner. EGFP-tau inclusions were counted in ∼24% of transfected cells after 4 h of treatment with MG132 (Supplementary Material, Fig. S1B). Quantification of detergent insoluble EGFP-tau aggregates following the formation of visible cellular inclusions revealed that while the co-expression of GSK3β alone or treatment with MG132 led to a ∼1.5-fold and ∼2-fold increase in the levels of aggregated EGFP-tau respectively, there was a ∼3-fold increase in the levels of aggregated EGFP-tau following both inhibition of the proteasome and co-expression of GSK3β (Fig. 2E). The tau inclusions were positive for thioflavin S (Supplementary Material, Fig. S1C). Moreover, both S396 and AT8 phosphorylated EGFP-tau co-localized with EGFP-tau inclusions in SK-N-SH cells (Fig. 2F, arrowheads). These data confirm the GSK3β-dependent phosphorylation of EGFP-tau on disease-associated epitopes and the aggregation of phosphorylated EGFP-tau that is exacerbated by inhibition of the proteasome (17,18,34).

NUB1 interacts with tau

SK-N-SH cells do not express endogenous NUB1 protein at readily detectable levels (Supplementary Material, Fig. S2A and B). Ectopic expression of GFP-NUB1 in SK-N-SH cells resulted in ∼73% nuclear localization, with the remainder being present in the cytoplasm (Supplementary Material, Fig. S2C). Proteasome inhibition induced a shift in the distribution of GFP-NUB1 with a decrease in nuclear fluorescence (∼52% of total) and a concomitant increase in cytoplasmic fluorescence (from ∼27 to ∼48% of total). This suggests an active translocation of NUB1 from the nucleus, and/or a relative stabilization of cytoplasmic NUB1 due to inhibition of the proteasome.

We next examined the association and co-localization of NUB1 with tau in SK-N-SH cells. We found a reciprocal co-immunoprecipitation of NUB1 in EGFP-tau immunoprecipitates (Fig. 3A) and of EGFP-tau in NUB1 immunoprecipitates (Fig. 3B), suggesting an interaction of these two proteins in neuroblastoma cells. The subcellular distribution of GFP-NUB1 in the presence of DsRed-tau was investigated (Fig. 3C). NUB1 exhibited primarily nuclear localization as previously observed. However, in contrast to the distribution of NUB1 alone (Supplementary Material, Fig. S2C), NUB1 co-localized with tau-positive microtubule bundles (Fig. 3C, arrows). Pearson’s coefficient (35), a measure of the co-recruitment of these
proteins, in the microtubule bundles was 0.67 ± 0.18 in the absence of MG132 (Fig. 3C). The distribution of tau was not altered by co-expression of NUB1 (compare Fig. 1B with Fig. 3C). MG132 treatment induced a shift of NUB1 towards the cytoplasm and formation of tau inclusions as before. NUB1 was recruited to both tau-positive microtubule bundles (Pearson’s coefficient = 0.60 ± 0.02) and inclusions (Pearson’s coefficient = 0.94 ± 0.05) following proteasome inhibition.

NUB1 interacts with GSK3β

Since we found that both NUB1 and GSK3β were associated with tau and recruited to tau inclusions, we next examined the association of NUB1 with GSK3β in SK-N-SH cells. We detected NUB1 in GSK3β immunoprecipitates (Fig. 3D) and reciprocally, GSK3β co-immunoprecipitated with NUB1 (Fig. 3E), suggesting that these two proteins are present in a complex. In addition, we found that NUB1 co-localized with GSK3β in the cytoplasm, and this became more prominent following the increased localization of NUB1 in the cytoplasm induced by inhibition of the proteasome (Fig. 3F). NUB1 expression also resulted in the enhanced localization of GSK3β in the nucleus (compare Fig. 3F with Fig. 2D). Therefore, NUB1 might directly influence the intracellular distribution of GSK3β.

NUB1 co-localizes with tau and GSK3β in primary neurons

NUB1 co-localized with exogenous tau and GSK3β and formed a complex with each in SK-N-SH cells. We next
examined the localization of NUB1 with endogenous tau and GSK3β in primary rat cortical neurons. Cultured neurons (5 days in vitro) were transfected with a plasmid expressing GFP-NUB1 (Fig. 4). NUB1 was localized prominently in the nucleus, and to a lesser extent in the cell body and neurites. NUB1 and endogenous tau were both detected in the cell body (Fig. 4A and I). Moreover, NUB1 co-localized with tau in primary neurites and noticeably in the varicosities (Fig. 4A, II, arrowheads). NUB1 expression also overlapped with that of endogenous GSK3β in the cytoplasm and the proximal neurites (Fig. 4B and I). GSK3β localization in the varicosities with NUB1 was not prominent (Fig. 4B, II, arrow). These data show that NUB1 co-localized with both endogenous tau and GSK3β in...
primary neurons, suggesting a potential interaction between the proteins.

NUB1 disrupts the tau–GSK3β interaction

Both NUB1 (Fig. 3C) and GSK3β (Fig. 2D) were independently recruited to tau inclusions and formed a complex with tau. Thus, the interplay between NUB1, GSK3β and tau were next examined in SK-N-SH cells following proteasome inhibition to maximise inclusion formation (Fig. 5). NUB1 co-localized with tau in the microtubule bundles (Fig. 5A, arrows) and inclusions (Fig. 5A, arrowheads). However, in the presence of NUB1, GSK3β was detected in some tau-containing bundles (arrows), but was excluded from all tau-positive inclusions. Since GSK3β is recruited to tau inclusions
in the absence of NUB1 (Fig. 2D), this suggests that NUB1 prevents the recruitment of GSK3β to tau inclusions.

Immunoprecipitation (IP) of tau and NUB1 from SK-N-SH cells co-expressing all three proteins was performed to assess a potential ternary association. When all three proteins were expressed together in SK-N-SH cells, less GSK3β co-immunoprecipitated with tau (compared with that present in GSK3β-tau complexes in the absence of NUB1) (Fig. 5B). The proportion of tau phosphorylated on S396 immunoprecipitated with the anti-tau antibody was similar when tau was co-expressed with NUB1 and GSK3β or with GSK3β alone. NUB1 co-immunoprecipitated pS396-tau only when GSK3β was present. When all the three proteins were co-expressed, the amount of GSK3β and tau co-immunoprecipitating with NUB1 was reduced by ~95% and ~75% respectively (Fig. 5C), whereas the amount of NUB1 immunoprecipitated was unchanged. These results show that NUB1 interferes with the association of GSK3β and tau, suggesting that the three proteins may compete for a common binding site on NUB1 within the ternary protein complex.

**NUB1 reduces tau phosphorylation and aggregation**

NUB1 disrupts the interaction between tau and GSK3β, and prevents the recruitment of GSK3β to tau inclusions. Therefore, we examined the influence of different NUB1 species, including those lacking the UBL and UBA domains, on the formation of tau inclusions in SK-N-SH cells. HA-tagged NUB1, NUB1L, and NUB1L lacking either the UBL domain (NUB1LΔUBL) or all three UBA domains (NUB1LΔUBA1-3) (Fig. 6A) (27) were expressed at similar levels in SK-N-SH cells (Fig. 6B). To analyze their effect on tau aggregation, the percentage of EGFP-tau transfected cells with inclusions was quantified with increasing amounts of each of the NUB1 constructs. While the number of EGFP-tau transfected cells remained unchanged (~36%), we found that increasing NUB1 expression induced a concentration-dependent decline in the percentage of inclusions for all NUB1 species with the exception of NUB1LΔUBA1-3 (Supplementary Material, Fig. S3). The ability of NUB1, NUB1L, NUB1LΔUBL and NUB1LΔUBA1-3 to suppress the formation of inclusions was directly compared (Fig. 6C). In the absence of proteasome inhibition, the percentage of EGFP-tau transfected cells with inclusions (control) was 16 ± 2%. This percentage was significantly decreased to 11 ± 1%, 12 ± 1% and 11 ± 1% (all P-values < 0.017) with NUB1, NUB1L and NUB1ΔUBL, respectively. In contrast, the percentage of EGFP-tau transfected cells with inclusions in the presence of NUB1LΔUBL was 16 ± 1% was unchanged compared with the control. Inhibiting the proteasome increased the percentage of EGFP-tau transfected cells with inclusions to 24 ± 2% (Fig. 6C, control). All of the NUB1 species with the exception of HA-NUB1LΔUBL (22 ± 2%, P = 0.11) decreased the percentage of transfected cells with inclusions.

We next examined the effect of the different NUB1 species on the aggregation of EGFP-tau (Fig. 6D). Similar to the quantitation of the percentage of EGFP-tau inclusions, we found that all of the NUB1 species with the exception of NUB1LΔUBA1-3 significantly decreased the levels of aggregated EGFP-tau in the absence of MG132. Proteasome inhibition increased the levels of aggregated EGFP-tau, but these levels were again significantly decreased by all the NUB1 species with the exception of NUB1LΔUBA1-3. Thus, NUB1 reduces the aggregation of tau and the formation of tau inclusions in a manner that is dependent on the presence of the UBA domains but not the UBL domain.

NUB1 might prevent tau aggregation by reducing tau phosphorylation. We therefore quantified the amount of total tau and S396 phosphorylated tau to determine whether NUB1 reduces tau phosphorylation at this site. Neither NUB1 nor any of the NUB1L species significantly affected the total amount of tau (phosphorylated and unphosphorylated) without proteasome inhibition (data not shown). The co-expression of GSK3β increased the level of S396 phosphorylated tau ∼18.4-fold (Fig. 6E). However, NUB1, NUB1L, NUB1LΔUBL or NUB1LΔUBA1-3 all significantly and efficiently reduced S396 phosphorylated tau by ∼75, ∼80, ∼78 and ∼81%, respectively, in the absence of MG132 (all P-values < 0.005) (Fig. 6E). In the presence of MG132, GSK3β-dependent phosphorylation of tau on S396 was increased slightly by ∼4%; however, all the NUB1 species significantly and effectively reduced the amount of tau phosphorylated on S396 (Fig. 6E). This result shows that NUB1 reduces the GSK3β-dependent phosphorylation of tau, independently of its UBL or UBA domains.

We next examined the interaction of the different NUB1 species with EGFP-tau (Fig. 6F) and GSK3β (Fig. 6G). All of the NUB1 species were able to interact with EGFP-tau. However, while the interaction of NUB1LΔUBL was considerably more efficient, the interaction of NUB1LΔUBA1-3 was significantly weaker (Fig. 6F). Similarly, the interaction of NUB1LΔUBL with GSK3β was more efficient, but an interaction between NUB1LΔUBA1-3 and GSK3β could not be detected (Fig. 6G). Hence, whilst the UBL domain is dispensable for the interaction with EGFP-tau and GSK3β, the UBA domain is necessary for the interaction with GSK3β.

**NUB1 regulates GSK3β stability**

NUB1 is a UBA/UBL domain protein that interacts directly with the proteasome to facilitate the degradation of target proteins. To determine whether NUB1 might induce GSK3β degradation and hence decrease tau phosphorylation, SK-N-SH cells were treated with cycloheximide (CHX) alone or in the presence of MG132, and the amount of GSK3β measured after 2 and 4 h in the absence and presence of NUB1 (Fig. 7A). The levels of GSK3β were normalized to the control (0 h CHX treatment) to plot the rate of turnover (Fig. 7B and Supplementary Material, Fig. S4A). In the absence of MG132, NUB1 accelerated the degradation of GSK3β (Fig. 7A and B). Interestingly, there was a biphasic degradation pattern of GSK3β with accelerated degradation in the presence of NUB1 within the first 2 h of CHX treatment. In the absence of NUB1, the amount of GSK3β present was unchanged after 2 h, but reduced to ∼55% of its initial value 4 h after CHX treatment. NUB1 resulted in significantly greater reductions in GSK3β to ∼88% (P = 0.041) and ∼15% (P = 0.005), respectively, at 2 and 4 h CHX treatment. This demonstrates that NUB1 was able to significantly reduce GSK3β levels (Fig. 7A) and accelerate its turnover (Fig. 7B).
Figure 6. NUB1 reduces tau aggregation and phosphorylation. (A) Schematic of NUB1, NUB1L, NUB1ΔUBL and NUB1ΔUBA1-3. (B) Cell lysates from SK-N-SH cells expressing HA-NUB1, HA-NUB1L, HA-NUB1ΔUBL or HA-NUB1ΔUBA1-3 were analysed by immunoblotting to detect the NUB1 species. Anti-NUB1 specifically detects the four recombinant NUB1 proteins, HA-NUB1, HA-NUB1L, HA-NUB1ΔUBL and HA-NUB1ΔUBA1-3 as single bands of ~70.5, ~71.9, ~61.5 and ~54.5 kDa, respectively. (C) SK-N-SH cells were transfected with plasmids expressing EGFP-tau and HA-GSK3β (control), and HA-NUB1, HA-NUB1L, HA-NUB1ΔUBL or HA-NUB1ΔUBA1-3 as indicated. Cells were fixed and the presence of EGFP-tau inclusions counted in at least 4 × 100 transfected cells for each experimental condition, blind to experimental status. Only HA-NUB1ΔUBA1-3 failed to reduce the number of transfected cells with inclusions. (D) SK-N-SH cells were transfected with plasmids expressing EGFP-tau and HA-GSK3β (control), and HA-NUB1, HA-NUB1L, HA-NUB1ΔUBL or HA-NUB1ΔUBA1-3 as indicated. Cell lysates were prepared and the levels of detergent insoluble aggregates of EGFP-tau quantitated by ImageJ analysis following the filter trap assay. HA-NUB1ΔUBA1-3 does not reduce the levels of aggregated EGFP-tau. (E) S396 phosphorylated EGFP-tau
Proteasome inhibition led to an ~1.5- and ~1.3-fold increase in GSK3β levels in the absence and presence of NUB1, respectively (0 h CHX treatment) (Fig. 7A). However, normalization of GSK3β levels revealed that the rate of GSK3β degradation in the absence and presence of NUB1 was unchanged by inhibition of the proteasome (Supplementary Material, Fig. S4). Interestingly, NUB1 levels also decreased following CHX treatment, with ~26% remaining after 4 h CHX treatment compared with the initial amount at 0 h.

In order to address whether the effect of NUB1 on GSK3β stability depends on the UBL and/or the UBA domains of NUB1, the CHX assays were repeated with HA-tagged species NUB1L, NUB1LΔUBL and NUB1LΔUBA1-3 in the absence and presence of MG132. Plotting the turnover of GSK3β in the absence of proteasome inhibition revealed that while NUB1L similarly accelerated the degradation of GSK3β (Fig. 7C), both NUB1LΔUBL (Fig. 7D) and NUB1LΔUBA1-3 (Fig. 7E) were defective.

**NUB1 depletion alters GSK3β levels and tau phosphorylation**

To confirm the effect of NUB1 on GSK3β, the turnover of endogenous GSK3β by NUB1 was explored in SH-SY5Y neuroblastoma cells which, in contrast to SK-N-SH cells, express both endogenous NUB1 and GSK3β. First, the association of NUB1 with endogenous GSK3β was confirmed in SH-SY5Y cells (Fig. 7F). Endogenous GSK3β was specifically co-immunoprecipitated with NUB1 and, reciprocally, endogenous GSK3β co-immunoprecipitated NUB1 (Fig. 7F). The effect of NUB1 on endogenous GSK3β levels was tested by silencing endogenous NUB1 with a small interfering RNA (siRNA) specific to NUB1 mRNA. NUB1 expression was specifically and effectively silenced by the NUB1-specific siRNA, but not by the control siRNA or in mock transfected cells (Fig. 7G). Quantification revealed that endogenous GSK3β was significantly stabilized (~1.6-fold) following the silencing of NUB1 expression compared with cells transfected with non-specific control siRNA and mock transfected cells (0 h CHX treatment) (Fig. 7H). Moreover, silencing of NUB1 expression delayed the degradation rate of GSK3β (Fig. 7I). Indeed, after 2 h of CHX treatment, the GSK3β levels following the silencing of NUB1 expression (103 ± 8.7%) were significantly higher than the GSK3β levels in control siRNA and mock transfected cells (62 ± 7.5% and 51 ± 5.1%, respectively, P = 0.001). After 6 h of CHX treatment, the levels of GSK3β were 18 ± 4% and 21 ± 3.8% in mock and control siRNA transfected cells, respectively, whereas they were significantly higher (47 ± 5.6%) following the silencing of NUB1 expression. The increased levels and stability of endogenous GSK3β following the siRNA-mediated silencing of NUB1 expression confirm a physiological role for NUB1 in GSK3β turnover and degradation.

The siRNA-mediated silencing of endogenous NUB1 in SH-SY5Y cells was further used to determine the effect on endogenous tau following the treatment of cells with okadaic acid (OA) to inhibit endogenous phosphatase activity and reveal phosphorylation. The OA treatment decreased the electrophoretic mobility of endogenous tau in SH-SY5Y cells detected with a pan-tau antibody; however, the total levels of tau were not affected by the silencing of endogenous NUB1 (Fig. 7J, pan-tau). Endogenous tau phosphorylated on the AT8 epitope (S199/S202/T205) was not detected in the absence of OA treatment (Fig. 7I, AT8). The OA treatment induced multiple high-molecular weight species of tau phosphorylated at this epitope, the formation of which was exacerbated by the silencing of NUB1 expression (Fig. 7J, AT8). A high AT8 immunoreactive tau was observed in the stacking gel in response to NUB1 siRNA. Therefore, in addition to the role of NUB1 in modulating the stability of GSK3β, NUB1 may also have an effect on endogenous tau, though whether this effect is direct or mediated via GSK3β has yet to be determined.

**DISCUSSION**

This study has revealed that the stability of the important cellular kinase, GSK3β, is regulated by the proteasome adaptor NUB1, which consequently modulates the effect of GSK3β on its substrate tau.

In neuroblastoma cells, tau induced the formation of microtubule bundles as reported previously and in accordance with its function as a microtubule-associated protein (36). The expression of GSK3β and the inhibition of the proteasomal activity decreased bundle formation and induced the formation of insoluble tau aggregates that were positive for thioflavin S. Indeed, GSK3β was found to interact with tau, and enhanced the formation of tau inclusions to which it was recruited. Moreover, the aggregation of tau in the presence of GSK3β correlated with an increase in the levels of tau phosphorylated on S396 and the AT8 epitope, characteristically detected following the pathological phosphorylation of tau (37). Indeed, tau phosphorylated on the AT8 epitope could not be detected in the absence of GSK3β co-expression, suggesting that the expression of endogenous kinases in SK-N-SH cells was not sufficient to induce tau hyperphosphorylation, or that their activity was compensated by the endogenous phosphatases. Therefore, GSK3β promoted the abnormal phosphorylation and aggregation of wild-type tau in neuroblastoma cells, and was used as a model to determine the role of NUB1.

NUB1 co-localized with tau in SK-N-SH cells, but also with endogenous tau in primary cortical neurons. In SK-N-SH cells, was detected by immunoblotting of SK-N-SH cell lysates prepared from cells expressing EGFP-tau alone, or co-expressing both EGFP-tau and HA-GSK3β in the presence of HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL or HA-NUB1LΔUBA1-3 as indicated. The levels of S396 phosphorylated EGFP-tau were quantified using ImageJ and normalized to the S396 phosphorylated GSK3β levels in the presence of HA-GSK3β and without proteasome inhibition. All the NUB1 species are able to strongly reduce the levels of GSK3β phosphorylated on S396, in the absence or presence of MG132. *P < 0.05, **P < 0.01, ***P < 0.001. (F, G) SK-N-SH cells were transfected with plasmids expressing EGFP-tau (F) or HA-GSK3β (G) and HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL or HA-NUB1LΔUBA1-3 as indicated. Proteins were immunoprecipitated with anti-tau (F) or anti-GSK3β (G) and protein complexes analysed by immunoblotting, as indicated. The asterisk represents the non-specific IgG band detected by the HA antibody. The UBA domain, but not the UBL domain, is required for efficient interaction.
NUB1 co-localized with tau in the microtubule bundles and was recruited to tau inclusions. Moreover, NUB1 and tau were reciprocally co-immunoprecipitated with one another, suggesting that the two proteins interact either directly or indirectly in a complex. In primary neurons, the localization of NUB1 overlapped with that of endogenous tau and,
Interestingly, NUB1 accumulated with tau in what appeared to be varicosities or presynaptic boutons. These boutons are swellings of the axons rich in neurotransmitter substances and involved in intercellular signal transduction (38). This raises the interesting possibility that NUB1 and tau could cooperate in the regulation of neurotransmitter trafficking or signal transduction at synapses. Indeed, NFT-bearing neurons in AD have reduced synaptic proteins such as synaptofysin compared with NFT-free neurons (39). A study of AD synaptosomes showed that Aβ peptides were found to aggregate in the synaptosomes which were also decorated with phosphorylated tau (40).

The expression of NUB1 reduced the formation of tau inclusions and decreased the levels of tau phosphorylated on S396. Similarly, the mutant NUB1ΔUBL had a significant effect on tau aggregation and decreased the overall levels of tau phosphorylated on S396; thus, it would seem that the UBL domain of NUB1 is not necessary for its effect on either tau aggregation or tau phosphorylation. In accordance with this finding, NUB1ΔUBL was able to interact strongly with both tau and GSK3β. In contrast, NUB1ΔUBA1-3 failed to reduce tau aggregation, suggesting that the UBA domains of NUB1 are necessary for its effect on tau aggregation. However, the mutant was able to decrease S396 phosphorylated tau levels. The reason why NUB1ΔUBA1-3 was defective in reducing the aggregation of tau despite decreasing the levels of phosphorylated tau is unclear. A potential explanation is that the interaction of NUB1ΔUBA1-3 with tau was sufficient to disrupt the interaction of GSK3β with tau, thereby reducing the levels of GSK3β-mediated tau phosphorylation on S396. In addition, it cannot be ruled out that NUB1 may exert a direct or independent effect on tau itself to affect its aggregation that requires the UBA domains and is therefore impaired in the NUB1ΔUBA1-3 mutant, effectively uncoupling the regulation of tau phosphorylation and aggregation. Indeed, the formation of high-molecular weight insoluble tau species was induced by the absence of NUB1 and increased hyperphosphorylation of tau, suggesting that NUB1 might function as a chaperone for tau, or independently influence cellular components involved in the conformational regulation of tau or in its cleavage by caspases, which have been implicated in tau aggregation (4).

Our data indicate that NUB1 could also influence tau phosphorylation and aggregation by modulating the tau kinase GSK3β. Indeed, the presence of NUB1 in SK-N-SH cells disrupted the interaction of tau and GSK3β, which correlated with the decreased levels of S396 phosphorylated tau and with the fact that GSK3β could no longer be detected in tau inclusions. NUB1 and GSK3β interacted reciprocally in both SK-N-SH and SH-SY5Y cells. Moreover, NUB1 co-localized with GSK3β in the cytoplasm in SK-N-SH cells and primary cortical neurons. Although GSK3β is essentially a cytoplasmic protein, GSK3β was also localized in the nucleus in the presence of NUB1 in SK-N-SH cells. Thus, similar to the translocation of p53 with NUB1 overexpression (32), NUB1 could regulate GSK3β function by influencing its cellular sub-localization.

NUB1 interacts directly with the proteasome to facilitate the degradation of target proteins (24–26); therefore, we examined whether NUB1 could target GSK3β. Indeed, NUB1 accelerated the turnover of GSK3β in SK-N-SH cells. Interestingly, both the UBL and UBA domains were necessary for NUB1 to accelerate the turnover of GSK3β, since this function was lost with either NUB1ΔUBL or NUB1ΔUBA1-3. The NUB1ΔUBA1-3 mutant was unable to interact with GSK3β, hence its inability to influence the turnover of GSK3β. However, the NUB1ΔUBL mutant was unable to accelerate the turnover of GSK3β despite the strong interaction of NUB1ΔUBL with GSK3β. Therefore, the UBL domain is necessary for the NUB1-mediated turnover of GSK3β. The UBL domain mediates the direct interaction of NUB1 with the proteasome (26) suggesting the proteasome-mediated degradation of GSK3β. However, proteasome inhibition induced increased levels of GSK3β, but the rate of turnover remained unchanged. The inhibition of proteasomal degradation may induce the subsequent removal via an alternative route, such as autophagy (41).

In comparison to the over-expression studies in SK-N-SH cells, the down regulation of endogenous NUB1 in SH-SY5Y cells by RNA interference greatly stabilized the levels of endogenous GSK3β and decreased the rate of turnover. Therefore, NUB1 might target GSK3β for proteasomal degradation. However, in this study, it has yet to be identified whether GSK3β is a substrate for modification by NEDD8 or FAT10, or whether the effect of NUB1 on GSK3β occurs independently of post-translational modification by ubiquitin-like modifiers.

In conclusion, this study has identified an important and novel role for NUB1 not only in the regulation of GSK3β, but also in the aggregation and phosphorylation of the GSK3β substrate tau. Therefore, NUB1 may play an important role in the neuropathogenesis of tauopathies and other neurodegenerative disorders. Moreover, NUB1 may influence the GSK3β-dependent phosphorylation and regulation of other GSK3β substrates, thereby implicating NUB1 in a multitude of important cellular processes.

MATERIALS AND METHODS

Antibodies

A rabbit polyclonal antibody to actin and mouse monoclonal antibodies to FLAG, HA, β-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Sigma (Poole, UK). A rabbit polyclonal antibody to tau was purchased from Dako (Cambridgeshire, UK). A mouse monoclonal antibody to GFP was purchased from Roche Diagnostic (Burgess Hill, UK). A rabbit polyclonal antibody to GSK3β was purchased from Cell Signalling (Hertfordshire, UK). Horseradish peroxidase-conjugated goat anti-mouse (GoM-HRP) and anti-rabbit secondary antibodies (GoR-HRP), and mouse monoclonal antibody AT8 were purchased from Thermo Scientific (Northumberland, UK). A rabbit polyclonal antibody to S396 phosphorylated tau, AlexaFluor 594 and 647 monoclonal anti-mouse secondary antibodies and AlexaFluor 594 polyclonal anti-rabbit secondary antibody were purchased from Invitrogen (Paisley, UK).

Plasmids

The expression plasmids pcDNA3.1-HA-NUB1, -HA-NUB1L, -HA-NUB1ΔUBL and -HA-NUB1ΔUBA1-3 were described
previously (28). NUB1-p3XFLAG-CMV (NUB1-FLAG) and pEGFP-C1-NUB1 (GFP-NUB1) were described previously (29,42). The plasmids pEGFP-C1-tau (EGFP-tau) and pCMV-DsRed-tau (DsRed-tau) that express the 0N4R tau isoform of human wild-type tau, and pMT2-HA-GSK3β were described previously (33,43).

Cell culture and transfection
SK-N-SH and SH-SYSY neuroblastoma cells (ECACC, Salisbury, UK) were maintained and grown as described (42). Cells were transfected 24 h after plating using Lipofectamine and Plus reagent according to the manufacturer’s instructions (Invitrogen, Paisley, UK). The cells were transfected as indicated with 100 ng of HA-NUB1, HA-NUB1L, HA-NUB1ΔUBL, HA-NUB1ΔUBAI-3, GFP-NUB1 or NUB1-FLAG, 150 ng of HA-GSK3β and 50 ng of EGFP-tau or DsRed-tau per well in eight-well chamber slides, and empty plasmid DNA added as necessary to equalize the total amount of DNA transfected. The amounts of DNA transfected were scaled up 8-fold per well for transfection of cells in six-well plates. The treatment of cells with 50 μM proteasome inhibitor MG132 (Z-Leu-Leu-Leu-al, Enzo Life Sciences, Plymouth, UK) or dimethyl sulfoxide (DMSO) was performed 28 h post-transfection for 4 h.

Embryonic day 18 rat cortical primary neurons were prepared and maintained as described (44). Cells were transfected 5 days after plating, using Lipofectamine 2000 (Invitrogen, Paisley, UK) and 3 μg of GFP-NUB1 per well in 12-well plates.

Immunocytochemistry
Twenty-eight hours post transfection, SK-N-SH cells were rinsed once with warm (37°C) phosphate buffered saline (PBS) and once with warm (37°C) PEM buffer (80 mM Pipes (pH 6.8), 5 mM EGTA, 1 mM MgCl2), and then fixed with 0.3% glutaraldehyde/0.5% Nonidet P-40 (NP-40) for 10 min at 37°C. Twenty-four hours after transfection, the rat cortical primary neurons were washed 3× with warm PBS (37°C) and fixed with 4% paraformaldehyde for 10 min at 37°C. Cells were permeabilized in 0.1% Triton X-100 for 5 min. SK-N-SH neuroblastoma cells and the rat cortical primary neurons were then processed in the same way for immunocytochemistry. Briefly, the cells were incubated in block solution (10% goat serum, 3% bovine serum albumin (BSA) in PBS) for 45 min, followed by incubation with the primary antibody anti-tau (1:2000), anti-β-tubulin (1:150), anti-GSK3β (1:100), anti-HA (1:500) or anti-pS396-tau (1:500) in block solution for 1 h. The cells were washed prior to incubation with the AlexaFluor 594 or 647 secondary antibody (1:1000) in block solution for 45 min. The cells were washed, incubated with 4’,6-diamidino-2-phenylindole (DAPI) (2 μg/ml in PBS) for 5 min and mounted with Dako mounting medium (Dako, Cambridgeshire, UK). For the detection of thioflavin S, the cells were fixed and treated with 0.05% thioflavin S for 8 min. The cells were washed three times in 80% ethanol and mounted with Dako mounting medium. All images were taken with a Carl Zeiss LSM700 confocal microscope (Carl Zeiss Ltd, Hertfordshire, UK).

Cell counts
To calculate the percentage of EGFP-tau inclusions in transfected cells, a minimum of four fields of 100 transfected cells were counted and scored for the presence of inclusions, blind to experimental status. ImageJ (http://rsbweb.nih.gov/ij/) was used to measure the fluorescence intensity of GFP-NUB1 in the nucleus and the cytoplasm (total fluorescence intensity minus nuclear fluorescence intensity) in at least 100 transfected cells blind to experimental status.

Pearson’s coefficient
Pearson’s coefficient was measured to determine the co-recruitment of GFP-NUB1 and DsRed-tau to microtubules and inclusions. Pearson’s coefficient was calculated using JACoP (http://rsbweb.nih.gov/ij/plugins/track/jacop.html) in ImageJ. At least three different cells for DsRed-tau and GFP-NUB1 co-localization were analysed. Pearson’s coefficient indicates the degree of overlap and scores between (−1) and (+1), where (−1) indicates total exclusion, (+1) a perfect image registration and (0) a random localization (35).

Immunoprecipitation
IPs were performed using Dynabeads (Invitrogen, Paisley, UK). The beads were pre-washed three times with RIPA buffer (1% (w/v) sodium deoxycholate, 150 mM NaCl, 1% (v/v) NP-40, 0.1% (w/v) sodium monododecyl sulphate (SDS), 50 mM Tris-HCl (pH7.5)) containing 0.02% (v/v) Tween 20 (RIPA-T buffer), and blocked overnight with 0.2% (w/v) BSA and Tween 20 (0.02% (v/v)). Cells were lysed 28 h post-transfection with 200 μl of RIPA buffer supplemented with 5% protease inhibitor cocktail (PIC) and 1% phosphatase inhibitor cocktail (PhIC). Thirty microlitres of cell lysate was removed, mixed with 10 μl of 4× loading buffer and kept at 4°C for the input fraction. The remaining supernatant was incubated with 25 μl of pre-washed beads and anti-FLAG (1:500), anti-tau (1:1000), anti-HA (1:500) or anti-GSK3β (1:100) primary antibodies overnight at 4°C. Alternatively, as controls, samples were incubated with a non-specific primary antibody (but raised in the same species) (IgG), or with the beads only (BO). The supernatant was removed and the Dynabeads were washed three times with RIPA-T buffer. Proteins were eluted in 40 μl of 4× loading buffer. Five microlitres of inputs, 5 μl of IP product and 10 μl of co-IP product were resolved on a polyacrylamide gel and analysed by western blotting.

Quantitative protein assay
Cells were lysed with 200 μl of SDS buffer (0.01% SDS in PBS) supplemented with 2% PIC and 1% PhIC. A nitrocellulose membrane was pre-equilibrated with SDS buffer on a dot-blot apparatus (Bio-Rad, Hertfordshire, UK). Cell lysates were quantified (BCA Protein Assay Kit, Thermo Scientific), serially diluted (final volume: 100 μl per sample) in SDS buffer, applied in quadruplicate to the nitrocellulose membrane and allowed to bind for 20 min. Proteins were detected by immunoblotting. Each experiment was performed at least
twice. A standard curve for the detection of S396 phosphorylated tau was performed to ensure that measurements were compared in the linear range. Serially diluted samples (1:20–1:5000) from cells expressing EGFP-tau and HA-GSK3β were applied to a nitrocellulose membrane in quadruplicate as described above and S396 phosphorylated tau levels were detected. Western blots were scanned and protein levels were analysed using ImageJ. The average intensity of the quadruplicate spots from the same sample was calculated along with the standard error of the mean, and either compared directly or normalized to the protein concentration. The values for non-normalized data or data normalized to protein concentration were not significantly different. Finally, all values were normalized to the reference intensity of tau phosphorylated in the presence of GSK3β and in the absence of proteasome inhibition.

The filter trap assay was performed as described previously (45). In brief, cell lysates were applied in quadruplicate to a pre-equilibrated 0.2 μm cellulose acetate membrane on a dot-blot apparatus (Bio-Rad). ImageJ was used to measure the spot intensity.

RNA interference

The ON-TARGETplus Specificity-Enhanced siRNA against NUB1 mRNA was obtained from Dharmacon with the sequence 5’-CGAUGGUUGCUUGAACUAAAU-3’ (28). In addition, a non-targeting siRNA was used as a negative control, with the sequence, 5’-UAGCGACUAAACACAUC AA-3’ (46). Briefly, SH-SY5Y neuroblastoma cells were transfected with 100 μl of 200 nm siRNA per well in a six-well plate using DharmaFECT according to the manufacturer’s protocol (Dharmacon, Thermo Scientific). The treatment of cells with OA (200 nm) was performed 28 h after transfection for 3 h.

Cycloheximide assay

Cells were treated with CHX (50 μg/ml) (Sigma, Poole, UK) 28 h after transfection for 2, 4 or 6 h, and with ether a vehicle (DMSO) or MG132 (50 μM) added 4 h before the end of the CHX treatment. Samples were applied to the same polyacrylamide gel (10%) in triplicate and analysed by ImageJ. For determination of the degradation rate, GSK3β was removed for protein quantification using the bicinchoninic acid (BCA) Protein Assay kit according to the manufacturer’s instructions (Thermos Scientific). Ten micrograms of proteins were resolved by denaturing SDS-PAGE (10%), and transferred to a nitrocellulose membrane using a Transblot semi-dry transfer cell (Bio-Rad). Membranes were blocked overnight at 4°C in 5% non-fat dried milk in PBS-Tween 20 (PBS-T). Proteins were detected with anti-tau (1:20 000), anti-GFP (1:2000), anti-HA (1:5000), anti-FLAG (1:5000), AT8 (1:1000), anti-pS396-tau (1:5000), anti-GAPDH (1:20 000), anti-actin (1:2000) or anti-GSK3β (1:2000) primary antibody diluted in 1% non-fat dried milk in PBS-T for 1 h at RT. Membranes were washed with PBS-T before incubation with GoM-HRP or GoR-HRP secondary antibody diluted in 1% dried milk in PBS-T for 45 min. Proteins were detected using ECL Plus reagent (GE Healthcare, Buckinghamshire, UK).

Statistical analysis

All statistical significance was determined using an unpaired Student’s t-test. The differences were considered significant if \( P < 0.05 \).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

Conflict of Interest statement. There are no conflicts of interest to declare.

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