Title

Negative Regulator of Ubiquitin-Like Protein 1 modulates the autophagy-lysosomal pathway via p62 to facilitate the extracellular release of tau following proteasome impairment

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Abstract

Negative Regulator of Ubiquitin-Like Protein 1 (NUB1) and its longer isoform NUB1L are ubiquitin-like (UBL)/ ubiquitin-associated (UBA) proteins that facilitate the targeting of proteasomal substrates, including tau, synphilin-1 and huntingtin. Previous data revealed that NUB1 also mediated a reduction in tau phosphorylation and aggregation following proteasome inhibition, suggesting a switch in NUB1 function from targeted proteasomal degradation to a role in autophagy. Here, we delineate the mechanisms of this switch and show that NUB1 interacted specifically with p62 and induced an increase in p62 levels in a manner facilitated by inhibition of the proteasome. NUB1 moreover increased autophagosomes and the recruitment of lysosomes to aggresomes following proteasome inhibition. Autophagy flux assays revealed that NUB1 affected the autophagy-lysosomal pathway primarily via the UBA domain. NUB1 localized to cytosolic inclusions with pathological forms of tau, as well as LAMP1 and p62 in the hippocampal neurons of tauopathy mice. Finally, NUB1 facilitated the extracellular release of tau following proteasome inhibition. This study thus shows that NUB1 plays a role in regulating the autophagy-lysosomal pathway when the ubiquitin proteasome system is compromised, thus contributing to the mechanisms targeting the removal of aggregation-prone proteins upon proteasomal impairment.

Introduction

The microtubule-associated protein tau is an intrinsically disordered protein that is predominantly expressed in the central and peripheral nervous system, where it is abundant in neuronal axons. Under normal physiological conditions, a major function of tau is to stabilise and facilitate the assembly of microtubules, with tau phosphorylation and dephosphorylation regulating microtubule affinity [1]. Hyperphosphorylated tau detaches from the microtubules and aggregates in the neuronal somatodendritic compartment forming neurofibrillary tangles (NFTs) [2], a hallmark of Alzheimer's disease (AD) and other tauopathies. One of the main factors contributing to the formation of NFTs is the failure of proteostasis mechanisms that aim to reduce the burden of tau aggregates in the cell. Deficits in the proteasome, autophagy and the lysosomal pathways have all been implicated in the progression of neurodegenerative disease and have been shown to contribute to tau pathology [3-8].

In this study, we investigated the role of negative regulator of ubiquitin-like modifier 1 (NUB1), a protein implicated in the aetiology of several neurodegenerative disorders, including Parkinson's disease, Huntington's disease and tauopathies [9-11]. NUB1 and its longer splicing isoform NUB1L are class III members of the ubiquitin-like (UBL) / ubiquitin-associated (UBA) family of proteins that interact with the proteasome via an N-terminal UBL domain to facilitate the proteasomal degradation of substrates [12]. The NUB1/NUB1L C-terminal and UBA domains mediate the interaction with the ubiquitin-like modifiers (ULM), NEDD8 and FAT10 respectively, but not ubiquitin [12, 13]. Similar to ubiquitin, NEDD8 is detected in pathological NFT lesions in AD patients [14].

We previously reported that NUB1/NUB1L interacts with and significantly reduces the levels of phosphorylated and aggregated tau leading to a reduction in intracellular tau inclusions [11]. Interestingly, this reduction in tau aggregation was evident even after inhibition of the proteasome and following deletion of the proteasome interacting UBL domain. Conversely, the ability of NUB1/NUB1L to reduce tau aggregation was lost following the deletion of the UBA domain. In this study, we therefore investigated the role of the autophagy and lysosomal pathways in the NUB1-mediated reduction of tau aggregation following proteasome inhibition. We confirmed that NUB1 reduces the level of insoluble phosphorylated tau upon proteasome inhibition. We found that proteasome impairment induced a specific interaction of NUB1 with p62, an important autophagy receptor, and led to a significant increase in p62 levels. Surprisingly, we found that NUB1 modulated the autophagy pathway downstream of autophagosome formation, altering both the number and size of autophagosomes and inducing the accumulation of lysosomes, with the UBA domain playing a prominent role. Finally, we found that NUB1 led to an increase in the extracellular release of tau under these conditions. These data show that NUB1 may participate in the proteostatic response to clear the cell of aggregated hyperphosphorylated tau following proteasome impairment via modulation of the autophagy-lysosomal pathway.

Results

Inhibition of the proteasome induces the accumulation of GFP-tau at aggresomes

The SH-SY5Y stable GFP-tau cell line was used to investigate the effect of NUB1 on tau following inhibition of the proteasome. GFP-tau expression was induced by tetracycline, and detected with the tau (pan-tau) and AT8 antibody (pS202/pT205), indicating the endogenous

phosphorylation of GFP-tau (Fig. 1A). In the absence of proteasome inhibition, GFP-tau co-localized with α tubulin confirming its microtubular localization (Fig. S1). Inhibition of the proteasome induced an increase in polyubiquitinated proteins and a reduction in monomeric ubiquitin (Fig. 1B), and the aggregation of GFP-tau inclusions at the microtubule organising centre (MTOC) (Fig. S1). The incidence of GFP-tau inclusions increased with time in MG132 treated cells (Fig. 1C and S1). After 4 h of treatment, inclusions were detected in only 7.3 ± 2.5 % of cells, and the percentage increased significantly at 6 h (30.9 ± 9.2 %) and 8 h (52.3 ± 4.4%) of treatment. GFP-tau inclusions were surrounded by a 'cage' of vimentin, and both ubiquitin and Hsp70, well-known markers for the accumulation of misfolded protein at aggresomes, co-localized with the GFP-tau inclusions at the MTOC (Fig. 1D). The GFP-tau inclusions were positive for AT8 (pS202/pT205) and pS396, indicating the presence of phosphorylated GFP-tau in the inclusions (Fig. 1D). All together, these data show that proteasome inhibition was sufficient to induce the accumulation of GFP-tau in aggresomes.

NUB1 induces a decrease in the level of insoluble phosphorylated tau

Filter trap assays were used to investigate the effect of NUB1 on GFP-tau. In these assays, insoluble protein aggregates larger than 2 μm are trapped on a cellulose acetate membrane whilst total protein levels (soluble + insoluble) are detected on nitrocellulose. NUB1 induced a decline in phosphorylated tau (pS396) relative to total tau (all tau species) in the total cellular fraction in both the absence and in the presence of proteasome inhibition (Fig. 2A). Increasing NUB1 also induced a significant decrease in the level of pS396 tau relative to total tau in the insoluble fraction trapped on the cellulose acetate membrane in both the absence and presence of MG132 (Fig. 2B). Noticeably, in both the total (Fig. 2A) and insoluble (Fig. 2B) cellular fractions, the overall levels of both pS396 tau and tau were comparatively lower with proteasome inhibition. Overall, the data confirmed the NUB1-dependent decrease in the levels of phosphorylated tau relative to total tau in the total and insoluble cell fractions, even after proteasome inhibition.

To determine whether the NUB1-dependent decrease of phosphorylated tau in the insoluble fraction (Fig. 2B) occurred as a function of the overall decline in the total cellular fraction (Fig. 2A), the ratio of insoluble protein detected on the cellulose acetate membrane was also quantified relative to the total level of protein detected on the nitrocellulose membrane (Fig. 2C, D). First, the level of total tau (all tau species) detected on the cellulose acetate membrane was determined relative to that detected on the nitrocellulose membrane (Fig. 2C). Interestingly, there was a significant reduction in insoluble tau in both the absence and

presence of proteasome inhibition only with the highest level of NUB1 expression (Fig. 2C). Similarly, the level of insoluble pS396 detected on the cellulose acetate membrane was measured relative to the level of total pS396 detected on the nitrocellulose membrane (Fig. 2D). A progressive decrease in the level of insoluble phosphorylated tau relative to total phosphorylated tau was detected with increasing expression of NUB1, in both the absence and presence of MG132 (Fig. 2D). These findings show that NUB1 can specifically target a reduction in insoluble phosphorylated tau, which persists after proteasome inhibition.

NUB1 leads to an increase in the number of large autophagosomes following proteasome inhibition

To clarify the mechanism behind the decrease in phosphorylated tau induced by NUB1 after proteasome inhibition, we assessed the effect of NUB1 on LC3B, a key protein in the formation of autophagosomes by western blotting in both DMSO (*lanes 1-7*) and MG132 (*lanes 8-14*) treated cells (Fig. 3A). In the absence of proteasome inhibition, increasing levels of NUB1 induced a decrease in phosphorylated pS396 tau as shown previously, but had no effect on the levels of LC3BII (*lanes 5-7*). Therefore, in the absence of proteasome inhibition, NUB1 has no effect on autophagy. Proteasome inhibition alone increased the level of LC3BII (*lane 8*), supporting the well known phenomenon that inhibition of the proteasome is sufficient to induce autophagy. However increasing levels of NUB1 with proteasome inhibition induced a decrease in GFP-tau and pS396 tau, and a concomitant increase in LC3BII levels that was not evident with MG132 treatment alone (*lanes 12-14* compared to *lane 8*). These findings were confirmed by quantification of the LC3BII levels (ImageJ) (Fig. 3B). Thus, proteasome inhibition appeared to facilitate a switch in NUB1 function to the autophagy pathway that correlated with declining levels of tau. Increasing levels of GFP had no effect on GFP-tau, pS396 tau or LC3B in either the absence or presence of proteasome inhibition (*lanes 2-4* and *lanes 9-11*) (Fig. 3A). LC3B positive autophagosomes were next analysed by immunocytochemistry as previously described [15, 16] (Fig. 3C). In agreement with the quantification of LC3BII levels, proteasome inhibition alone was sufficient to induce an increase in LC3B positive autophagosomes, however NUB1 induced an even further increase in autophagosomes following proteasome inhibition (Fig. 3C). Quantification of the number and size of LC3B positive autophagosomes confirmed these findings (Fig. 3D). In the absence of MG132, significantly less puncta were quantified with few larger than 1 μm^2 (Fig. 3D), and NUB1 alone had no effect. Interestingly, upon proteasome inhibition, quantification of the percentage of cells with puncta bigger than 1 μm^2 [15] revealed a

significant NUB1-mediated increase in puncta size compared to MG132 treatment alone (Fig. 3D).

NUB1 affects components of the autophagy and lysosomal pathway

As NUB1 induced an increase in LC3BII levels and autophagosome size following proteasome inhibition, we investigated the effect of NUB1 on the autophagy receptor p62 and the lysosomal marker LAMP1 (Fig. 4). p62 was observed predominantly in the cytoplasm, and co-localized with GFP-tau at the aggresomes following proteasome inhibition (Fig. 4A). Interestingly, p62 increased dramatically in MG132 treated cells in both the absence and presence of NUB1, but a greater increase in p62 was observed in cells expressing NUB1 following proteasome inhibition, and NUB1 alone had no effect (Fig. 4A, B). We also analysed the p62 protein level by western blotting and confirmed that NUB1 led to a significant upregulation of p62 upon proteasome inhibition, exceeding the levels observed with proteasome inhibition alone (Fig. 4 C, D).

The effect of NUB1 on the lysosomal marker LAMP1 was also investigated (Fig. 4E-G). In DMSO treated cells, LAMP1 was detected as cytosolic intracellular spots, which predominantly localized at the MTOC (Fig. 4E). In MG132 treated cells expressing NUB1, there was a drastic and significant increase in the LAMP1 positive lysosomes in the vicinity of the MTOC. Moreover, in these cells, LAMP1 appeared to surround the aggresomes, demarcating a large cage-like area filled with numerous LAMP1 positive puncta (Fig. 4E). Quantification of the number of LAMP1 puncta per cell (Fig. 4F) and the LAMP1 positive area per cell (Fig. 4G) confirmed that NUB1 induced a significant increase in the number of and area occupied by LAMP1 positive lysosomes at the MTOC following proteasome inhibition compared to cells treated with MG132 only. Interestingly, NUB1 alone in the absence of MG132 appeared to induce a small increase in p62 levels (Fig. 4C, D) and LAMP1 puncta (Fig. 4F, G) but this did not reach significance, thus the effect is significantly enhanced by proteasome inhibition.

NUB1 co-localizes with phosphorylated tau, p62 and LAMP1 in TAU mice

We investigated the localization of endogenous NUB1 and tau in the hippocampus of wild type and TAU mice expressing P301L tau (0N4R) [17, 18] (Fig. 5 and S3). In the WT animals, NUB1 localization was predominantly nuclear but was also detected along the neuronal axons, where NUB1 co-localized with axonal tau (Fig. 5A, WT/arrowhead). In high (hi-TAU) and low (low-TAU) copy TAU mice, the axonal localization of tau was lost or severely affected (Fig. 5A, hi-TAU, low-TAU). Moreover, tau was detected in NFTs in hi-

TAU mice reflecting the severe tau pathology in these animals at 12 months with a less severe pathology in the low-TAU mice of the same age as indicated by fewer NFTs [18]. While NUB1 localization was predominantly nuclear in both the low-TAU and hi-TAU animals, a weaker NUB1 signal could also be detected in tau NFTs in hi-TAU mice (Fig. 5A, hi-TAU/arrowheads), and cytosolic intracellular aggregates positive for NUB1 were visible in the pyramidal neurons of low-TAU mice (Fig. 5A, low-TAU/asterisks). Brain sections of WT and low-TAU animals were also analysed using the AT8 and MC1 antibodies (Fig. 5B). While AT8 recognizes phosphorylated PHF tau, MC1 recognises conformationally abnormal tau, and both antibodies have been widely used to detect pathological tau in AD. NUB1 co-localized with both AT8 and MC1 in the cytosolic aggregates in pyramidal neurons of the CA1 area, particularly noticeable in the low-TAU animals (Fig. 5B, low-TAU/AT8/asterisks, low-TAU/MC1/asterisks). Interestingly, in low-TAU mice, NUB1 cytosolic inclusions were also positive for p62 (Fig. 5B, low-TAU/p62/asterisks), although not all the p62 positive puncta were positive for NUB1 (Fig. 5B, low-TAU/p62/arrows). LAMP1 positive vesicles detected prominently in cytosolic puncta in the low-TAU mice were also positive for NUB1 (Fig. 5B, low-TAU/LAMP1/asterisks). These data show the co-localization of NUB1 with pathological tau, and markers for autophagy and lysosomes, suggesting that NUB1 may play a role in the autophagy-lysosomal pathway *in vivo*.

NUB1 affects the number of autophagosomes through the UBA domains

We investigated the role of the UBL and UBA domains using lentiviral vectors with the in-frame deletion of these domains in NUB1L. We analysed the expression of HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL or HA-NUB1LΔ1-3UBA by immunoblotting (Fig. 6A). HA-NUB1 and HA-NUB1L differ in size by only 14 amino acids, giving respective molecular weights of ~73 and ~74 kDa, with the predicted molecular weights of HA-NUB1ΔUBL and HA-NUB1LΔ1-3UBA ~64 kDa and ~54 kDa respectively. The HA-tagged proteins were expressed at similar levels and MG132 inhibition did not induce a further increase in levels (Fig. 6A). The effect of HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA on LC3B puncta following proteasome inhibition was quantified as described [15, 16] (Fig. 6B, C, D). All of the NUB1 variants induced a significant increase in the percentage of cells with large puncta (Fig. 6C). Analysis of the number of LC3B puncta per cell revealed a significant increase induced by HA-NUB1, HA-NUB1L and HA-NUB1LΔUBL, but HA-NUB1LΔ1-3UBA was less effective (Fig. 6D). The UBA domain may thus contribute to the NUB1-mediated increase in LC3B puncta.

The UBA domains affect the interaction of NUB1 with p62

We investigated the involvement of the UBA and UBL domains in the NUB1-mediated regulation of p62 levels in the absence or presence of MG132 (Fig. 6E). HA-NUB1L, HA-NUB1LΔ1-3UBA, and HA-NUB1LΔUBL induced the upregulation of p62 levels in both the absence and presence of MG132, however in the absence of MG132, this upregulation was significant only for HA-NUB1LΔUBL. Proteasome inhibition alone induced a significant increase in p62 levels, and HA-NUB1L, HA-NUB1LΔ1-3UBA, and HA-NUB1LΔUBL all induced a further upregulation of p62 with MG132 treatment. However, this upregulation was less efficient following deletion of the UBA domain.

We investigated the interaction of the HA-tagged proteins with p62 by co-immunoprecipitation (Fig. 6F, G). Proteasome inhibition did not induce a noticeable increase in the levels of HA-NUB1 or HA-NUB1L in the inputs, and both HA-NUB1 and HA-NUB1L were efficiently and specifically immunoprecipitated (Fig. 6F). The level of endogenous p62 in the input increased noticeably in cells expressing HA-NUB1 or HA-NUB1L and treated with MG132. Interestingly, p62 was specifically co-immunoprecipitated with HA-NUB1 and HA-NUB1L when the proteasome was blocked, and HA-NUB1L, which has an extra UBA domain compared to HA-NUB1, co-immunoprecipitated p62 more efficiently (Fig. 6F). We also investigated which domain was important for the interaction with p62 (Fig. 6G). Inhibition of the proteasome did not induce an increase in the levels of HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA in the inputs, and both HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA were specifically immunoprecipitated with high efficiency (Fig. 6G). p62 was specifically co-immunoprecipitated with both HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA, however the co-immunoprecipitation was more efficient in cells treated with MG132. Interestingly, the co-immunoprecipitation of p62 was noticeably more efficient with HA-NUB1LΔUBL in the presence of MG132 (Fig. 6G). Therefore, the UBA domain may be important for mediating an efficient interaction with p62 dependent on proteasome inhibition, whilst the UBL domain may inhibit it.

NUB1 induces a block in the autophagy flux

As NUB1 influenced both the number of autophagosomes and lysosomes following proteasome inhibition, we performed autophagy flux assays using bafilomycin A1 (BAF) and 3-methyladenine (3-MA). BAF inhibits vacuolar H+ ATPase (V-ATPase) thereby inhibiting the fusion between autophagosomes and lysosomes and preventing the maturation of autophagic vacuoles at the late phase of autophagy, whilst 3-MA blocks flux at the initial

stage during the formation of autophagosomes via inhibition of type III phosphatidylinositol 3-kinases (PI-3K). To understand the role of the UBA and UBL domains, we compared the deletion mutants to HA-NUB1L. The LC3BII and HA levels were normalised to actin and the normalised levels of LC3BII levels plotted relative to HA expression (Fig. 7).

Fig. 7A shows the effect of proteasome inhibition alone on autophagy flux in untransduced cells. In cells treated with DMSO, LC3BII was almost undetectable. Proteasome inhibition alone increased LC3BII levels. A further small increase in LC3BII levels occurred following the treatment of cells with BAF after proteasome inhibition, whilst the LC3BII levels following treatment with 3-MA and MG132 and were not significantly different to that in untransduced control cells. The analysis of autophagy flux in cells expressing HA-NUB1L (Fig. 7B) revealed that HA-NUB1L induced a significant increase in LC3BII levels following proteasome inhibition. Interestingly, the LC3BII levels decreased significantly following the treatment of cells with MG123 and BAF compared to MG132 treated cells, suggesting that the NUB1L-mediated increase in LC3BII levels is not due to an increased induction of autophagosome formation, but likely due to a downstream block in the autophagy-lysosome pathway. As expected, 3-MA treatment reduced LC3BII levels back to control levels. The analysis of autophagy flux in cells expressing HA-NUB1LΔ1-3UBA (Fig. 7C) showed that following proteasome inhibition, HA-NUB1LΔ1-3UBA also induced an increase in LC3BII levels, and an even greater increase in LC3BII levels was induced following the treatment of cells with MG132 and BAF. This suggests that HA-NUB1LΔ1-3UBA does not induce a block in autophagy flux. The level of LC3BII in cells treated with MG132 plus 3-MA decreased to control baseline levels. Investigation of the effect of HA-NUB1LΔUBL showed similar trends to that of HA-NUB1L, suggesting that HA-NUB1ΔUBL may induce a downstream block in autophagy similar to HA-NUB1L (Fig. 7D). The treatment of HA-NUB1ΔUBL cells with MG132 plus 3-MA led to a significant decrease in the level of LC3BII although this did not reach baseline levels.

The differences between HA-NUB1L (light grey bars), HA-NUB1LΔ1-3UBA (medium grey bars) and HA-NUB1LΔUBL (dark grey bars) are highlighted in figure 7E. In this comparison, it is evident that in the absence of proteasome inhibition, only HA-NUB1L induced a small increase in LC3BII levels. In the presence of MG132 (no BAF or 3-MA), all three HA-NUB1L constructs induced an increase in LC3BII levels. However, HA-NUB1LΔ1-3UBA was significantly less effective than either HA-NUB1L or HA-NUB1LΔUBL, and HA-NUB1LΔUBL was only slightly less effective than HA-NUB1L.

Hence, the UBA domain in particular is important for mediating an increase in LC3BII levels. Moreover, autophagy flux assays conducted with MG132 and BAF suggest that the UBA domain plays an important role in mediating the block in autophagy flux, which is lost upon deletion of the UBA domain.

NUB1 facilitates the extracellular release of tau

As NUB1 induced a downregulation in the insoluble levels of pS396 tau and a block in autophagy flux following proteasome inhibition, we investigated the extracellular release of tau. The supernatant from cells overexpressing NUB1 in presence or absence of proteasomal inhibition was analysed by dot blot (Fig 7F). There was no significant difference in the number of cells for each experimental condition upon collection of the supernatants (data not shown). Tau or pS396 were barely detected in the supernatant of uninduced cells, and very low levels of extracellular tau or pS396 were detected in the absence of proteasome inhibition or with NUB1 overexpression alone. Interestingly, proteasome inhibition alone induced a significant increase in the extracellular levels of tau and NUB1 induced a further increase in the extracellular release of tau following proteasome inhibition (Fig. 7F). Similarly, NUB1 significantly increased the levels of extracellular pS396 tau compared to proteasome inhibition alone.

Discussion

In this study, we show that NUB1 plays an important role in the proteostasis of tau, and may more generally contribute to the proteostasis of proteotoxic proteins via modulation of the autophagy-lysosomal pathway. A schematic of the role of NUB1 in tau proteostasis is depicted in Fig. 8.

Previously, it was reported that NUB1 interacts with the proteasome via its N-terminal UBL domain [12] to facilitate the proteasomal degradation of ubiquitinated synphilin-1, which interacts directly with the C-terminus of NUB1, and NUB1 thus reduces the formation of synphilin-1 positive cellular inclusions [9]. NUB1 was also found to interact with and enhance the polyubiquitination and proteasomal degradation of normal full length and mutant polyglutamine expanded huntingtin [10]. This reduced the levels, aggregation and cellular toxicity of mutant huntingtin *in vitro* and *in vivo*. Similarly, we have previously shown that NUB1 can interact with tau in a manner requiring the NUB1 C-terminal UBA domain to

reduce the levels of aggregated tau and the formation of tau inclusions *in vitro* [11]. Collectively, these data suggest that in the absence of proteasome inhibition, NUB1 can facilitate the proteasome-mediated turnover of ubiquitinated substrates (Fig. 8).

Soluble and insoluble tau isolated from PHFs and *in vitro* recombinant tau is a substrate for E3 ubiquitin ligase-mediated ubiquitination and proteasomal degradation via lysine 6-, 11-, 48- and 63-linked ubiquitin and polyubiquitin chains [19, 20, 21, 22]. The identified ubiquitination sites within tau are in the microtubule binding domain, suggesting that similar to phosphorylation, tau ubiquitination may weaken microtubule binding affinity and promote tau aggregation. Indeed, NFT-tau isolated from AD brains can directly inhibit endogenous proteasome activity [4]. It is also thought that tau can readily be degraded by the proteasome in the absence of ubiquitination, and proteasomes have been shown to directly degrade natively unfolded or intrinsically disordered proteins *in vivo*, including α-synuclein and tau [23]. Thus the presiding consensus is that the UPS is important for the clearance of soluble tau, which could be processed via canonical and non-canonical ubiquitin-chain mediated proteasomal degradation, but also autophagic clearance following proteasome impairment (Fig. 8). In support of this proposal, it has been reported that the inhibition of the proteasome in cultured primary neurons induces a reduction rather than an elevation in endogenous tau levels irrespective of phosphorylation status via autophagy [24], suggesting that the UPS is important in the clearance of soluble tau at the early stages but not of late hyperphosphorylated tau aggregates.

In accordance with this data, our previous findings revealed that NUB1 might target the clearance of substrates in a proteasome-independent manner following the inhibition of the proteasome [11]. In particular, NUB1 was shown to reduce the aggregation of tau following inhibition of the proteasome or deletion of the proteasome-interacting UBL domain. Moreover, NUB1 was also able to reduce the levels of phosphorylated tau both in the absence and presence of proteasome inhibition. One mechanism by which NUB1 may reduce the phosphorylation of tau is by modulating GSK3β-mediated tau phosphorylation. We previously reported that NUB1 interacts with GSK3β to facilitate its proteasome-mediated turnover [11]. We showed that the downregulation of endogenous NUB1 stabilised endogenous GSK3β to proteasomal degradation. The accelerated turnover of GSK3β by NUB1 was dependent on both the proteasome-interacting UBL domain as well as the UBA

domain, which mediated the interaction of NUB1 with GSK3β. Thus, both the NUB1 UBL and UBA domains were required to shuttle GSK3β to the proteasome to facilitate GSK3β turnover, and GSK3β is likely another substrate for NUB1-mediated proteasomal turnover. However, not all effects of NUB1 on phosphorylated tau were mediated via GSK3β. In particular, we found that NUB1 was able to downregulate the level of phosphorylated tau after inhibition of the proteasome or deletion of the proteasome-interacting UBL domain. We also found that NUB1 could downregulate the levels of phosphorylated tau following deletion of the GSK3β-interacting UBA domain. Our current study suggests that one way in which NUB1 can reduce phosphorylated tau following inhibition of the proteasome is by redirecting it towards the autophagy-lysosomal pathway. This study also suggests that the primary mechanism by which this is achieved is via p62.

In the absence of proteasome inhibition, p62 interacts non-covalently with lysine 48- and 63-linked ubiquitinated or polyubiquitinated tau through the p62 UBA domain, and delivers the cargo to the proteasome via the N-terminal Phox-BEM1 (PB1) domain, which shares considerable structural homology with the UBL domain [20, 25]. The PB1 domain is also involved in the self-oligomerization of p62 or heterodimerization of p62 with NBR1 or other PB1-domain containing proteins, and p62 oligomerization prevents proteasomal degradation of the p62 substrate by limiting access to the catalytic core of the proteasome. p62 thus plays a role in the phase separation of ubiquitinated proteins into larger aggregates or condensates, and facilitates the autophagic degradation of ubiquitinated proteins by tethering the ubiquitinated cargo to the nascent autophagosomal membranes via the interaction of the p62 LC3-interacting region (LIR) with ATG8 proteins such as LC3B [26-30] (Fig. 8).

In our study, we show that NUB1 induced an increase in the accumulation of large autophagosomes as well as the accumulation of lysosomes at the aggresomes following proteasome inhibition. Our data also show that NUB1 mediated an increase in the levels of p62 following proteasome inhibition. Moreover, we report a specific interaction of NUB1 with p62, which was more efficient following proteasome inhibition. We propose that NUB1 interacts with and helps to target tau to the UPS but that upon proteasome inhibition, NUB1 facilitates the delivery of pathological tau to the autophagy-lysosomal pathway via an interaction with p62 (Fig. 8). In our study, the deficit in protein degradation upon proteasome inhibition combined with an increase in p62 could facilitate the formation of a NUB1-p62

complex. This complex might promote a cellular switch from proteasomal degradation to autophagy to facilitate the targeting of aggregation-prone polyubiquitinated and hyperphosphorylated tau to the autophagy machinery. Moreover, our data suggest that the UBA domain may promote the NUB1-mediated increase in autophagosomes and interaction with p62 whilst the UBL domain may impede this function. Interestingly, the *in vivo* data from a Tauopathy model also showed the involvement of NUB1 in the autophagy and lysosomal pathways. In the hippocampus of aged TAU mice with a low copy number of P301L *MAPT*, NUB1 co-localized with conformationally abnormal phosphorylated tau in small cytoplasmic inclusions that were also positive for p62 and LAMP1.

Overall, our data suggest that NUB1 may mediate an upregulation of the autophagy-lysosomal pathway following proteasomal impairment to facilitate the clearance of proteotoxic species of tau. However, we consistently and surprisingly found that when blocking the autophagy flux with bafilomycin A1, NUB1 did not induce further formation of autophagosomes. These data suggested that NUB1 might instead induce a block in autophagy flux downstream of autophagosome formation by impairing the fusion of autophagosomes with lysosomes, consequently inducing the accumulation of lysosomes at the aggresome. Deletion of the UBA domain impaired the NUB1-mediated block in autophagy flux, suggesting that this domain may thus be important for mediating the switch in NUB1 function to the autophagy pathway following proteasome inhibition. Interestingly, it has been shown that tau pathology may similarly lead to impairment of the autophagy-lysosomal system. The expression of mutant human tau in cultured neurons has been reported to induce a dramatic accumulation of autophagosomes and lysosomes [31]. Autophagic vacuoles have been found to accumulate in neurons from transgenic mice overexpressing human P301L tau [32], and an increase in the number of lysosomes has been detected in neurons from transgenic mice overexpressing G272V, P301L and R406W tau [33]. Finally, AD brains show both accumulated autophagosomes and autolysosomes [34]. By facilitating the recruitment of pathological tau to the autophagy-lysosomal pathway, NUB1 may also facilitate a deficit in this pathway. The exact mechanisms by which this deficit might occur is not well understood, however a recent report showed that the accumulation of tau inhibited the expression of IST1, which facilitates the formation of the Endosomal Sorting Complex Required for Transport (ESCRT)-III [35]. The ESCRT-III complex in turn is required for autophagosome-lysosome fusion. Via this mechanism, overexpression of human wild-type

full-length tau induced autophagy deficits by repressing autophagy-lysosome fusion leading to significantly increased LC3 and p62 protein levels with autophagosome accumulation.

Finally, our data suggest that the NUB1-facilitated impairment of both proteasomal and autophagy-lysosomal degradation might trigger the extracellular release of pathological tau (Fig. 8). The exact cellular mechanisms by which deficits in the autophagy-lysosomal pathway induce the extracellular release of proteotoxic substrates is poorly defined. However, evidence from several studies show that autophagy-lysosomal dysregulation can increase the release and intercellular propagation of pathogenic aggregates [36,37]. In AD, lysosomal dysfunction has been shown to increase the presence of Aβ in multivesicular bodies and promote the release of APP C-terminal fragments via exosomes, and in PD, the impairment of autophagosome-lysosomal fusion has been shown to promote the secretion of aggregated α-synuclein [38-40]. The exosomal release of tau has been implicated in the spread of tau pathology, and the transfer of pathological tau assemblies via tunnelling nanotubes, the formation of which is upregulated by lysosomal stress, has also been reported [41-44].

In summary, our experimental data show a role for NUB1 in regulating the level of insoluble phosphorylated tau following inhibition of the proteasome. We report a role for NUB1 in the autophagy-lysosomal pathway following proteasome inhibition. As NUB1 has been implicated in the aetiology of several neurodegenerative diseases, it is possible that the role of NUB1 identified in this study could be relevant to other neurodegenerative diseases in which the UPS and autophagy-lysosomal pathways are compromised, and may generally play a role in proteostasis mechanisms in these diseases aimed at alleviating cellular proteotoxicity.

Materials & Methods

Cloning and generation of TetR-GFP-tau SH-SY5Y cell line

A stable tetracycline inducible GFP-tau (0N4R) SH-SY5Y neuroblastoma cell line was generated (TetR-GFP-tau). The attL-containing *pENTR3C-GFP-tau* plasmid was generated by subcloning GFP-tau (0N4R) PCR products in a pENTR3C vector (Invitrogen) digested with BamHI and EcoRV. The GFP-tau PCR products were amplified from a pEGFP-C1-tau plasmid. The attB-containing destination plasmid *pT-Rex-DEST30-GFP-tau* was generated by Gateway® technology (Invitrogen). SH-SY5Y cells were transfected with

pcDNA6_TetR_IRES_blast and pT-Rex-DEST30-GFP-tau as previously described to generate TetR-GFP-tau cells [45].

Cell culture

Human SH-SY5Y cells, SH-SY5Y stable TetR-GFP-tau cells and HEK-293T cells were grown at 37°C in 5% CO_2. SH-SY5Y cells were cultured in DMEM/F12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). SH-SY5Y stable TetR-GFP-tau cells were cultured in the same media supplemented with blasticidin S (3 μg/ml) (Invitrogen) and G418 (700 μg/ml) (Sigma). HEK-293T cells were cultured in DMEM/glutaMAX (Invitrogen) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cloning and production of lentiviral vectors

pRRLSIN.cPPT.PGK-NUB1 (pRRL-NUB1) was generated by subcloning NUB1 cDNA into a pRRLSIN.cPPT.PGK backbone. pRRLSIN.cPPT.PGK-GFP.WPRE (pRLL-GFP) was a gift from Didier Trono (Addgene plasmid no. 12252; http://n2t.net/addgene:12252; RRID:Addgene_12252). pRLL-GFP was double digested with BamHI and SalI to cut out the GFP sequence, while pEGFP-C1-NUB1 was double digested with BglI and SalI to release NUB1. pRRLSIN.cPPT.PGK-HA-NUB1, -HA-NUB1L, -HA-NUB1LΔ1-3UBA and -HA-NUB1LΔUBL were generated by subcloning the respective HA-NUB1 cDNAs into a pRRLSIN.cPPT.PGK backbone. HA-NUB1, HA-NUB1L, HA-NUB1LΔ1-3UBA and HA-NUB1LΔUBL cDNAs were amplified by PCR from pcDNA3.1-HA-NUB1, -HA-NUB1L, -HA-NUB1LΔ1-3UBA and -HA-NUB1ΔUBL [12] and subcloned using StrataClone Vector Mix amp/kan (Agilent) into pRLL-GFP double digested with BamHI and SalI to remove the GFP sequence. Viral particles were produced using HEK-293T cells as described [46], and the filtered conditioned medium was centrifuged by ultracentrifugation at 20,000 rpm for 2 h or treated with PEG-*it*™ Virus Precipitation Solution (5X) (System Biosciences). The lentiviral pellet was resuspended in cold sterile phosphate buffered saline (PBS). Titration of the lentivirus (viral genome/ml) was performed by real time quantitative PCR (qPCR) using pAlb (Addgene plasmid no. 22037) to generate a standard curve as described by the Trono lab (https://tronolab.epfl.ch/research/).

Transduction and treatment of TetR-GFP-tau cells

TetR-GFP-tau cells were seeded at $5x10^4$ cells per well in a 8-well chamber slide or $6.5x10^5$ cells per well in a 6-well plate at day 0 and treated with tetracycline (1 μg/μl) the following day (day 1) to induce the expression of GFP-tau. Cells were induced for 24 h and treated with

MG132 (50 μM) (Enzo Life Sciences) or vehicle (DMSO, Sigma) for 4, 6, or 8 h as indicated prior to the end of the induction period. For the transduction of TetR-GFP-tau cells with lentivirus (LV), cells were seeded at $3x10^4$ cells per well in a 8-well-chamber slide or $3.5x10^5$ cells per well in a 6-well plate on day 0. On day 1, cells were transduced with 10, 20 or 30 multiplicity of infection (MOI) of lentiviral vectors or were untransduced (NT). On day 3, the cells were treated with 1 μg/μl of tetracycline and the expression of GFP-tau induced for 12-14 h prior to treatment with different drugs. For inhibition of the proteasome, cells were treated with MG132 (50 μM) (Enzo Life Sciences) for 8 h. For the autophagy flux assay, cells were treated with MG132 for 8 h, and with bafilomycin A1 (BAF) (100 nM) (Chalbiochem, Millipore) or 3-methyladenine (3-MA) (5 mM) (Sigma) for 3 h prior to the end of the 8 h MG132 treatment.

Immunoblotting

SH-SY5Y cells were washed in PBS and lysed in RIPA buffer (1% sodium deoxycholate, 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS), 50mM Tris-HCl pH7.5) supplemented with 2% protease inhibitor cocktail (PIC) (Sigma) and 1% phosphatase inhibitor (PhIC) (Sigma) at 4ºC. Protein quantification was performed using the bicinchoninic acid (BCA) assay kit (Thermo-scientific). After adding 4X sample buffer (SB) (0.025% bromophenol blue, 10% β-mercaptoethanol, 20% glycerol, 5% SDS, 125 mM Tris-HCl pH 6.8), samples were boiled for 3-4 min and centrifuged at 12000 x g. 10-20 μg of proteins were resolved by SDS-PAGE and analysed by western blotting. Protein samples were resolved on a 10-15% polyacrylamide gel and transferred to a nitrocellulose membrane or to a previously activated 0.45 μm PDVF membrane (GE Healthcare Amersham™ Hybond™-P membrane). Membranes were blocked in 5% non-fat milk powder in PBS (w/v) or 5% BSA in PBS and incubated overnight at 4ºC with the primary antibody. Membranes were developed using an ECL solution (Luminata Crescendo Western HRP substrate, Millipore) and analysed using the Bio-Rad ChemiDoc MP imaging system. The amount of a specific protein was calculated by measuring the intensity of the relative band with ImageJ software (NIH; https://imagej.nih.gov/ij/). Values were normalised to the reference intensity of a loading control or to the total amount of protein.

Immunoprecipitation

For immunoprecipitation, $3.5x10^5$ cells per well were seeded in 6-well plates 96 h prior to the lysis of cells in 250 μl RIPA buffer supplemented with 5% PIC and 1% PhIC at 4ºC. 30 μl of protein lysate were kept as input, mixed with 4X SB and boiled for 3 min. HA-constructs

were immunoprecipitated from 180 µl lysate using a mouse monoclonal anti-HA antibody (1:250) (H3663, Sigma) by incubating the cell lysates overnight at 4ºC with pre-washed magnetic beads (Dynabeads™ Protein G, Invitrogen). The magnetic beads were washed with RIPA buffer, resuspended in 4X SB and the proteins analysed by SDS-PAGE and immunoblotting as described above.

Filter trap assays

$3.5x10^5$ cells per well were seeded in 6-well plates 96 h prior to the lysis in 200 µl SDS buffer (10 mM Tris-HCl pH 8, 10 mM NaCl, 0.1 % SDS) supplemented with 2% PIC and 1% PhIC at 4ºC. Cells were sonicated for 10 sec, boiled for 2 min and immediately applied to a 0.22 µm cellulose acetate membrane or a nitrocellulose membrane in a dot blot apparatus at the appropriate dilutions (made up to a final volume of 100 µl in SDS buffer). In order to define the appropriate dilution factor for detection of tau and pS396 tau, a standard curve was performed. Serial dilutions of induced TetR-GFP-tau lysate were prepared (1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000) and applied to the previously equilibrated membrane. The samples were allowed to bind the membrane for 15-20 min before washing the membrane twice with SDS buffer. For the analysis of total proteins, equivalent aliquots of samples were applied to a nitrocellulose membrane and treated in the exactly the same way as the acetate membrane. Once the appropriate dilution of sample for detection of the protein of interest was determined from the standard curve, this dilution was used in all subsequent filter trap experiments. Samples diluted 1:200 were used to detect total tau or 1:50 to detect pS396 tau by immunoblotting.

Supernatant dot blot assays

$3.5x10^5$ cells per well were seeded in 6-well plates and treated as described above (transduction and treatment of TetR-GFP-tau cells). The cells were treated with MG132 (50 µM) or DMSO for 8 h prior to collection of the supernatants. The total supernatant from each experimental condition was collected, centrifuged at 1000 rpm for 5 min, and applied to a nitrocellulose membrane previously equilibrated in SDS buffer for 15-20 min. Total tau or pS396 tau were detected by immunoblotting.

Immunocytochemistry

$3x10^4$ cells per well were seeded in 8-well-chamber slides for 96 h before performing immunocytochemistry. Cells were washed in PBS and fixed in pre-cooled methanol for 10 min at -20ºC. After washing the cells in PBS, permeabilization was performed in 0.1% Triton X-100 for 5 min. Blocking was performed for 1 h in blocking buffer (3% BSA, 10% normal

donkey serum in PBS). Cells were incubated with the primary antibody for 1 h, washed 3 times in PBS and then incubated with the secondary antibody for 45 min at RT. Primary and secondary antibody were diluted in blocking buffer. DAPI (Sigma) was used to detect the nuclei. Images were acquired with the Carl Zeiss LMS700 confocal microscope, processed using Adobe Photoshop CS4 and analysed using Image J.

Animals

The TAU mouse is a model of tauopathy that encodes the 0N4R isoform of human microtubule-associated tau (MAPT) containing the P301L mutation, that causes frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), and was generated by GSK. Hi-TAU mice have approximately 10 copies of the transgene, about twice the copy number of low-TAU mice [17, 18]. The expression of P301L mutant tau is driven by the CaMKII promoter heterozygously expressed on a C57BL/6j background [17, 18]. Tissue from the hippocampus was prepared as described [17, 18]. C57BL/6j wild type age-matched littermates were used as the control.

Immunohistochemistry

Mouse brain cryosections were washed in PBS and incubated for 30 min in PBS with 0.1% SDS, 5 mM DTT. Antigen retrieval was performed by heating the cryosections to 75°C in 0.1 M sodium citrate, pH 9.0 for 3 h, and then permeabilising the sections in 0.03% Triton X-100/PBS. Sections were blocked in 8% serum in 0.03% Triton X-100/PBS for 1 h at room temperature, followed by incubation overnight at 4°C with the primary antibody diluted in blocking buffer. The sections were incubated with secondary antibodies at RT for 1 h. Low magnification images were taken with a Nikon ECLIPSE 80i microscope, while high magnification high-resolution images were taken with a Carl Zeiss LMS700 confocal microscope. All the images were processed with Adobe Photoshop CS4.

Antibodies

NUB1 was detected using a rabbit polyclonal anti-NUB1 [47] (WB = 1:2000, IHC = 1:200). Tau was detected using a mouse monoclonal anti-tau (WB = 1:2000, IHC/ICC = 1:1000) (MA515108, Invitrogen) and a rabbit polyclonal anti-tau (WB = 1:40000, IHC/ICC = 1:2000) (1HC, Dako). Mouse monoclonal AT8 antibody was used to detect tau phosphorylated on S202 and T205 (WB = 1:1000, IHC = 1:100, ICC = 1:250) (MN1020, Invitrogen), MC1 antibody was used to detect a pathological conformation of tau (IHC = 1:400) (kindly provided by Dr Peter Davies, Department of Pathology, The Feinstein Institute for Medical Research, NY), while a rabbit polyclonal antibody was used to detect tau phosphorylated on S396 (WB = 1:5000, ICC = 1:500) (44-752G, Invitrogen). A rabbit polyclonal antibody was

used to detect GFP (WB = 1:1000) (632376, Clontech). Ubiquitin was detected with a mouse monoclonal FK2 antibody (WB = 1:1000, ICC = 1:100) (BML-PW8810, Enzo Life Sciences), Hsp70 (ICC = 1:50) (H5147) and α-tubulin (ICC = 1:2000) (T5168) were detected with mouse monoclonal antibodies from Sigma. Monoclonal antibodies from Abcam were used for vimentin (ICC = 1:100) and pericentrin (ICC = 1:1000) (ab19044). A rabbit polyclonal anti-LC3B was purchased from Cell Signaling, NEB (WB = 1:1000, ICC = 1:200) (3868), a mouse monoclonal anti-p62 from Abcam (WB = 1000, IHC = 1:100, ICC = 1:500) (Ab56416) and a mouse monoclonal anti-LAMP1 from Santa Cruz (IHC = 1:50, ICC = 1:10) (sc-20011) to detect autophagy markers. The HA tagged constructs were detected using a mouse monoclonal anti-HA (WB = 1:1000, ICC = 1:500) (H3663, Sigma). For WB, the loading controls were detected using mouse monoclonal anti-GAPDH (1:40000) (G8795, Sigma) and mouse monoclonal anti-actin (1:5000) (MAB1501R, Millipore). Goat anti-mouse (1:30000) and anti-rabbit (1:30000) secondary antibodies conjugated to horseradish peroxidase (HRP) from Pierce Biotechnology were used. For IHC and ICC cells were incubated with donkey anti-mouse or anti-rabbit Alexa-Fluor secondary antibodies (1:600) (Invitrogen).

Puncta quantification

To measure the size and number of puncta positive for LC3B or LAMP1, RGB images were split into the 3 channels and a threshold was introduced for the channel corresponding to LC3B or LAMP1 signal to exclude the background. The same threshold settings were applied for all measurements and fields were selected at random. In order to count the number of cells in the field with puncta bigger than 1 μm^2 [15] the "Analyze Particles" tool (ImageJ) was used and only elliptic spots with a size between 1-infinity μm^2 were included in the analysis. The obtained image was merged with the DAPI channel to identify the cells of interest. In order to count the total number of puncta per cell and the averaged size of the puncta or only the total number of puncta, the "Analyze Particles" tool was used to include puncta with a size between 0-infinity μm^2.

Intensity analysis

To evaluate the intensity of p62 signal, RGB images were split into the 3 channels, and a threshold was introduced for the channel corresponding to p62 signal to exclude the background. The same threshold settings were applied for all measurements. The averaged intensity of the signal was normalised for the number of cells per field to obtain the averaged intensity per cell (ImageJ).

Statistical analysis

One-way Analysis of Variance (ANOVA) and two-way ANOVA were used as appropriate to assess statistical significance. One-way ANOVA was applied to compare multiple experimental groups for a single independent variable, while two-way ANOVA was applied to compare multiple experimental groups for two independent variables. Bonferroni, Tukey's or Newman-Keuls corrections were performed to test pairwise differences within or between the experimental groups. The level of statistical significance was set at 0.05. Where applicable, experiments were conducted at least 3 times.

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Conflict of interest statement

The authors do not have any conflicts of interest to declare.

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Figure Legends

Figure 1. GFP-tau forms aggresomes upon proteasome inhibition. (**A**) Tetracycline (TET)-inducible GFP-tau expression was analysed by western blotting using antibodies to total tau (anti-tau) and phosphorylated tau (AT8, pS202/pT205). The parental neuroblastoma cell line was used as a control (CTR). (**B**) Uubiquitin was detected in TetR-GFP-tau cells treated the with MG132 for 8 h. The asterisk demarcates the ubiquitin monomer. (**C**) Cells treated with MG132 for 4, 6 and 8 h were analysed (ImageJ) by counting the percentage of GFP-tau expressing cells with aggresomes. The quantification of aggresomes was conducted blind to experimental status. Data represent the mean percentage ± SD. One-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**D**) Aggresomes in tetracycline induced cells treated with MG132 for 8 h were analysed by immunocytochemistry for total tau, phosphorylated tau (AT8, pS202/pT205 and pS396), vimentin, ubiquitin and Hsp70. Arrows indicate the cells highlighted in the zoomed insets. Scale bar 20 μm.

Figure 2. NUB1 affects the level of insoluble phosphorylated tau. Cell lysates were analysed by filter trap on a nitrocellulose membrane (total fraction) (**A**) or a cellulose acetate membrane (0.22 μm) (insoluble fraction) (**B**) using antibodies to total tau and phosphorylated tau (pS396), and the intensity of each spot was measured using ImageJ. The ratio of pS396 tau to total tau in the total cellular fraction (**A**) and in the insoluble fraction (**B**) was calculated and plotted. The ratio between the level detected on the acetate membrane (Ins, insoluble fraction) relative to the nitrocellulose membrane (Tot, total fraction) was also calculated and plotted for both tau (tau ratio (Ins/Tot)) (**C**) and pS396 tau (pS396 ratio (Ins/Tot)) (**D**). All lysates were analysed in triplicate. Data (n=3) represent the averaged ratio ± SD. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

Figure 3. NUB1 affects the level of LC3BII and the size of autophagosomes. (**A**) Lysates were analysed by immunoblotting using antibodies for NUB1, GFP, total tau, phosphorylated tau (pS202/pT205 and pS396) and LC3B. (**B**) The intensity of the LC3BII band was measured using ImageJ. Data (n=3) represent the average level of LC3BII ± SD normalized to the loading control and plotted relative to LC3BII in the absence of both MG132 and LV.

Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**C**) Cells were fixed and analysed by immunocytochemistry to detect tau and LC3B. Arrows demarcate the zoomed cells shown in the inset. Scale Bar 20 μm. (**D**) The size of the LC3B positive puncta was measured by ImageJ and a cut off of $1\mu m^2$ was applied. Data represent the % of cells with puncta bigger than $1\mu m^2$ ± SD of 250 cells per treatment. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

Figure 4. NUB1 affects the level of p62 and LAMP1. (**A**) Cells were analysed by immunocytochemistry using antibodies to tau and p62. Arrows demarcate the zoomed cells shown in the insets. Scale bar 20 μm. (**B**) The relative intensity of p62 signal per cell was measured (ImageJ). Data represent the averaged intensity per cell ± SD for 150 cells per treatment. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**C**) Cells were analysed by immunoblotting to detect p62 and actin as a loading control. (**D**) p62 levels detected by immunoblotting were measured (ImageJ), normalised to actin and plotted. Data (n=3) represents the averaged value of triplicate samples ± SD. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**E**) Cells were analysed by immunohistochemistry to detect tau and LAMP1. Arrows demarcate the zoomed cells shown in the insets. Scale Bar 20 μm. (**F**) The number of LAMP1 positive puncta per cell was measured (ImageJ). Data (n=3) represent the averaged number of LAMP1 positive puncta per cell ± SD for 130 cells per treatment. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**G)** The total area per cell (μm^2) positive for LAMP1 signal was measured in 140-250 cells per treatment by ImageJ. Data (n=3) represent the averaged area per cell ± SD. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$.

Figure 5. NUB1 co-localizes with phosphorylated tau, p62 and LAMP1 in TAU mice. (**A**) NUB1 and tau localization was investigated in the pyramidal neurons in the CA1 area of the hippocampus of WT, hi-TAU and low-TAU mice at 12 months of age. NUB1 localized predominantly to the nuclei and axons (arrowhead) of hippocampal neurons in WT mice. NUB1 was detected in NFTs in hi-TAU mice (arrowhead) and in cytosolic aggregates (asterisks) observed in low-TAU mice. Scale bar 10 μm. (**B**) NUB1, phosphorylated tau (AT8, pS202/pT205), conformationally abnormal tau (MC1), p62 and LAMP1 localization was investigated in the pyramidal neurons of low-TAU mice at 12 months. Asterisks indicate cytoplasmic aggregates, arrows indicate cytoplasmic inclusion positive only for p62. Scale bar 10 μm.

Figure 6. The UBA domain affects the NUB1-mediated increase in the number of LC3B puncta and the interaction of NUB1 with p62. (**A**) HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA cell lysates were analysed by immunoblotting using anti HA and anti actin antibodies. (**B**) Cells were fixed and analysed by immunocytochemistry to detect the HA-tagged proteins and LC3B. Inserts demarcated by white squares highlight the zoomed cells shown to the right. Scale Bar 20 μm. (**C**) The percentage of cells with large puncta and (**D**) the number of LC3B puncta per cell were measured in 100-125 cells per treatment (ImageJ). Data presents the averaged value ± SD. One-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**E**) HA-NUB1, HA-NUB1L, HA-NUB1LΔUBL and HA-NUB1LΔ1-3UBA cell lysates were analysed by immunoblotting using anti p62. p62 levels were measured (ImageJ), normalised to the total amount of protein and plotted relative to p62 in the absence of both MG132 and LV. Data presents the averaged value of triplicate samples ± SD. One-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. HA-NUB1 and HA-NUB1L lysates (**F**) or HA-NUB1LΔ1-3UBA and HA-NUB1LΔUBL lysates (**G**) were incubated overnight at 4^{o}C with an anti HA antibody to immunoprecipitate the HA tagged proteins. The asterisk demarcates actin in the input and the IgG heavy chain (Hc) is indicated.

Figure 7. NUB1L induces a block in autophagy flux requiring the UBA domain (**A**-**E**) and facilitates the extracellular release of tau (**F**). TetR-GFP-tau cells were untransduced (**A**) or transduced with pRRL-HA-NUB1L (**B**), pRRL-HA-NUB1LΔ1-3UBA (**C**) or pRRL-HA-NUB1LΔUBL (**D**). Cells were treated with DMSO or MG132 for 8 h before cell lysis.

Bafilomycin A1 (BAF) or 3'-methyladenine (3-MA) were added to MG132 treated cells 3 h before cell lysis. Cell lysates were analysed by immunoblotting using anti LC3B, anti HA and anti actin. The experiment (**A-D**) was conducted simultaneously under identical experimental conditions, and all western blots were analysed simultaneously using identical settings. To measure the effect of proteasome inhibition alone (**A**), LC3BII band intensity was measured (Image J), normalised to actin and plotted. To measure the effect of HA-NUB1L (**B**), HA-NUB1LΔ1-3UBA (**C**) and HA-NUB1LΔUBL (**D**), LC3BII and HA band intensity were measured (ImageJ), normalised to actin and LC3BII levels plotted relative to HA expression. (**E**) LC3BII levels are plotted together to compare the effect of HA-NUB1L (light grey bars), HA-NUB1LΔ1-3UBA (medium grey bars) or HA-NUB1LΔUBL (dark grey bars) to levels in untransduced cells (white bars). For **A**-**E**, data (n=3) represents the averaged value of triplicate samples ± SD. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. (**F**) The total supernatants from TetR-GFP-tau cells overexpressing HA-NUB1 in comparison to untransduced cells in the absence or presence of MG132 were analysed by dot blot on a nitrocellulose membrane using antibodies to total tau and phosphorylated tau (pS396). The intensity of each spot (in triplicate) per condition was measured in 3 independent experiments (n=3) using ImageJ, averaged and plotted relative to the untransduced control. Two-way ANOVA was applied: (*) $p<0.05$; (**) $p<0.01$; (***) $p<0.001$. The blots shown beneath each graph are from samples analysed on the same blot and are representative of 1 experiment.

Figure 8. The role of NUB1 in tau proteostasis. Abnormal hyperphosphorylation of tau is an early pathological event regulated by kinases such as GSK3β, and phosphatases such as PP2A. Tau is also ubiquitinated by various ubiquitin ligases, however in the early stages, tau may be degraded by the proteasome in either an ubiquitin-independent or ubiquitin-dependent manner. NUB1 may facilitate the targeted proteasomal degradation of phosphorylated ubiquitinated tau by facilitating tau ubiquitination and delivery to the proteasome via the NUB1 ubiquitin-like domain (UBL). Tau oligomers and aggregates are inaccessible to the proteasome and are targeted to the autophagy-lysosome system. NUB1 may facilitate the delivery of tau to the autophagy-lysosomal system via interaction with the autophagy receptor p62 when the activity of the proteasome is compromised. The NUB1-facilitated accumulation of tau in the autophagy-lysosomal system may in turn lead to an

impairment of the fusion of autophagosomes with lysosomes, redirecting the pathological tau species towards extracellular release.

Supplementary figures

Figure S1. GFP-tau predominantly localizes to microtubules but accumulates in aggresomes following proteasome inhibition. (**A**) The localization of GFP-tau was analysed following the treatment of cells with DMSO or MG132 for 8 h by immunocytochemistry to detect tau, α-tubulin, pericentrin and vimentin. Scale Bar 20 μm. (**B**) The localization of GFP-tau at the MTOC positive for pericentrin (*) after 8 h of MG132 treatment is shown to highlight the number of cells with tau positive aggresomes. Scale Bar 20 μm.

Figure S2. Localization of NUB1 and tau in the hippocampus of young and aged hi-TAU mice. NUB1 and tau localization was investigated in the dentate gyrus (infrapyramidal and suprapyramidal blades) and the CA1, CA2 and CA3 regions of the hippocampus of WT and hi-TAU mice at 4 and 12 months by immunohistochemistry using anti NUB1 and anti tau antibodies. NUB1 localizes to the hippocampal nuclei and axons in WT and hi-TAU animals at 4 and 12 months of age, while tau localizes along neuronal axons in WT mice at 4 and 12 months, and to the axons of the stratum lucidum of the CA3 area at 4 months and NFTs at 12 months in hi-TAU mice. Arrows indicate examples of NFTs. gcl, granule cell layer; sgz, subgranular zone; pcl, pyramidal cell layer, sl, stratum lucidum, so, stratum oriens. Scale bar 20 μm

Abbreviations

3-methyladenine (3-MA)

Alzheimer's disease (AD)

Analysis of Variance (ANOVA)

bafilomycin A1 (BAF)

bicinchoninic acid (BCA)

microtubule organising centre (MTOC)

multiplicity of infection (MOI)

Negative Regulator of Ubiquitin-Like Protein 1 (NUB1)

neurofibrillary tangles (NFTs)

phosphatase inhibitor (PhIC)

phosphatidylinositol 3-kinases (PI-3K)

protease inhibitor cocktail (PIC)

sodium dodecyl sulphate (SDS)

tetracycline (TET)

tunneling nanotubes (TNTs)

ubiquitin-associated (UBA)

ubiquitin-like (UBL)

vacuolar H+ ATPase (V-ATPase)