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RESEARCH ARTICLE

FRMD4A–cytohesin signaling modulates the cellular release of tau

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ABSTRACT

One of the defining pathological features of Alzheimer's disease is the intraneuronal accumulation of tau (also known as MAPT) protein. Tau is also secreted from neurons in response to various stimuli and accumulates in the cerebrospinal fluid of Alzheimer's disease patients. Tau pathology might spread from cell to cell through a mechanism involving secretion and uptake. Here, we developed an assay to follow cellular release and uptake of tau dimers. Individual silencing of ten common late-onset Alzheimer's disease risk genes in HEK293T cells expressing the tau reporters suggested that *FRMD4A* is functionally linked to tau secretion. FRMD4A depletion by using RNA interference (RNAi) reduced and overexpression increased tau secretion. The activity of cytohesins, interactors of FRMD4A and guanine-nucleotide-exchange factors of Arf6, was necessary for FRMD4A-induced tau secretion. Increased Arf6 and cell polarity signaling through Par6 and atypical protein kinase Cζ (aPKCζ) stimulated tau secretion. In mature cortical neurons, FRMD4A RNAi or inhibition of cytohesins strongly upregulated secretion of endogenous tau. These results suggest that FRMD4A, a genetic risk factor for late-onset Alzheimer's disease, regulates tau secretion by activating cytohesin–Arf6 signaling. We conclude that genetic risk factors of Alzheimer's disease might modulate disease progression by altering tau secretion.

KEY WORDS: Alzheimer's disease, Neurodegenerative disease, Functional genomics, Polarity signaling, Risk gene, PARD6A

INTRODUCTION

Tau (also known as MAPT) is a microtubule-associated protein normally localized in the cytosol of neurons and other cells (reviewed by Buée et al., 2000). Intraneuronal accumulation of hyperphosphorylated and fibrillar aggregated forms of tau results in the formation of pathological neurofibrillary tangles that characterize several neurodegenerative diseases, including Alzheimer's disease (Ballatore et al., 2007; Morris et al., 2011). In patients suffering from Alzheimer's disease, and also other forms of central nervous system (CNS) injury, increased tau levels are found in the cerebrospinal fluid (CSF) (Ost et al., 2006; Vigo-Pelfrey et al., 1995). Previously, CSF-tau was thought to mostly originate from degenerated axons and dead neurons (Blennow et al., 1995). However, tau is also found in the CSF of healthy human subjects (Blennow et al., 1995; Vandermeeren et al., 1993; Vigo-Pelfrey et al., 1995) and interstitial fluid of healthy wild-type mice (Yamada et al., 2011).

Recent data suggest that misfolded tau can propagate from cell to cell in a prion-like fashion (Frost et al., 2009; Sanders et al., 2014). Tau can be released from cells, including neurons, in the absence of disease or toxicity, and tau release is enhanced by neuronal activity (Pooler et al., 2013) and modified by changes in the tau protein that are associated with tauopathies (Karch et al., 2012). Presynaptic glutamate release is associated with tau secretion (Yamada et al., 2014), and synaptic contacts enhance cell-to-cell propagation of tau (Calafate et al., 2015). It has been suggested that full-length non-vesicle-associated tau is released by an unconventional temperature-dependent secretion process that is not blocked by inhibitors of the conventional secretory pathway, such as monensin or brefeldin A (Chai et al., 2012).

Tau misfolds into low molecular weight (LMW) oligomers prior to assembly into fibrils. Tau dimers appear to be a crucial building block for the formation of tau aggregates, including paired-helical filaments (PHFs) (Friedhoff et al., 1998). Both extracellular LMW tau oligomers and short fibrils, but not monomers or long fibrils, can be taken up by neurons (Wu et al., 2013). Frost et al. have shown that extracellular tau aggregates, but not tau monomers, are taken up by cultured cells and can induce fibrillization of intracellular full-length tau (Frost et al., 2009). Macropinocytosis, an endocytic process characterized by actin-driven membrane ruffling that allows internalization of extracellular fluids and macromolecular structures, has been shown to be involved in cellular uptake of tau fibrils (Holmes et al., 2013).

The question of how genes associated with late-onset Alzheimer's disease (LOAD) confer the risk for sporadic disease is important for better understanding of the etiology of the disease and for developing effective therapies. We recently used a protein-fragment complementation assay (PCA)-based *in vitro* system combined with individual knockdown of the top ten LOAD risk genes following RNA interference (RNAi) to study their potential functional connection to amyloid-beta precursor protein (APP) metabolism and regulation of tau phosphorylation (Martiskainen et al., 2015). Here, we developed a sensitive live-cell assay system for monitoring cellular release and uptake of tau dimers, based on the PCA principle (Remy and Michnick, 2006). Using a combination of these assays and small interfering RNAs (siRNAs) against selected LOAD risk genes, we found that a pathway involving FRMD4A, a LOAD risk gene (Lambert et al., 2013), together with its partners in the atypical protein kinase C (aPKC)–Par cell polarity signaling complex and Arf6, regulate tau secretion.

RESULTS

A sensitive live-cell assay for monitoring secretion of tau dimers

We developed a sensitive live-cell assay system as a quantitative *in vitro* model for studying cellular release and uptake of tau dimers.

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PCA allows detection of interactions of proteins fused with complementary reporter protein fragments. We have previously used a PCA based on humanized *Gaussia princeps* luciferase (GLuc) (Remy and Michnick, 2006) for studying cellular regulation of both APP and tau (Martiskainen et al., 2015; Merezhko et al., 2014; Nykänen et al., 2012). Here, we used a tau dimer PCA reporter based on the tau 0N4R isoform carrying complementary GLuc fragments (Fig. 1). Luminescence generated in washed cell monolayers served as a quantitative measure of the amount of intracellular tau dimers, whereas the luminescence signal in cell- and debris-cleared conditioned medium was used as a readout of tau secretion.

Expression of tau–GLuc reporter proteins was compared to non-tagged tau in HEK293T cells (Fig. 2A). Coexpression of tau–GLuc1 and tau–GLuc2 in HEK293T cells resulted in a high level of PCA signal. As the highest PCA signals were generated by reporter constructs that carried the GLuc fragment at the C-termini (Fig. 2B), we proceeded with assay development based on tau–GLuc1C and tau–GLuc2C constructs. Expression of tau reporters alone or in combination with free GLuc fragments did not generate luminescence signal (Fig. 2C,D). When tau–GLuc1 and tau–GLuc2 (hereafter tau–GLuc1/2) constructs were expressed together, there was high luminescence generated by washed cell monolayers but also a strong luminescence signal present in the cleared culture medium (Fig. 2D). The signal from the medium was independent of changes in lactate dehydrogenase (LDH) release, suggesting that the appearance of tau reporters in media was not due to passive leakage and disruption of cells (Fig. 2D). Both intracellular and secreted tau levels correlated with the level of tau–GLuc1/2 reporter gene dose (Fig. 2E). During a 24-h observation period, tau–GLuc1/2 reporters accumulated in serum-free culture medium with linear kinetics for up to 20 h, as shown by a LDH-normalized tau secretion PCA (Pearson r^2=0.9715; Fig. 2F).

A previous report using the split GFP system to study tau oligomerization found that aggregation reduced the signal-generating ability of the tau reporter system (Chun et al., 2007). Western blot analysis of tau–GLuc species from the cell lysate showed that monomeric tau–GLuc1/2 reporters migrated at ∼70 kDa and with a minor band at ∼140 kDa representing dimeric tau, and some lower bands likely representing truncated tau species (Fig. 2G). Chemical crosslinking (BS3) was used to preserve higher oligomeric tau species in both the cell lysate and conditioned medium. In the crosslinked, concentrated medium sample, there was a weak band corresponding to monomeric tau, and the rest of the tau immunoreactivity appeared as a high molecular mass smear (Fig. 2G), similar to previously described *in vitro* generated tau fibrils (Wu et al., 2013). In cell lysates, the monomeric tau band remained strong even after crosslinking, with some additional dimeric and trimeric and tetrameric bands visible. When the tau–GLuc1/2 conditioned medium was resolved on a native gel, a roughly equal ratio of tau–GLuc monomers and dimers was observed (Fig. 2H). In addition, some tau–GLuc was trapped in the stacking gel suggesting that some tau–GLuc1/2 in the conditioned medium was in an aggregated form. These results show that the majority of the secreted tau–GLuc reporters are in the form of monomers, dimers and soluble pre-aggregates or fibrils, and thus the GLuc tag does not interfere with the normal oligomerization and aggregation behavior of tau.

Next, we studied the reversibility of the tau–GLuc PCA system in a detergent titration experiment. As expected, addition of SDS to samples containing either full-length GLuc enzyme or tau–GLuc1/2 reporter complexes completely suppressed all luminescence signal starting from 0.01% concentration of the detergent (Fig. 2I, left panel). Addition of Triton X-100, a milder non-ionic detergent capable of dissociating protein complexes, had no effect on the activity of the GLuc enzyme even up to 1% concentration, whereas luminescence derived from tau–GLuc1/2 reporter complexes was completely suppressed by only 0.1% detergent concentration (Fig. 2I, middle panel). A similar effect was observed with saponin, a glucoside with detergent properties (Fig. 2I, right panel). These data suggest that the majority of tau–GLuc1/2-derived luminescence signal is generated by tau dimers that are reversibly

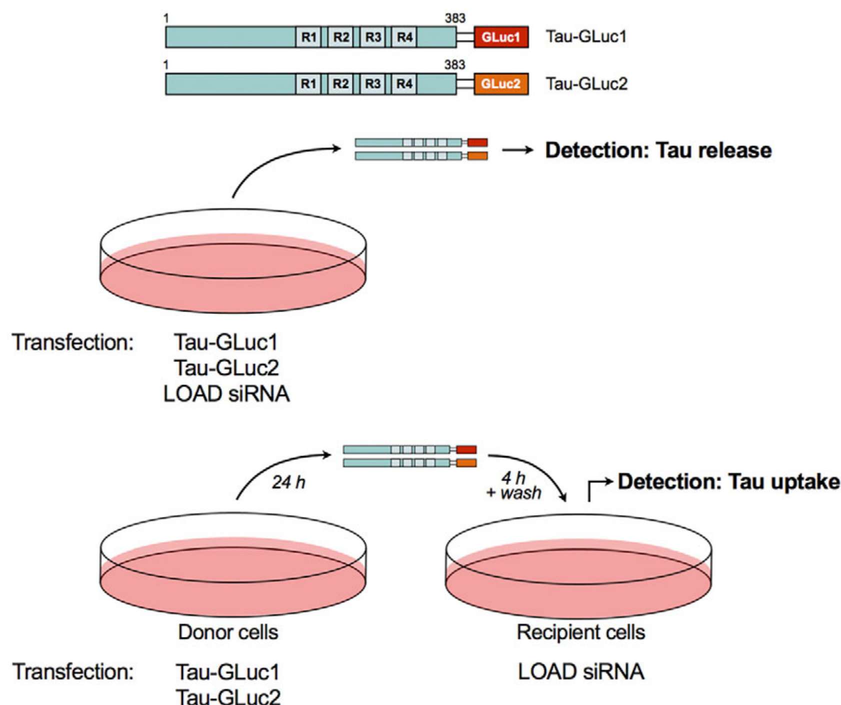

Fig. 1. Experimental design used in this study. GLuc PCA-based detection of tau dimer release and uptake, and the screening strategy for studying the effects of LOAD susceptibility genes on tau release and uptake.

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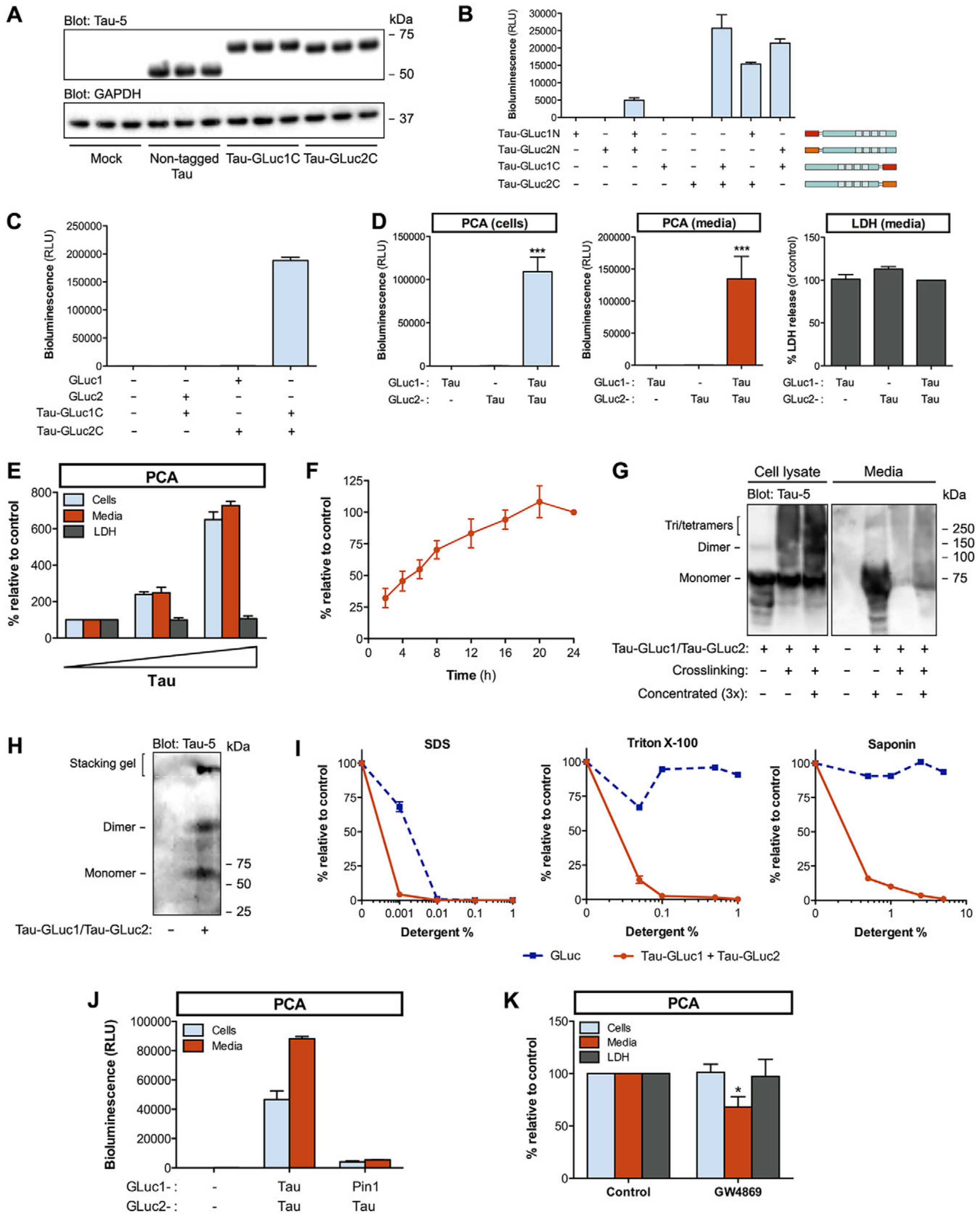

Fig. 2. See next page for legend.

associated. Moreover, given that it is highly unlikely that the soluble tau–GLuc aggregates observed in Fig. 2H would be dissolved by 0.1% Triton X-100, it seems that the tau–GLuc in the aggregate form is not capable of generating luminescence.

Whereas the tau–GLuc reporter dimers accumulated in the culture medium, a PCA reporter complex consisting of tau and the peptidyl-prolyl cis-trans-isomerase Pin1 (Nykänen et al., 2012), was not secreted at high levels (Fig. 2J). Ceramide regulates exocytosis

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Fig. 2. GLuc PCA-based detection of tau dimer release. (A) Western blot analysis of tau–GLuc reporter constructs and non-tagged tau in HEK293T cells. GAPDH was used as loading control. (B) Expression of different combinations of tau–GLuc reporters carrying the luciferase fragments either at the N- or C-termini. (C) GLuc fragments expressed alone or with a single luciferase fragment attached to tau do not generate significant background signal. (D) The tau–GLuc1C and tau–GLuc2C PCA signal in washed cell monolayers (left) and in conditioned medium (middle) is shown. Relative LDH release (right) was used as a release control. (E) The tau PCA signal intensity in cells and medium is dependent on the reporter gene dosage (reporter plasmids at 60 ng, 80 ng and 100 ng per well). (F) Tau release kinetics during a 24-h incubation. The tau dimer level in the medium was determined by PCA, and the PCA signal was normalized to the LDH release. All values are relative to the 24-h timepoint. (G) Western blot analysis of tau–GLuc reporter constructs in cell lysates and conditioned medium. Concentration (3×) and crosslinking was used to facilitate detection of secreted tau–GLuc reporters. (H) Native PAGE analysis of tau–GLuc1/2 in 100× concentrated conditioned medium. 100× concentrated medium from mock-transfected cells (–) was used as a control. (I) The reversibility of the tau–GLuc1/2 interaction (red) in conditioned medium was studied by titrating with SDS (left panel), Triton X-100 (middle panel) and saponin (right panel). Full-length *Gaussia* luciferase (blue) was used as a control. (J) Comparison of cellular and medium PCA signals from cells expressing tau–GLuc1/2 and Pin1–GLuc1 and tau–GLuc2 reporters. (K) The effect of GW4869 (10 µM, 24 h) on tau–GLuc1/2 reporter release. RLU, relative light units. Results are mean±s.e.m. from three or four replicate experiments. **P*<0.05, ****P*<0.001 (ANOVA).

(Rohrbough et al., 2004) and exosomal secretion (Trajkovic et al., 2008), and tau might be secreted in association with small vesicles such as exosomes (Saman et al., 2012) and other microvesicles (Dujardin et al., 2014). GW4869, an inhibitor of ceramide generation by neutral sphingomyelinase (nSMase), reduced tau–GLuc1/2 reporter secretion from HEK293T cells by 32% (Fig. 2K) suggesting that tau secretion could be linked to sphingomyelin metabolism and membrane microdomains associated with ceramide signaling.

The total tau content in the cell lysate and conditioned medium was determined by enzyme-linked immunosorbent assay (ELISA) and normalized to the total protein content. Cell lysates contained on average 16,700±1300 pg of tau per µg of protein whereas the medium contained 77.6±7.3 pg of tau per µg of protein (mean±s.e.m.; Fig. 3A). Based on these values, it seems that less than 0.5% of the cellular tau is released in our HEK293T cell system. This is comparable to a previous report showing that 0.1–0.3% of cellular tau is released from transfected HEK293 T-Rex cells and from human induced neurons (Chai et al., 2012).

It remains currently unclear whether tau is secreted inside or in association with microvesicles (Dujardin et al., 2014; Saman et al., 2012; Simón et al., 2012), in vesicle-free form (Chai et al., 2012; Kim et al., 2010a) or as a mixture of those. In the HEK293T cell-based system, 99.8% of the tau dimer-derived PCA signal was abolished after addition of 0.005% (v/v) trypsin to the conditioned medium (Fig. 3B). Addition of 0.005% saponin (v/v) together with 0.005% trypsin resulted in a 99.9% loss of the PCA signal. These data suggest that the majority of tau dimers are in vesicle-free form, with less than 0.5% of secreted tau being inside vesicles. tau–GLuc1/2- conditioned medium was next fractionated into ectosomal (larger microvesicles), exosomal and vesicle-free fractions (Fig. 3C), and their tau content was analyzed by western blotting (Fig. 3D). Owing to the low level of vesicular tau, the unfractionated and vesicle-free media fractions were further concentrated by performing an Amicon filter centrifugation before loading onto the gel. Semi-quantitative analysis of the western blots confirmed that the majority (99.7%) of tau secreted by HEK293T cells was in vesicle-free form whereas ectosomal (0.22%) and exosomal (0.05%) tau represented a small minority of the extracellular tau produced by these cells (Fig. 3E). PCA analysis also confirmed that the vast majority of the tau-dimer-derived PCA signal remained in the vesicle-free fraction after the ultracentrifugation steps (Fig. 3F). Taken together, these data characterize a PCA-based assay for monitoring cellular release of soluble tau dimers.

Studying cellular uptake of tau dimers using the tau–GLuc PCA

In order to study cellular uptake of our tau reporters, we first conditioned medium for 24 h with HEK293T cells expressing tau–GLuc1 and tau–GLuc2. When naïve recipient cells were exposed to the tau–GLuc1/2-conditioned medium for 4 h, washed with PBS and immunostained for tau, a punctate intracellular tau pattern was observed (Fig. 4A, right) suggesting that some of the tau–GLuc1/2 reporters had been internalized by the cells. Cells exposed to conditioned medium generated by mock-transfected cells did not show similar tau-immunoreactive puncta (Fig. 4A, left).

PCA data further showed that a simple washing step by PBS abolished >99% of the cell-bound PCA signal (Fig. 4B), suggesting that although extracellular tau species bind to the cell surface, the tau uptake process in HEK293T cells is not very rapid or efficient. Heparan sulfate proteoglycans (HSPGs), which are ubiquitously expressed on cell surfaces, mediate uptake of tau assemblies from monomers to oligomers and fibrils (Holmes et al., 2013; Mirbaha et al., 2015). An additional washing step with 20 µg/ml heparin further reduced the signal. Addition of trypsin to the cells after these washing steps showed that majority of the cell-surface-bound tau had been removed, suggesting that the remaining PCA signal represents internalized tau species. Further addition of saponin, to allow cell-membrane penetration of trypsin, abolished the remaining cell-bound signal (Fig. 4B). Thus, a washing procedure including three steps [PBS, heparin (20 µg/ml in DMEM) and PBS] was used in all subsequent tau uptake experiments.

As shown in Fig. 4C, the accumulation kinetics of the tau PCA signal suggest a saturable mechanism, with the uptake rate slowing down after 4 h. Therefore, an exposure time of 4 h was used in all subsequent tau uptake experiments. Overall, the internalized tau PCA signal was low, possibly due to re-secretion of a significant portion of uptaken tau (data not shown). Addition of GW4869, previously shown to reduce tau secretion (Fig. 2K), to the cells 16 h before and during incubation with tau-conditioned medium significantly increased the tau PCA signal retained by the cells (by up to 184%; Fig. 4D), supporting the idea of rapid re-secretion of internalized tau dimers.

RNAi screen of LOAD susceptibility genes

In order to study whether LOAD risk genes are functionally linked to tau secretion and uptake, we used a panel of siRNAs for knocking down ApoE, BIN1, CLU, ABCA7, CR1, PICALM, CD33, CD2AP, FRMD4A and TREM2 expression (Martiskainen et al., 2015) (Fig. 1). These top ten LOAD risk genes were selected based on meta-analyses of LOAD genetic association studies (Bertram et al., 2007), with the addition of two recently identified risk genes (Guerreiro et al., 2013; Jonsson et al., 2013; Lambert et al., 2013). MS4A6A and MS4A4E were excluded as they are not expressed in HEK293T cells. The knockdown efficiencies of the selected siRNAs were determined by quantitative real-time PCR (qPCR) in our previous study (Martiskainen et al., 2015). We co-transfected tau–GLuc1 and tau–GLuc2 PCA reporters with the selected

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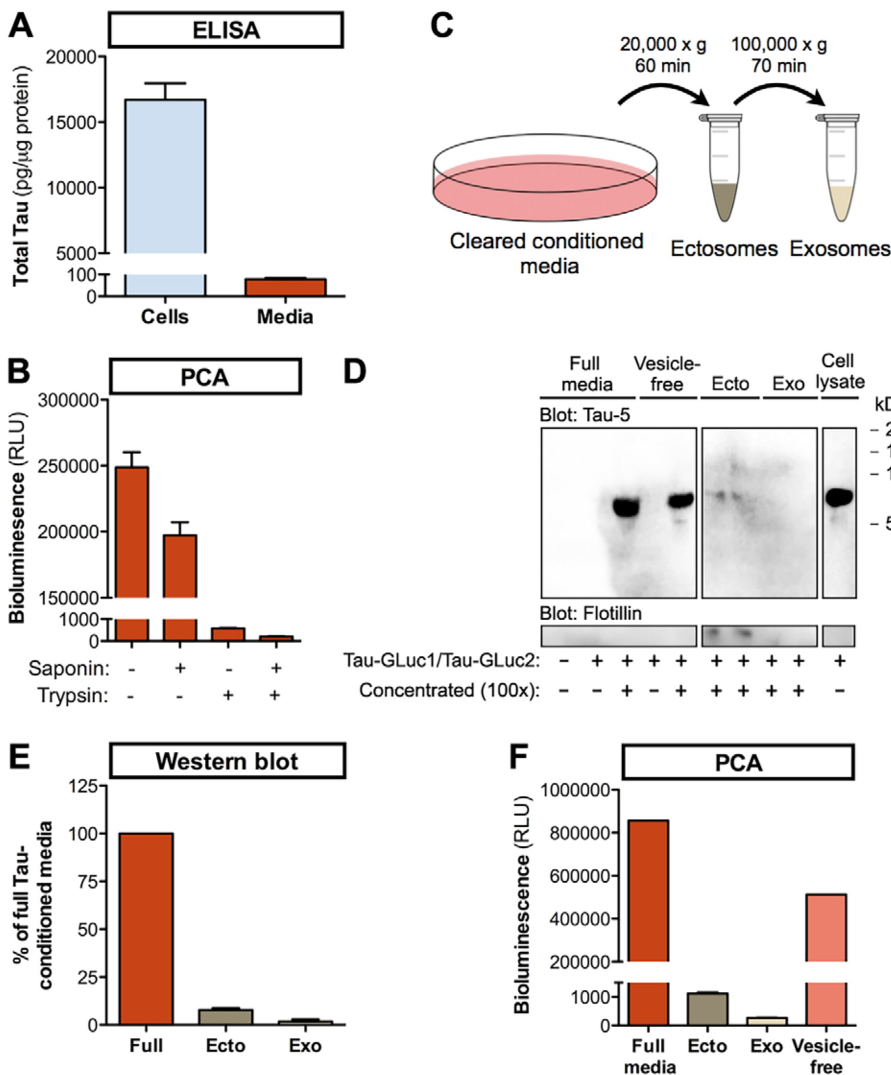

Fig. 3. Characterization of the tau–GLuc reporters in conditioned medium. (A) ELISA analysis of total tau levels in HEK293T cell lysates and conditioned medium after transient transfection with tau–GLuc1/2 reporters. (B) Addition of 0.005% (v/v) trypsin to the tau-reporter-conditioned medium shows that the majority of HEK293T-secreted tau is free-floating and not protected by vesicle membrane. Addition of 0.005% (v/v) saponin and trypsin shows that permeabilization of the vesicle membrane allows nearly complete degradation of the reporter proteins in the medium. Results in A and B are means±s.e.m. (*n*=3). (C) Schematic presentation of the conditioned medium fractionation process. Ectosomal, exosomal and vesicle-free fractions were separated by sequential centrifugation. (D) Western blot analysis of tau–GLuc1/2 reporter proteins in conditioned medium and isolated media fractions. Vesicle-free, ectosomal, exosomal and unfractionated full media were analyzed. Cell lysate served as a positive control of tau expression. Some samples were concentrated 100× by filter centrifugation to facilitate detection of tau. Flotillin-1 was used as a marker of ectosomes. (E) Optical-density-based quantification of tau–GLuc1/2 levels (from experiments as in D). All values are relative to the tau–GLuc1/2 level in full medium (100%). (F) PCA analysis of the tau–GLuc1/2 dimer signal in unfractionated medium and isolated media fractions. RLU, relative light units. Results in E and F show the mean from two replicate experiments.

siRNAs in HEK293T cells. Knockdown of any of the LOAD genes did not result in significant changes in the level of intracellular tau dimers (Fig. 5A). For assessing tau secretion, conditioned medium was analyzed for both tau dimer content, by PCA, and LDH release, as a specificity control. Based on the LDH-normalized tau PCA signal in the medium, knockdown of CD33, CD2AP, FRMD4A and TREM2 reduced tau secretion (Fig. 5B). TREM2 knockdown had the strongest effect (−55%), whereas the effect of CD33, CD2AP and FRMD4A was between −19% and −27% (mean values from cells transfected with two independent siRNAs per target gene). For the tau uptake assay, we transfected recipient cells with siRNAs against LOAD risk genes, exposed them to tau–GLuc1/2-conditioned medium for 4 h, washed them and measured the level of the internalized tau dimer PCA signal (Fig. 5C). Only ApoE knockdown caused a significant change in tau uptake (+29%).

TREM2 and CD33 are strongly expressed in myelomonocytic cells and their expression level in HEK293T cells is low. Next, we used the tau release PCA assay and siRNA in the CHME-5 cell line, which is derived from primary cultures of human fetal microglial cells (Janabi et al., 1995). Because tau accumulates in microglial cells during aging, overexpression of tau in microglial cells induces their activation (Wang et al., 2013) and microglia might facilitate spreading of tau pathology (Asai et al., 2015), the effect of TREM2 and CD33 on tau secretion could be a potentially interesting lead. However, in CHME-5 cells transfected with both tau–GLuc1 and tau–GLuc2 PCA reporters and either TREM2 or CD33 siRNA, there was no change in either cellular tau dimerization or secreted tau dimer content (Fig. 5D). This suggests that the effects of TREM2 and CD33 on tau release seen in HEK293T cells are not recapitulated in a microglial cell line.

FRMD4A–cytohesin signaling regulates cellular release of tau

As our previous study has shown that FRMD4A is reduced in the brain of LOAD patients and functionally linked to tau (Martiskainen et al., 2015), we wanted to further explore the role of FRMD4A in tau secretion. For FRMD4A overexpression studies, we first verified that tagged FRMD4A constructs are properly expressed and localized in HEK293T cells. Expression of FRMD4A–HA and FRMD4A–GFP resulted in similar localization patterns, mostly in cytosolic vesicle-like structures (Fig. 6A). Coexpression of FRMD4A–GFP with tau–GLuc2 did not reveal substantial colocalization of the two proteins. In HEK293T cells, overexpressed tau was present close to the plasma membrane, in regions where occasional FRMD4A-positive vesicles were also observed (Fig. 6B, arrowheads). The FRMD4A–HA construct was also used to verify the silencing efficiency of the FRMD4A siRNA at the protein level. Coexpression of the FRMD4A siRNA with the FRMD4A–HA plasmid resulted in a 39% reduction

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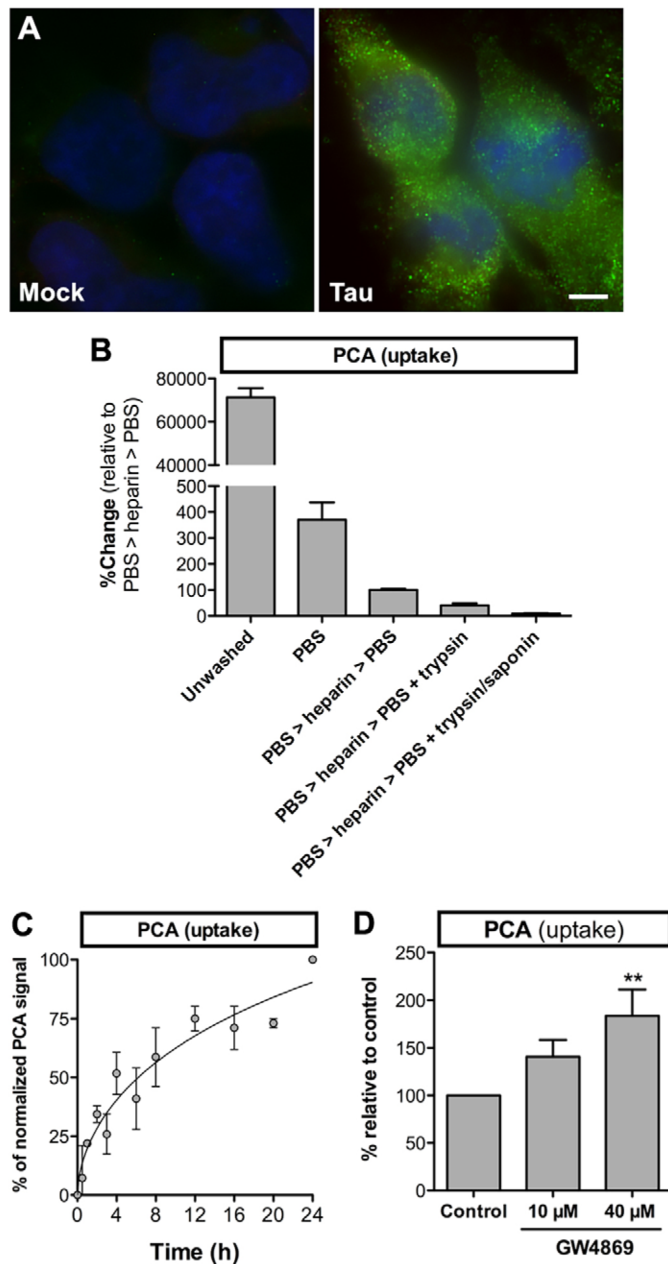

Fig. 4. Tau–GLuc1/2 reporter uptake in naïve HEK293T recipient cells. (A) Immunofluorescence micrographs of tau–GLuc1/2 internalized by naïve HEK293T cells. After 4 h incubation of naïve recipient cells in the mock- (left) or tau–Gluc1/2- (right) conditioned medium, cells were washed, fixed and immunostained with the tau-5 antibody. Nuclei were counterstained with Hoechst 33342. Scale bar: 10 µm. (B) Comparison of the effects of wash conditions on cell-bound tau as determined by the PCA. Naïve HEK293T cells were exposed to tau–GLuc1/2 conditioned medium for 24 h. Before PCA measurement, conditioned medium was removed from the wells and the cells were washed as indicated. (C) Tau dimer uptake kinetics over a 24-h period. Values are relative to maximal uptake at 24 h. (D) Addition of GW4869 to the conditioned medium enhances the cellular PCA signal, and thus the amount of internalized tau–GLuc1/2 reporters. Recipient cells were pretreated with GW4869 or an equal volume of DMSO for 16 h before a medium change and during the 4-h exposure to tau–GLuc1/2-conditioned medium. Results are mean±s.e.m. (*n*=3). $^{**}P<0.01$ (ANOVA).

of the FRMD4A–HA protein level (Fig. 6C). This is in line with the reduction of FRMD4A mRNA level (−41%) with this siRNA (Martiskainen et al., 2015).

Increasing levels of FRMD4A expression dose-dependently increased the levels of tau dimer PCA signal in medium (maximum +146%), whereas the cellular tau dimer PCA level remained almost unchanged (Fig. 6D). These data, together with that shown in Figs 5B and 6C, show that the level of FRMD4A expression correlates with the level of tau release in HEK293T cells. In epithelial cells, FRMD4A has been shown to act as a scaffolding protein connecting the cell polarity complex between Par3 and Par6 (PARD3 and PARD6A, respectively) to Arf6 signaling mediated by cytohesin-1 (Ikenouchi and Umeda, 2010). SecinH3, a small-molecule antagonist of cytohesin and Sec7 (the yeast cytohesin) guanine-nucleotide-exchange factor (GEF) activity (Hafner et al., 2006), suppressed the tau secretion induced by FRMD4A overexpression while increasing intracellular tau dimer levels (Fig. 6E). In cells expressing endogenous levels of FRMD4A, SecinH3 also increased intracellular tau levels but had only a mild effect on tau secretion (Fig. 6F).

FRMD4A–cytohesin signaling enhances translocation of active Arf6 to the plasma membrane (Ashery et al., 1999; Hafner et al., 2006; Ikenouchi and Umeda, 2010). Similar to overexpression of FRMD4A, overexpression of wild-type Arf6 in HEK293T cells expressing tau–GLuc1/2 cells strongly enhanced both intracellular tau dimerization and tau secretion. Overexpression of wild-type Arf6 caused a strong increase in tau secretion (Fig. 6G), which was almost completely blocked by coexpression of Arf6 siRNA but was not affected by SecinH3 treatment. Expression of the dominant-negative mutant Arf6 T27N also mildly increased tau secretion, but, in comparison to wild-type Arf6, the effect was significantly lower (−69%; Fig. 6H). The constitutively active Arf6 Q79L mutant had the strongest impact on tau secretion, with a more than 30-fold increase compared to endogenous Arf6 levels.

aPKC subtype ζ (aPKCζ) is a ceramide-binding protein and, as a part of the Par polarity signaling complex, regulates several membrane trafficking events also related to exocytosis (Horikoshi et al., 2009; Joberty et al., 2000; Wang et al., 2009). Overexpression of aPKCζ enhanced tau secretion with an effect comparable to FRMD4A overexpression (Fig. 7A). Expression of the C-terminal ceramide-binding region of aPKCζ (C20ζ, amino acids 405–592) (Wang et al., 2009) did not significantly affect basal tau secretion but coexpression of C20ζ suppressed aPKCζ-overexpression-induced tau secretion.

Par6 links aPKCζ to Par3 (Joberty et al., 2000), and Par3 has been shown to activate Arf6 through FRMD4A (Ikenouchi and Umeda, 2010). Expression of wild-type Par6 resulted in a sevenfold increase in tau secretion whereas expression of Par6 (S345A), an inactive mutant not phosphorylated by aPKC (Gunaratne et al., 2013), had little effect (Fig. 7C). SecinH3 treatment had no effect on aPKCζ- or Par6-induced tau secretion, suggesting that cytohesin GEF activity is not required for this effect of the aPKC–Par6 complex (Fig. 7B,D). These results show that tau secretion is regulated by the activity of the FRMD4A–cytohesin–Arf6 pathway and the associated aPKC–Par6 polarity complex signaling.

Whereas cytohesins (also known as mSec7 proteins) have a role in vesicle transport at the presynaptic terminal (Ashery et al., 1999; Neeb et al., 1999), the role of FRMD4A in neurons remains poorly understood. We transduced cultures of mouse cortical neurons with lentivirus expressing FRMD4A short hairpin RNA (shRNA), resulting in 99% reduction of endogenous FRMD4A mRNA levels (Fig. 8A). Mature [21 days *in vitro* (DIV)] cortical neurons were transduced with a GFP-expressing lentivirus, an FRMD4A shRNA lentivirus or were left untreated, and medium was

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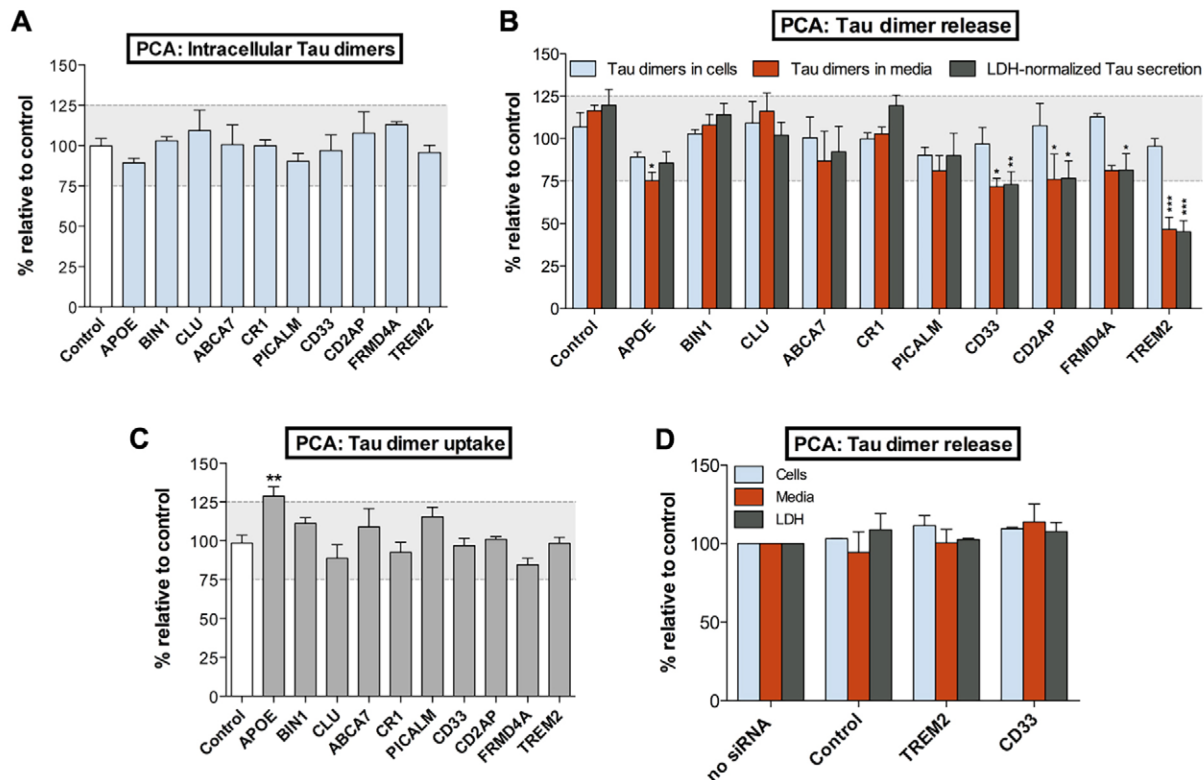

Fig. 5. Effect of selected LOAD risk genes on tau secretion and uptake. (A) Levels of intracellular tau dimers. HEK293T cells were co-transfected with tau-GLuc1/2 PCA reporters and indicated siRNAs. No alterations in intracellular tau dimerization were observed. Control cells were transfected with 5 nM control siRNA. Two control siRNAs were used, and their average effect is reported as the control. (B) Cellular release of tau dimers. HEK293T cells were transfected as in A. LDH release was used to normalize tau secretion values. (C) The effect of gene silencing on cellular uptake of tau dimers. HEK293T cells were transfected with indicated siRNAs and exposed to conditioned medium containing the tau–GLuc1/2 dimers. (D) Tau dimer release in CHME-5 cells co-transfected with control, TREM2 and CD33 siRNA. Results are mean±s.e.m. (n=3). *P<0.05, **P<0.01, ***P<0.001 (ANOVA).

conditioned for 3 days. A mouse-specific ELISA was used to measure secretion of endogenous tau. As shown in Fig. 8B, silencing of FRMD4A expression in neurons significantly promoted tau secretion (+390%, P=0.046 versus untransduced control cells). As the effect was opposite to what was observed in HEK293T cells, we also tested the effect of the cytohesin inhibitor SecinH3. Compared to vehicle-treated mature neurons, SecinH3 also enhanced tau secretion (Fig. 8C), similar to the FRMD4A shRNA. The maximum effect was reached at 20 µM (+204%, P=0.031). These results suggest that altering the activity of FRMD4A–cytohesin signaling significantly affects tau secretion, but compared to non-neuronal cells, such as HEK293T, the effect is unexpectedly in the opposite direction.

DISCUSSION

Here, we report development of a new assay system for monitoring the cellular release and uptake of soluble tau dimers. Although the assay was designed to be a minimally invasive live-cell system, there are two potential caveats: the use of GLuc reporter fusion fragments at the C-terminus of tau and the requirement of overexpression of tau–GLuc reporter constructs. However, the C-terminal GLuc fusion of tau did not interfere with the normal expression, localization and oligomerization or aggregation behavior of tau. In our transient-transfection-based setup in HEK293T cells, tau reporter expression was not associated with significant toxicity. LDH release was used to control for unspecific release of cytosolic proteins.

The PCA signal generated by the secreted tau–GLuc1/2 reporters appears to be largely derived from tau dimers, which have been previously shown to be the crucial building blocks for formation of tau aggregates and PHFs (Friedhoff et al., 1998). Native gel and western blot analyses suggested that the secreted tau–GLuc reporters spontaneously assembled into dimers and, to some degree, into soluble aggregates or fibrils. Importantly, the tau–GLuc1/2 dimers could be dissociated with low concentrations of Triton X-100 and saponin suggesting that they are reversibly associated. The association of GLuc PCA reporters has previously shown to be reversible (Remy and Michnick, 2006). However, in a bimolecular fluorescence complementation (BiFC) assay, which is using complementary fragments of fluorescent reporter proteins, the reporter proteins tend to lock the interacting proteins together (Kodama and Hu, 2012). Moreover, given that 0.1% Triton X-100 is unlikely to dissolve aggregated or fibrillar forms of tau, and, at this concentration, all of the tau–GLuc1/2 PCA signal was lost from the conditioned medium samples, it seems that the aggregated tau reporters are incapable of generation of luminescence. In this regard, our GLuc PCA would be similar to the previously reported tau BiFC assay that showed a reduced signal upon tau aggregation (Chun et al., 2007). Thus, based on available evidence, we conclude that the tau–GLuc PCA assay is capable of detection of secreted and internalized tau species that are mostly in dimeric form.

In our system, more than 99% of secreted tau was found in vesicle-free form with only a minor fraction pelleted together with ectosomes and exosomes. It is possible that vesicle-free tau is overrepresented owing to post-secretion release of tau from microvesicles such as exosomes (Barten et al., 2011; Kim et al., 2010b; Saman et al., 2012). Interestingly, we noted that vesicle-

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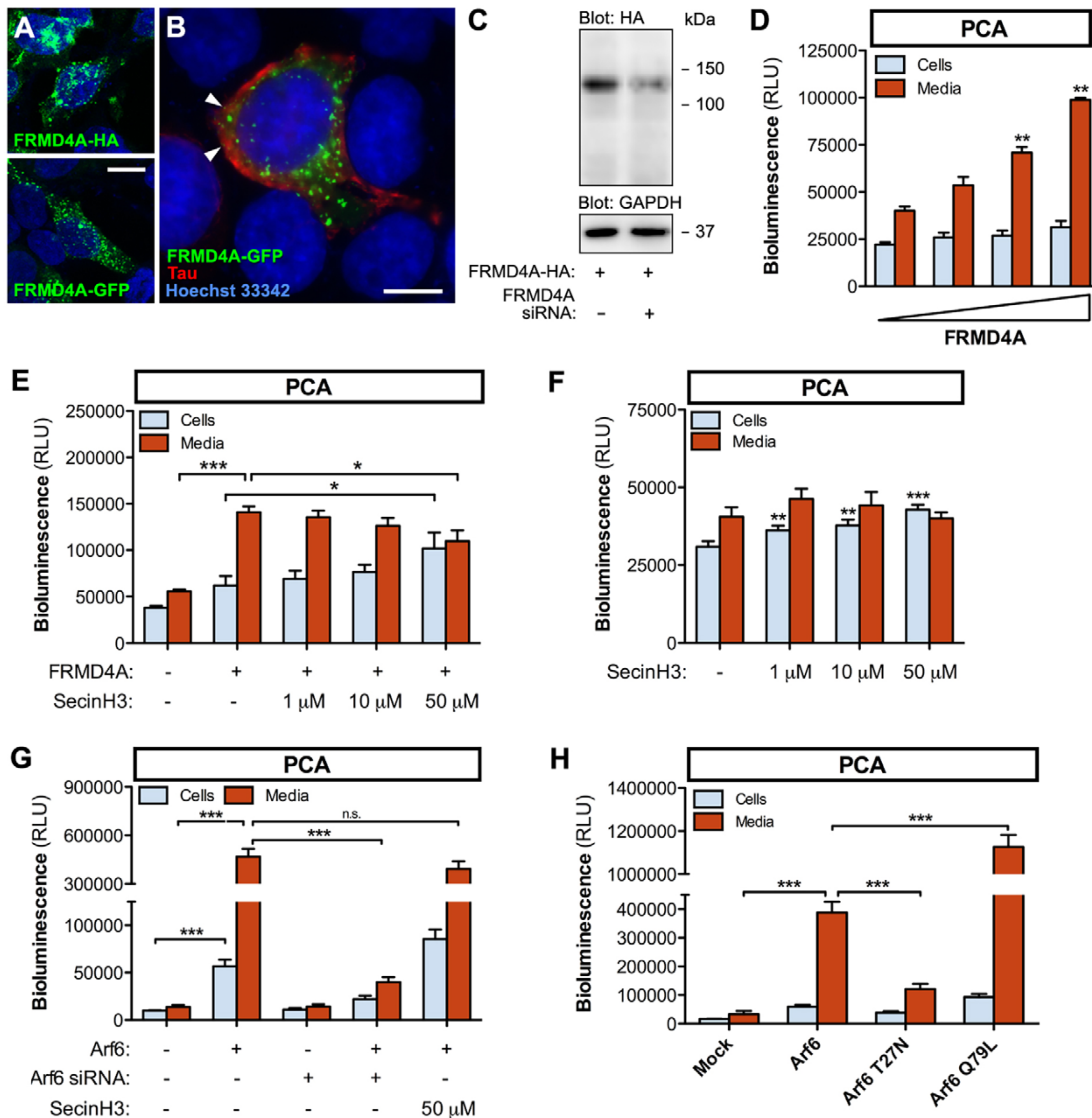

Fig. 6. FRMD4A modulates cellular release of tau through cytohesins and Arf6. (A) FRMD4A–HA (top) and FRMD4A–GFP (bottom) localization in transiently transfected HEK293T cells. The intense vesicle-like staining pattern suggests that FRMD4A is linked to membrane trafficking in HEK293T cells. Scale bar: 10 µm. (B) FRMD4A–GFP and tau–GLuc2 coexpression in HEK293T does not reveal significant colocalization. Tau localization was revealed by tau-5 immunostaining and nuclei were counterstained with Hoechst 33342. Arrowheads indicate FRMD4A-containing puncta in tau-enriched areas near the plasma membrane. Scale bar: 10 µm. (C) Western blot analysis showing that FRMD4A siRNA reduced the expression of FRMD4A–HA protein. Cells were transfected with FRMD4A–HA and FRMD4A siRNA as in Fig. 5. (D) Overexpression of FRMD4A in HEK293T cells promotes secretion of tau–GLuc1/2 reporters. FRMD4A plasmid was transfected at 0, 10 ng, 30 ng and 50 ng per well (the total plasmid amount with the tau–GLuc1/2 reporters was 100 ng). Values are relative to mock-transfected cells with endogenous levels of FRMD4A. (E) Inhibition of cytohesin activity by addition of SecinH3 at the indicated concentrations to the culture medium reduces FRMD4A-stimulated tau secretion in HEK293T cells overexpressing FRMD4A and tau–GLuc1/2 reporters. (F) SecinH3 increases intracellular tau dimer levels and has a subtle effect on secretion in HEK293T cells expressing tau–GLuc1/2 reporters (and endogenous FRMD4A). (G) Overexpression of wild-type Arf6 strongly enhances tau secretion, which is effectively blocked by Arf6 siRNA. (H) The effect of the dominant-negative mutant (T27N) and constitutively active mutant (Q79L) of Arf6 on tau secretion. RLU, relative light units. Results are mean±s.e.m. (n=3). *P<0.05, **P<0.01, ***P<0.001; n.s., not significant (ANOVA).

bound tau is more actively internalized than vesicle-free tau. As tau internalized via different routes might have a different intracellular fate, future studies are needed for addressing specific cellular uptake mechanisms of vesicle-bound versus vesicle-free extracellular tau species.

Although the tau secretion assay was highly sensitive, in the uptake assay, the levels of internalized tau were quite low. This might be partially explained by rapid re-secretion of internalized tau in HEK293T cells. Macropinocytosis is an endocytic process used for bulk uptake of macrosolutes and appears to be a major route of entry for extracellular tau fibrils (Holmes et al., 2013). Different from endosomes, macropinosomes themselves undergo rapid exocytosis making their traffic bidirectional and dependent on regulated coordination of endocytic and exocytic events (Falcone

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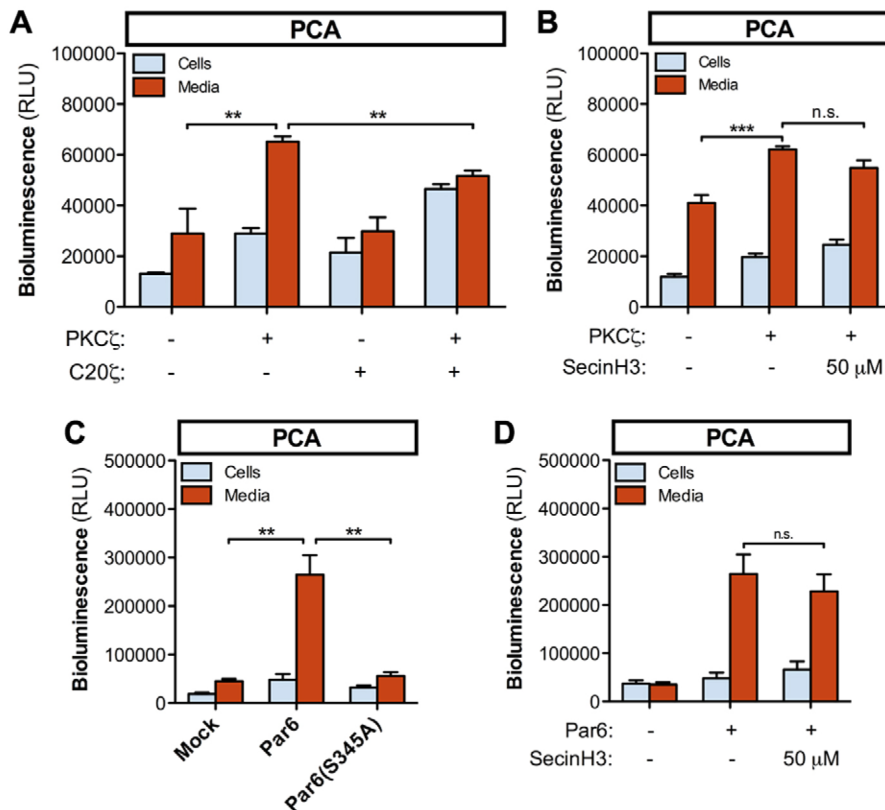

Fig. 7. Polarity signaling by aPKCζ and Par6 stimulate tau secretion. (A) aPKCζ overexpression promotes tau–GLuc1/2 reporter secretion, which is partially inhibited by expression of the ceramide-binding C-terminus of aPKCζ (C20ζ; amino acids 405–592 of aPKCζ). C20ζ expression alone does not significantly alter tau secretion. (B) Inhibition of cytohesin activity by SecinH3 does not reduce tau secretion stimulated by overexpression of aPKCζ in HEK293T cells. (C) Overexpression of wild-type Par6 but not the inactive Par6(S345A) promotes tau secretion but has no effect on intracellular tau dimers in HEK293T cells. (D) SecinH3 does not suppress the tau secretion induced by Par6 overexpression. RLU, relative light units. Results are mean±s.e.m. (n=3). ** P<0.01, *** P<0.001; n.s., not significant (ANOVA).

et al., 2006). This might explain the relative inefficiency of tau uptake and the observed re-secretion of the tau reporters in our system.

Avila et al. recently hypothesized that, given that several of the proteins encoded by the top LOAD risk genes interact with tau (ApoE, BIN1, clusterin and PICALM), these proteins potentially modify cell-to-cell propagation of tau (Avila et al., 2015). In our *in vitro* study, ApoE siRNA affected uptake of tau. This might be related to the tau-binding property of ApoE (Fleming et al., 1996; Strittmatter et al., 1994). Unexpectedly, genes functionally connected to endocytic pathways and modification of tau pathology, such as BIN1 (Chapuis et al., 2013) and PICALM (Xiao et al., 2012), did not affect tau secretion or uptake significantly. Instead, CD2AP, FRMD4A, TREM2 and CD33 knockdown caused a significant decrease in tau secretion. *TREM2* and *CD33* are predominantly expressed in myelomonocytic cells, and their expression level in HEK293T cells is low. Moreover, their proximal signaling partners, such as DAP12 (also known as

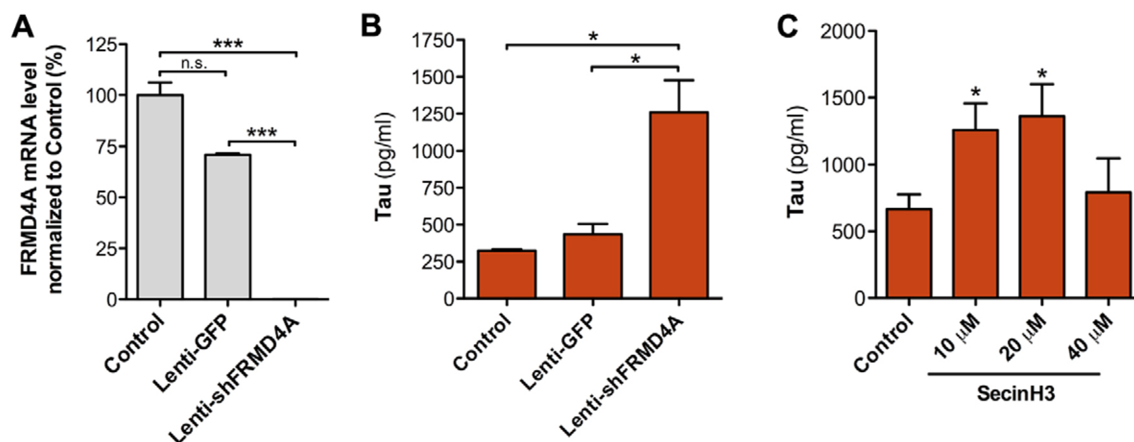

Fig. 8. Reduced FRMD4A level or cytohesin activity promotes secretion of endogenous tau in primary cortical neurons. (A) Mouse cortical neurons were transduced with lentivirus encoding for GFP (Lenti-GFP) or shRNA against FRMD4A (Lenti-shFRMD4A) or were left untreated (control) at 21 DIV. At 2 days post transduction, mRNA levels of FRMD4A were analyzed by qPCR and normalized to the GAPDH mRNA levels. (B) The effect of FRMD4A silencing on tau secretion in cortical neurons. Mouse cortical neurons were prepared and transduced in the same way as in A. Medium was collected 3 days after transduction. ELISA was used to analyze the tau level in cleared (cell-debris-free) conditioned medium. (C) Mouse cortical neurons were incubated with different concentrations of SecinH3 or DMSO (control) at 21 DIV. Medium was collected 3 days later. ELISA was used to study the tau level in cleared conditioned medium. Results are mean±s.e.m. (n=3). * P<0.05, *** P<0.001; n.s., not significant (ANOVA).

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TYROBP), might be lacking from cells that are not of myeloid origin, limiting or altering their function in commonly used cell lines like HEK293T. The fact that knockdown of *TREM2* or *CD33* in a microglial cell line, with higher endogenous expression levels of both genes, failed to show any effects on tau secretion suggests that the HEK293T findings might be false-positive hits, possibly related to off-target effects of siRNA than TREM2 and CD33 function. This also shows that although the *in vitro* screening system has certain benefits, functional verification of hits is important.

Using a specific haplotype-based genome-wide association approach, FRMD4A was identified as a genetic susceptibility factor for LOAD (Lambert et al., 2013). The functional connections between FRMD4A protein and Alzheimer's disease pathophysiology are currently poorly understood. Single-nucleotide polymorphisms (SNPs) within the FRMD4A locus are associated with alterations in the plasma amyloid β peptide (Aβ) levels and the $A\beta_{42}$ to $A\beta_{40}$ ratio in three independent populations from patients not displaying dementia (Lambert et al., 2013). We recently reported that the expression of FRMD4A declines in relation to increasing neurofibrillary pathology in Alzheimer's disease patients, and that FRMD4A is functionally linked to both APP metabolism and tau phosphorylation status *in vitro* (Martiskainen et al., 2015). Our current results clearly show that FRMD4A levels in cells are connected to the level of tau secretion. In non-neuronal cells, decreased FRMD4A levels reduce the ability of cells to secrete tau, which might lead to intracellular accumulation, hyperphosphorylation and toxicity of tau (Gendreau and Hall, 2013; Hall and Saman, 2012). However, in a more physiological and disease-relevant context, in mature cortical neurons, reduced FRMD4A levels and cytohesin inhibition, strongly promoted the secretion of endogenous tau. The difference in the behavior between our model systems might be explained by the highly specialized and very tightly regulated secretion machinery of neurons (Südhof, 2013). Thus, reduced FRMD4A levels, as observed in the LOAD patient brains (Martiskainen et al., 2015), might drive tau secretion and accelerate disease progression.

FRMD4A regulates epithelial cell polarity by connecting the Par3–Par6–aPKCζ complex to Arf6 activation through cytohesins (Ikenouchi and Umeda, 2010). Par polarity complex signaling plays a crucial role in neuronal polarization (Insolera et al., 2011) but also in membrane trafficking, including vesicular secretion (Balklava et al., 2007). Interestingly, cytohesin-1 has been shown to facilitate synaptic transmission in *Xenopus laevis* neuromuscular junction, most likely by making more presynaptic vesicles available for fusion at the plasma membrane through a direct interaction with Munc13-1 (also known as Unc13a) (Ashery et al., 1999; Neeb et al., 1999). By contrast, in hippocampal neurons, Arf6 silencing has recently been reported to increase synaptic exocytosis, and both Arf6 silencing and cytohesin inhibition by SecinH3 results in an increase in the number of docked synaptic vesicles (Tagliatti et al., 2016). As neuronal tau release appears to be related to plasma membrane fusion of presynaptic vesicles (Pooler et al., 2013; Yamada et al., 2014), it seems plausible that FRMD4A, cytohesin and Arf6 levels and activity in neurons serve as regulators of tau secretion. Our results thus establish a previously unknown connection between the activity of the Par6–aPKCζ–FRMD4A–cytohesin–Arf6 signaling pathway and tau secretion. Interestingly, inhibition of cytohesin activity has recently been shown to enhance autophagic flux and to reduce the burden of misfolded SOD1 in amyotrophic lateral sclerosis (ALS) models (Zhai et al., 2015). It remains to be seen whether FRMD4A–cytohesin signaling plays a more general role in the maintenance of neuronal proteostasis.

Polarized delivery of proteins and lipids to specific subdomains of the plasma membrane is important to a wide range of biological processes such as epithelial cell polarization and neuronal synaptogenesis. Our results suggest that the release of tau from cells is linked to cell polarity signaling through the Par6–aPKC complex. Defining the molecular mechanisms that regulate tau secretion will help understanding of how tau pathology propagates in Alzheimer's disease and other tauopathies.

MATERIALS AND METHODS

DNA constructs and siRNA

The split *Gaussia princeps* luciferase (GLuc) system used in this study has been previously described (Remy and Michnick, 2006). The human cDNAs for tau (isoform 0N4R) and CD2AP were purchased from Thermo Scientific. The FRMD4A–GFP plasmid was a gift from Junichi Ikenouchi (Kyushu University, Fukuoka, Japan) (Ikenouchi and Umeda, 2010), pcDNA3/HA-Arf6 was a gift from Thomas Roberts (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA) (Addgene plasmid # 10834) (Furman et al., 2002), and pCMV5B-Flag-Par6 wt (Addgene plasmid # 11748) and pMEP5-Flag Par6 S345A (Addgene plasmid # 24648) were gifts from Jeff Wrana (Department of Molecular Genetics, University of Toronto, Toronto, Canada) (Ozdamar et al., 2005). aPKCζ cDNA was acquired from the ORFeome library by the Genome Biology Unit at the University of Helsinki, and C20ζ (amino acids 405–592) was cloned into the pcDNA6-V5/His expression plasmid (Invitrogen). shRNA clones (in pLKO.1) for human TREM2 and CD33, and mouse FRMD4A were acquired from the TRC1.0 library by the Functional Genomics Unit Biomedicum Helsinki. All plasmids were sequenced to confirm their identity.

Three siRNAs per each LOAD risk gene and two control siRNAs were purchased from Invitrogen (Ambion Silencer Select Predesigned siRNA). Gene-silencing efficiency of siRNAs was determined using qPCR, and the two most effective siRNAs per target gene were selected for further experiments (Martiskainen et al., 2015).

Cell culture and transfection

Human embryonic kidney 293T cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium [DMEM with 10% (v/v) FBS (Gibco, Invitrogen)] and 1% (v/v) L-glutamine-penicillin-streptomycin solution (Lonza) at 37°C, in 5% CO_2 and water-saturated air. Transfection of HEK293T cells was performed using JetPei and Jetprime reagents (Polyplus) according to manufacturer's instructions. Human microglial CHME-5 cells (Janabi et al., 1995) were cultured and transfected with JetPrime. Mouse primary cortical neurons were prepared and cultured as described previously (Nykänen et al., 2012). Neurons were transduced with lentiviruses after a medium change at 21 DIV. At 3 days post transduction, the medium was collected for analysis.

Lentivirus production

Four shRNA clones targeting FRMD4A were tested by transient transfection in N2A mouse neuroblastoma cells. Based on qPCR analysis, one clone was selected for production of lentiviral particles. Lentiviruses were produced, stored and used in neuron cultures as described previously (Kysenius et al., 2012).

Protein-fragment complementation assay

PCA was performed as previously described (Martiskainen et al., 2015). HEK293T cells were plated on poly-L-lysine-coated white-walled 96-well plates (Perkin Elmer) at a density of 10,000 cells per well. GLuc reporter plasmids and siRNA were transfected (100 ng of total plasmid DNA, siRNA at 5 nM) at 24 h post plating. The culture medium was changed to Phenol-Red-free DMEM (Gibco, Invitrogen) without serum 30 min before the measurement. The PCA signal was read with a Wallac 1420 Victor[3] fluorescence multiplate reader (PerkinElmer) or Varioskan Flash multiplate reader (Thermo Scientific) 48 h post plating by injecting 25 µl of native coelenterazine (Nanolight Technology) per well (final concentration 20 µM). For a standard experimental condition, four replicate wells were

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used, and three or four independent experiments were performed. GW4869 (Sigma) and SecinH3 (R&D Systems) were dissolved in DMSO, and further diluted in culture medium.

Tau secretion assay

At 16 h before measurement, cells transfected with tau–GLuc1/2 reporters were washed once with PBS and changed to Phenol-Red-free DMEM (Gibco, Invitrogen) without serum (140 µl per well). At 30 min before measurement, the plate was spun at 200–300 ***g*** for 3–5 min using a swing bucket rotor (Eppendorf, centrifuge 5810) and conditioned Phenol-Red-free DMEM was recollected. A total of 75 µl of this conditioned medium was used for measuring the PCA signal in conditioned medium (secreted tau dimers), and 50 µl was used for lactate dehydrogenase (LDH) release measurement (Promega CytoTox 96® Assay, G1781) according to the manufacturer's instructions. After collection of conditioned medium, the cells were changed to 75 µl of fresh Phenol-Red-free DMEM and the PCA signal was detected as described for a standard PCA (intracellular tau dimers). PCA signal in the conditioned medium was normalized by dividing with the corresponding LDH level from the same well. For the RNAi screen of tau secretion, tau–GLuc1, tau–GLuc2 and LOAD risk gene siRNAs (at 5 nM) were co-transfected into HEK293T cells.

Tau uptake assay

Conditioned medium (Phenol-Red-free DMEM) was collected from HEK293T cells transfected with tau–GLuc reporters after 24 h conditioning and cleared by centrifugation at 3000 ***g*** for 30 min to remove cell debris. The level of tau–GLuc1/2 dimers in the medium was determined by PCA. Untransfected naïve HEK293T cells on 96-well plates were washed once with pre-warmed PBS and changed to conditioned medium containing tau–Gluc1/2. After 4 h incubation, the medium was completely removed by gently pipetting and the cells were washed once with PBS, then incubated with 20 µg/ml heparin (Sigma) for 5 min to remove cell-surface-associated tau. Heparin solution was removed, cells were washed with PBS and 75 µl of Phenol-Red-free DMEM was added to the wells and the cellular PCA signal was measured as described above. For the RNAi screen of tau uptake, siRNAs against LOAD risk genes were transfected into HEK293T cells using JetPrime, and after 24 h cells were changed to fully supplemented DMEM. Next, at 44 h post plating, the medium was replaced with tau–GLuc1/2-conditioned medium and incubated for 4 h. Cells were washed and processed for PCA detection as described above.

Immunofluorescence imaging

Immunofluorescence imaging was performed as previously described (Kysenius et al., 2012). Cells fixed on glass coverslips were incubated with tau-5 (Invitrogen, cat. no. AHB0042, 1:600) and anti-HA (HA-7, Sigma, cat. no. H3663, 1:600) antibodies. After PBS washes, coverslips were incubated with Alexa-Fluor-568-conjugated anti-mouse-IgG secondary antibody (Invitrogen, 1:2000). Nuclei were stained with Hoechst 33342 (Invitrogen) and coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen). Images were taken with a Zeiss AxioImager M1 epifluorescence microscope.

Western blotting

Western blotting was performed as previously described (Nykänen et al., 2012). Cells grown on poly-L-lysine-coated six-well plates and transfected with 3 µg of total DNA per well were washed twice with ice-cold PBS at 48 h post transfection. Then, cells were lysed and equal amounts of lysate were resolved on 4–12% gradient Bis-Tris gels (Novex, Invitrogen) under reducing conditions. Proteins were transferred onto PVDF membranes (GE Healthcare) with semidry blotting (Bio-Rad). Tau-5 (Invitrogen #AHB0042, 1:1000), anti-HA (HA-7, Sigma #H3663, 1:1000) and anti-GAPDH (6C5, Millipore #MAB374, 1:1000) antibodies, horseradish-peroxidase-conjugated secondary antibodies and ECL western blotting detection reagent (Thermo) were used to detect the chemiluminescence signal. QuantityOne software (Bio-Rad) was used for quantitative analysis of western blots.

Native PAGE

Conditioned media were collected and concentrated to 100× with Amicon Ultra-15 Centrifugal Filters (Merck Millipore, UFC903008). Samples were prepared using the Novex™ Tris-Glycine Native Sample Buffer (LC2673), and resolved on NuPAGE™ Novex™ 3-8% Tris-Acetate Protein Gels (EA0375BOX) with an equal volume of sample per well, using Novex™ Tris-Glycine Native Running Buffer (LC2672; Novex, ThermoFisher Scientific) according to the manufacturer's instructions. The gel was blotted and analyzed as described above.

Protein crosslinking

Protein samples from cell lysates and conditioned media were processed as described above. When needed, conditioned medium was concentrated by using Amicon Ultra-15 Centrifugal Filters (Merck Millipore, UFC903008). Samples with equal amounts of total protein were incubated for 30 min in room temperature with the non-cleavable and membrane-impermeable crosslinker BS3 [bis(sulfosuccinimidyl) suberate; Pierce, Thermo] according to the manufacturer's instructions. BS3 was pre-diluted in milli-Q H_2O, and added to samples at a final concentration of 5 mM. Tris-HCl (pH 7.5) was used as quenching buffer and added to samples at a final concentration of 50 mM for 15 min at room temperature. Equal volumes of samples were analyzed by western blotting.

Media fractionation

For separation of larger microvesicles or ectosomes and exosomes, a widely used method was employed (Théry et al., 2006). Medium was first cleared at 3000 ***g*** for 30 min, followed by centrifugation at 20,000 ***g*** for 60 min to pellet the ectosomal fraction of conditioned medium (Sorvall WX Floor Ultra centrifuge). The supernatant containing the exosomal fraction of the medium was carefully transferred to a fresh tube without disturbing the pellet (not visible). The pellet was washed once with PBS and spun again at 20,000 ***g*** for 60 min. After gently removing the supernatant, the pellet was resuspended either in Phenol-Red-free DMEM for PCA measurement, or PBS or 1.5× Laemmli buffer for western blot analysis. The volume used for the resuspension of the pellet was adjusted proportionally according to the amount of medium loaded for centrifugation. The supernatant was centrifuged at 100,000 ***g*** for 70 min to pellet the exosomal fraction (Beckman Coulter ultracentrifuge with an SW41 Ti rotor). The exosomal pellet (not visible) was washed with PBS, spun again at 100,000 ***g*** for 70 min and resuspended in the same way as the ectosomal fraction. Flotillin-1 (BD Transduction Labs #F65020, 1:500) was used as a marker for ectosomal vesicles (Kowal et al., 2016).

ELISA

Human tau ELISA measurements were performed by using a commercial total human-specific tau ELISA kit (KHB0041, Novex, ThermoFisher). HEK293T cells transfected with tau–GLuc reporters were incubated in DMEM for 24 h. Medium was then collected and centrifuged at 900 ***g*** for 10 min at 4°C and the cells were lysed in western blot extraction buffer. The protein concentration was determined by a BCA protein assay kit (Thermo) for both lysate and medium. The ELISA values were normalized to cell lysate total protein concentration values. Mouse tau ELISA measurements were performed using a commercial mouse-specific tau ELISA kit (KMB7011, Novex, ThermoFisher).

Statistical analyses

A minimum of three independent repetitions were used for each experiment. Microsoft Excel and GraphPad Prism software were used for statistical analyses and generation of graphs. Statistical significance was evaluated with two-tailed Student's *t*-tests and two-way ANOVA, where appropriate, with the significance threshold set at $P<0.05$.

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Competing interests
H.J.H. is an employee and shareholder of Herantis Pharma Plc, which is unrelated to this study.

Author contributions
X.Y., N.-P.N., C.A.B., A.H., M.H., R.-L.U. and H.J.H. designed and performed experiments, analyzed the data and wrote the manuscript.

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