LRP::FLAG reduces phosphorylated tau levels in Alzheimer's Disease

Authors: Katelyn Cuttler^{1,2}, Monique J. Bignoux¹, Tyrone C. Otgaar¹, Stephanie Chigumba¹, Eloise Ferreira¹ and Stefan F.T. Weiss^{1*}

¹School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa

²Present Address: Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, Republic of South Africa

*E-mail: stefan.weiss@wits.ac.za

Abstract

Alzheimer's disease (AD) is characterized by amyloid beta (A β) plaque and neurofibrillary tangle formation, respectively. Neurofibrillary tangles form as a result of the intracellular accumulation of hyperphosphorylated tau. Telomerase activity and levels of the human reverse transcriptase (hTERT) subunit of telomerase are significantly decreased in AD. Recently it has been demonstrated that the 37kDa/67kDa laminin receptor (LRP/LR) interacts with telomerase and is implicated in A β pathology. Here we show that LRP/LR co-localizes with tau in the perinuclear cell compartment and FRET confirmed a direct interaction between LRP/LR and tau in HEK-293 cells. Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreased total and phosphorylated tau levels with a concomitant decrease in PrP^c levels, a tauopathy-related protein. Additionally, LRP::FLAG overexpression resulted in increased hTERT levels. These data indicate for the first time a role of LRP/LR in tauopathy of Alzheimer's Disease and recommend LRP::FLAG as a potential alternative therapeutic tool for Alzheimer's Disease treatment through rescuing cells from A β induced cytotoxicity and, as shown in this report, decreased phosphorylated tau levels.

Key Words: 37 kDa/67 kDa laminin receptor (LRP/LR), tau, Alzheimer's disease, telomerase, PrP^c, hTERT

Introduction

Alzheimer's disease (AD) which affects approximately 50 million people worldwide [1]. It is the most prevalent form of dementia and incidence is rapidly increasing. Since AD mainly affects the elderly, it is an increasing concern due to increased global life expectancy. In addition, only long-term palliative care is available for AD patients and therefore, AD is a significant socio-economic burden. Indeed, the cost of specialized care for AD amounted to \$1 trillion dollars worldwide in 2018 [1]. Thus, there is a great need to develop effective disease-modifying treatments for AD.

There are two key neuropathological hallmarks in AD, namely amyloid plaques formed from the extracellular aggregation of amyloid beta ($A\beta$) and neurofibrillary tangles formed from the intracellular accumulation of hyperphosphorylated tau [2]. While $A\beta$ is considered by some to be the candidate etiological agent of AD [3,4], hyperphosphorylated tau is equally important in the progression of AD. Neurofibrillary tangles accumulate in specific brain regions involved in memory and block the microtubular transport system, which harms synaptic communication [2], while amyloid plaques disrupt neuronal functions, resulting in apoptosis. Taken together, these agents are able to damage many areas of the brain, ultimately resulting in death. Therefore, it is vital to develop treatments that target hyperphosphorylated tau as well as $A\beta$.

Tau is a microtubule-associated protein that normally functions to promote the assembly of tubulin into microtubules and to bind to and stabilize assembled microtubules [5]. Since these microtubules are situated along cytoskeletal tracks, they mediate vital cell processes, including the transport of nutrients and neurotransmitters. In healthy cells, the level of tau phosphorylation is highly modulated and regulates microtubule plasticity [6], motor activity and axonal transport [7], as well as neurite outgrowth [8]. In AD, however, this process is dysregulated, and tau is hyperphosphorylated and dissociates from microtubules. This hyperphosphorylated tau then diffuses into the cytoplasm and associates to form neurofibrillary tangles [9]. This causes cytoskeletal tracks to disintegrate, thereby inhibiting the vital cell processes they mediate, which ultimately leads to cell death [9]. Overall, hyperphosphorylation of tau negatively impacts synaptic health, plasma membrane cell signalling and the protection of DNA from cell stressors [9], all of which may contribute to the progression of AD. Indeed, studies have shown that the degree of dementia is correlated with the number of neurofibrillary tangles present within the brain tissue [10–14].

Furthermore, hyperphosphorylation of tau can be caused by highly neurotoxic $A\beta$ fibrils which activate the tau kinase, glycogen synthase kinase 3 (GSK3) [5,15]. Therefore, tau hyperphosphorylation is dependent on the extracellular aggregation of $A\beta$ [16]. Thus, the aggregation of $A\beta$ is necessary in order for both the $A\beta$ pathology and tauopathy of AD. In order to do this, $A\beta$ needs to interact with lipid membranes and cellular receptors [17]. One such receptor is the 37 kDa/ 67 kDa Laminin Receptor Precursor/ High Affinity Laminin Receptor (LRP/LR) [18], also known as LamR1, RPSA and p40 [19]. LRP/LR is a multifunctional receptor mainly found in the lipid raft regions of plasma membranes, as well as in the nucleus [20] and cytoplasm

[21]. LRP/LR is implicated in numerous diseases, including cancer, prion disorders, ageing and AD. LRP/LR is upregulated in multiple metastatic cancers causing increased metastasis, increased angiogenesis and the impediment of apoptosis [22-34]. Furthermore, LRP/LR is also involved in both cancer and ageing via a functional relationship with telomerase [35-37].

In AD, LRP/LR, PrP^c and A β have been shown to function together in order for the disease to progress. LRP/LR acts as a receptor for A β ₄₂ internalization, co-localizes with amyloid precursor protein (APP), γ -secretase and β -secretase, and interacts directly with the presenilin 1 (PS1) subunit of γ -secretase during the amyloidogenic processing of APP [18,38-40]. Moreover, blockage or downregulation of LRP/LR with the anti-LRP/LR specific antibody, IgG1-iS18, or short hairpin RNAs (shRNAs), respectively, has successfully impeded A β shedding and rescued cells from A β -induced cytotoxicity [38,39]. Furthermore, it was found that the IgG1-iS18 antibody-mediated rescuing of cells from cytotoxicity is dependent on PrP^c due to the interaction between PrP^c and A β ₄₂ [41]. Recently, an *in vivo* study, whereby B6SJL-Tg6799 AD transgenic mice were injected with the IgG1-iS18 antibody, confirmed the *in vitro* findings whereby a decrease in amyloid plaque formation and A β ₄₂ levels with a concomitant increase in both short-term and learning memory was observed [42].

Another protein recently found to be associated with AD is telomerase. Telomerase is a multi-subunit ribonucleoprotein which functions to add TTAGGG repeats to the ends of chromosomes [43]. In highly proliferative cells, the addition of these repeats protects telomeres from erosion [43]. There are two essential components to the human telomerase enzyme, namely hTERC, the RNA component, and hTERT, the reverse transcriptase [44]. Telomerase activity is thus important in cellular senescence and is implicated in both ageing and cancer where it has opposing effects [45,46]. Importantly, cellular ageing occurs as a result of limited telomerase activity. The resultant telomere erosion causes cells to enter replicative senescence where they are no longer able to divide [46]. This process is supposed to act as a defense mechanism against cancer formation [47-49], however, it can lead to the development of age-related disorders. Furthermore, hTERT has numerous extra-telomeric functions. It is known to function as a transcription factor in several pathways, such as the Wnt- β -catenin pathway and NF- κ B pathway, which are both involved in cellular proliferation [50]. Additionally, hTERT is able to migrate to mitochondria when cells are under oxidative stress, in order to convey protection against reactive oxygen species (ROS) [51,52]. Furthermore, hTERT plays a role in the replication and repair of mitochondrial DNA (mtDNA), thereby protecting cells from mtDNA damage [53]. Since AD is considered an age-related disorder, telomerase has also been implicated in AD. Indeed, telomerase activity is known to be inhibited by A β and cells affected by an aggregation of A β have critically shortened telomeres [54,55]. Additionally, hTERT is able to persist in neurons where it may play a neuroprotective role against hyperphosphorylated tau and the resultant neurofibrillary tangles [56]. Indeed, the absence of hTERT further contributes to neurodegeneration as it can no longer protect mitochondria from oxidative stress [56]. Furthermore, overexpressing hTERT is known to protect cells against A β -induced apoptosis [57]. Therefore, an upregulation of telomerase, and specifically hTERT, may

be a potential treatment strategy for AD as it may be able to protect neurons from both A β and tau-induced damage [55,56].

Recently, Otgaar *et al.*, [36] found that an overexpression of LRP::FLAG increased both hTERT levels and telomerase activity with a concomitant reduction in senescent markers. Furthermore, Ferreira *et al.*, [42] observed an increase in mTERT in mice treated with the anti-LRP/LR specific antibody, IgG1-iS18 with a concomitant decrease in A β peptides and amyloid plaques. In addition, an increase in telomerase activity and hTERT expression was observed with a concomitant reduction in A β shedding and intracellular A β levels in cells overexpressing LRP::FLAG [58]. Furthermore, this increase in hTERT rescued cells from A β -mediated cytotoxicity [58]. Therefore, LRP/LR has a role in the upregulation of telomerase activity and hTERT expression. Since both LRP/LR and telomerase are known to play a role in the A β facet of AD, it was hypothesized that they could also play a role in tauopathy. Thus, this study aimed to determine if LRP/LR has a relationship with tau and whether overexpression of LRP::FLAG has an effect on tau and tauopathy-related proteins.

Results and Discussion

LRP/LR is essential for A β mediated cytotoxicity during the development and progression of AD. However, it is not known whether LRP/LR has an effect on the other neuropathological agent of AD, the hyperphosphorylated tau. Therefore, this study demonstrates that LRP/LR interacts directly with tau and that LRP::FLAG overexpression decreases the levels of total and phosphorylated forms of tau, as well as PrP^c. Concurrently, the overexpression of LRP::FLAG also increased the levels of hTERT and phosphorylated hTERT.

LRP/LR interacts directly with tau

In order to determine whether LRP/LR interacts with tau, confocal microscopy with Airyscan™ was first utilized to confirm whether these two proteins co-localized in HEK-293 cells (Figure 1A). Figure 1A d-f represent a merge of tau (red) with LRP/LR (green), and areas of yellow indicate spatial overlap. Since both LRP/LR and tau are reportedly found in the cytoplasm and the nucleus, our results confirm this and further indicate that these proteins co-localise in those cell compartments [20,21]. Since we observed the co-localisation of LRP/LR and tau, we wanted to investigate whether there was an interaction between these proteins. FRET analysis was employed in HEK-293 cells and confirmed that LRP and tau interact. FRET occurs between fluorescently labelled proteins if they are within 10 nm of each other (Förster, 1948), resulting in the fluorescence shift which we observed in this study (Figure 1B f). Therefore, not only do LRP/LR and tau co-localise, our results indicate that these proteins directly interact, as they exist within 10 nm of each other.

Since, LRP/LR and tau were shown to interact directly (Figure 1B), it is thus possible that LRP/LR is involved in the main function of tau; to assemble and maintain microtubules in healthy cells. Indeed, cytoplasmic LRP is known to tightly associate with tubulin structures and may serve to tether ribosomes to microtubules in order to assist in protein synthesis [59,60]. Therefore, it is possible that cytoplasmic LRP could also bind to tau peptides in order to assist in stabilizing microtubules during their assembly and normal functioning. In AD, however, this interaction could be misappropriated to worsen the destabilizing effects of tau hyperphosphorylation. Cytoplasmic LRP may no longer be able to stabilize microtubules and this could contribute to the disintegration of cytoskeletal tracks. Importantly, though, strategies targeting LRP/LR could make use of this interaction for therapeutic intervention in AD by counteracting the microtubule destabilizing effects of tau hyperphosphorylation.

LRP::FLAG overexpression decreases total and phosphorylated tau levels

Since a direct interaction was confirmed between LRP/LR and tau, we wanted to investigate whether LRP/LR plays a role in tauopathy. HEK-293 and SH-SY5Y cells were stably transfected with the pCIneo-moLRP-FLAG plasmid [61] to induce an overexpression of LRP::FLAG. Western blotting confirmed the transfection of both HEK-293 and SH-SY5Y cells (Figure 2A; C; Figure S2). After confirming transfection of both cell lines, western blotting was utilized to determine the effect of LRP::FLAG overexpression on both total and phosphorylated tau levels (Figure 2A; C; Figure S3-S5). A significant decrease in total tau of 45% and 35% was observed in transfected HEK-293 (Figure 2B) and transfected SH-SY5Y cells (Figure 2D), respectively. Since it is not possible to investigate hyperphosphorylation of tau *in vitro*, two antibodies were used which target tau at two different phosphorylation sites (pS404 and pT231). In transfected HEK-293 cells there was a significant 68% reduction in tau phosphorylated at S404 and a significant 98% reduction in tau phosphorylated at T231 (Figure 2B). In transfected SH-SY5Y cells, a significant 63% and 92% reduction was observed, respectively (Figure 2D). Since these are two of the major culprits of tauopathy, the decreased phosphorylation at these two sites together may indicate a possible decrease in hyperphosphorylated tau [62–65]. This decrease in phosphorylated tau was also observed in the cytoplasm and the nucleus of both HEK-293 and SH-SY5Y cells (Figure 3) by using confocal microscopy. Therefore the overexpression of LRP::FLAG might decrease the overall hyperphosphorylation of tau.

Hyperphosphorylation of tau is a major problem in AD where it forms tangles that disrupt the internal structure of neuronal cells by de-stabilizing the cytoskeletal tracks [9]. Thus the significant decrease observed in both tau phosphorylation sites (pS404 and pT231) may result in a reduced amount of aggregation of hyperphosphorylated tau, resulting in less damage to neuronal cells. [62–65] Since this aggregation is known to cause disintegration of cytoskeletal tracks, a decrease in hyperphosphorylated tau may hinder cytoskeleton disintegration and improve vital cell processes, such as axonal transport, thereby permitting neurons to retain normal functioning in AD patients [9]. In particular, the phosphorylation of tau at S404 and T231, which was investigated in this study, is known to negatively affect the binding of the tau protein to the microtubules, thereby de-

stabilizing them and inhibiting cytoskeletal signalling and transport [65,66]. Indeed, T231 mutant N18 neuroblastoma cells abolished phosphorylation of tau by the serine/threonine kinase GSK3, indicating that phosphorylation at T231 plays an important role in the hyperphosphorylation of tau [63]. Therefore, a decrease in phosphorylation at these sites may prevent the dissociation of tau and promote tubulin polymerization and assembly into microtubules [63,66]. Thus, the structural integrity of the cells would be maintained, and their signalling and transport capabilities would remain intact, allowing them to better resist the damaging effects of AD. In particular, DNA damage repair proteins can be trafficked to the sites of cytotoxicity in order to rescue the cells [67]. The DNA damage caused by the cytotoxicity present in AD results in neuronal apoptosis via oxidative stress [68,69]. In addition, hyperphosphorylation of tau is able to enhance A β cytotoxicity by sequestering Fyn kinase [9]. Fyn is thus re-distributed to the neuron whereby it can strengthen the excitotoxic signalling of the neurotransmitter glutamate, which is known to enhance the cytotoxicity of A β oligomers [9,70]. Therefore, a decrease in phosphorylated tau and the resultant decrease in neurofibrillary tangles could both lessen cytotoxicity as well as allow DNA repair to occur. Overall, the decrease in phosphorylated tau observed could be acting as a potential mitigator for AD development and progression.

Furthermore, a decrease in total tau levels was observed in both cell lines (Figure 2, Figure 3). Wild-type tau is essential for the functioning of microtubules and cell signalling, therefore a decrease in total tau may be detrimental to the cells. However, we have seen that LRP::FLAG overexpression rescues cells from cytotoxicity after treatment with A β_{42} [58]. Therefore, we suggest that the effect of the decreased tau is minimal, otherwise the cells would enter apoptosis as a result of microtubule disintegration and loss of structural integrity. Indeed, there is a known redundancy between tau and other microtubule-associated proteins [71], such as the redundancy between MAP-1B and tau during axonal growth [72,73]. Therefore, it is possible that another protein could function in the place of tau in order to maintain the internal structure of the cell. In addition, the decreases in total tau are more finite than those seen in phosphorylated tau. Thus, while overexpression of LRP::FLAG does not specifically target phosphorylated tau, it seems to have a much more notable effect on the phosphorylated form. In this regard, it is known that the interaction between LRP and laminin is able to affect the phosphorylation of ERK, JNK and p38 proteins [74]. Therefore, LRP may be involved in kinase/phosphatase axes and laminin cell signalling. Thus, it is possible that the LRP-laminin interaction could also affect the phosphorylation of tau, especially as LRP/LR was shown to directly interact with tau. Furthermore, LRP/LR and focal adhesion kinase (FAK) have been shown to interact after LRP/LR binds to laminin [75]. Indeed, in neurons treated with A β there is also increased phosphorylation of FAK and an increased association between FAK and Fyn kinase in addition to the increased phosphorylation of tau. Taken together, these increases are known to enhance cytotoxicity in AD [76]. Therefore, it is possible that the interaction between LRP and FAK, and the subsequent activation of signalling pathways, such as PI3-kinase/Akt and MAPK [75], may affect the phosphorylation of tau in AD. Therefore, given the potential role of LRP in kinase/phosphatase

axes and that LRP and tau directly interact, it is plausible that LRP could likewise affect the phosphorylation of tau.

LRP::FLAG overexpression increases hTERT and phospho-TERT levels

LRP/LR has also recently been found to interact with the anti-ageing related proteins telomerase and hTERT [36]. In AD, neuronal cells have critically shortened telomeres as an accumulation of A β is able to inhibit the activity of telomerase [54,55]. Furthermore, there is an absence of hTERT in cells affected by hyperphosphorylated tau [56]. Since both LRP/LR and telomerase are known to play a role in the A β facet of AD, it was hypothesized that they could also play a role in tauopathy. It is known that overexpression of LRP::FLAG increases hTERT levels in HEK-293 cells [36] and SH-SY5Y cells [58]. It has also been shown that this increase results in an increase in telomerase activity [36,58]. Thus, western blotting was performed to examine the effect of LRP::FLAG overexpression on hTERT levels, together with the active form of hTERT, phospho-TERT (pS824) (Figure 4A; C; Figure S6-S7), as this is indicative of an increase in telomerase activity. A significant increase of 120% and 125% in hTERT levels was observed in transfected HEK-293 (Figure 4B) and SH-SY5Y (Figure 4D) cells, respectively. This is consistent with previous observations [36,58]. A significant increase in phospho-TERT levels was also observed both cell lines, 112% in transfected HEK-293 cells (Figure 4B) and 96% in transfected SH-SY5Y cells (Figure 4D). It is known that an increase in hTERT induces/ causes a corresponding increase in telomerase activity [36,77,78]. However, in order to be active, hTERT has to be present in the nucleus [79]. Therefore, in order for nuclear localisation of the full length 120kDa hTERT variant to occur, it firstly undergoes phosphorylation at the serine residues 227 and/or 824 by PI3-kinase/AKT signalling [79]. This suggests that the increase in telomerase activity is as a result of not only increased hTERT levels but also hTERT import to the nucleus. Therefore, the increase in both hTERT and phospho-TERT observed in transfected HEK-293 and SH-SY5Y cells is indicative of an increase in telomerase activity as previously observed by Otgaar et al. [36] and Bignoux et al. [58].

Indeed, by comparing phospho-TERT and hTERT levels in HEK-293 and SH-SY5Y cells, it is clear that not all hTERT is being converted into phospho-TERT. This hTERT may thus be involved in extra-telomeric functions. Of note is the protective function of hTERT against ROS. hTERT is able to migrate to the mitochondria when cells are under oxidative stress, which allows hTERT to interact with mtDNA in order to convey protection [51,52]. In AD, there is compromised energy production in the brain due to mitochondrial dysfunction [80]. This dysfunction can increase ROS production which has been shown to promote the hyperphosphorylation of tau [81], although it has not yet been determined how this occurs. Conversely, hyperphosphorylation of tau and the formation of neurofibrillary tangles could also impair the movement of mitochondria along microtubules [82]. In neurons, mitochondria are distributed to areas of the axon where metabolic demand is high, such as synapses [83,84], and active growth cones or branches [85,86]. As a result, ATP depletion can occur in other areas of axons and dendrites which can worsen synaptic dysfunction [87]. Importantly, in AD, the absence of hTERT has been shown to increase the levels

of mitochondrial superoxide in neurons [56] and truncated tau expressed *in vivo* generates increased oxidative stress over time through an accumulation of ROS [88]. Furthermore, pathological tau and hTERT have been observed to be mutually exclusive [56], suggesting that neurons expressing hTERT may be prevented from developing tau pathology. Indeed, it has previously been observed that LRP::FLAG overexpression causes an increase in hTERT in the cytoplasm and nucleus [36,58] and it is this increase in the cytoplasm and nucleus which could correspond to an increase in the extra-telomeric functions of hTERT. Therefore, in AD, the increase in hTERT observed, particularly in the cytoplasm, could improve mitochondrial function and protect against ROS production. Furthermore, nuclear hTERT is able to regulate inflammatory and proliferative pathways, such as Wnt and NF- κ B [50]. Therefore, the increased nuclear hTERT observed could regulate pro-proliferative and anti-inflammatory genes via these pathways which could prevent apoptosis and aid cell survival in AD. Therefore, an increase in hTERT may prevent cell cycle arrest and apoptosis in a number of ways; through increased telomerase activity and telomere elongation, by reducing ROS production thereby improving mitochondrial function, as well as by regulating cell survival pathways. Overall, an increase in hTERT could slow the progression of tauopathy and AD.

LRP::FLAG overexpression decreases PrP^c levels

Although the role of PrP^c in tau hyperphosphorylation is poorly understood, it is the primary receptor for A β ₄₂ and has an important role in A β ₄₂ pathology [89]. Thus, it was hypothesized that PrP^c may also be important for tauopathy.

The effect of LRP::FLAG overexpression on PrP^c was therefore investigated using western blotting (Figure 5A, 5C; Figure S8). Endogenous PrP^c can exist as three isoforms: non-glycosylated, monoglycosylated and diglycosylated and thus ranges in size from approximately 20 kDa to approximately 35 kDa (Vana and Weiss, 2006). In HEK-293 cells, both the di- and monoglycosylated forms were seen (Figure 5A), while in SH-SY5Y cells only the monoglycosylated form was observed (Figure 5C). It is important to note that PrP^c glycoform ratios differ across brain regions and across cell lines, thereby explaining the different banding patterns [90,91]. Therefore, the differences in total PrP^c levels were used for comparison. A significant decrease in total PrP^c in both transfected HEK-293 and SH-SY5Y cells of 31% (Figure 5B) and 39% (Figure 5D), respectively, was observed.

It has been suggested that PrP^c has a protective role against tau hyperphosphorylation and is able to downregulate tau expression [92,93]. Contrary to this, we observed a decrease in both PrP^c and phosphorylated tau in cells overexpressing LRP::FLAG. However, it has previously been observed that LRP::FLAG overexpression specifically reduced both intracellular and extracellular A β ₄₂ levels [58]. Indeed, Larson *et al.* [94] identified a signalling cascade between soluble A β and PrP^c which activates Fyn kinase. This activated Fyn kinase is known to cause synaptic and cognitive impairment in transgenic mice when dysregulated [95,96]. Furthermore, Larson *et al.* [94] discovered that this PrP^c-dependent activation of Fyn can also cause the missorting and

hyperphosphorylation of tau. However, high levels of A β are required for this PrP^c-mediated Fyn activation and this may only occur during the later stages of AD [94]. Therefore, we postulate that PrP^c may switch roles as AD progresses due to the increasing levels of cytotoxic A β . Thus, in the current study, the decrease in PrP^c observed may be beneficial in preventing the A β -PrP^c signalling cascade occurring in the later stages of AD. Therefore LRP::FLAG overexpression is able to decrease phosphorylated tau, A β ₄₂ and PrP^c levels. These proteins interact with each other during progression of AD, whereby A β ₄₂ binds to PrP^c causing cytotoxicity and increased A β ₄₂ shedding, which results in hyperphosphorylation of tau and the formation of neurofibrillary tangles. Furthermore, as the diagnosis and knowledge of AD improves, it may become easier to determine when PrP^c becomes detrimental and thus a potential target of treatment. Overall, we believe that the decrease in PrP^c caused by LRP::FLAG overexpression could be beneficial; however more research would need to be done to better understand the role of PrP^c in tauopathy.

Conclusion

In conclusion, LRP/LR and tau share the same sub-cellular locations and directly interact with each other. LRP::FLAG overexpression caused a decrease in phosphorylated tau and PrP^c levels. Taken together with previous results showing that LRP::FLAG overexpression decreases A β ₄₂ levels as well as rescuing cells from A β ₄₂-mediated cytotoxicity [58], this study shows that the overexpression of LRP::FLAG is able to affect each of the major proteins in the A β ₄₂-PrP^c-hyperphosphorylated tau signalling cascade and thereby decrease the cytotoxic effects observed in AD. Furthermore, hTERT levels increased after LRP::FLAG overexpression and thus could provide protection against the cytotoxicity caused by tau hyperphosphorylation. LRP::FLAG might act as a potential alternative therapeutic tool for Alzheimer's Disease treatment through a rescuing mechanism of A- β mediated cytotoxicity and a decrease of phosphorylated tau levels which might be mediated by increased hTERT and decreased PrP^c levels, respectively.

Methods

Cell Culture

HEK293 and SH-SY5Y cells were cultured in DMEM with high glucose (4.5 g/l) and 4 mM L-Glutamine (Hyclone): Ham's F12 with 1 mM L-Glutamine (Hyclone) in a 1:1 ratio. In addition, the media was supplemented with 15% FBS (Hyclone) and 2% penicillin/streptomycin (Biowest). Cells were maintained at 37 °C in a humidified incubator.

Confocal Microscopy with the addition of Airyscan™

Cells were seeded onto microscope coverslips within a 6-well plate and incubated overnight to reach 50-70% confluency. All further steps were performed with gentle agitation. Cells were fixed

with 4% formaldehyde (VWR) in PBS for 20 minutes at room temperature. Cells were then washed 3 times in 1X PBS, after which they were permeabilized with 0.25% Triton X-100 (Sigma Aldrich) in PBS for 20 minutes. Thereafter, cells were washed 3 times in 1X PBS and blocked in 0.5% BSA (Amresco) in PBS for 20 minutes. Cells were then incubated in primary antibody (1:200 in 0.5% BSA in PBS) and incubated overnight at 4 °C. Subsequently, the coverslips were washed 3 times with 0.5% BSA in PBS and then incubated with the appropriate secondary antibody (1:500 in 0.5 % BSA in PBS) for an hour in the dark, at room temperature. The primary and secondary antibodies that were used are listed in Table 1. The cells were then counterstained with 0.1 µg/ml DAPI (Sigma Aldrich) for 5 minutes in the dark for nuclear staining. Coverslips were then washed 3 times with PBS. Coverslips were then mounted onto clean microscope slides using Fluoromount™ Aqueous Mounting Medium (Sigma Aldrich) and incubated at room temperature for 1.5 hours in order to allow the mounting medium to set. Controls were prepared as above, using secondary antibodies only. All images were acquired using the Zeiss LSM 780 confocal microscope at 630x magnification, with the addition of Airyscan™ to obtain resolution of 140 nm. Images were analyzed using Zen 2010 imaging software v2.1.

Table 1: Antibodies used for confocal microscopy

Target Protein	Primary Antibody	Secondary Antibody
LRP/LR	Human anti-LRP/LR (IgG1-iS18)	Anti-human IgG-FITC (Sigma Aldrich: F9512)
Total Tau (Tau-5)	Mouse anti-Tau (Tau-5) (EMD: MAB361)	Anti-mouse IgG-CF™ 647 (Sigma Aldrich: SAB4600182)
Phospho-Tau (pS404)	Rabbit anti-p-Tau (S404) (Cell Signalling: 20194S)	Anti-rabbit IgG-CF™ 647 (Sigma Aldrich: SAB4600184)
PHF-Tau (pT231)	Mouse anti-Phospho-Tau (Thr231) (AT180) (Invitrogen: MN1040)	Anti-mouse IgG-CF™ 647 (Sigma Aldrich: SAB4600182)

Flow Cytometric Analysis of Försters Resonance Energy Transfer (FRET)

FRET is a very sensitive technique that is used to assess protein interactions *in vitro*. In this experiment the donor/ acceptor pair PE/ APC was used to immunolabel the proteins of interest. The presence of FRET was examined using the FL3 filter set of the Accuri C6 Flow Cytometer (BD Biosciences). CF™ 647, which is an APC contemporary, is excited by the 650 nm neon/ helium laser and emits at 660 nm. Within the FL3 channel, excitation is achieved with the 488 nm argon laser and emission is detected at 660 nm. CF™ 647 is not excited by the 488 nm laser and therefore does not exhibit fluorescence within this channel. However, if CF™ 647 is in close proximity to PE, it may be indirectly excited via FRET resulting in enhanced fluorescence emission in FL3.

The experimental design was based off of the FRET analysis performed by Da Costa Dias *et al.* [18] with a few alterations. Briefly, after a 3 hour incubation in serum-free media, HEK-293 cells were detached and harvested via centrifugation at 150 xg for 10 minutes. Since tau is an intracellular protein, following a 20 minute fixation with 4% formaldehyde at 4 °C, the cells were permeabilized by incubating them in 0.1 % Triton X-100 in PBS at 4 °C for 20 minutes. Thereafter, the cells were blocked in 0.5% BSA in PBS for 10 minutes. Cells were centrifuged at 2700 xg for 10 minutes after each step in order to remove the supernatant. The cells were then incubated in primary antibody solutions for 2 hours, washed thrice with PBS and incubated in secondary antibody solutions for 2 hours. Primary and secondary antibodies were used at a concentration of 1:100 in 0.05% BSA in PBS. The primary and secondary antibodies used are listed in Table 2. Cells were washed in PBS three times prior to analysis. Wash steps were performed by centrifuging cells at 2700 xg for 5 minutes and removing the supernatant. Three biological repeats were performed, and 15 000 cells were analyzed per sample. PrP^c was used as a positive control, as there is a direct interaction between LRP/LR and PrP^c [97], and chloramphenical acetyl transferase (CAT) was used as a negative control, as LRP/LR has been shown not to interact with it [38].

Table 2: Antibodies used for Försters Resonance Energy Transfer (FRET)

Target Protein	Primary Antibody	Secondary Antibody
LRP/LR	Human anti-LRP/LR (IgG1-iS18)	Anti-human IgG-PE (Abcam: ab7006)
Total Tau (Tau-5)	Mouse anti-Tau (Tau-5) (EMD: MAB361)	Anti-mouse IgG-CF TM 647 (Sigma Aldrich: SAB4600182)
Prion Protein (PrP ^c)	Mouse anti-Prion Protein (Sigma Aldrich: P0110)	Anti-mouse IgG-CF TM 647 (Sigma Aldrich: SAB4600182)
CAT	Rabbit anti-CAT (Sigma Aldrich: C9336)	Anti-rabbit IgG-CF TM 647 (Sigma Aldrich: SAB4600184)

Stable Overexpression of LRP::FLAG

In order to determine the effect of LRP::FLAG overexpression on tau levels, cells were transfected with the plasmid, pCIneo-moLRP-FLAG [61]. Cells were cultured until 40-50% confluency was achieved. The cells were then transfected using the XfectTM Transfection protocol (Takara). The transfected cells were incubated for 48 hours. Thereafter, cell media was replenished, and the cells were treated with 600-800 ng/ml Geneticin, as a selective treatment. Subsequently, cells were cultured in 200-400 ng/ml of Geneticin to ensure the maintenance of LRP::FLAG expression. Stable transfection was achieved after six weeks of Geneticin treatment.

Western Blotting

Western blotting was used to confirm expression of LRP::FLAG as well as to detect total protein levels of tau, phospho-tau (pS404 and pT231), hTERT, phospho-TERT and PrP^C, post-transfection. β -actin was used as the loading control. Briefly, cell lysates were prepared using 1X RIPA buffer and protein levels quantified using a BCA assay. Proteins were resolved on a 12% SDS-PAGE gel for approximately 1 hour at 150 V in 1X electrophoresis buffer (25mM Tris, 192 mM glycine and 0.1% SDS). A total of 30 μ g (LRP::FLAG) and 60 μ g (Tau, phospho-Tau (pS404), phospho-Tau (pT231), hTERT, phospho-TERT (pS824) and PrP^C) protein was resolved. Thereafter, the separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus at 300 mV for 45-50 minutes in 1X transfer buffer (20% methanol in 25 mM Tris and 19.2 mM glycine). The proteins were fixed to the membrane in 0.4% formaldehyde for 30 minutes. Membranes were blocked in 3% BSA in PBS for 1 hour. The blots were incubated overnight with the appropriate primary antibody, diluted in 3% BSA in PBS, with gentle shaking at 4 °C. Subsequently, the membranes were washed in 0.1% Tween 20 in PBS (PBST). The membranes were then incubated with the appropriate secondary antibody, diluted in 3% BSA in PBS, for 1 hour in the dark and, thereafter, washed with PBST. Membranes were incubated with Thermo Scientific chemiluminescent substrate and imaged using the Bio-Rad GelDoc XR Imager. Densitometric analysis was performed using Image Lab 5.1 software (Bio-Rad). Primary and secondary antibodies are listed in Table 3.

Table 3: Antibodies used for western blotting

Target Protein	Primary Antibody	Dilution	Secondary Antibody	Dilution
β -Actin	Mouse anti- β -Actin Peroxidase (Sigma Aldrich: A3854)	1:10000	-	-
LRP::FLAG	Mouse anti-FLAG [®] M2 (Sigma Aldrich: F-3165)	1:6666	Anti-mouse IgG-HRP (Abcam: ab97023)	1:10000
LRP::FLAG	Mouse anti-FLAG [®] M2-Cy3 [™] (Sigma Aldrich: A-9594)	1:5000	-	-
Total Tau (Tau-5)	Mouse anti-Tau (Tau-5) (EMD: MAB361)	1:500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333
Phospho-Tau (pS404)	Rabbit anti-p-Tau (S404) (Cell Signalling: 20194S)	1:1000	Anti-rabbit IgG-HRP (Cell Signalling: 7074S)	1:3333
PHF-Tau (pT231)	Mouse anti-Phospho-Tau (Thr231) (AT180) (Invitrogen: MN1040)	1:500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333
hTERT	Mouse anti-hTERT	1:2500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333

	(Santa Cruz Biotechnology: sc-77511)			
Phospho-hTERT (pS824)	Rabbit anti-phospho-Telomerase (pSer ⁸²⁴) (Sigma Aldrich: SAB4504295)	1:1000	Anti-rabbit IgG-HRP (Cell Signalling: 7074S)	1:3333
Prion Protein (PrP ^c)	Mouse anti-Prion Protein (Sigma Aldrich: P0110)	1:5000	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333

Statistical Analysis

All statistical analysis of the data obtained was carried out using Microsoft Excel 2016 (Microsoft Corporation). All experiments were performed with three biological repeats and error bars represent standard deviation. The two-tailed Student's *t*-test was performed at a 95% confidence interval. Values of **p* < 0.05 were considered significant, while values of ***p* < 0.01 and ****p* < 0.001 were considered very significant.

Acknowledgements

This work is based upon research supported by the National Research Foundation (NRF), the Republic of South Africa (RSA), grant numbers 105832, 99061, 118851, awarded to SFTW. Any opinions, findings and conclusions or recommendations expressed in this material are those of the author(s), and therefore, the National Research Foundation does not accept any liability in this regard thereto.

Author Contributions

KC performed all experiments, analysed the data and wrote the manuscript. MJB and SC assisted with experimental aspects. MJB, TCO and SC assisted with preparation and editing of the manuscript. SFTW and EF conceptualized the study and edited the manuscript.

Conflict of Interest

The authors declare that they have no competing interests that might be perceived to influence the results and discussion reported in this paper. The authors declare no competing financial interests.

References

1. Alzheimer's Disease International (2018) *World Alzheimer Report 2018: The state of the art of dementia research: New frontiers*.
2. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT (2011) Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med* **1**: a006189.
3. Reitz C (2012) Alzheimer's disease and the amyloid cascade hypothesis: a critical review. *Int J Alzheimers Dis* **2012**: 1–11.
4. Choi SH, Kim YH, Hebisch M, Sliwinski C, Lee S, D'Avanzo C, Chen H, Hooli B, Asselin C, Muffat J, et al. (2014) A three-dimensional human neural cell culture model of Alzheimer's disease. *Nature* **515**: 274–278.
5. Avila J (2006) Tau phosphorylation and aggregation in Alzheimer's disease pathology. *FEBS Lett* **580**: 2922–2927.
6. Brion JP, Octave JN, Couck AM (1994) Distribution of the phosphorylated microtubule-associated protein tau in developing cortical neurons. *Neuroscience* **63**: 895–909.
7. Spittaels K, C V den H, Van Dorpe J, Geerts H, Mercken M, Bruynseels K, Lasrado R, Vandezande K, Laenen I, Boon T, et al. (2000) Glycogen synthase kinase-3 β phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J Biol Chem* **275**: 41340–41349.
8. Biernat J, Wu YZ, Timm T, Zheng-Fischhofer Q, Mandelkow E, Meijer L, Mandelkow EM (2002) Protein kinase MARK/PAK-1 is required for neurite outgrowth and establishment of neuronal polarity. *Mol Biol Cell* **13**: 4013–4028.
9. Haass C, Mandelkow E (2010) Fyn-tau-amyloid: a toxic triad. *Cell* **142**: 356–358.
10. Tomlinson BE, Blessed G, Roth M (1970) Observations on the brains of demented old people. *J Neurol Sci* **11**: 205–242.
11. Alafuzoff I, Iqbal K, Frieden H, Adolfson R, Winblad B (1987) Histopathological criteria for progressive dementia disorders. Clinico-pathological correlation and classification by multivariate data analysis. *Acta Neuropathol* **74**: 209–223.
12. Arriagada P V, Growdon JH, Hedley-Whyte ET, Hyman BT (1992) Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology* **42**: 631–639.
13. Hampel H, Burger K, Pruessner JC, Zinkowski R, DeBernardis J, Kerkman D, Leinsinger G, Evans AC, Davies P, Moller HJ, et al. (2005) Correlation of cerebrospinal fluid levels of tau protein phosphorylated at threonine 231 with rates of hippocampal atrophy in Alzheimer disease. *Arch Neurol* **62**: 770–773.
14. Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with

- mild cognitive impairment: a follow-up study. *Lancet Neurol* **5**: 228–234.
15. DaRocha-Souto B, Coma M, Perez-Nievas PG, Scotton TC, Siao M, Sánchez-Ferrer P, Hashimoto T, Fan Z, Hudry E, Barroeta I, et al. (2012) Activation of glycogen synthase kinase-3 beta mediates β -amyloid induced neuritic damage in Alzheimer's disease. *Neurobiol Dis* **45**: 425–437.
 16. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* (80-) **297**: 353–365.
 17. Mucke L, Selkoe DJ (2012) Neurotoxicity of amyloid β -protein: synaptic and network dysfunction. *Cold Spring Harb Perspect Med* **2**: a006338.
 18. Da Costa Dias B, Jovanovic K, Gonsalves D, Moodley K, Reusch U, Knackmuss S, Weinberg MS, Little M, Weiss SFT (2014) The 37kDa/67kDa Laminin Receptor acts as a receptor for A β 42 internalization. *Sci Rep* **4**: 5556.
 19. Jovanovic K, Chetty CJ, Khumalo T, Da Costa Dias B, Ferreira E, Malindisa ST, Caveney R, Letsolo BT, Weiss SFT (2015) Novel patented therapeutic approaches targeting the 37/67 kDa laminin receptor for treatment of cancer and Alzheimer's disease. *Expert Opin Ther Pat* **25**: 567–582.
 20. Ford CL, Randal-Whitis L, Ellis SR (1999) Yeast proteins related to the p40/laminin receptor precursor are required for 20S ribosomal RNA processing and the maturation of 40S ribosomal subunits. *Cancer Res* **59**: 704–710.
 21. Kinoshita K, Kaneda Y, Sato M, Saeki Y, Wataya-Kaneda M, Hoffmann A (1998) LBP-p40 binds DNA tightly through associations with histones H2A, H2B, and H4. *Biochem Biophys Res Commun* **253**: 277–282.
 22. Zuber C, Knackmuss S, Zemora G, Reusch U, Vlasova E, Diehl D, Mick V, Hoffmann K, Nikles D, Fröhlich T, et al. (2008) Invasion of tumorigenic HT1080 cells is impeded by blocking or downregulating the 37-kDa/67-kDa laminin receptor. *J Mol Biol* **378**: 530–539.
 23. Omar A, Reusch U, Knackmuss S, Little M, Weiss SFT (2012) Anti-LRP/LR-specific antibody IgG1-iS18 significantly reduces adhesion and invasion of metastatic lung, cervix, colon and prostate cancer cells. *J Mol Biol* **419**: 102–109.
 24. Khumalo T, Reusch U, Knackmuss S, Little M, Veale RB, Weiss SFT (2013) Adhesion and invasion of breast and oesophageal cancer cells are impeded by anti-LRP/LR-specific antibody IgG1-iS18. *PLoS One* **8**: e66297.
 25. Khusal R, da Costa Dias B, Moodley K, Penny C, Reusch U, Knackmuss S, Little M, Weiss SFT (2013) In Vitro Inhibition of Angiogenesis by Antibodies Directed against the 37kDa/67kDa Laminin Receptor. *PLoS One* **8**: e58888.
 26. Moodley K, Weiss SFT (2013) Downregulation of the non-integrin laminin receptor reduces cellular viability by inducing apoptosis in lung and cervical cancer cells. *PLoS One* **8**: e57409.

27. Chetty C, Khumalo T, Da Costa Dias B, Reusch U, Knackmuss S, Little M, Weiss SFT (2014) Anti-LRP/LR specific antibody IgG1-iS18 impedes adhesion and invasion of liver cancer cells. *PLoS One* **9**: e96268.
28. Chetty CJ, Ferreira E, Jovanovic K, Weiss SFT (2017) Knockdown of LRP/LR induces apoptosis in pancreatic cancer and neuroblastoma cells through activation of caspases. *Exp Cell Res* **360**: 264–272.
29. Munien C, Rebelo TM, Ferreira E, Weiss SFT (2017) IgG1-iS18 impedes the adhesive and invasive potential of early and late stage malignant melanoma cells. *Exp Cell Res* **351**: 135–141.
30. Rebelo TM, Chetty CJ, Ferreira E, Weiss SFT (2016) Anti-LRP/LR-specific antibody IgG1-iS18 impedes adhesion and invasion of pancreatic cancer and neuroblastoma cells. *BMC Cancer* **16**: 917.
31. Rebelo TM, Vania L, Ferreira E, Weiss SFT (2018) siRNA - mediated LRP/LR knock-down reduces cellular viability of malignant melanoma cells through the activation of apoptotic caspases. *Exp Cell Res* **368**: 1–12.
32. Vania L, Chetty CJ, Ferreira E, Weiss SFT (2016) Anti-LRP/LR specific antibody IgG1-iS18 significantly impedes adhesion and invasion in early and late stage colorectal carcinoma cells. *Mol Med* **22**: 664–673.
33. Vania L, Rebelo TM, Ferreira E, Weiss SFT (2018) Knock-down of LRP/LR promotes apoptosis in early and late stage colorectal carcinoma cells via caspase activation. *BMC Cancer* **18**: 602.
34. Vania L, Morris G, Otgaar TC, Bignoux MJ, Bernert M, Burns J, Gabathuse A, Singh E, Ferreira E, Weiss SFT (2019) Patented therapeutic approaches targeting LRP/LR for cancer treatment. *Expert Opin Ther Pat* **29**: 987–1009.
35. Naidoo K, Malindisa ST, Otgaar TC, Bernert M, Da Costa Dias B, Ferreira E, Reusch U, Knackmuss S, Little M, Weiss SFT, et al. (2015) Knock-Down of the 37kDa/67kDa Laminin Receptor LRP/LR Impedes Telomerase Activity. *PLoS One* **10**: e0141618.
36. Otgaar TC, Ferreira E, Malindisa S, Bernert M, Letsolo BT, Weiss SFT (2017) 37kDa LRP::FLAG enhances telomerase activity and reduces senescent markers in vitro. *Oncotarget* **8**: 86646–86656.
37. Weiss SFT (2017) Bad Boy with a Twist: Targeting the 37kDa/67 kDa Laminin Receptor for Treatment of Cancer and Neurodegenerative Diseases and for Changing Telomere Dynamics. *Cell Cell Life Sci J* **2**: 114.
38. Da Costa Dias B, Jovanovic K, Gonsalves D, Moodley K, Reusch U, Knackmuss S, Penny C, Weinberg MS, Little M, Weiss SFT (2013) Anti-LRP/LR specific antibody IgG1-iS18 and knock-down of LRP/LR by shRNAs rescue cells from A β 42 induced cytotoxicity. *Sci Rep* **3**: 2702.
39. Jovanovic K, Gonsalves D, Da Costa Dias B, Moodley K, Reusch U, Knackmuss S, Penny

- C, Weinberg MS, Little M, Weiss SFT (2013) Anti-LRP/LR specific antibodies and shRNAs impede amyloid beta shedding in Alzheimer's disease. *Sci Rep* **3**: 2699.
40. Jovanovic K, Loos B, Da Costa Dias B, Penny C, Weiss SFT (2014) High resolution imaging study of interactions between the 37 kDa/67 kDa laminin receptor and APP, beta-secretase and gamma-secretase in Alzheimer's disease. *PLoS One* **9**: e100373.
41. Pinnock EC, Jovanovic K, Pinto MG, Ferreira E, Da Costa Dias B, Penny C, Knackmuss S, Reusch U, Little M, Schatzl HM, et al. (2016) LRP-LR Antibody Mediated Rescuing of Amyloid- β -Induced Cytotoxicity is Dependent on PrPc in Alzheimer's Disease. *J Alzheimers Dis* **49**: 645–657.
42. Ferreira E, Bignoux MJ, Otgaar TC, Tagliatti N, Jovanovic K, Letsolo BT, Weiss SFT (2018) LRP/LR specific antibody IgG1-iS18 impedes neurodegeneration in Alzheimer's disease mice. *Oncotarget* **9**: 27059–27073.
43. Greider CW, Blackburn EH (1989) A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* **337**: 331–337.
44. Greider CW, Blackburn EH (1996) Telomeres, telomerase and cancer. *Sci Am* **274**: 92–97.
45. Harley CB, Villeponteau B (1995) Telomeres and telomerase in aging and cancer. *Curr Opin Genet Dev* **5**: 249–255.
46. Shay JW, Wright WE (2007) Hallmarks of telomeres in ageing research. *J Pathol* **211**: 114–123.
47. Greenberg RA, Chin L, Femino A, Lee K-H, Gottlieb GJ, Singer RH, Greider CW, DePinho RA (1999) Short Dysfunctional Telomeres Impair Tumorigenesis in the INK4a Δ 2/3 Cancer-Prone Mouse. *Cell* **97**: 515–525.
48. González-Suárez E, Samper E, Flores JM, Blasco MA (2000) Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. *Nat Genet* **26**: 114–117.
49. Feldser DM, Greider CW (2007) Short Telomeres Limit Tumor Progression In Vivo by Inducing Senescence. *Cancer Cell* **11**: 461–469.
50. Li Y, Tergaonkar V (2014) Noncanonical functions of telomerase: implications in telomerase-targeted cancer therapies. *Cancer Res* **74**: 1639–1644.
51. Ahmed S, Passo JF, Birket MJ, Beckmann T, Brings S, Peters H, Birch-Machin MA, von Zglinicki T, Saretzki G (2008) Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress. *J Cell Sci* **17**: 1046–1053.
52. Saretzki G (2009) Telomerase, mitochondria and oxidative stress. *Exp Gerontol* **44**: 485–492.
53. Sharma NK, Reyes A, Green P, Caron MJ, Bonini MG, Gordon DM, Holt IJ, Santos JH (2012) Human telomerase acts as a hTR-independent reverse transcriptase in mitochondria. *Nucleic Acids Res* **40**: 712–725.

54. Panossian LA, Porter VR, Valenzuela HF, Zhu X, Reback E, Masterman D, Cummings JL, Effros RB (2003) Telomere shortening in T cells correlates with Alzheimer's disease status. *Neurobiol Aging* **24**: 77–84.
55. Wang J, Zhao C, Zhao A, Li M, Ren J, Qu X (2015) New insights in amyloid beta interactions with human telomerase. *J Am Chem Soc* **137**: 1213–1219.
56. Spilisbury A, Miwa S, Attems J, Saretzki G (2015) The role of telomerase protein TERT in Alzheimer's disease and in tau-related pathology in vitro. *J Neurosci* **35**: 1659–1674.
57. Zhu H, Fu W, Mattson MP (2000) The Catalytic Subunit of Telomerase Protects Neurons Against Amyloid β -Peptide-Induced Apoptosis. *J Neurochem* **75**: 117–124.
58. Bignoux MJ, Cuttler K, Otgaar TC, Ferreira E, Letsolo BT, Weiss SFT (2019) LRP::FLAG Rescues Cells from Amyloid- β -Mediated Cytotoxicity Through Increased TERT Levels and Telomerase Activity. *J Alzheimers Dis* **69**: 1–13.
59. Auth D, Brawerman G (1992) A 33-kda polypeptide with homology to the laminin receptor: Component of translation machinery. *Proc Natl Acad Sci U S A* **89**: 4368–4372.
60. Venticinque L, Meruelo D (2012) Comprehensive proteomic analysis of nonintegrin laminin receptor interacting proteins. *J Proteome Res* **11**: 4863–4872.
61. Vana K, Weiss S (2006) A Trans-dominant Negative 37 kDa/67 kDa Laminin Receptor Mutant Impairs PrPSc Propagation in Scrapie-infected Neuronal Cells. *J Mol Biol* **358**: 57–66.
62. Evans DB, Rank KB, Bhattacharya K, Thomsen DR, Gurney ME, Sharma SK (2000) Tau phosphorylation at serine 396 and serine 404 by human recombinant tau protein kinase II inhibits tau's ability to promote microtubule assembly. *J Biol Chem* **275**: 24977–24983.
63. Lin YT, Cheng JT, Liang LC, Ko CY, Lo YK, Lu PJ (2007) The binding and phosphorylation of Thr231 is critical for Tau's hyperphosphorylation and functional regulation by glycogen synthase kinase 3 β . *J Neurochem* **103**: 802–813.
64. Gu J, Congdon EE, Sigurdsson EM (2013) Two novel Tau antibodies targeting the 396/404 region are primarily taken up by neurons and reduce Tau protein pathology. *J Biol Chem* **288**: 33081–33095.
65. Mondragón-Rodríguez S, Perry G, Luna-Muñoz J, Acevedo-Aquino MC, Williams S (2014) Phosphorylation of tau protein at sites Ser 396–404 is one of the earliest events in Alzheimer's disease and Down syndrome. *Neuropath Appl Neurobiol* **40**: 121–135.
66. Cho JH, Johnson G V (2003) Glycogen synthase kinase 3 β phosphorylates tau at both primed and unprimed sites. Differential impact on microtubule binding. *J Biol Chem* **278**: 187–193.
67. Poruchynsky MS, Komlodi-Pasztor E, Trostel S, Wilkerson J, Regairaz M, Pommier Y, Zhang X, Maity TK, Robey R, Burotto M, et al. (2015) Microtubule-targeting agents augment the toxicity of DNA-damaging agents by disrupting intracellular trafficking of

- DNA repair proteins. *Proc Natl Acad Sci U S A* **112**: 1571–1576.
68. Zhang J, Pieper A, Snyder SH (1995) Poly(ADP-Ribose) Synthetase Activation: An Early Indicator of Neurotoxic DNA Damage. *J Neurochem* **65**: 1411–1414.
69. Deng G, Su JH, Ivins KJ, Van Houten B, Cotman CW (1999) Bcl-2 facilitates recovery from DNA damage after oxidative stress. *Exp Neurol* **159**: 309–318.
70. Ittner LM, Ke YD, Delerue F, Bi M, Gladbach A, van Eersel J, Wolfing H, Chieng BC, Christie MJ, Napier IA, et al. (2010) Dendritic function of tau mediates amyloid-beta toxicity in Alzheimer's disease mouse models. *Cell* **142**: 387–397.
71. Dehmelt L, Halpain S (2005) The MAP2/Tau family of microtubule-associated proteins. *Genome Biol* **6**: 204.
72. DiTella MC, Feiguin F, Carri N, Kosik KS, Caceres A (1996) MAP-1B/TAU functional redundancy during laminin-enhanced axonal growth. *J Cell Sci* **109**: 467–477.
73. Takei Y, Teng J, Harada A, Hirokawa N (2000) Defects in axonal elongation and neuronal migration in mice with disrupted tau and map1b genes. *J Cell Biol* **150**: 989–1000.
74. Givant-Horwitz V, Davidson B, Reich R (2004) Laminin-induced signaling in tumor cells: The role of the m(r) 67,000 laminin receptor. *Cancer Res* **64**: 3572–3579.
75. Sun L, Liu L, Liu X, Wang Y, Li M, Yao L, Yang J, Ji G, Guo C, Pan Y, et al. (2014) MG r1-Ag/37 LRP induces cell adhesion-mediated drug resistance through FAK/PI 3K and MAPK pathway in gastric cancer. *Cancer Sci* **105**: 651–659.
76. Williamson R, Scales T, Clark BR, Gibb G, Reynolds CH, Kellie S, Bird IN, Varndell IM, Sheppard PW, Everall I, et al. (2002) Rapid tyrosine phosphorylation of neuronal proteins including tau and focal adhesion kinase in response to amyloid- β peptide exposure: involvement of Src family protein kinases. *J Neurosci* **22**: 10–20.
77. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science (80-)* **279**: 349–352.
78. Tomas-Loba A, Flores I, Fernández-Marcos PJ, Cayuela ML, Maraver A, Tejera A, Borrás C, Matheu A, Klatt P, Flores JM, et al. (2008) Telomerase Reverse Transcriptase Delays Aging in Cancer-Resistant Mice. *Cell* **135**: 609–622.
79. Chung J, Khadka P, Chung IK (2012) Nuclear import of hTERT requires a bipartite nuclear localization signal and Akt-mediated phosphorylation. *J Cell Sci* **125**: 2684–2697.
80. Atamna H, Frey II WH (2007) Mechanisms of mitochondrial dysfunction and energy deficiency in Alzheimer's disease. *Mitochondrion* **7**: 297–310.
81. Melov S, Adlard PA, Morten K, Johnson F, Golden TR, Hinerfeld D, Schilling B, Mavros C, Masters CL, Volitakis I, et al. (2007) Mitochondrial oxidative stress causes hyperphosphorylation of tau. *PLoS One* **2**: e536.

82. Ebner A, Godemann R, Stamer K, Illenberger S, Trinczek B, Mandelkow EM, Mandelkow E (1998) Overexpression of tau protein inhibits kinesin-dependent trafficking of vesicles, mitochondria, and endoplasmic reticulum: implications for Alzheimer's disease. *J Cell Biol* **143**: 777–794.
83. Treeck HH, Pirsig W (1979) Differentiation of nerve endings in the cochlear nucleus on morphological and experimental basis. *Acta Otolaryngol* **87**: 47–60.
84. Gotow T, Miyaguchi K, Hashimoto PH (1991) Cytoplasmic architecture of the axon terminal: filamentous strands specifically associated with synaptic vesicles. *Neuroscience* **40**: 587–598.
85. Morris RL, Hollenbeck PJ (1993) The regulation of bidirectional mitochondrial transport is coordinated with axonal outgrowth. *J Cell Sci* **104**: 917–927.
86. Ruthel G, Hollenbeck PJ (2003) Response of mitochondrial traffic to axon determination and differential branch growth. *J Neurosci* **23**: 8618–8624.
87. Mattson MP, Liu D (2002) Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders. *Neuromolecular Med* **2**: 215–231.
88. Cente M, Filipcik P, Pevalova M, Novak M (2006) Expression of a truncated tau protein induces oxidative stress in a rodent model of tauopathy. *Eur J Neurosci* **24**: 1085–1090.
89. Freir DB, Nicoll AJ, Klyubin I, Panico S, McDonald JM, Risse E, Asante EA, Farrow MA, Sessions RB, Saibil HR, et al. (2011) Interaction between prion protein and toxic amyloid β assemblies can be therapeutically targeted at multiple sites. *Nat Commun* **2**: 1–9.
90. Beringue V, Mallinson G, Kaisar M, Tayebi M, Sattar Z, Jackson G, Anstee D, Collinge J, Hawke S (2003) Regional heterogeneity of cellular prion protein isoforms in the mouse brain. *Brain* **126**: 2065–2073.
91. Kuczius T, Koch R, Keyvani K, Karch H, Grassi J, Groschup MH (2007) Regional and phenotype heterogeneity of cellular prion proteins in the human brain. *Eur J Neurosci* **25**: 2649–2655.
92. Schmitz M, Wulf K, Signore SC, Schulz-Schaeffer WJ, Kermer P, Bähr M, Wouters FS, Zafar S, Zerr I (2014) Impact of the cellular prion protein on amyloid- β and 3PO-tau processing. *J Alzheimers Dis* **38**: 551–565.
93. Vergara C, Ordóñez-Gutiérrez L, Wandosell F, Ferrer IF, Río Fernández JA, Gavín Marín R (2014) Role of PrP^C in tau protein levels and phosphorylation in Alzheimer's disease evolution. *Mol Neurobiol* **51**: 1206–1220.
94. Larson M, Sherman MA, Amar F, Nuvolone M, Schneider JA, Bennett DA, Aguzzi A, Lesné SE (2012) The complex PrP^C-Fyn couples human oligomeric A β with pathological Tau changes in Alzheimer's disease. *J Neurosci* **32**: 16857–16871.
95. Chin J, Palop JJ, Puolivali J, Massaro C, Bien-Ly N, Gerstein H, Searce-Levie K, Masliah E, Mucke L (2005) Fyn kinase induces synaptic and cognitive impairments in a

transgenic mouse model of Alzheimer's disease. *J Neurosci* **25**: 9694–9703.

96. Roberson ED, Halabisky B, Yoo JW, Yao J, Chin J, Yan F, Wu T, Hamto P, Devidze N, Yu GQ, et al. (2011) Amyloid- β /Fyn-induced synaptic, network, and cognitive impairments depend on tau levels in multiple mouse models of Alzheimer's disease. *J Neurosci* **31**: 700–711.
97. Gauczynski S, Peyrin JM, Haïk S, Leucht C, Hundt C, Rieger R, Krasemann S, Deslys JP, Dormont D, Lasmézas CI, et al. (2001) The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J* **20**: 5863–5875.

Figure Legends:

Figure 1. LRP/LR and tau co-localize and interact in HEK-293 cells. (A) Image showing the co-localization between tau (red) (a) and LRP/LR (green) (b). Nuclei are stained with DAPI (blue) (c). Co-localization occurs between LRP/LR and tau in the perinuclear compartment, represented by yellow fluorescence in the merged image (d), as white areas (e) and as fluorescence in the third quadrant of the 2D cytofluorogram (f). All images were taken at 630x magnification with the addition of Airyscan™. Scale bars represent 20 μ m. (B) Flow cytometric analysis of FRET between intracellular LRP/LR and tau in HEK-293 cells shows a shift in fluorescence which is indicative of a direct interaction. The fluorescence intensity of the unstained cells (dark blue histogram) was superimposed with that of cells labelled with the CF™ 647 secondary antibody only (yellow histogram) as well as with cells in which the proteins of interest; PrP^C, CAT and tau, were labelled with CF™ 647 (a, c, e). (b) Co-labelling of LRP/LR-PE and PrP^C-CF™ 647 (positive control) (pink histogram). (d) Co-labelling of LRP/LR-PE and CAT-CF™ 647 (negative control) (green histogram). (f) Co-labelling of LRP/LR-PE and Tau-CF™ 647 (red histogram). Each panel is a representative image. n = 3 biological repeats.

Figure 2: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases levels of total Tau as well as phospho-Tau. A, B) A 45% decrease in total Tau, 68% decrease in phospho-Tau (pS404) and 98% decrease in phospho-Tau (pT231) was observed in transfected HEK-293 cells. C, D) A 35% decrease in total Tau, 63% decrease in phospho-Tau (pS404) and 92% decrease in phospho-Tau (pT231) was observed in transfected SH-SY5Y cells. A, C) HEK-293 and SH-SY5Y cells were confirmed to be overexpressing LRP::FLAG after stable transfection with the pCneo-moLRP-FLAG plasmid. LRP::FLAG was detected in transfected HEK-293 cells only (A). LRP::FLAG was detected in transfected SHSY-5Y cells only (C). A, C) β -Actin was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (B) and SH-SY5Y (C) cells which were set to 100%. Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001; Student's *t*-test.

Figure 3: Intracellular expression of tau and phospho-Tau in transfected and non-transfected HEK-293 and SH-SY5Y cells. b, e) Nuclei are stained with DAPI (blue). A, B) Expression of total tau decreases in transfected HEK-293 and transfected SH-SY5Y cells. Endogenous levels of total tau (red) (a). Levels of total tau (red) after transfection with pCneo-moLRP-FLAG plasmid (d). Merge of total tau with DAPI staining (c, f). C, D) Expression of phospho-Tau (pS404) is decreased in transfected HEK-293 and transfected SH-SY5Y cells. Endogenous levels of phospho-Tau (pS404) (red) (a). Levels of phospho-Tau (pS404) (red) after transfection with pCneo-moLRP-FLAG plasmid (d).

Merge of phospho-Tau (pS404) with DAPI staining (c, f). E, F) Expression of phospho-Tau (pT231) is decreased in transfected HEK-293 and transfected SH-SY5Y cells. Endogenous levels of phospho-Tau (pT231) (red) (a). Levels of phospho-Tau (pT231) (red) after transfection with pCIneo-moLRP-FLAG plasmid (d). Merge of phospho-Tau (pT231) with DAPI staining (c, f). All images were taken at 630x magnification with the addition of Airyscan™. Scale bars represent 20 μ m.

Figure 4: LRP::FLAG overexpression increases hTERT levels in HEK-293 and SH-SY5Y cells and increases phospho-TERT levels in SH-SY5Y cells. A, B) A 120% increase in hTERT and 112% increase in phospho-TERT was observed in transfected HEK-293 cells. C, D) A 125% increase in hTERT and 96% increase in phospho-TERT was observed in transfected SH-SY5Y cells. β -Actin was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (B) and SH-SY5Y (D) cells which were set to 100%. Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001; Student's *t*-test.

Figure 5: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases PrP^c levels. A, B) A 31% decrease in total PrP^c (di- and monoglycosylated), 34% decrease in diglycosylated PrP^c and 27% decrease in monoglycosylated PrP^c was observed in transfected HEK-293 cells. (C, D) A 39% decrease in total PrP^c (monoglycosylated) was observed in transfected SH-SY5Y cells. β -Actin was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (B) and SH-SY5Y (D) cells which were set to 100%. Error bars represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001; Student's *t*-test.

A

HEK-293 Cells

B

Non-transfected HEK-293 Cells	Transfected HEK-293 Cells
----------------------------------	------------------------------

B

C

Non-transfected SH-SY5Y Cells	Transfected SH-SY5Y Cells
----------------------------------	---------------------------------

D

LRP::FLAG reduces phosphorylated tau levels in Alzheimer's Disease

Authors: Katelyn Cuttler^{1,2}, Monique J. Bignoux¹, Tyrone C. Otgaar¹, Stephanie Chigumba¹, Eloise Ferreira¹ and Stefan F.T. Weiss^{1*}

¹School of Molecular and Cell Biology, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, Republic of South Africa

²Present Address: Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, Republic of South Africa

*E-mail: stefan.weiss@wits.ac.za

Supplementary Material

Figure S1: Full β -Actin western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S2: Full FLAG western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S3: Full total tau western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S4: Full phosphorylated tau (pS404) western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S5: Full phosphorylated tau (pT231) western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S6: Full hTERT western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S7: Full phosphorylated TERT (pS824) western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells.

Figure S8: Full PrP^c western blots for HEK-293 cells (A) and SH-SY5Y cells (B). Lanes 1-3: non-transfected cells; lanes 4-6: transfected cells; lane 7: repeat of transfected SH-SY5Y cells not included in analysis.