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Investigating the effect of LRP/LR and telomerase on tauopathy in alzheimer's disease cell culture models

Cuttler, Katelyn

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SCHOOL OF MOLECULAR CELL BIOLOGY
UNIVERSITY OF THE WITWATERSRAND
BRAAMFONTEIN
JOHANNESBURG

Investigating the Effect of LRP/LR and Telomerase on Tauopathy In Alzheimer's Disease Cell Culture Models

Katelyn Cuttler (746330)

DISSERTATION
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in
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in the
FACULTY OF SCIENCE

Supervisor: Prof. Stefan F.T. Weiss

Co-Supervisor: Dr. Eloise van der Merwe

Declaration

I, Katelyn Cuttler (746330), am a student registered for the degree of Masters of Science (MSc) by dissertation in the academic year 2019.

I hereby declare the following:

I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.

I confirm that the work submitted for assessment for the above degree is my own unaided work except where explicitly indicated otherwise.

I have not submitted this work before for any other degree or examination at this or any other University.

I have followed the required conventions in referencing the thoughts and ideas of others.

I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.

Signed  on the 21st day of May 2019

Abstract

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder resulting in dementia in the elderly. Currently, approximately 50 million people worldwide are suffering from AD. This disease is a result of two neuropathological features, extracellular amyloid plaques caused by aggregation of amyloid beta (Aβ) peptides and intracellular neurofibrillary tangles caused by accumulation of hyperphosphorylated tau protein. Tau is a microtubule-associated protein that normally functions to stabilize microtubules. Hyperphosphorylation of tau causes its dissociation from microtubules, ultimately leading to cell death. Furthermore, levels of the human reverse transcriptase ((hTERT) subunit of telomerase, has been shown to be significantly decreased in tau-affected neuronal cells. Normally, hTERT would function to protect cells from oxidative stress. Therefore, the absence of hTERT enhances neurodegeneration. It has recently been demonstrated that the 37 kDa/ 67 kDa laminin receptor (LRP/LR) interacts with telomerase leading to increases in both telomerase activity and hTERT levels. Furthermore, LRP/LR is known to play a role in Alzheimer's disease as a receptor for $A\beta_{42}$ internalization and is involved in the amyloidogenic processing of amyloid precursor protein (APP). This study investigates whether overexpression of LRP::FLAG, with a concomitant increase in hTERT levels, will change total tau and phosphorylated tau levels *in vitro*. To address this question, we overexpressed LRP::FLAG *in vitro* and investigated total tau, phosphorylated tau, hTERT, $A\beta_{42}$ and PrP^c levels. LRP/LR and tau co-localize in the perinuclear cell compartment and Försters Resonance Energy Transfer (FRET) confirmed a direct interaction between LRP/LR and tau. Thereafter, cells were transfected with the pCIneo-moLRP-FLAG plasmid in order to overexpress LRP::FLAG. Western blotting and confocal microscopy confirmed a decrease in total tau and phosphorylated tau levels in cells overexpressing LRP::FLAG. Therefore, LRP::FLAG overexpression could lead to a decrease in neurofibrillary tangle formation. Western blotting also confirmed an increase in hTERT levels, which could rescue AD-affected cells from the cytotoxicity caused by $A\beta_{42}$ and tau. Furthermore, the levels of $A\beta_{42}$ and PrP^c, two tauopathy-related proteins, were also shown to decrease. Therefore, a reduction in both $A\beta_{42}$ and PrP^c could result in less tau hyperphosphorylation and neurofibrillary tangle formation. This is the first study to show that there is an interaction between LRP/LR and tau and the results suggest that overexpression of LRP::FLAG may be a possible treatment strategy for the tauopathy of AD.

In loving memory of my mother

Jean Lynch

1959-2017

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Own contribution: assisted with Aβ-shedding ELISA experiments and analysis

Patents:

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Inventors: Burns J, Cuttler K, Bignoux MJ, Ferreira E, Weiss SFT
Own contribution: conceptualized and designed the study with the help of Dr. Eloise Ferreira
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Table of Contents

Declaration ii
Abstract iii
Acknowledgements v
Research Outputs vi
List of Abbreviations ix
List of Figures xi
List of Tables xii
1. Introduction 1
1.1. Alzheimer's Disease 1
1.2. Neuropathology of Alzheimer's Disease 2
1.2.1 Tau 3
1.2.1.1 Hyperphosphorylation of Tau 5
1.2.2 Amyloid Beta 7
1.2.2.1 Aβ and Cellular Receptors 8
1.3. Cellular Prion Protein (PrP^c) 9
1.3.1 PrP^c in Alzheimer's Disease 10
1.4. The 37 kDa/ 67 kDa Laminin Receptor (LRP/LR) 12
1.4.1 LRP/LR in Alzheimer's Disease 13
1.5. Telomere Biology and Telomerase 14
1.5.1 Telomere Biology 14
1.5.2 Telomerase 16
1.5.3 Telomeres, Telomerase and Alzheimer's Disease 18
1.6. Justification of Study 18
2. Aims and Objectives 20
2.1. Main Aim 20
2.2. Objectives 20
3. Experimental Procedures 21
3.1. Cell Culture 21
3.2. Confocal Microscopy with the addition of Airyscan™ 22
3.3. Flow Cytometric Analysis of Försters Resonance Energy Transfer (FRET) 24
3.4. Stable Overexpression of LRP::FLAG 27
3.5. Western Blotting 27
3.5.1 Bicinchoninic Acid (BCA) Assay 27

3.5.2 SDS-PAGE and Western Blotting 28
3.6. Statistical Analysis 30
4. Results 31
4.1. LRP/LR co-localizes with tau and hTERT 31
4.2. LRP/LR and tau interact directly 31
4.3. Confirmation of LRP::FLAG overexpression in HEK-293 and SH-SY5Y cells 34
4.4. Overexpression of LRP::FLAG decreases total tau and phospho-tau levels 35
4.5. Overexpression of LRP::FLAG increases total hTERT levels 37
4.6. Overexpression of LRP::FLAG decreases Aβ expression and PrP^c levels 39
5. Discussion 41
5.1. LRP/LR and Tau co-localization and FRET analysis 42
5.2. LRP::FLAG overexpression decreases total and phosphorylated tau levels 43
5.3. LRP::FLAG overexpression affects hTERT and phospho-TERT levels 46
5.4. LRP::FLAG overexpression decreases $A\beta_{42}$ and PrP^c levels 48
5.5. Conclusion 50
5.6. Future Work 50
6. References 52
Appendix 68

List of Abbreviations

α7nAChR:	A7 Nicotininc Acetylcholine receptor
Aβ:	Amyloid beta
AD:	Alzheimer's disease
AICD:	APP intracellular domain
APC:	Allophycocyanin
APP:	Amyloid precursor protein
BBB:	Blood brain barrier
BCA Assay:	Bicinchoninic acid assay
BSA:	Bovine serum albumin
CAT:	Chloramphenical acetyl transferase
CDK5:	Cyclin-dependent protein kinase 5
CNS:	Central nervous system
DAPI:	4',6'-diamidino-2-phenylindole
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl sulfoxide
ECM:	Extracellular matrix
FAK:	Focal adhesion kinase
FBS:	Foetal bovine serum
FITC:	Fluorescein isothiocyanate
FRET:	Försters Resonance Energy Transfer
FPRL1:	Formyl peptide receptor-like 1
GPI Anchor:	Glycosylphosphatidylinositol anchor
GSK3:	Glycogen synthase kinase 3
HRP:	Horse radish peroxidase
HSPG:	Heparin sulphate proteoglycan
hTERC:	Human telomerase RNA component
hTERT:	Human telomerase reverse transcriptase
IgG:	Immunoglobulin G
LRP/LR:	Laminin receptor precursor/ high affinity laminin receptor
MAPT:	Microtubule-associated protein tau
mtDNA:	Mitochondrial DNA
mTERT:	Mouse telomerase reverse transcriptase
NMDA-R:	N-methyl-D-aspartate receptor
$P75^{NTR}$:	P75 Neurotrophin receptor
PBS:	Phosphate buffered saline
PE:	Phycoerythrin
POT1:	Protection of telomeres protein 1
PP2A:	Protein phosphatase 2A
PP2B:	Protein phosphatase 2B
PrP^{c}:	Cellular prion protein
PS1:	Presenilin 1
PVDF:	Polyvinylidene fluoride

RAGE:	Receptor for advanced glycosylation end products
RIPA Buffer:	Radioimmunoprecipitation buffer
ROS:	Reactive oxygen species
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA:	Short hairpin RNA
siRNA:	Short interfering RNA
SRA:	Aβ-scavenging receptor A
t-loop:	Telomere loop
TCR:	T-cell antigen receptor
TRF1:	Telomeric repeat factor 1
TRF2:	Telomeric repeat factor 2

List of Figures

Figure 1.1: Stages of Alzheimer's disease showing how atrophy spreads....2
Figure 1.2: Brain changes and neuropathological agents in Alzheimer's disease 3
Figure 1.3: Model illustration of A) a healthy neuron and B) a diseased neuron showing how tau molecules stabilize microtubules....5
Figure 1.4: Central pathways affecting phosphorylation of tau....6
Figure 1.5: Model showing the amyloid beta cascade hypothesis of Alzheimer's disease development....8
Figure 1.6: PrP^c mediates Aβ-induced synaptic toxicity. 10
Figure 1.7: Schematic illustration of the transmembrane 37 kDa/ 67 kDa Laminin Receptor (LRP/LR) showing the binding sites of various ligands 12
Figure 1.8: Schematic depiction of the t-loop and shelterin complex of proteins that cooperate to protect the ends of telomeres 15
Figure 1.9: Schematic depiction of telomerase ribonucleoprotein. 17
Figure 3.1: A schematic representation of the experimental design employed for FRET analysis of the potential LRP/LR-Tau interaction. 25
Figure 4.1: LRP/LR, tau and hTERT localization and co-localization within HEK-293 cells....32
Figure 4.2: Flow cytometric analysis of FRET between intracellular LRP/LR and tau. 34
Figure 4.3: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells transfected with the pCIneo-moLRP-FLAG plasmid 35
Figure 4.4: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases levels of total Tau as well phospho-Tau....36
Figure 4.5: Intracellular expression of Tau and phospho-Tau in transfected and non-transfected HEK-293 cells 37
Figure 4.6: Overexpression of LRP::FLAG increases hTERT levels in HEK-293 and SH-SY5Y cells and phospho-TERT levels in SH-SY5Y cells 38
Figure 4.7: Intracellular expression of $A\beta_{42}$ in transfected and non-transfected HEK-293 cells. Expression of $A\beta_{42}$ decreases in transfected HEK-293 cells. 39
Figure 4.8: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases PrP^c levels 40
Figure A1: Enlarged LRP/LR and tau localization and co-localization within HEK-293 cells. 68
Figure A2: Enlarged LRP/LR and hTERT localization and co-localization within HEK-293 cells....69
Figure A3: Flow cytometric analysis of FRET between intracellular LRP/LR and tau – Biological replicates....70
Figure A4: Proteins of interest are successfully labelled during FRET analysis 71
Figure A5: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells transfected with the pCIneo-moLRP-FLAG plasmid – Full Blots....72
Figure A6: Overexpression of LRP::FLAG in HEK-293 cells decreases levels of total Tau as well phospho-Tau – Full Blots....73
Figure A7: Overexpression of LRP::FLAG in SH-SY5Y cells decreases levels of total Tau as well phospho-Tau – Full Blots....75
Figure A9: Overexpression of LRP::FLAG increases hTERT levels in HEK-293 and SH-SY5Y cells and phospho-TERT levels in SH-SY5Y cells -Full Blots....76
Figure A10: Intracellular expression of $A\beta_{42}$ in transfected and non-transfected HEK-293 cells with negative contols....77

Figure A11: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases PrP^c levels – Full Blots……78

List of Tables

Table 1.1: Membrane associated proteins to which Aβ may bind. 9
Table 3.1: Antibodies used for confocal microscopy 23
Table 3.2: Antibodies used for Försters Resonance Energy Transfer (FRET) 26
Table 3.3: Antibodies used for western blotting 29
Table 4.1: Ratio of phospho-TERT to hTERT in transfected SH-SY5Y cells shows that not all hTERT is converted to phospho-TERT 39
Table A1: 1X RIPA Buffer 79
Table A2: 12% (w/v) SDS-PAGE Gel 79
Table A3: 30% Acrylamide/ 0.8% Bisacrylamide 79
Table A4: 2X Tris-HCl/ SDS pH 8.8 80
Table A5: 4X Tris-HCl/ SDS pH 6.8 80
Table A6: 1X Electrophoresis Buffer pH 8.8 80
Table A7: Transfer Buffer pH 8.3 80
Table A8: 20X Phosphate Buffered Saline (PBS) pH 7.2/7.4 81

1. Introduction

1.1. Alzheimer's Disease

Alzheimer's disease (AD) is a type of dementia which affects approximately 50 million people worldwide (Alzheimer's Disease International, 2018). It is the most prevalent form of dementia and incidence is rapidly increasing. Indeed, the incidence of AD is expected to increase to 82 million by 2030 (Alzheimer's Disease International, 2018). AD mainly affects the elderly and thus is an increasing concern due to increased global life expectancy. In addition, only long-term palliative care is available for AD patients. As the disease can take many years to become fatal, it places a huge economic burden on health care systems. The cost of specialized care for AD amounted to $1 trillion dollars in 2018 (Alzheimer's Disease International, 2018). The disease also places a huge social burden on relatives. Therefore, there is a great need to develop effective non-palliative treatments for AD.

The disease is characterized by cognitive impairments, such as memory loss, disorientation and behavioural changes (Verdier and Penke, 2004). In addition, there is a diminishment in higher-order brain functions, such as thinking and learning (Gimbel *et al.,* 2010) which causes dementia and eventually death (Amieva *et al.,* 2008). AD has been defined as a three-stage disease (Sperling *et al.,* 2011) (Figure 1.1). In the first stage amyloid plaques are evident; however, there are no evident cognitive or behavioural changes (Sperling *et al.,* 2011). In stage two these plaques have become cytotoxic leading to neurodegeneration and synaptic dysfunction (Sperling *et al.,* 2011). The neurodegeneration mainly occurs in the cortex and hippocampal regions of the brain, resulting in memory loss and behavioural changes (Sperling *et al.,* 2011). In the final stage, severe neurological damage is present and, in addition to memory loss, verbal changes and lack of muscle control is seen (Sperling *et al.,* 2011). However, the process of AD begins long before there is clinical evidence for the disease. In the most widely accepted hypothesis, the amyloid beta (Aβ) cascade hypothesis, it is accumulation of cytotoxic Aβ peptides that results in neuronal death and synaptic loss (Sperling *et al.,* 2011). This can occur decades before amyloid plaques are evident in stage one (Sperling *et al.,* 2011). Therefore, it is important understand the neuropathology of AD in order to treat the disease in the preclinical phase.

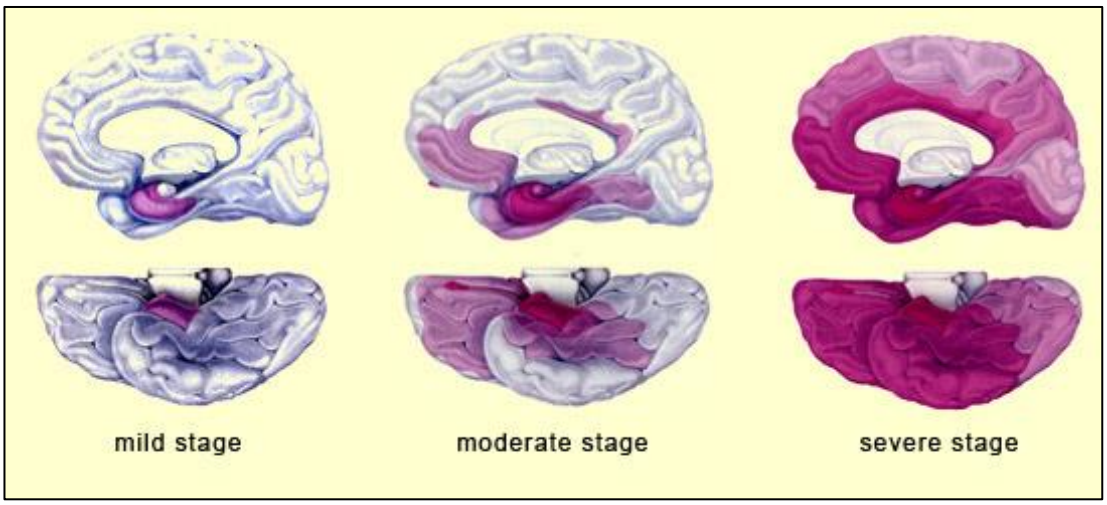


Figure 1.1: Stages of Alzheimer's disease showing how atrophy spreads. Initially atrophy is observed around the hippocampus. As the disease progress, atrophy extends to the temporal cortex. Finally, in the severe stage the cortex as a whole is affected.
(Adopted from http://thebrain.mcgill.ca/flash/i/i_08/i_08_cr/i_08_cr_alz/i_08_cr_alz.html [Accessed 18/01/2018]).

1.2. Neuropathology of Alzheimer's Disease

AD is characterized by disruption of neuronal circuits, loss of synapses and neurotoxicity (Serrano-Pozo *et al.*, 2011) as a result of the two neuropathological agents, amyloid plaques and neurofibrillary tangles. Amyloid plaques are due to an extracellular aggregation of Aβ peptides while neurofibrillary tangles form from the intracellular accumulation of hyperphosphorylated tau (Serrano-Pozo *et al.*, 2011) (Figure 1.2). The extracellular amyloid plaques are neurotoxic and aggregate between neurons, disrupting their functions and leading to apoptosis (Serrano-Pozo *et al.*, 2011). Meanwhile, neurofibrillary tangles accumulate in specific brain regions involved in memory and block the microtubular transport system, which harms synaptic communication (Serrano-Pozo *et al.*, 2011). Overall, these agents are able to damage many areas of the brain which ultimately results in death (Serrano-Pozo *et al.*, 2011).

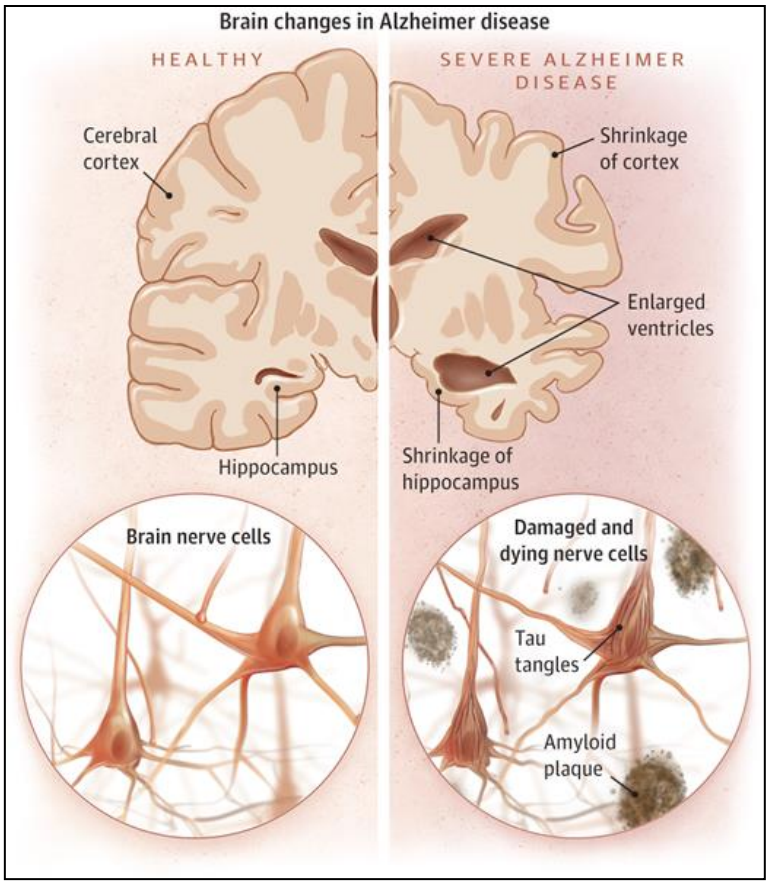


Figure 1.2: Brain changes and neuropathological agents in Alzheimer's disease. The neurotoxicity seen in Alzheimer's is a result of two neuropathological agents; extracellular amyloid plaques and intracellular tau neurofibrillary tangles.
(Adopted from Jin, 2015).

1.2.1 Tau

Tau is a microtubule-associated protein that functions to stimulate the assembly of tubulin into microtubules. Furthermore, tau binds to and stabilizes the microtubules once they are assembled (Avila, 2006). Since these microtubules are situated along the cytoskeletal tracks they mediate vital cell processes, such as the transport of nutrients and neurotransmitters (Avila, 2006). There are six different isoforms of tau that are present in the adult human brain and they stimulate microtubule assembly with varying efficiency. In healthy cells, the level of tau phosphorylation is highly modulated and regulates microtubule plasticity (Brion *et al.,* 1994), motor activity and axonal transport (Spittaels *et al.,* 2000), and neurite outgrowth (Biernat *et al.,* 2002).

In AD, however, tau is hyperphosphorylated and forms tangles, thereby disrupting the internal structure of neuronal cells (Haass and Mandelkow, 2010) (Figure 1.3). In addition,

phosphorylation of tau at specific sites, such as S262, S356 and S422, inhibits the ability of the hyperphosphorylated tau to be recognized by proteases, thereby sparing it from proteasomal degradation (Guillozet-Bongaarts *et al.,* 2006; Dickey *et al.,* 2007). Therefore, the hyperphosphorylation of tau is able to induce the AD pathology in multiple ways. Firstly, this hyperphosphorylation can induce the missorting of tau from the axons to the dendritic spines, resulting in inhibition of transport and loss of spines (Thies and Mandelkow, 2007; Hoover *et al.,* 2010). Hyperphosphorylated tau is also known to interact with the kinesin-associated protein, c-Jun N-terminal kinase-interacting protein (JIP1) and impair the formation of the kinesin complex. This complex is important in mediating axonal transport (Ittner *et al.,* 2009). Lastly, neurofibrillary tangles are formed when this hyperphosphorylation causes tau to dissociate from microtubules and diffuse into the cytoplasm (Haass and Mandelkow, 2010) (Figure 3). This causes the cytoskeletal tracks to disintegrate (Figure 3), thereby inhibiting vital cell processes, such as the transport of nutrients and neurotransmitters, which ultimately leads to cell death (Haass and Mandelkow, 2010). Therefore, the hyperphosphorylation of tau negatively impacts synaptic health, plasma membrane cell signalling and the protection of DNA from cell stressors (Haass and Mandelkow, 2010), all of which may contribute to the development or progression of neurodegeneration. Indeed, the degree of dementia observed has been correlated with the number of neurofibrillary tangles present within the brain tissue (Tomlinson *et al.,* 1970; Alafuzoff *et al.,* 1987; Arriagada *et al.,* 1992; Hampel *et al.,* 2005; Hansson *et al.,* 2006).

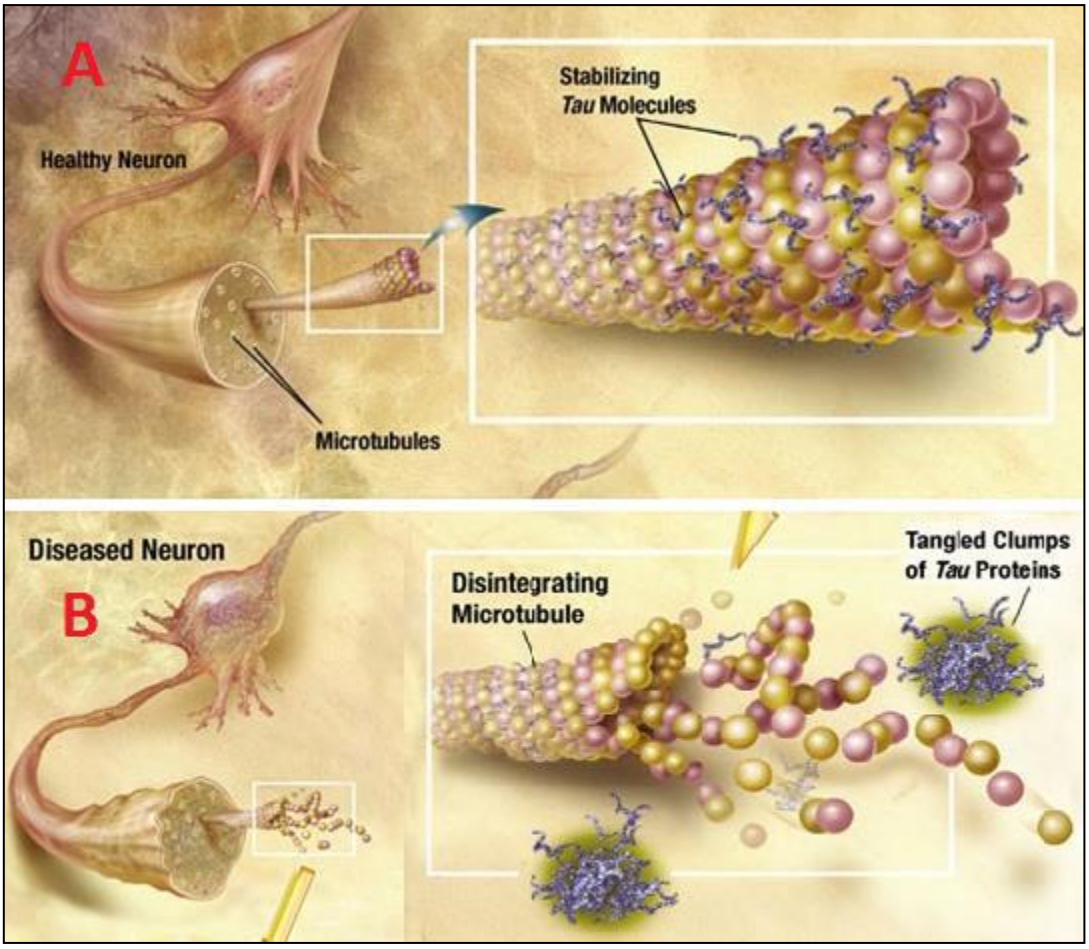


Figure 1.3: Model illustration of A) a healthy neuron and B) a diseased neuron showing how tau molecules stabilize microtubules. In healthy neurons, tau binds to microtubules and stabilizes them. In diseased neurons, tau dissociates from microtubules to form tangled clumps. This destabilizes microtubules and causes them to disintegrate.
(Adopted from www.dddmag.com/article/2015/01 [Accessed 20/07/2017]).

1.2.1.1 Hyperphosphorylation of Tau

There are 85 putative serine, threonine or tyrosine phosphorylation sites on the human tau protein. As a result, the normal level of tau phosphorylation is the result of a dynamic interplay between tau kinases and tau phosphatases (Gong and Iqbal, 2008). Tau kinases, such as glycogen synthase kinase 3 (GSK3) and cyclin-dependent protein kinase 5 (CDK5) (Ferrer *et al.*, 2005; Mazanetz and Fischer, 2007) work to phosphorylate tau while phosphatases, such as protein phosphatase 2A and 2B (PP2A and PP2B), reverse this phosphorylation (Goedert *et al.*, 1995; Sontag *et al.*, 1996; Gong *et al.*, 2000) (Figure 1.4). In tau pathologies, prior phosphorylation of tau by CDK5 enhances its subsequent phosphorylation by GSK3. GSK3 then phosphorylates residues which affect the binding of

tau to microtubules (Cho and Johnson, 2002). The residues threonine-231, serine-396 and serine-404 are particularly important in this role (Cho and Johnson, 2002). Furthermore, the phosphorylation of tau by GSK3 may be required in order for tau polymers to form (Perez *et al.*, 2000; Perez *et al.*, 2002). In AD, CDK5 may be upregulated (Patrick *et al.*, 1999), while PP2A is often downregulated (Gong *et al.*, 1995; Loring *et al.*, 2001; Vogelsberg-Ragaglia *et al.*, 2001; Sontag *et al.*, 2004). Since PP2A has broad substrate specificity, the downregulation of PP2A can result in the hyperphosphorylation of several other neuronal proteins, such as β-tubulin and β-catenin, which further exacerbates neuronal damage by inhibiting the assembly of these molecules.

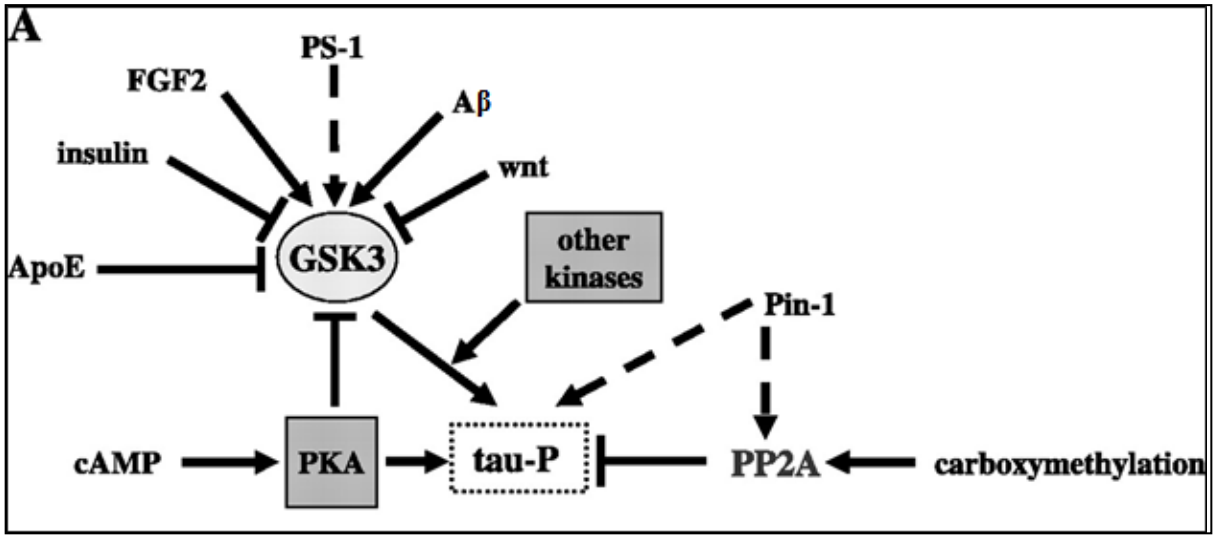


Figure 1.4: Central pathways affecting phosphorylation of tau. Several molecules can activate GSK3, which is one of the main tau kinases. Conversely, PP2A and other tau phosphatases can repress tau phosphorylation. In Alzheimer's disease, the dynamic interplay between kinases and phosphatases is disrupted, leading to hyperphosphorylation of tau.
(Adapted from Avila *et al.,* 2004).

Highly neurotoxic Aβ fibrils can induce hyperphosphorylation by activating GSK3 (Avila, 2006; DaRocha-Souto *et al.*, 2012) (Figure 1.4) however, the exact mechanism is not known. Additionally, the antagonistic action of Aβ on the insulin receptor may also promote GSK3 activation (Xie *et al.*, 2002). GSK3 can then phosphorylate serine and threonine residues on tau and inhibit its ability to bind to microtubules (Avila, 2006). Tau hyperphosphorylation is therefore dependent on the extracellular aggregation of $A\beta_{42}$ (Hardy and Selkoe, 2012).

1.2.2 Amyloid Beta

Although Aβ is a normal physiological peptide and present in healthy cells, its functions are not well understood (Hiltunen *et al.*, 2009), but it has been implicated in neuronal survival and viability (Plant *et al.*, 2003). It is produced by the sequential cleavage of the amyloid precursor protein (APP). In healthy cells, APP can be processed by two distinct pathways, namely non-amyloidogenic and amyloidogenic processing. In the non-amyloidogenic pathway, APP is sequentially cleaved by α-secretase and γ-secretase to yield sAPPα, a soluble non-pathogenic protein, and a peptide termed P3 (Querfurth and LaFerla, 2010). Importantly, the initial cleavage of APP by α-secretase occurs within the Aβ amino acid sequence, thereby preventing the generation of Aβ. However, during amyloidogenic processing, sequential cleavage of APP by β-secretase and γ-secretase yields Aβ and the APP intracellular domain (AICD) (Vassar *et al.*, 2009). In AD, misappropriation of this amyloidogenic pathway or a decline in Aβ clearance causes an increase in the concentration of Aβ peptide resulting in amyloid plaque formation and the development of AD. There is substantial evidence that Aβ is the primary pathological agent of AD (Reitz, 2012; Choi *et al.*, 2014) (Figure 1.5); however, there are still limitations to this hypothesis.

The Aβ monomers formed from amyloidogenic processing then misfold, associate and aggregate in various ways (Demuro *et al.*, 2010). The soluble $A\beta_{42}$ oligomers, in particular, accumulate in the cell as they have a higher aggregation propensity than the other forms (Jarret *et al.* 1993). This oligomer also has a higher neurotoxicity than the other forms of Aβ (Sado, 1998). Thus, plaques form due to this aggregation of Aβ and are responsible for the initial neuronal loss seen in AD (Kudo *et al.*, 2012). Additionally, these plaques are able to interfere with the inter-neuronal synaptic signalling and induce inflammation of the brain, which activates an immune response (Querfurth and LaFerla, 2010). These immune cells will further exacerbate the disease due to their inflammatory effects (Sperling *et al.*, 2011).

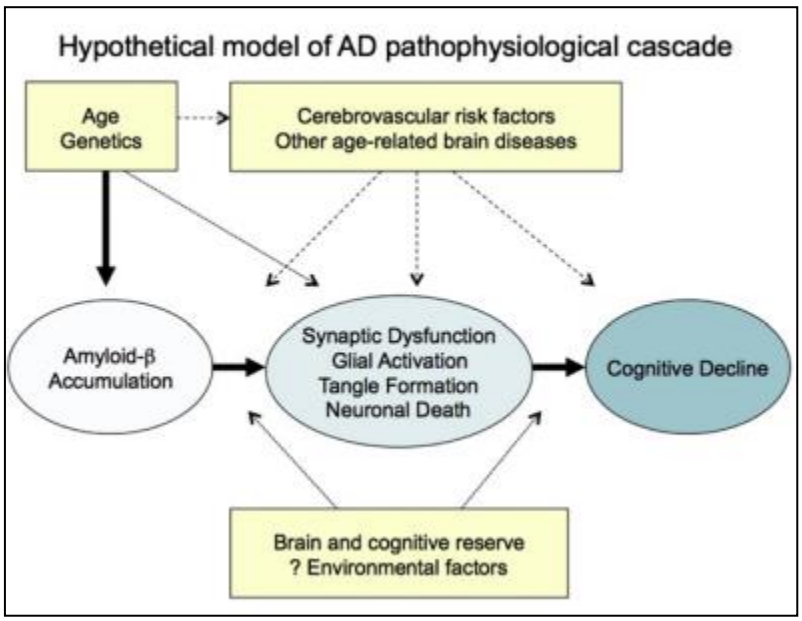


Figure 1.5: Model showing the amyloid beta cascade hypothesis of Alzheimer's disease development. Risk factors can lead to an accumulation of cytotoxic Aβ which causes the other aspects of Alzheimer's, include tangle formation and neuronal death. Overall, these effects lead to cognitive decline.
(Adopted from Sperling *et al.,* 2011).

1.2.2.1 Aβ and Cellular Receptors

In order for AD to progress, $A\beta_{42}$ needs to interact with lipid membranes and cellular receptors (Mucke and Selkoe, 2012). These $A\beta_{42}$ oligomers associate with the plasma membrane of neuronal cells resulting in distortion of the membrane and the formation of ion channels (Lin *et al.,* 2001). This causes an influx of calcium (Ca^{2+}) ions which disrupt synaptic plasticity and enhances mitochondrial permeability, leading to cytotoxicity (Lin *et al.,* 2001). Indeed, destabilization of the Ca^{2+} levels triggers free radical formation (Brustovetsky *et al.,* 2005), lipid peroxidation (Lovell *et al.,* 2001) and apoptosis (Bernardi *et al.,* 2006) which could contribute to cytotoxicity.

In order to disrupt the plasma membrane, $A\beta_{42}$ interacts with multiple receptors. These include Aβ-scavenging receptor A (SRA) and receptor for advanced glycosylation end products (RAGE) on the surface of microglial cells (Le *et al.,* 2001) (Table 1.1). Microglial cells normally have a neuroprotective function as they are responsible for neuronal immune responses and can secrete Aβ-clearance enzymes, such as neprilysin and insulin–degrading enzyme (Le *et al.,* 2001). However, in AD, the neuroprotection is diminished as a result of the pro-inflammatory cytokines associated with amyloid plaque formation (Querfurth and

LaFerla, 2010). Chronically activated microglial cells release damaging cytokines, such as interleukin-1 and tumour necrosis factor α (TNF-α) which results in increased cell death (Akiyama *et al.,* 2000). The most detrimental association of $A\beta_{42}$ is with the cellular prion protein (PrP^c). The binding of $A\beta_{42}$ to PrP^c is vital for disease development as it causes cognitive damage in the learning and memory systems of the brain (Gimbel *et al.,* 2010).

Table 1.1: Membrane associated proteins to which Aβ may bind.

Adapted from Da Costa Dias *et al.,* 2011.

Amyloid Precursor Protein (APP)	N-methyl-D-aspartate receptor (NMDA-R)
A7 Nicotininc Acetylcholine receptor (α7nAChR)	P75 Neurotrophin receptor ($P75^{NTR}$)
Receptor for advanced glycosylation end products (RAGE)	Integrins (particularly $\alpha_5\beta_1$)
Insulin receptor	Formyl peptide receptor-like-1 (FPRL1)
Scavenger receptors (classes A, B, B1)	Heparin sulphate proteoglycans (HSPGs)
Cellular prion protein (PrP^c)	Laminin receptor precursor/ high affinity laminin receptor (LRP/LR)

1.3. Cellular Prion Protein (PrP^c)

PrP^c is a glycoprotein that occurs in high concentrations in the brain (Westaway *et al.,* 2011). PrP^c attaches to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Linden *et al.,* 2008) and functions as a cell surface receptor with neuroprotective roles against apoptotic signals and oxidative stress (Biasini *et al.,* 2012). PrP^c is also proposed to function in cellular adhesion, differentiation and signalling (Resenberger *et al.,* 2011) as well as synaptogenesis (Kanaani *et al.,* 2005) and neurite growth (Santuccione *et al.,* 2005) via various signalling cascades and interactions with an array of proteins (Da Costa Dias *et al.,* 2011). Additionally, PrP^c is important in sleep memory and other cognitive processes, such as latent learning and long-term memory (Nishida *et al.,* 1997). In AD, however, the high concentrations of Aβ bind with high affinity to PrP^c (Laurén *et al.,* 2009). This may be due to the fact that both proteins are implicated in neurodegeneration.

1.3.1 PrP^c in Alzheimer's Disease

In AD, $A\beta_{42}$ oligomers, but not Aβ monomers or higher ordered fibrils, bind to PrP^c, suggesting that PrP^c mediates $A\beta_{42}$-induced synaptoxicity (Laurén *et al.*, 2009) (Figure 1.6). Indeed, Bate and Williams (2011) showed that the reduction of synaptic plasticity and increased neuronal loss in PrP^c-knockout cells is dependent on PrP^c. Additionally, there are two Aβ binding sites on PrP^c, and the site found between amino acids 95-110 is considered critically important for AD progression (Freir *et al.*, 2011). Thus, PrP^c is considered to be the primary receptor for Aβ (Figure 1.6).

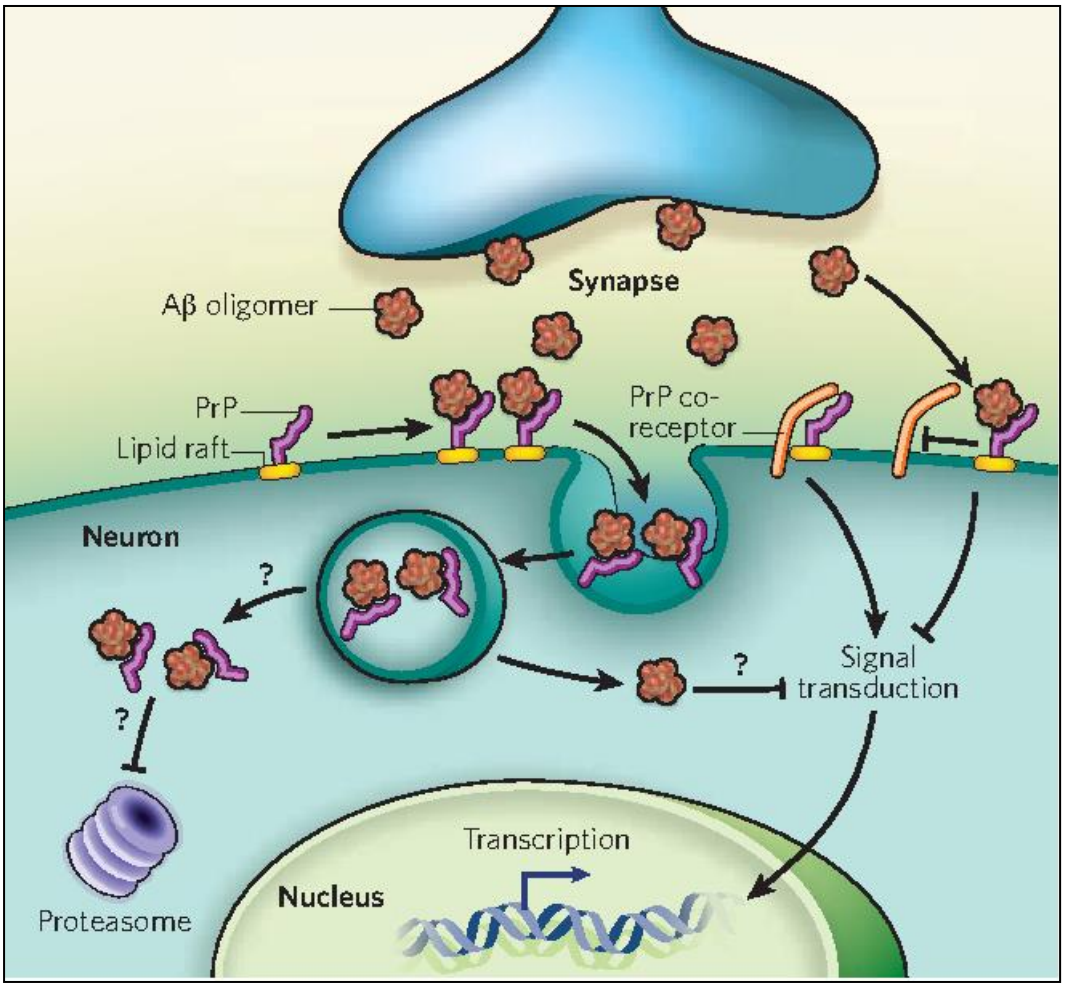


Figure 1.6: PrP^c mediates Aβ-induced synaptic toxicity. Aβ oligomers interact with the membrane bound prion protein disrupting the interaction between PrP^c and its co-receptor. This can impair the signal transaction pathways. Alternatively, internalization of the Aβ-PrP^c complex allows Aβ oligomers to reach intracellular components and interfere with cellular functions. (Adopted from Cisse and Mucke, 2009).

Under normal conditions, PrP^c has also been shown to regulate the amyloidogenic processing of APP through a negative feedback loop (Parkin *et al.*, 2007). PrP^c hinders β-secretase cleavage, thereby preventing amyloidogenic processing of APP and the production of Aβ (Da

Costa Dias *et al.*, 2011). Therefore, PrP^c is able to inhibit Aβ shedding (Da Costa Dias *et al.*, 2011). However, in AD, the high concentrations of Aβ bind to and sequester PrP^c, thus preventing PrP^c from inhibiting the β-secretase cleavage of APP (Kellett and Hooper, 2009). This results in the internalization of the Aβ-PrP^c complex, further reducing the inhibitory effects of PrP^c (Pinnock *et al.*, 2016) (Figure 1.6). In addition, this complex is vital for the occurrence of cognitive damage to the learning and memory systems during AD as Aβ oligomers can reach intracellular components and interfere directly with their cellular functions, resulting in cell death (Gimbel *et al.*, 2010) (Figure 1.6).

The role of PrP^c in tau hyperphosphorylation, however, is poorly understood. Some papers suggest that PrP^c has a protective role as it is able to downregulate tau expression (Schmitz *et al.*, 2013; Vergara *et al.*, 2015). However, since PrP^c mediates Aβ-induced deficits in AD, it is also possible that PrP^c may indirectly affect the phosphorylation of tau. Indeed, Larson *et al.* (2012) identified a signalling cascade whereby soluble Aβ binds to PrP^c, causing it to form a complex with the kinase Fyn and activate it. This PrP^c-dependent activation of Fyn leads to the missorting and hyperphosphorylation of tau (Larson *et al.*, 2012). It is possible that PrP^c can play both a protective and a detrimental role in AD as the PrP^c-mediated Fyn activation may only occur in the late stages of AD, when Aβ levels are at their highest (Larson *et al.*, 2012). Thus, in the early stages of AD, PrP^c may have a protective role by attenuating tau production (Vergara *et al.*, 2015). Since it is unknown whether PrP^c modulates tau levels in both healthy and AD-affected neurons, it is not yet possible to determine if PrP^c can switch from a protective role to a detrimental role as AD progresses.

Since PrP^c is tethered to the plasma membrane via a GPI anchor, and does not have a transmembrane domain, it relies on receptors to mediate its cytotoxic role in AD. The major cell surface receptor for PrP^c is the 37 kDa/67 kDa laminin receptor precursor/ high affinity laminin receptor (LRP/LR) (Gauczynski *et al.*, 2001). In addition, the PrP^c co-receptors, HSPGs, also bind to LRP/LR (Hundt *et al.*, 2001). Therefore, LRP/LR was hypothesized to play a role in AD.

1.4. The 37 kDa/ 67 kDa Laminin Receptor (LRP/LR)

The main function of LRP/LR is as a non-integrin receptor for laminin-1. It is found on the cell surface, in the nucleus and in the cytoplasm (Jovanovic *et al.,* 2015). In the nucleus, LRP/LR supports nuclear structures (Ford *et al.,* 1999), while in the cytoplasm it supports translation (Kinoshita *et al.,* 1998). On the cell surface, LRP/LR is a multifunctional receptor binding multiple ligands, including: laminin-1, elastin, HSPGs, PrP^c and IgG antibodies (Jovanovic *et al.,* 2015) (Figure 1.7). Additionally, LRP/LR is a receptor for multiple viruses and bacteria (Mbazima *et al.,* 2010; Jovanovic *et al.,* 2015).

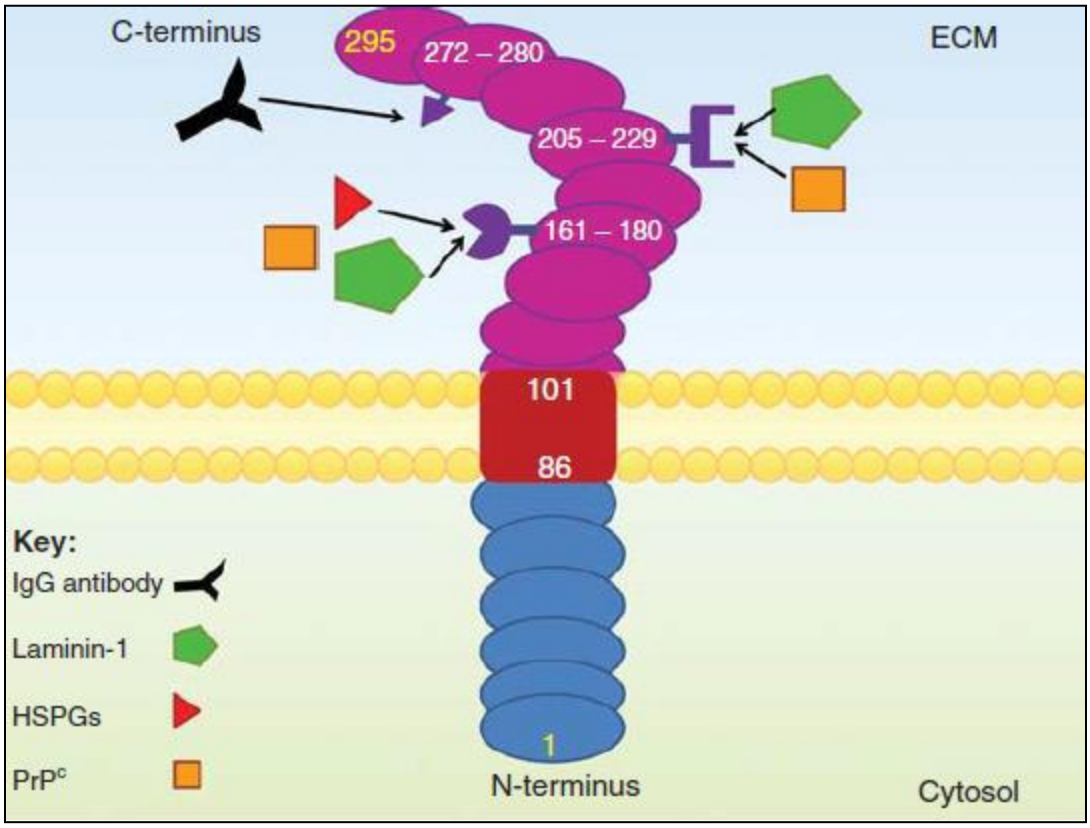


Figure 1.7: Schematic illustration of the transmembrane 37 kDa/ 67 kDa Laminin Receptor (LRP/LR) showing the binding sites of various ligands.
(Adopted from Jovanovic *et al.,* 2015).

The main ligand of LRP/LR, however, is the extracellular matrix (ECM) protein, laminin-1 (Jovanovic *et al.,* 2015). This interaction is important for cell survival, adhesion, proliferation and mobility, which are also key factors in tumorigenesis (DiGiacomo and Meruelo, 2016). As a result, LRP/LR is upregulated in multiple metastatic cancers (Zuber *et al.,* 2008; Omar *et al.,* 2012; Khumalo *et al.,* 2013; Chetty *et al.,* 2014) and contributes to the impediment of apoptosis in tumorigenic cells (Moodley and Weiss, 2013; Chetty *et al.,* 2017; Rebelo *et al.,*

2018; Vania *et al.,* 2018). However, blockade of LRP/LR with the anti-LRP/LR specific antibody, IgG1-iS18, has been shown to reduce metastasis (Omar *et al.,* 2012; Chetty *et al.,* 2013; Khumalo *et al.,* 2013; Rebelo *et al.,* 2016; Vania *et al.,* 2016; Munien *et al.,* 2017; Weiss 2017) and reduce angiogenesis (Khusal *et al.,* 2013) in a number of cell lines. Alternatively, downregulation of LRP/LR using short interfering RNAs (siRNAs) has been shown to promote apoptosis (Moodley and Weiss, 2013; Khumalo *et al.,* 2015; Chetty *et al.,* 2017; Weiss 2017; Rebelo *et al.,* 2018; Vania *et al.,* 2018) and decrease telomerase activity (Naidoo *et al.,* 2015; Weiss 2017).

1.4.1 LRP/LR in Alzheimer's Disease

Since Aβ and LRP/LR share binding partners, such as PrP^c, HSPGS and laminin-1 (Jovanovic *et al.,* 2014), possible interactions between these proteins have been examined.

LRP/LR, PrP^c and Aβ have been shown to function together in order for AD to progress. This has been shown where LRP/LR and Aβ co-localize on the cell surface and in cytoplasmic regions and directly associate with each other (Da Costa Dias *et al.,* 2013). Furthermore, LRP/LR acts as a receptor for $A\beta_{42}$ internalization into the cell (Da Costa Dias *et al.,* 2014). Additionally, LRP/LR co-localizes with APP, γ-secretase and β-secretase (Jovanovic *et al.,* 2013) and interacts directly with the presenilin 1 (PS1) subunit of γ-secretase during the amyloidogenic processing of APP (Jovanovic *et al.,* 2014). These studies therefore implicate LRP/LR in AD development and progression.

Moreover, these and other studies, have blocked or downregulated LRP/LR via the use of the LRP/LR specific antibody, IgG1-iS18, and short hairpin RNAs (shRNAs), respectively. Both of these methods were successful at impeding Aβ shedding (Jovanovic *et al.,* 2013) and rescuing cells from Aβ-induced cytotoxicity (Da Costa Dias *et al.,* 2013). In addition, it was found that the IgG1-iS18 antibody-mediated rescuing of cells from this cytotoxicity is dependent on PrP^c (Pinnock *et al.,* 2016).

In order to further elucidate the role of LRP/LR in AD, a recent *in vivo* study using B6SJL-Tg6799 AD transgenic mice was performed (Ferreira *et al.,* 2018). Mice were intranasally treated with the anti-LRP/LR specific antibody, IgG1-iS18, over a period of eight weeks. The mice showed an improvement in both short-term and learning memory, which corresponded with a decrease in amyloid plaque generation (Ferreira *et al.,* 2018). Interestingly, an increase

in mouse telomerase reverse transcriptase (mTERT) levels was also observed (Ferreira *et al.*, 2018). Previous publications have shown that LRP/LR is implicated in cancer and ageing via an interaction with telomerase and its catalytic component, human telomerase reverse transcriptase (hTERT) (Naidoo *et al.*, 2015; Otgaar *et al.*, 2017). Therefore, Ferreira *et al.*, (2018) showed that this interaction may also be implicated in AD.

AD may be considered an ageing disorder, as its incidence greatly increases after the age of 65 (Alzheimer's Disease International, 2018). In addition, Aβ and telomerase are considered antagonistic as cells affected by Aβ aggregation have critically shortened telomeres (Wang *et al.*, 2015). Furthermore, a correlation between high levels of Aβ and low levels of telomerase activity has been reported (Wang *et al.*, 2015). Recently, it was shown that overexpression of LRP::FLAG increases hTERT levels and telomerase activity, even in the presence of cytotoxic $A\beta_{42}$, which has a protective effect on cells and decreases $A\beta_{42}$ shedding (Bignoux *et al.*, 2019) Therefore, it is important to continue to investigate the effect of the LRP/LR-telomerase interaction in AD in order to better understand how the interaction occurs and to determine if it is an appropriate target for therapeutic intervention.

1.5. Telomere Biology and Telomerase

1.5.1 Telomere Biology

Telomeres are repeated TTAGGG elements found at the end of linear chromosomes (Moyzis *et al.*, 1988) that play an essential role in the maintenance of chromosome structure and function (Harley and Villeponteau, 1995). Telomeric repeats are required as a protective measure against the "end replication problem". Briefly, DNA polymerases require 3' primers on the lagging strand in order to initiate DNA synthesis (Harley *et al.*, 1990). When these 3' primers are excised by the 5' to 3' exonuclease activity of DNA polymerase, they leave a gap of single stranded DNA (ssDNA) that must be joined together by ligases (Harley *et al.*, 1990). However, this DNA is unstable and often degraded by nucleases. As a result, the DNA polymerases cannot fully replicate linear chromosomes and the chromosome ends are shortened by each cell division (Harley *et al.*, 1990). Thus, the telomeres are able to protect the ends of chromosomes from degradation and illegitimate processing, thereby preventing the loss of functional genetic material (Harley and Villeponteau, 1995). However, there is

still a limit to the number of times a normal cell population can divide, known as the Hayflick limit (Hayflick and Moorhead, 1961). Once this limit has been reached, cells enter senescence, a metabolically active but non-dividing phase of the cell cycle (Hayflick and Moorhead, 1961).

In order to prevent the loss of functional genetic material, telomeric DNA and telomere-associated proteins interact with each other to form a telomere loop (t-loop) (Figure 1.8). The telomere is thus not recognized as a DNA break and resists degradation (Griffith *et al.*, 1999). These associated proteins, such as telomeric repeat factor 1 and 2 (TRF1 and TRF2), and protection of telomeres protein 1 (POT1), among others, also interact with each other to form the "shelterin" complex, which is able to prevent chromosome end fusions, maintain telomere structure and length, and regulate the binding of telomerase on telomeres (van Steensel *et al.*, 1998; Epel *et al.*, 2004; Mattern *et al.*, 2004).

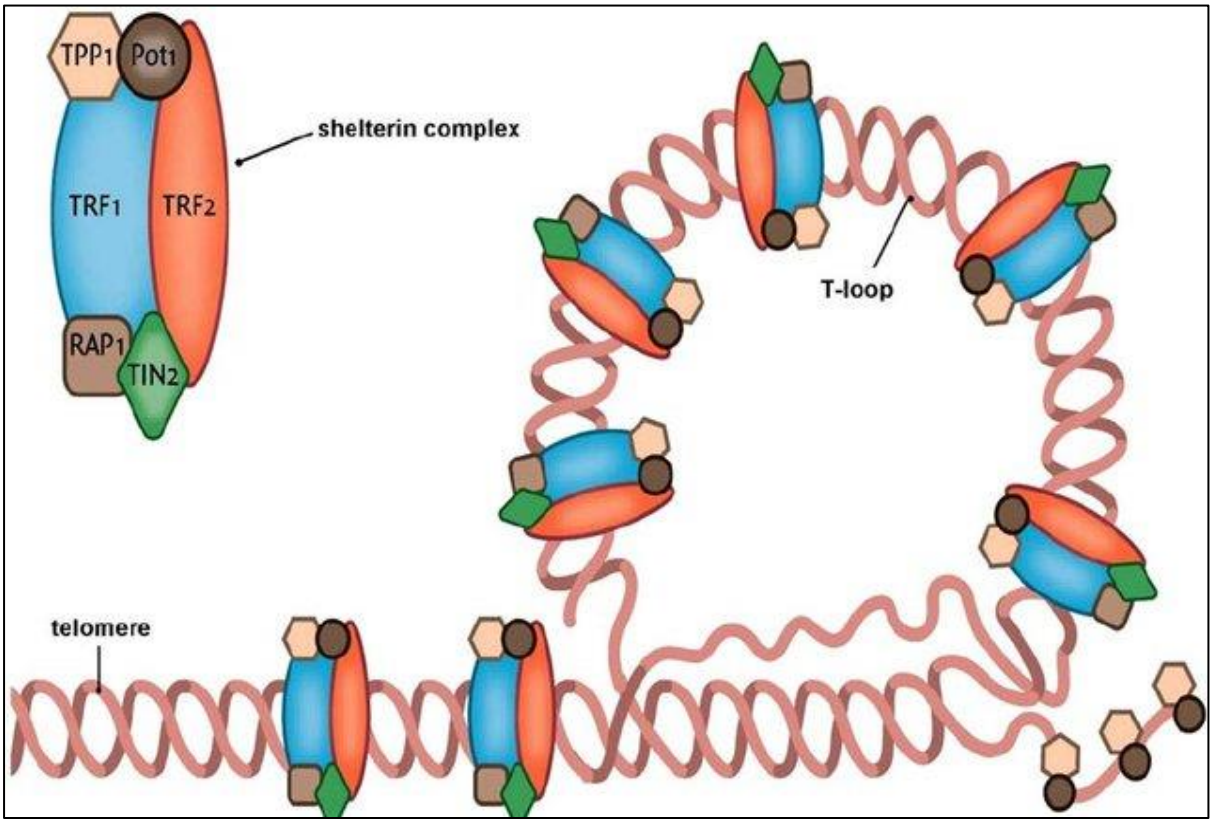


Figure 1.8: Schematic depiction of the t-loop and shelterin complex of proteins that cooperate to protect the ends of telomeres.
(Adopted from Huzen *et al.,* 2010).

1.5.2 Telomerase

Telomerase is a multi-subunit ribonucleoprotein which functions to add TTAGGG telomeric repeats to the ends of chromosomes (Greider and Blackburn, 1989). This allows telomere length to be partially maintained after each cell division, thereby maintaining chromosome function (Greider and Blackburn, 1989). Additionally, by increasing total telomere length, telomerase is able to increase the replicative potential of a cell (Bodnar *et al.,* 1998). There are two essential components to the human telomerase enzyme, namely hTERC and hTERT. The reverse transcriptase component, hTERT, uses the RNA component, hTERC, as a template for telomere extension (Greider and Blackburn, 1996) (Figure 1.9). As hTERT is generally expressed in low levels in the nucleus, it is considered to be the rate-limiting factor and regulator of telomerase. Indeed, certain cells can often express hTERC but have no detectable telomerase activity (Greider and Blackburn, 1996; Nakayama *et al.,* 1998). Furthermore, different cells display differential levels of telomerase activity. Most somatic cells display little or no telomerase activity while germline cells, embryonic stem cells and cancerous cells show high levels of telomerase activity (Saretzki, 2009).

Telomerase has additional functions that are independent of telomere maintenance. Indeed, significant levels of hTERT have been detected with no corresponding increase in telomere length (Liu and Yung, 1998; Wick *et al.,* 1999). hTERT is known to function as a transcription factor in several pathways, such as the Wnt-β-catenin pathway and NF-κB pathway (Li and Tergaonkar, 2014). These pathways are both involved in cellular proliferation and stem cell maintenance (Li and Tergaonkar, 2014). Additionally, telomerase has a protective effect against reactive oxygen species (ROS). This is accomplished when cells are under oxidative stress and telomerase migrates to the mitochondria, allowing hTERT to interact with mitochondrial DNA (mtDNA) to convey protection against ROS (Saretzki, 2009). Once oxidative stress has decreased to a manageable level, telomerase returns to the nucleus (Saretzki, 2009). Furthermore, hTERT plays a role in the replication and repair of mtDNA, thereby further protecting cells from mtDNA damage (Sharma *et al.*, 2012).

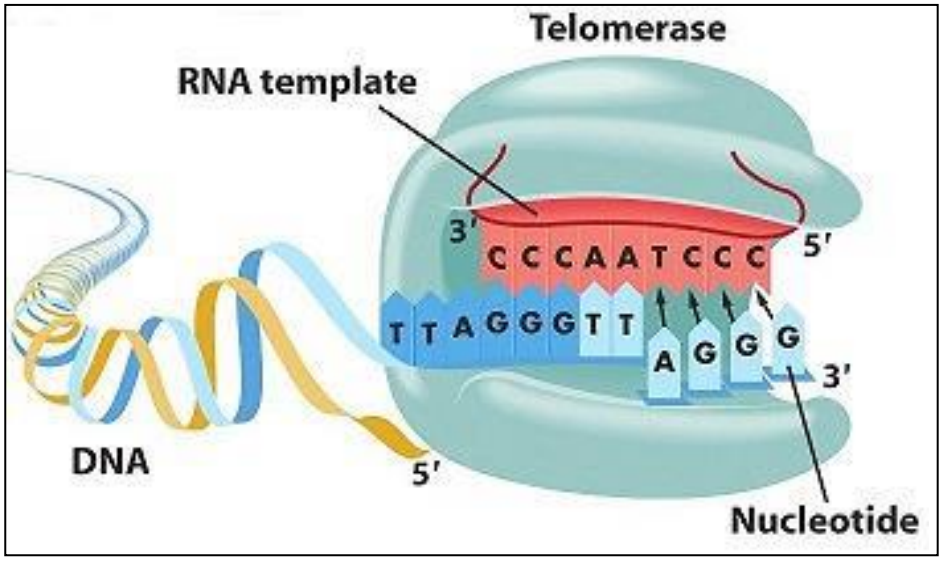


Figure 1.9: Schematic depiction of telomerase ribonucleoprotein. The RNA component, hTERC, is used by hTERT as a template to extend telomeres.
(Adopted from https://bioinfoworld.files.wordpress.com/2013/03/tert1.jpg [Accessed 18/01/2018]).

Telomerase plays antagonistic effects in cancer and ageing. Cancerous cells have high telomerase activity, while ageing cells have lower activity (Harley and Villeponteau, 1995). Highly active telomerase can trigger proliferation beyond the Hayflick limit and, in conjunction with carcinogenic mutations, aids in the promotion of tumorigenesis. In fact, elevation of hTERT at any stage of the cell cycle leads to immortalization (Bodnar *et al.*, 1998). As a result of this extended proliferation, mutations can accumulate leading to the initiation of cancer (Harley and Villeponteau, 1995). Indeed, most cancers show higher levels of telomerase activity and this can, in some instances, confer resistance to chemotherapy (Kuranaga *et al.*, 2001; Shoup *et al.*, 2004).

Conversely, 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 (Shay and Wright, 2007). This process is supposed to act as a defence mechanism against cancer formation (Greenberg *et al.*, 1999; González-Suárez *et al.*, 2000; Feldser and Greider, 2007); however it can lead to the development of age-related disorders. Indeed, ageing disorders, such as rheumatoid arthritis and AD, display short telomere profiles and limited telomerase activity, similar to that of cellular ageing (Panossian *et al.*, 2003; Schönland *et al.*, 2003).

1.5.3 Telomeres, Telomerase and Alzheimer's Disease

There is still limited information on the importance of telomeres in the brain. Neuronal cells of the central nervous system (CNS) are unable to divide (Allsopp *et al.,* 1995), however neurogenesis can occur in certain regions of the brain, such as the lateral ventricles and the gyrus (Jin *et al.,* 2005). In fact, increased hippocampal neurogenesis has been observed in AD patients and may be an attempt to replace neuronal loss (Jin *et al.,* 2005). However, decreased neurogenesis caused by telomere erosion, amongst others, can negate this protective effect. Therefore, a decrease in neurogenesis due to telomere shortening may be a pre-disposing factor for AD (Ming and Song, 2005).

Comparatively, the actively dividing microglial cells of the brain can be affected by telomere erosion (Weng, 2012). Telomeric shortening can affect the proliferation of microglia resulting in dysregulated inflammatory signalling within the brain. This may enhance oxidative stress-dependent cell senescence and lead to the progression of neurodegenerative disease, such as AD (Weng, 2012). Information regarding the role of telomerase in AD is still limited. It is known that the accumulation of Aβ inhibits telomerase activity since cells affected by an aggregation of Aβ have critically shortened telomeres (Panossian *et al.,* 2003). Additionally, hTERT is able to persist in neurons where it may play a neuroprotective role against hyperphosphorylated tau and the resultant neurofibrillary tangles (Spilsbury *et al.,* 2015). Indeed, the co-localization of hTERT with hippocampal mitochondria may inhibit tau hyperphosphorylation (Spilsbury *et al.,* 2015). Conversely, the absence of hTERT contributes to neurodegeneration as it can no longer protect mitochondria from oxidative stress. 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 Aβ and tau-induced damage (Spilsbury *et al.,* 2015; Wang *et al.,* 2015).

1.6. Justification of Study

The relationship between LRP/LR and telomerase in AD is not fully understood. It is known that LRP/LR and telomerase have similar functions in preventing apoptosis and promoting cell viability, therefore a relationship between these proteins was investigated. Naidoo *et al.* (2015) showed that siRNA downregulation of LRP/LR corresponded with a decrease in hTERT levels and telomerase activity. Moreover, Otgaar *et al.* (2017) showed that an

overexpression of LRP::FLAG increased both hTERT levels and telomerase activity with a concomitant reduction in senescent markers. Furthermore, telomerase and hTERT are known to have a role in AD (Panossian *et al.,* 2003; Spilsbury *et al.,* 2015; Wang *et al.,* 2015). Indeed, AD transgenic mice treated with the LRP/LR specific antibody, IgG1-iS18, not only displayed decreased amyloid plaque generation but also increased mTERT levels (Ferreira *et al.,* 2018). Therefore, the possible effect of increased hTERT levels on Aβ was investigated, as hTERT was predicted to have a neuroprotective role in AD. Bignoux *et al.* (2019) observed a significant decrease in $A\beta_{42}$ shedding following overexpression of LRP::FLAG and the subsequent increase in hTERT levels, in the presence of cytotoxic levels of $A\beta_{42}$. Thus, hTERT was shown to have a neuroprotective role against Aβ. Indeed, since tau hyperphosphorylation is dependent on Aβ aggregation (Hardy and Selkoe, 2012), hTERT is predicted to have a protective role in tau pathology (Spilsbury *et al.,* 2015). Thus, since LRP/LR and telomerase play a role in AD, investigation of the possible interactions between LPR/LR, telomerase and tau will improve our understanding of AD. Therefore, this project will elucidate if there is an interaction, whether direct or indirect, between LRP/LR and tau and will investigate possible therapeutic strategies for the treatment of AD and other tau pathologies.

2. Aims and Objectives

2.1. Main Aim

To determine the effect of LRP/LR and telomerase on tauopathy *in vitro*

2.2. Objectives

- To investigate whether there is co-localization between endogenous LRP/LR, hTERT and/or tau in HEK-293 cells using confocal microscopy
- To investigate whether there is an interaction between endogenous LRP/LR and tau in HEK-293 cells using Försters Resonance Energy Transfer (FRET)
- To achieve a stable LRP::FLAG overexpression in HEK-293 and SH-SY5Y cells
- To analyze the effect of LRP::FLAG overexpression on the protein levels of LRP/LR, hTERT and tau in HEK-293 and SH-SY5Y cells using western blotting and confocal microscopy
- To analyze the effect of LRP::FLAG overexpression on the protein levels of tauopathy-related proteins in HEK-293 and SH-SY5Y cells using western blotting and confocal microscopy

3. Experimental Procedures

3.1. Cell Culture

Cell culture is an *in vitro* model that attempts to mimic the *in vivo* conditions in which cells grow, allowing for the investigation into the biochemical and biological processes of the cells. For this study, the following cell lines were used:

SH-SH5Y: human neuroblastoma cells from Sigma Aldrich

HEK-293: human embryonic kidney cells from ATCC

(Human Research Ethics Committee: W-CJ-140804-1)

SH-SY5Y cells are neuronal cells that are commonly used to investigate neurodegenerative diseases (Agholme *et al.*, 2010; Carolindah *et al.*, 2013; Zheng *et al.*, 2006) while HEK-293 cells were used as a control since they express neuronal markers and have detectable levels of telomerase activity (Shaw *et al.*, 2002).

The cells were cultured in Dulbecco's Modified Eagle Medium (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% foetal bovine serum (FBS) (Hyclone) and 2% penicillin/streptomycin (Biowest). The cells were incubated in a humidified incubator at 37 °C with 5% CO_2 to permit optimal proliferation.

In order to maintain the cell line, the cells were passaged when they reached a confluency of 70-80%. First, the cells were washed with phosphate buffered saline (PBS) (Table A8) and then incubated with trypsin at 37 °C for 5 minutes in order to detach the cells from the cell culture flask. Thereafter, fresh media was added for total volume of 10 ml, in order to inactivate the trypsin. Cells were then split in a 1:2 ratio (SH-SH5Y) or 1:10 ratio (HEK-293) and, again, fresh media was added to a total volume of 10 ml.

In addition, cell stocks were prepared in order to cryopreserve cells. After trypsinization, cells were centrifuged at 2700 x g for 10 minutes. Cell pellets were then resuspended in a mixture of 80% FBS and 20% dimethyl sulfoxide (DMSO) (Merck Millipore) and stored at -70 °C.

Cell harvesting was performed on cells with 70-80% confluency in order to collect samples for downstream applications. After trypsinization cells were centrifuged at 2700 x g for 10 minutes. Thereafter, the pellets were stored at -20 °C until required.

3.2. Confocal Microscopy with the addition of Airyscan™

Confocal microscopy is a qualitative technique that allows for the localization of fluorescently labelled proteins to be determined (Miller and Shakes, 1995). This technique was performed to determine whether LRP/LR and tau co-localize and to confirm the co-localization between LRP/LR and hTERT. Additionally, this technique was performed to observe the levels of the tauopathy-related protein, $A\beta_{42}$.

Cells were grown to a confluency of 70-80% and 500 µl was seeded into a total volume of 3 ml onto microscope coverslips within a 6-well plate. Thereafter, cells were incubated for 24 hours in a humidified incubator in order to reach 50-70% confluency. All further steps were performed with gentle agitation. Cells were fixed with 4% formaldehyde (VWF) in PBS for 20 minutes. In order to view the intracellular structure of cells, they were permeabilized with 0.25% Triton X-100 (Sigma Aldrich) in PBS for 20 minutes. Cells were then blocked in 0.5% bovine serum albumin (BSA) (Amresco) in PBS for 20 minutes, and thereafter primary antibody (1:200 in 0.5 % BSA in PBS) was added. The cells were incubated overnight at 4 °C. The following day, the coverslips were washed with PBS for 5 minutes and then incubated with the appropriate fluorescein isothiocyanate (FITC)-coupled or the CF™ 647-coupled secondary antibody (1:500 in 0.5 % BSA in PBS) and incubated for an hour in the dark. The primary and secondary antibodies used are listed in Table 3.1. The cells were then counterstained with 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich) for 5 minutes in the dark in order to stain the nuclei. Coverslips were then washed and mounted onto clean microscope slides using Fluoromount™ Aqueous Mounting Medium (Sigma Aldrich). The slides were incubated at room temperature for 1.5 hours to allow the mounting medium to set. All images were acquired at room temperature using the Zeiss LSM 780 confocal microscope at 630x magnification. In addition, Airyscan™ was used to improve the resolution. Airyscan™ is an addition to the Zeiss LSM 780 confocal microscope that increases the resolution from 200nm to 140nm.

Table 3.1: Antibodies used for confocal microscopy

Target Protein	**Primary Antibody**	**Secondary Antibody**
LRP/LR	Human anti-LRP/LR (Monoclonal) (IgG1-iS18)	Anti-human IgG-FITC (Sigma Aldrich: F9512)
Total Tau (Tau-5)	Mouse anti-Tau (Tau-5) (Monoclonal) (EMD: MAB361)	Anti-mouse IgG-CF™ 647 (Sigma Aldrich: SAB4600182)
Phospho-Tau (pS404)	Rabbit anti-p-Tau (S404) (Monoclonal) (Cell Signalling: 20194S)	Anti-rabbit IgG-CF™ 647 (Sigma Aldrich: SAB4600184)
PHF-Tau (pT231)	Mouse anti-Phospho-Tau (Thr231) (AT180) (Monoclonal) (Invitrogen: MN1040)	Anti-mouse IgG-CF™ 647 (Sigma Aldrich: SAB4600182)
hTERT	Mouse anti-hTERT (Polyclonal) (Santa Cruz Biotechnology: sc-77511)	Anti-mouse IgG-CF™ 647 (Sigma Aldrich: SAB4600182)
$A\beta_{42}$	Rabbit anti-beta-Amyloid (1-42) (Polyclonal) (Abcam: ab39377)	Anti-rabbit IgG- FITC (Sigma Aldrich: F0382

3.3. 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 technique, a non-radiative transfer of energy will only occur if the fluorochromes are within 1-10 nm of each other (Förster, 1948). This distance-dependent transfer of energy occurs between a donor and acceptor molecule (Förster, 1948). In this experiment the donor/ acceptor pair phycoerythrin (PE)/ allophycocyanin (APC) was used to immunolabel the proteins of interest. PE is excited by the 488 nm argon laser and emits at 575 nm. Thus, it may be detected using the FL2 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, and can be detected using the FL4 filter set. The presence of FRET between the proteins of interest was evaluated using the FL3 filter set. Within this 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. Thus, FL3 is considered the optimal channel for PE/ APC FRET detection.

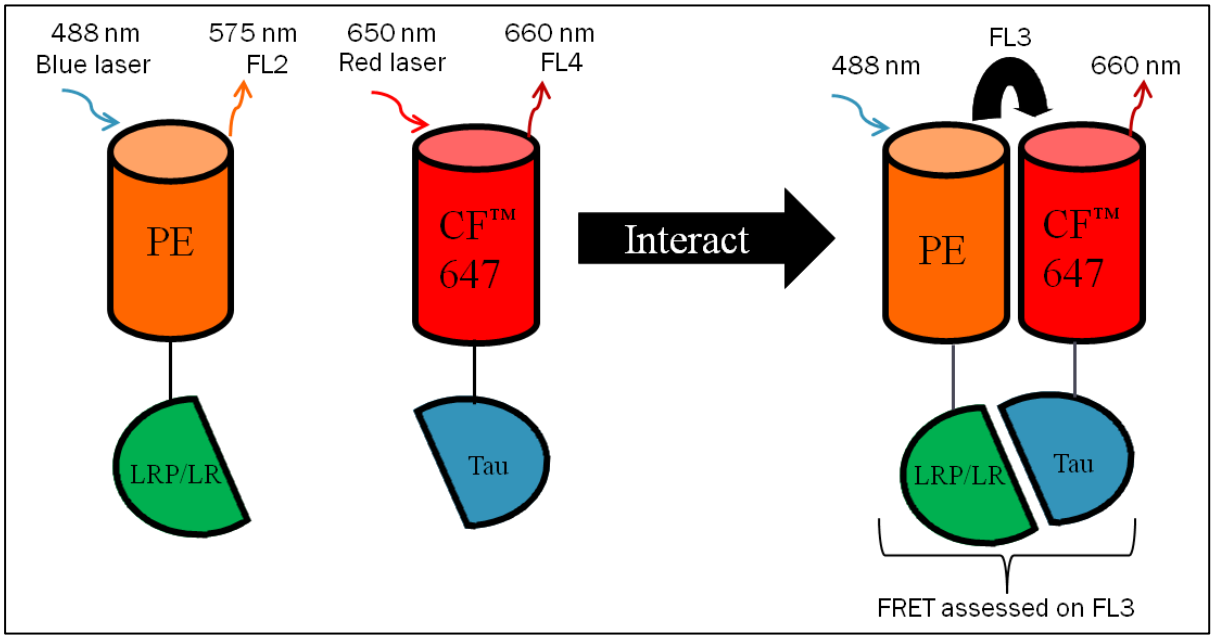


Figure 3.1: A schematic representation of the experimental design employed for FRET analysis of the potential LRP/LR-Tau interaction.
Adapted from Da Costa Dias *et al.* (2014) (Suppl).

The experimental design was based on the FRET analysis performed by Da Costa Dias *et al.* (2014). HEK-293 cells were incubated in serum-free media for 3 hours prior to assessment. Cells were then detached and harvested by centrifugation at 150 x g for 10 minutes. The cells were washed twice with PBS. All wash steps were carried out at 2700 x g for 5 minutes. Cells were then incubated in 4% formaldehyde at 4 °C for 20 minutes in order to fix them. Thereafter, cells were centrifuged at 2700 x g for 10 minutes to remove the formaldehyde. Since tau is an intracellular protein, the cells were permeabilized by incubating them in 0.1 % Triton X-100 in PBS at 4 °C for 20 minutes. Again, the cells were centrifuged at 2700 x g for 10 minutes in order to remove the Triton X-100. Cells were washed once with PBS and then blocked in 0.5 % BSA in PBS for 10 minutes at room temperature. Thereafter, primary antibodies (1:100 in 0.05 % BSA in PBS) were added and the cells were incubated for 2 hours at room temperature. Cells were then washed once with PBS and once with 0.5 % BSA in PBS. Thereafter, secondary antibodies (1:100 in 0.05 % BSA in PBS) were added and the cells were again incubated for 2 hours at room temperature. The primary and secondary antibodies used are listed in Table 3.2. The cells were then washed twice with PBS and resuspended in PBS for detection. Three biological repeats were performed and 15 000 cells were analyzed per sample. The efficacy of the assay was investigated by using PrP^{c} as a positive control as LRP/LR binds to it with high affinity (Gauczynski *et al.*, 2001) and chloramphenical acetyl transferase (CAT) as a negative control as LRP/LR has been shown not to bind to it (Da Costa Dias *et al.*, 2013).

The following samples were prepared:

1. Unstained cells

2. Cells labelled with anti-human PE only

3. Cells labelled with anti-mouse CF™ 647 only

4. Cells labelled with anti-rabbit CF™ 647 only

5. Cells labelled with IgG1-iS18-PE (LRP/LR detection)

6. Cells labelled with anti-PrP^C-CF™ 647 (PrP^C detection)

7. Cells labelled with anti-CAT-CF™ 647 (CAT detection)

8. Cells labelled with anti-Tau-CF™ 647 (Tau detection)

9. Cells labelled with both IgG1-iS18-PE and anti-PrP^C-CF™ 647 (positive control)

10. Cells labelled with both IgG1-iS18-PE and anti-CAT-CF™ 647 (negative control)

11. Cells labelled with both IgG1-iS18-PE and anti-Tau-CF™ 647

Table 3.2: Antibodies used for Försters Resonance Energy Transfer (FRET)

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

3.4. 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 (Vana and Weiss, 2006). Cells were cultured until 40-50% confluency was achieved. The cells were then transfected using Xfect™ Transfection Reagents (Takara). Briefly, 5 µg of plasmid DNA was mixed with 100 µl Xfect Reaction Buffer and 1.5 µl Xfect Polymer and incubated at room temperature for 10 minutes. This allowed the lipophilic nanoparticle complexes to form around the construct. The mixture was then added to the cells prior to a three-day incubation. Thereafter, a media change was performed and the cells were permitted to proliferate for two days, giving the cells sufficient time to recover from the transfection process and produce the LRP::FLAG protein. The antibiotic Geneticin was then used as a selective treatment. Initially, a high concentration of the antibiotic (6000-8000 ng/µl) was administered until all the non-transfected cells had died. Thereafter, cells were cultured in 2000-4000 ng/µl of Geneticin to ensure the maintenance of LRP::FLAG expression. Stable transfection was achieved after six weeks of Geneticin treatment.

3.5. Western Blotting

3.5.1 Bicinchoninic Acid (BCA) Assay

BCA assays were performed in order to quantify the total protein concentration present in a cell lysate post protein extraction. This ensured that equal protein concentrations were used for the subsequent sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting procedures. The peptide bonds present in protein cause the Cu^{2+} ions in BCA to reduce to Cu^{+} ions, which results in a colorimetric change that can be measured using an ELISA plate reader.

Cells were harvested as described in 3.1. Thereafter, they were incubated in cold radioimmunoprecipitation (RIPA) buffer (Table A1) for 10 minutes in order to lyse the cells. The cell lysates were diluted 1:5 with distilled water and 25 µl was loaded into individual wells on a 96-well plate in triplicate. The samples were incubated in 200 µl of 98% BCA and 2% copper (II) sulphate ($CuSO_4$) at 37 °C for 20 minutes. BSA of known concentrations was used to construct a standard curve. BSA standards were prepared by dilution to produce

dilutions ranging from 0 mg/ml to 1 mg/ml, in 0.2 mg/ml increments. Thereafter, 25 µl were loaded in triplicate on the same plate and were incubated in the same mixture. Thereafter, the plate was measured at a wavelength of 562 nm using the Multiskan® GO ELISA plate reader (Thermo Scientific). Total protein concentrations were then extrapolated from the BSA standard curve.

3.5.2 SDS-PAGE and Western Blotting

SDS-PAGE was used in order to separate the whole protein extract by size, allowing the approximate size of the target protein to be assessed (Towbin *et al.*, 1979). Thereafter, the proteins were transferred to a membrane via western blotting. Western blotting uses specific antibodies to immunologically detect and quantify the protein levels of the target protein (Towbin *et al.*, 1979).

The SDS-PAGE procedure was performed using 12% (w/v) Tris-HCl gels (Table A2), allowing the resolving of proteins of 10-200 kDa. All samples were assayed in triplicate. Loading Buffer (40 mM Dithiothreitol) was added to the soluble protein samples and they were heated at 95 °C for 5 minutes prior to loading. A total of 30 µg (LRP::FLAG) and 60 µg (Tau, phospho-Tau (pS404), phospho-Tau (pT231), hTERT, phospho-TERT (pS824) and PrP^{c}) was then separated on the gel. A prestained molecular weight marker was loaded onto each gel.

The proteins were separated at 150 V for approximately 1 hour in 1X electrophoresis buffer (25mM Tris, 192 mM glycine and 0.1% SDS – Table A6). Thereafter, the separated proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer apparatus. Transfer was performed at 300 mV for 45-50 minutes using 1X transfer buffer (20% methanol in 25mM Tris and 192 mM glycine – Table A7). The proteins were fixed to the membrane in 0.4% formaldehyde for 30 minutes. The membrane was then blocked in 3% BSA in 0.1% PBS-Tween for an hour in order to prevent non-specific antibody binding. The membranes were incubated overnight at 4 °C with primary antibody as per Table 3.3. The next day, five 0.1% PBS-Tween washes were performed for 5 minutes each to remove any unbound antibody. The membranes were then incubated for an hour at room temperature with the appropriate horse radish peroxidise (HRP)-coupled secondary antibody as per Table 3.3. Again, the membrane was washed with 0.1% PBS-Tween as above. The membranes were then incubated with Thermo Scientific chemiluminescent substrate, which reacts with the

HRP enzyme to form a precipitate that can be detected using radiation exposure. The membranes were then imaged using the Bio-Rad GelDoc XR Imager. In SH-SY5Y cells, LRP::FLAG was detected using mouse anti-FLAG® conjugated to the fluorescent probe, Cy3 (Sigma Aldrich: A-9594) (Table 3.3) as the anti-mouse HRP antibody (Abcam: ab97023) bound non-specifically. Thus, this blot was not treated with chemiluminescent substrate and was instead viewed on the GelDoc using Cy3 fluorescence. The blots were then analyzed using the Bio-Rad Image Lab 4.0 software and densitometry was performed in order to obtain protein levels relative to the loading control, β-Actin.

Table 3.3: Antibodies used for western blotting

Target Protein	Primary Antibody	Dilution	Secondary Antibody	Dilution
β-Actin	Mouse anti-β-Actin Peroxidase (Monoclonal) (Sigma Aldrich: A3854)	1:10000	-	-
LRP::FLAG	Mouse anti-FLAG® M2 (Monoclonal) (Sigma Aldrich: F-3165)	1:6666	Anti-mouse IgG-HRP (Abcam: ab97023)	1:10000
LRP::FLAG	Mouse anti-FLAG® M2-Cy3™ (Monoclonal) (Sigma Aldrich: A-9594)	1:5000	-	-
Total Tau (Tau-5)	Mouse anti-Tau (Tau-5) (Monoclonal) (EMD: MAB361)	1:500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333
Phospho-Tau (pS404)	Rabbit anti-p-Tau (S404) (Monoclonal) (Cell Signalling: 20194S)	1:1000	Anti-rabbit IgG-HRP (Cell Signalling: 7074S)	1:3333
PHF-Tau (pT231)	Mouse anti-Phospho-Tau (Monoclonal) (Thr231) (AT180) (Invitrogen: MN1040)	1:500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333
hTERT	Mouse anti-hTERT (Polyclonal) (Santa Cruz Biotechnology: sc-77511)	1:2500	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333

Phospho-hTERT (pS824)	Rabbit anti-phospho-Telomerase ($pSer^{824}$) (Polyclonal) (Sigma Aldrich: SAB4504295)	1:1000	Anti-rabbit IgG-HRP (Cell Signalling: 7074S)	1:3333
Prion Protein (PrP^{c})	Mouse anti-Prion Protein (Monoclonal) (Sigma Aldrich: P0110)	1:5000	Anti-mouse IgG-HRP (Abcam: ab97023)	1:3333

3.6. Statistical Analysis

All experiments were performed in triplicate, allowing standard deviation to be calculated and statistical analysis to be performed. All statistical analysis of the data obtained was carried out using Microsoft Excel 2016 (Microsoft Corporation). Analysis of results was performed in order to determine whether the results collected were significantly relevant. The two-tailed Student's *t*-test (equal variance) 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 and highly significant, respectively.

4. Results

4.1. LRP/LR co-localizes with tau and hTERT

In order to determine whether LRP/LR interacts with tau, it was important to investigate the sub-cellular localization of these proteins. Since hTERT interacts with LRP/LR and plays a role in tauopathy, its localization was also examined.

Confocal microscopy was employed to confirm the intracellular co-localization of LRP/LR and hTERT (Naidoo *et al.*, 2015). Figure 4.1 shows that there is indeed co-localization between LPR/LR and hTERT in the nucleus and perinuclear compartment of HEK-293 cells. Further, confocal microscopy was performed to determine whether LRP/LR and tau co-localize (Figure 4.1). The merged panels (Figure 4.1: D, J) represent a merge of the red tau or hTERT signal with the green LRP/LR signal, with areas of yellow representing overlap. The co-localization panels (Figure 4.1: E, K) clearly label the areas of overlap with white dots, making co-localization easier to visualize. The 2D cytofluorogram (Figure 4.1: F, L) is a diagrammatic representation of the laser signals whereby a diagonal in the third quadrant represents a strong co-localization of the proteins. Therefore, as seen in figure 4.1, LRP/LR and tau co-localize in the perinuclear compartment of HEK-293 cells.

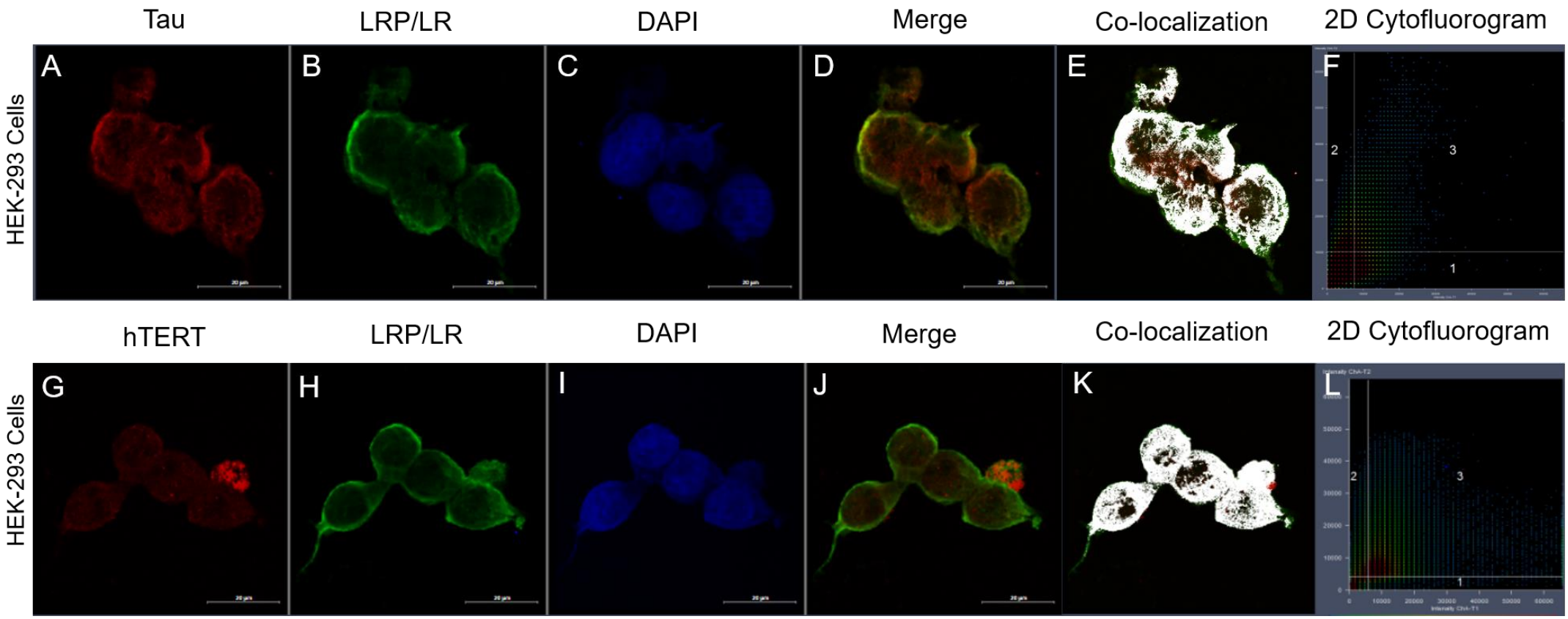


Figure 4.1: LRP/LR, tau and hTERT localization and co-localization within HEK-293 cells. This figure illustrates the co-localization between LRP/LR (green) and tau (red) as well as LRP/LR (green) and hTERT (red). A) Endogenous tau levels in HEK-293 cells. Tau localizes to the cytosol. B, H) Endogenous LRP/LR levels in HEK-293 cells. LRP/LR localizes to the cytosol, nucleus and cell surface. G) Endogenous hTERT levels in HEK-293 cells. hTERT is localized to the cytosol and nucleus. C, I) Nuclei are stained with DAPI (blue). 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). Co-localization occurs between LRP/LR and hTERT in the nucleus and perinuclear compartment, represented by yellow fluorescence in the merged image (J), as white dots (K) and as fluorescence in the third quadrant of the 2D cytofluorogram (L). All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.

4.2. LRP/LR and tau interact directly

Since LRP/LR co-localized with tau, it was investigated whether the proteins interact directly with each other by detecting if FRET occurred between the two immunolabelled proteins.

Flow cytometry was employed in order to examine FRET in HEK-293 cells. The shift observed in CF™ 647 fluorescence intensity when cells were co-labelled with LRP/LR-PE and Tau-CF™ 647 (Figure 4.2: F) suggests that FRET has occurred between these two proteins. In this experiment, PrP^c was used as a positive control as LRP/LR binds to it with high affinity (Gauczynski *et al.,* 2001) and CAT was used as a negative control as LRP/LR has been shown not to bind to it (Da Costa Dias *et al.,* 2013). The CF™ 647 fluorochrome is not excited by the 488 nm laser and did not exhibit a fluorescence emission in the FL3 channel as the fluorescence intensity of the proteins labelled with the antibody (PrP^c, CAT and Tau) was able to be superimposed on that of the unstained cells (Figure 4.2: A,C,E). Upon co-labelling of cells with LRP/LR-PE and PrP^c-CF™ 647 (Figure 4.2: B), there was shift in the CF™ 647 histogram along the FL3 fluorescence intensity axis, which is indicative of the interaction between these proteins. Conversely, upon co-labelling with LRP/LR-PE and CAT-CF™ 647 (Figure 4.2: D), there was no shift in the CF™ 647 histogram, thus no effect on the emission of CF™ 647 was observed. These observations agree with literature (Gauczynski *et al.,* 2001; Da Costa Dias *et al.,* 2013) and therefore prove that the FRET analysis was reliable.

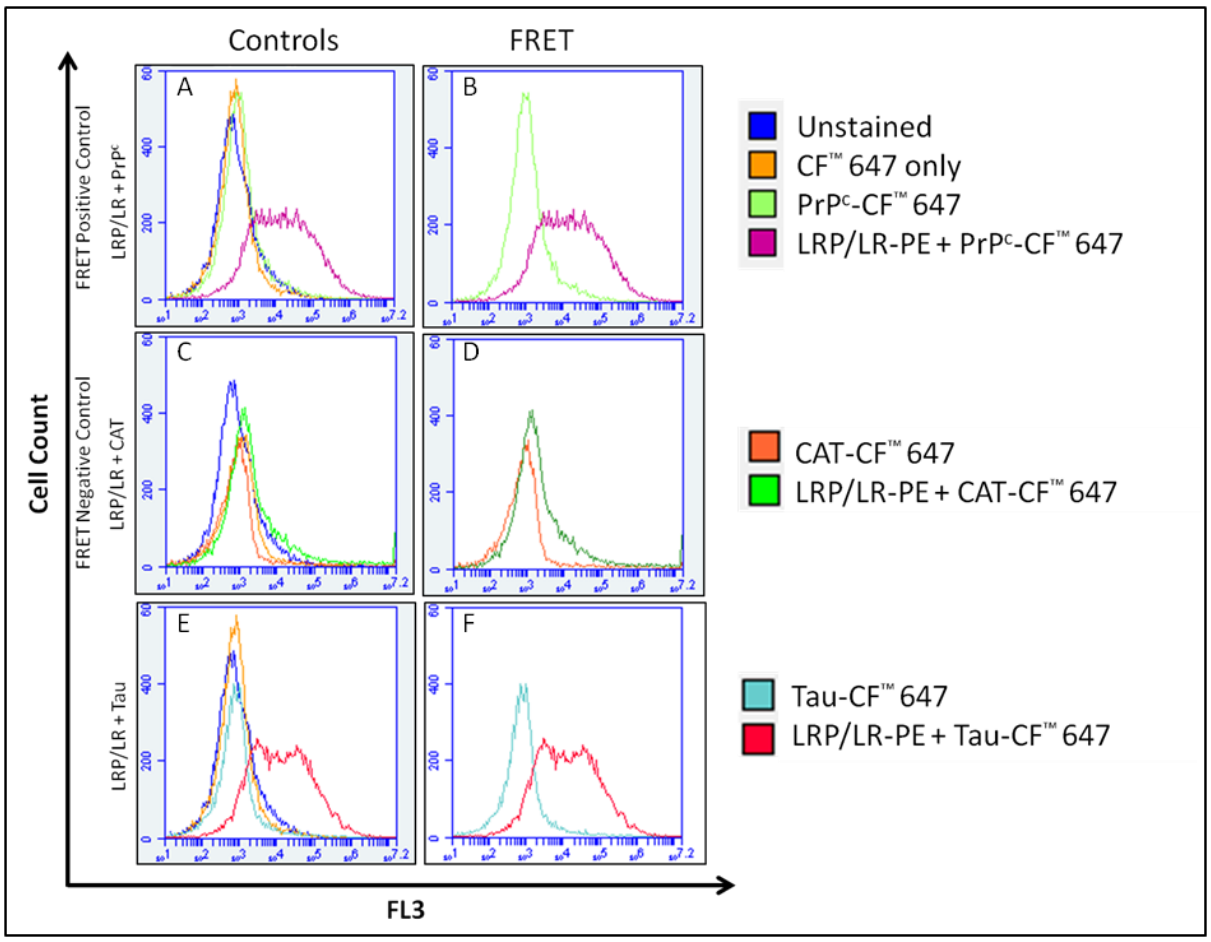


Figure 4.2: Flow cytometric analysis of FRET between intracellular LRP/LR and tau. The fluorescence intensity of the unstained HEK-293 cells (dark blue histogram) was superimposed with that of cells labelled with the CF™ 647 secondary antibody only (yellow histogram) as well as with cells in which the 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. Three biological replicates were conducted (Figure A3).

4.3. Confirmation of LRP::FLAG overexpression in HEK-293 and SH-SY5Y cells

Since a direct interaction occurs 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. This was done in order to overexpress LRP::FLAG. For clarification purposes, cells transfected with the pCIneo-moLRP-FLAG plasmid will be labelled as transfected cells, while those that have not been transfected with the plasmid will be labelled as non-transfected cells.

Western blotting was used to determine whether the cells had been successfully transfected with the plasmid (Figure 4.3). The detection of the LRP::FLAG protein in the transfected cells shows that both the HEK-293 (Figure 4.3: A) and SH-SY5Y (Figure 4.3: B) cells were stably transfected.

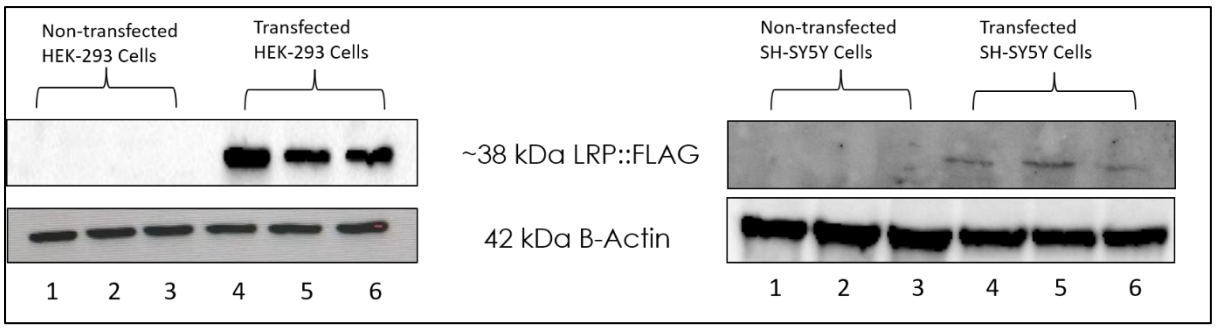


Figure 4.3: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells transfected with the pCIneo-moLRP-FLAG plasmid. Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells. A) LRP::FLAG (HRP) was detected in transfected HEK-293 cells but not non-transfected HEK-293 cells B) LRP::FLAG (Cy3) was detected in transfected SHSY-5Y cells but not non-transfected SH-SY5Y cells. B-Actin (HRP) was used as a loading control. n=3 biological repeats.

4.4. Overexpression of LRP::FLAG decreases total tau and phospho-tau levels

In order to determine the effect of LRP::FLAG overexpression on the levels of tau and phospho-tau (Figure 4.4: A1 and B1), western blotting was utilized (Figure 4.4: A1 and B1).

A significant decrease in total tau was observed in both HEK-293 and SH-SY5Y cells after overexpression of LRP::FLAG when compared to non-transfected cells. A highly significant decrease of 45% was observed in transfected HEK-293 (Figure 4.4: A2) cells and 35% was observed in transfected SH-SY5Y (Figure 4.4: B2) cells. The pattern of expression of tau depends on the developmental and functional state of cells (Uberti *et al.,* 1997). Therefore, tau may be functioning slightly differently in HEK-293 cells compared to SH-SY5Y cells, thereby explaining the slightly different banding pattern observed.

Since hyperphosphorylation of tau causes neurofibrillary tangle formation (Serrano-Pozo *et al.,* 2011), the effect of LRP::FLAG overexpression on phosphorylated tau levels was investigated. It is not possible to investigate hyperphosphorylation *in vitro,* therefore two antibodies targeting tau phosphorylated at different sites (pS404 and pT231) were used. Furthermore, this allowed, off-target effects to be disregarded as a potential reason for any observed difference.

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 4.4: A2). In transfected SH-SY5Y cells, a significant 63% and 92% reduction was observed (Figure 4.4: B2), respectively. The decrease in these two phosphorylation sites together is indicative of a decrease in hyperphosphorylated tau.

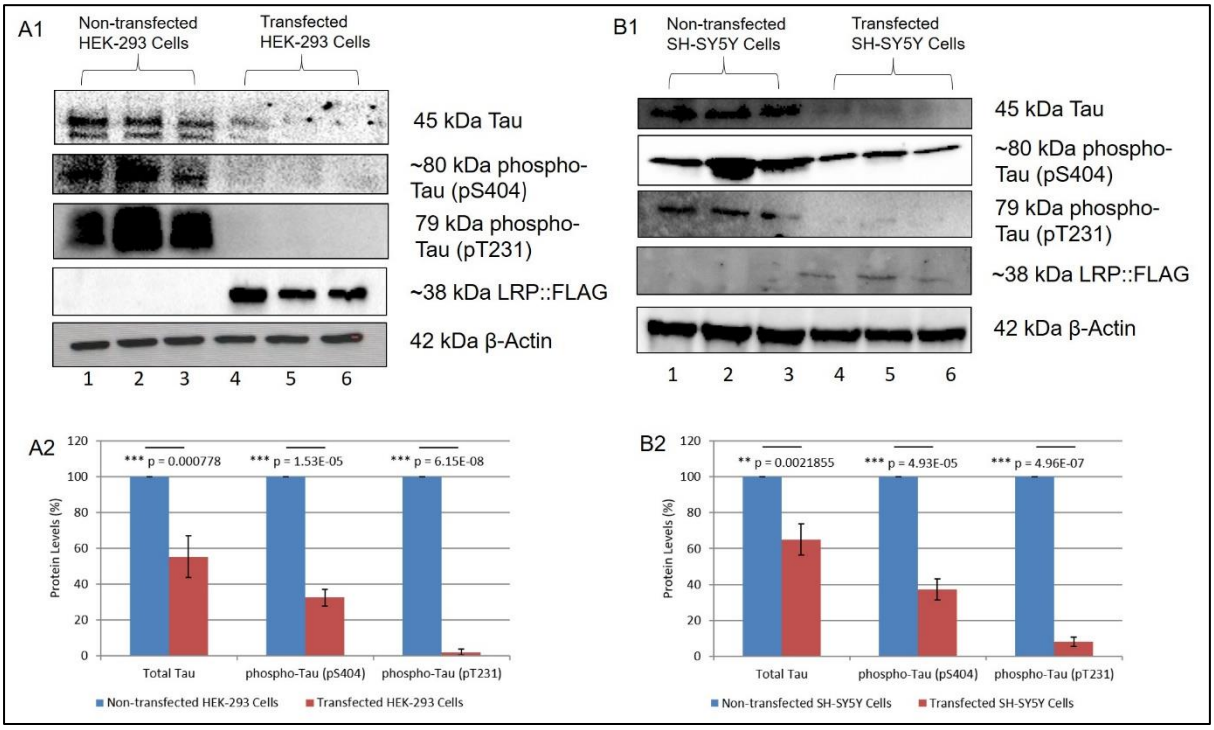


Figure 4.4: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases levels of total Tau as well phospho-Tau. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells. B-Actin was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (A2) and SH-SY5Y (B2) cells which were set to 100%. Error bars represent one standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001; Student's *t*-test.

Confocal microscopy was further employed to analyze the expression of tau and phospho-tau intracellularly as these proteins are normally found in the cytoplasm and nucleus (Figure 4.5). The merged panels represent the merge of the red staining, representing tau or phospho-tau, with the blue nuclear DAPI staining. These results confirm the reduction of total tau and phospho-tau in all cellular locations in transfected cells relative to the non-transfected cells.

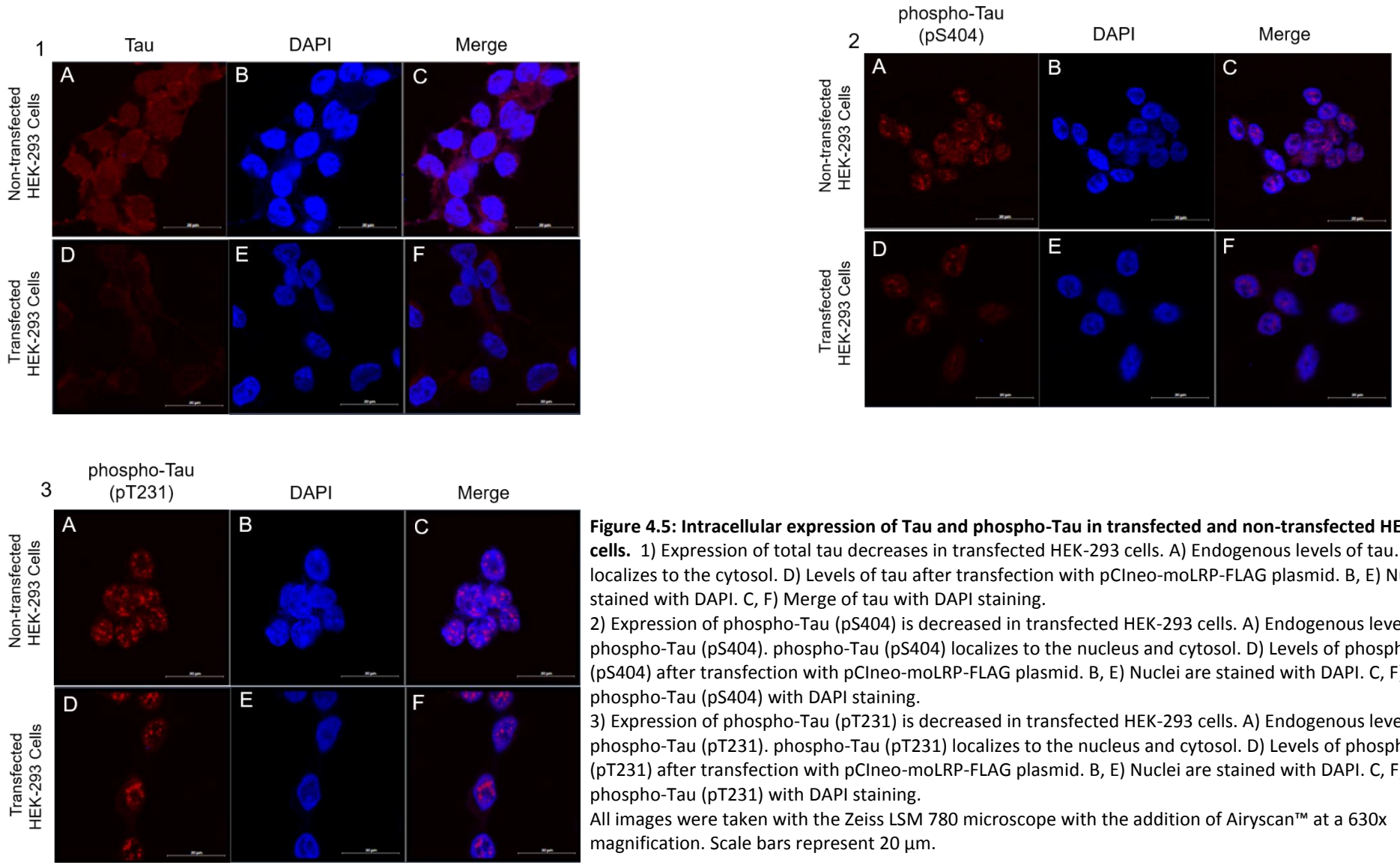


Figure 4.5: Intracellular expression of Tau and phospho-Tau in transfected and non-transfected HEK-293 cells. 1) Expression of total tau decreases in transfected HEK-293 cells. A) Endogenous levels of tau. Tau localizes to the cytosol. D) Levels of tau after transfection with pCIneo-moLRP-FLAG plasmid. B, E) Nuclei are stained with DAPI. C, F) Merge of tau with DAPI staining.
2) Expression of phospho-Tau (pS404) is decreased in transfected HEK-293 cells. A) Endogenous levels of phospho-Tau (pS404). phospho-Tau (pS404) localizes to the nucleus and cytosol. D) Levels of phospho-Tau (pS404) after transfection with pCIneo-moLRP-FLAG plasmid. B, E) Nuclei are stained with DAPI. C, F) Merge of phospho-Tau (pS404) with DAPI staining.
3) Expression of phospho-Tau (pT231) is decreased in transfected HEK-293 cells. A) Endogenous levels of phospho-Tau (pT231). phospho-Tau (pT231) localizes to the nucleus and cytosol. D) Levels of phospho-Tau (pT231) after transfection with pCIneo-moLRP-FLAG plasmid. B, E) Nuclei are stained with DAPI. C, F) Merge of phospho-Tau (pT231) with DAPI staining.
All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.

4.5. Overexpression of LRP::FLAG increases total hTERT levels

It is known that overexpression of LRP::FLAG increases hTERT levels in HEK-293 cells (Otgaar *et al.*, 2017) and SH-SY5Y cells (Bignoux *et al.*, 2019). It has also been shown that this increase results in an increase in telomerase activity (Otgaar *et al.*, 2017; Bignoux *et al.*, 2019). Thus, we wished to examine the effect of LRP::FLAG overexpression on hTERT levels together with the active form of hTERT, phospho-TERT, as this is indicative of an increase in telomerase activity. This was done by western blotting (Figure 4.6: A1 and B1).

There was a significant increase in hTERT levels in transfected HEK-293 and SH-SY5Y cells of 120% (Figure 4.6: A2) and 125% (Figure 4.6: B2), respectively. There was also a significant 96% increase in phospho-TERT levels in transfected SH-SH5Y cells (Figure 4.6: B2). However, no change in phospho-TERT was observed in transfected HEK-293 cells (Figure 4.6: A2). The increases in hTERT and phospho-TERT are indicative of an increase in telomerase activity (Bodnar *et al.*, 1998; Tomas-Loba *et al.*, 2008; Otgaar *et al.*, 2017).

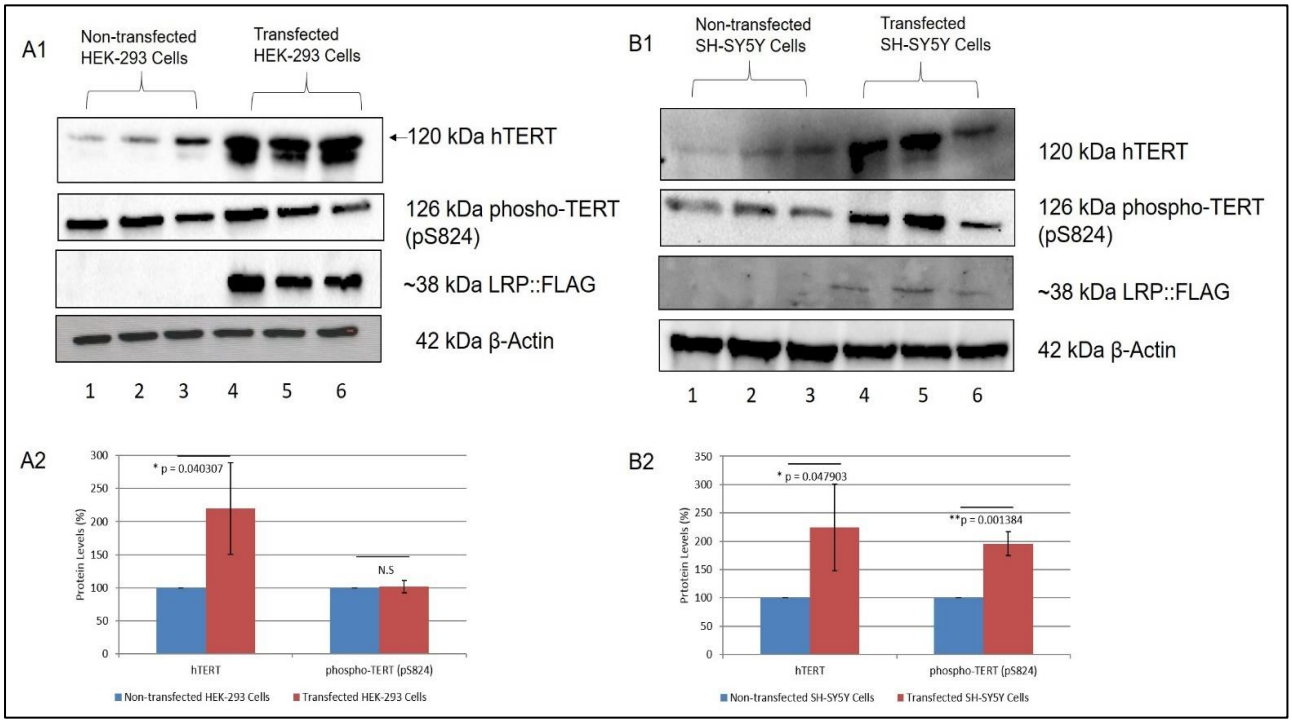


Figure 4.6: Overexpression of LRP::FLAG increases hTERT levels in HEK-293 and SH-SY5Y cells and phospho-TERT levels in SH-SY5Y cells. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells. B-Actin (HRP) was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (A2) and SH-SY5Y (B2) cells which were set to 100%. Error bars one represent standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001 N.S.: non-significant; Student's *t*-test.

In addition, by examining the ratio between phospho-TERT and hTERT levels in transfected SH-SY5Y cells, it is clear that not all hTERT is being converted into phospho-TERT (Table 4.1).

Table 4.1: Ratio of phospho-TERT to hTERT in transfected SH-SY5Y cells shows that not all hTERT is converted to phospho-TERT. Analysis was performed relative to hTERT levels which were set to 1. n = 3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001

Transfected SH-SY5Y Cells		
	Ratio	*p*-value
phospho-TERT: hTERT	0.516 ± 0.037	2.20571E-05 (***)

4.6. Overexpression of LRP::FLAG decreases Aβ expression and PrP^c levels

It is known that the hyperphosphorylation of tau is dependent on the extracellular aggregation of $A\beta_{42}$ as the neurotoxic Aβ fibrils can upregulate kinases which induce phosphorylation (Hardy and Selkoe, 2012). Thus, $A\beta_{42}$ expression was investigated using confocal microscopy.

An overall decrease in $A\beta_{42}$ expression was observed in transfected HEK-293 cells compared to non-transfected cells (Figure 4.7). The merged panels represent the merge of the green staining, representing $A\beta_{42}$, with the blue DAPI nuclear staining. Therefore, the decrease in $A\beta_{42}$ expression may contribute to the decreases in phosphorylated tau previously seen (Avila, 2006, Hardy and Selkoe, 2012).

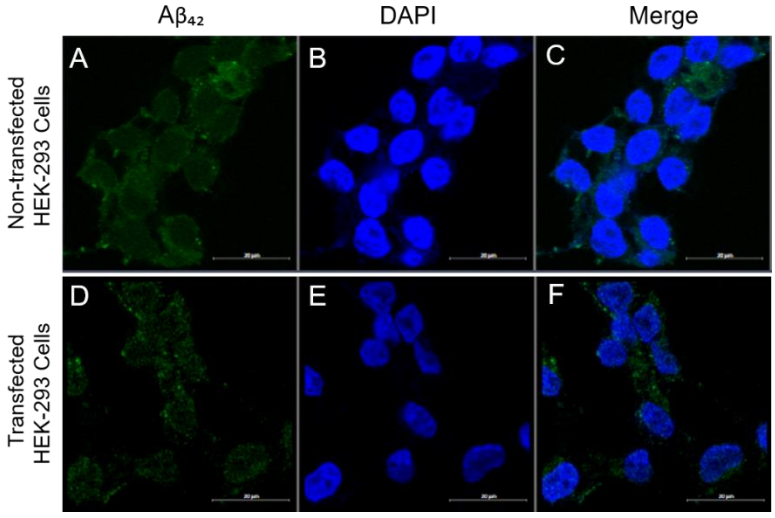


Figure 4.7: Intracellular expression of $A\beta_{42}$ in transfected and non-transfected HEK-293 cells. Expression of $A\beta_{42}$ decreases in transfected HEK-293 cells. A) Endogenous levels of $A\beta_{42}$. $A\beta_{42}$ localizes to the cytosol and cell surface. D) Levels of $A\beta_{42}$ after transfection with pCIneo-moLRP-FLAG plasmid. B, E) Nuclei are stained with DAPI. C, F) Merge of $A\beta_{42}$ with DAPI staining. All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.

PrP^c is known to play a role in AD as it is considered to be the primary receptor for $A\beta_{42}$ (Freir *et al.*, 2011). Thus, it was hypothesized that PrP^c is also important for tauopathy. Therefore, the effect of LRP::FLAG overexpression on PrP^c was investigated using western blotting (Figure 4.8: A1 and B1).

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 4.8: A1), while in SH-SY5Y cells only the monoglycosylated form was observed (Figure 4.8: B1). It is important to note that glycosylation is not necessary for encoding strain information in prions (Piro *et al.*, 2009) and that PrP^c glycoform ratios differ across brain regions and across cell lines (Beringue *et al.*, 2003; Kuczius *et al.*, 2007). Therefore, the differences in total PrP^c levels were examined.

A significant decrease in total PrP^c in both transfected HEK-293 and SH-SY5Y cells of 34% (Figure 4.8: A2) and 39% (Figure 4.8: B2), respectively, was observed.

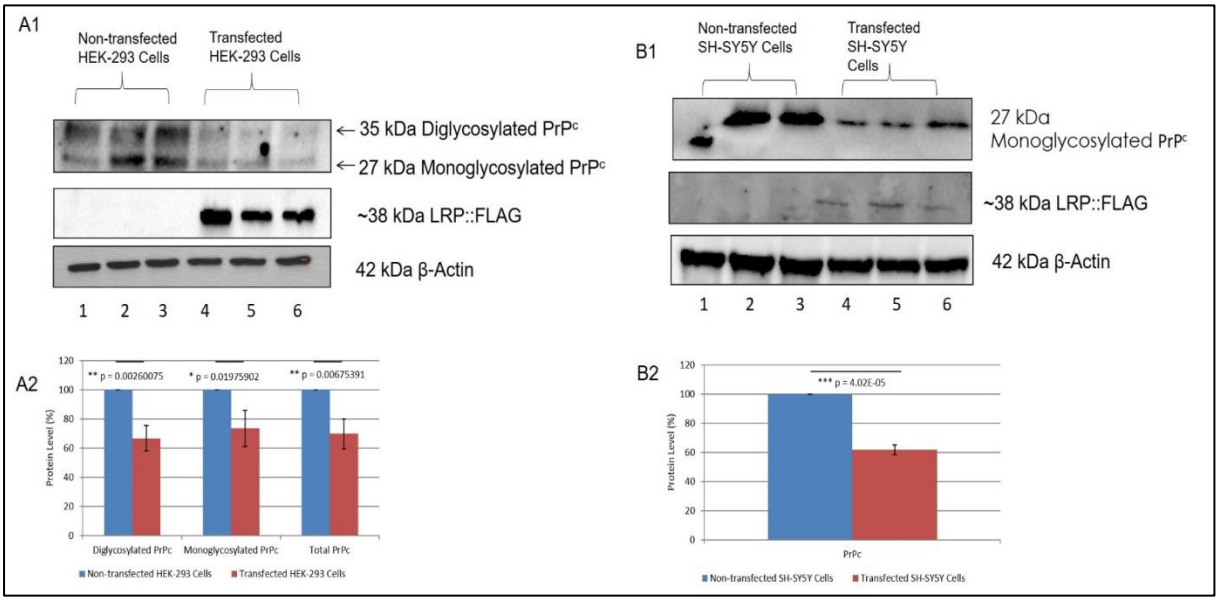


Figure 4.8: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases PrP^c levels. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells. B-Actin (HRP) was used as a loading control. Densitometric analysis performed was relative to the non-transfected HEK-293 (A2) and SH-SY5Y (B2) cells which were set to 100%. Error bars represent one standard deviation, n=3 biological repeats. *p: 0.05, **p: 0.01, ***p: 0.001; Student's *t*-test.

5. Discussion

AD is a devastating form of dementia that mainly affects the elderly. In addition, AD is a huge social and economic burden as it can take many years to be fatal. Only long-term palliative care is able for this disease, therefore non-palliative treatments are urgently required. LRP/LR may be a potential therapeutic target for this disease as it has been shown to play a role in AD development and progression. LRP/LR is a known receptor for $A\beta_{42}$ internalization (Da Costa Dias *et al.,* 2014) and is involved in the amyloidogenic processing of APP, which generates the cytotoxic Aβ peptide (Jovanovic *et al.,* 2013; Jovanovic *et al.,* 2014). Indeed, studies targeting LRP/LR have successfully impeded Aβ shedding and rescued cells from Aβ-induced cytotoxicity (Da Costa Dias *et al.,* 2013; Jovanovic *et al.,* 2013; Pinnock *et al.,* 2016; Bignoux *et al.,* 2019). These observations have also been confirmed *in vivo*, whereby AD transgenic mice treated with the anti-LRP/LR antibody, IgG1-iS18, which blocks cell surface LRP/LR, showed decreased amyloid plaque generation with a corresponding improvement in short-term and learning memory (Ferreira *et al.,* 2018). However, it is not known whether LRP/LR has an effect on the other neuropathological agent of AD, hyperphosphorylated tau. Therefore, this project aimed to determine if targeting LRP/LR could have an effect on the tauopathy of AD in addition to its affects on Aβ pathology.

LRP/LR has also recently been implicated in ageing through an interaction with telomerase and hTERT (Otgaar *et al.,* 2017). The length of telomeres regulates the replicative lifespan of a cell (Harley and Villeponteau, 1995), thus telomeres become shorter as cellular divisions progress during an organism's lifespan. Eventually, cells can no longer divide and enter a state known as replicative senescence. In this state, cells are still metabolically active but are permanently in cell cycle arrest (Allsopp *et al.,* 1995). Recently, it was shown that upregulation of LRP/LR can impede this ageing process by increasing hTERT levels and telomerase activity (Otgaar *et al.,* 2017). In AD, neuronal cells have critically shortened telomeres as an accumulation of Aβ is able to inhibit the activity of telomerase (Panossian *et al.,* 2003; Wang *et al.,* 2015). Furthermore, there is an absence of hTERT in cells affected by hyperphosphorylated tau (Spilsbury *et al.,* 2015). This can worsen oxidative damage as hTERT can no longer convey protection against ROS (Spilsbury *et al.,* 2015). Therefore, the upregulation of telomerase and hTERT is a potential treatment strategy for AD. Indeed,

hTERT has been shown to have a neuroprotective role against Aβ, whereby an increase in hTERT resulted in decreased $A\beta_{42}$ shedding and increased cellular viability *in vitro* (Bignoux *et al.,* 2019) while *in vivo* an increase in mTERT correlated with a decrease in amyloid plaque generation (Ferreira et al., 2018).

Since both LRP/LR and telomerase are known to play a role in the Aβ facet of AD, it was hypothesized that they could also play a role in tauopathy. Indeed, tau hyperphosphorylation is known to be dependent on Aβ aggregation (Hardy and Selkoe, 2012). In addition, hTERT has a neuroprotective role against Aβ (Wang *et al.,* 2015; Bignoux *et al.,* 2019) and the absence of hTERT worsens tau-induced oxidative damage (Spilsbury *et al.,* 2015). Hyperphosphorylated tau and hTERT appear to be mutually exclusive (Spilsbury *et al.,* 2015). Therefore, overexpression of hTERT could also protect neurons from the damaging effects of hyperphosphorylated tau.

5.1. LRP/LR and Tau co-localization and FRET analysis

In order to assess this hypothesis, HEK-293 and SH-SY5Y cell lines were used as they are commonly used to investigate neurodegenerative diseases (Shaw *et al.,* 2002; Zheng *et al.,* 2006; Agholme *et al.,* 2010; Carolindah *et al.,* 2013). We first wanted to determine if our proteins of interest were present in the same sub-cellular locations, as this is a prerequisite for potential interactions. LRP/LR and hTERT were confirmed to co-localize, as has previously been seen (Figure 4.1: J-L) (Naidoo *et al.,* 2015; Otgaar *et al.,* 2017). Co-localization was also observed between LRP/LR and tau (Figure 4.1: D-F), as confirmed by the strong diagonal in the 2D cytofluorogram (Figure 4.1: F). This co-localization occurs mainly in the perinuclear compartment and indicates that a spatial overlap occurs between LRP/LR and tau. Therefore, it implies that an association between these two proteins is possible. Furthermore, if these proteins do associate in the perinuclear compartment the nuclear functions of both LRP/LR and tau could be affected in AD.

Since the co-localization detected by confocal microscopy has a resolution limit of 140 nm, the co-localization observed between LRP/LR and tau does not necessarily demonstrate that the proteins interact with each other. Indeed, LRP/LR is a fairly ubiquitous protein as it can be found in the lipid raft region, the cytoplasm (Kinoshita *et al.,* 1998) and the nucleus (Ford *et al.,* 1999) while tau is found in the cytoplasm and the nucleus. Thus, the proteins could just share the same sub-cellular locations. Therefore, in order to determine if a reaction could

occur, FRET was employed using HEK-293 cells. Upon co-labelling of cells with Tau-CF™ 647 and LRP/LR-PE there is an augmentation of FL3 fluorescence intensity (Figure 4.2: F). This suggests that FRET has occurred between these two proteins. Thus, these proteins are found within 10 nm of each other and it is highly probable that they interact with each other. It is thus possible that LRP/LR is involved in the assembly and maintenance of microtubules in healthy cells. Indeed, cytoplasmic LRP is known to bind to histones to assist in transcription (Kinoshita *et al.*, 1998). Furthermore, LRP tightly associates with tubulin structures and may serve to tether ribosomes to microtubules in order to assist in protein synthesis (Auth and Brawerman, 1992; Venticinque *et al*., 2011). Therefore, 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, it is thus possible that strategies targeting LRP/LR could also target this interaction as a therapeutic intervention.

5.2. LRP::FLAG overexpression decreases total and phosphorylated tau levels

Since it is highly probable that LRP/LR and tau directly interact, it was investigated whether LRP/LR plays a role in the tauopathy of AD. Therefore, HEK-293 and SH-SY5Y cells were stably transfected with the pCIneo-moLRP-FLAG plasmid in order to increase total LRP levels in the cells by overexpressing LRP::FLAG. The overexpression of LRP::FLAG in the transfected HEK-293 and SH-SY5Y cells was confirmed using western blotting (Figure 4.3). Thereafter, the levels of tau and phospho-tau were examined in the transfected cells. Both confocal microscopy and western blotting showed that total tau as well as tau phosphorylated at S404 and tau phosphorylated at T231 decreased in transfected cells (Figure 4.4). In addition, confocal microscopy showed that there was on overall decrease in all the cellular locations where tau and the phosphorylated tau proteins are expressed (Figure 4.5). In AD, tau is hyperphosphorylated and forms tangles that disrupt the internal structure of neuronal cells (Haass and Mandelkow, 2010). Since it is not possible to investigate hyperphosphorylated tau *in vitro*, tau phosphorylated at two different amino acids, namely S404 and T231, was investigated. Therefore the significant decrease of both isoforms is indicative of a decrease in hyperphosphorylated tau. However, more isoforms could be investigated to strengthen this argument. Since hyperphosphorylated tau disrupts the internal

structure of cells by affecting the cytoskeletal tracks (Haass and Mandelkow, 2010) it may be possible that the decrease in phosphorylated tau seen after LRP::FLAG overexpression may result in less damage to neuronal cells. Thus, there may be a decrease in the aggregation of hyperphosphorylated tau. Since this aggregation is known to cause disintegration of cytoskeletal tracks, a decrease may no longer inhibit vital cell processes, such as axonal transport, thereby permitting neurons to retain normal functioning (Haass and Mandelkow, 2010). In particular, the phosphorylation of tau at S404 and T231, which was investigated in this study, are known to negatively affect the binding of the tau protein to microtubules, thereby de-stabilizing them and inhibiting cytoskeletal signalling and transport (Cho and Johnson, 2002; Mondragón-Rodriguez *et al.,* 2009). Indeed, T231 mutant N18 neuroblastoma cells abolished phosphorylation of tau by GSK3, indicating that phosphorylation at T231 plays an important role in the hyperphosphorylation of tau (Lin *et al.,* 2007). Therefore, a decrease in phosphorylation at these sites may prevent the dissociation of tau and promote tubulin polymerization and assembly into microtubules (Cho and Johnson, 2002; Lin *et al.,* 2007). This, in turn, could lead to cells being better able to resist the damaging effects of AD as their structural integrity is maintained and their signalling and transport capabilities are intact. In particular, DNA damage repair proteins can be trafficked to the sites of cytotoxicity in order to rescue cells (Poruchynsky *et al.,* 2015). In addition, hyperphosphorylation of tau is able to enhance Aβ cytotoxicity by sequestering Fyn kinase (Haass and Mandelkow, 2010). 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 Aβ oligomers (Haass and Mandelkow, 2010; Ittner *et al.,* 2010). Therefore a decrease in phosphorylated tau could both lessen cytotoxicity as well as allowing DNA repair to occur. Microtubules are a vital component in cytoskeletal tracks and their dysfunction can lead to the disintegration of the cytoskeletal tracks as a whole. In turn, it is known that the hyperphosphorylation of tau can negatively impact plasma membrane signalling and synaptic health (Haass and Mandelkow, 2010) due to the effects of microtubule disintegration on the cellular architectural framework, which can lead to disintegration of the neuron as a whole. Therefore, if less tau hyperphosphorylation occurs, the general synaptic health of neurons will be maintained. 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 also observed (Figure 4.4; Figure 4.5). As previously mentioned, 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 cell viability remains the same in cells overexpressing LRP::FLAG compared to those that do not (Bignoux *et al.,* 2019). Therefore we can assume 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 known redundancy between tau and other microtubule-associated proteins (Dehmelt and Halpain, 2005) such as the redundancy between MAP-1B and tau during axonal growth (DiTella *et al.,* 1996; Takei *et al.,* 2000). 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 smaller to those seen in phosphorylated tau (45% versus 68-98% in transfected HEK-293 cells; 35% versus 63-92% in transfected SH-SY5Y cells). Thus, while overexpression of LRP::FLAG does not specifically target phosphorylated tau, it has a much more notable effect on the phosphorylated form. It is known that the interaction between LRP and laminin is able to affect the phosphorylation of ERK, JNK and p38 proteins (Givant-Horwitz *et al*., 2004). 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 (Sun *et al.,* 2014). In neurons treated with Aβ, in addition to the increased phosphorylation of tau, there is also increased phosphorylation of FAK and an increased association between FAK and Fyn kinase, which is known to enhance cytotoxicity in AD (Williamson *et al.,* 2002). Therefore it is possible interaction between LRP and FAK, and the subsequent activation of signalling pathways, such as PI3-kinase/Akt and MAPK (Sun *et al.,* 2014), may affect the phosphorylation of tau in AD. Therefore, LRP::FLAG, or the upregulation of LRP, has the potential to be optimized as a therapeutic strategy. Furthermore, since there are no current non-palliative treatments available for tauopathy and AD, it is beneficial to continue investigating any potential disease-modifying therapeutics. Thus, more studies would have to be performed to better determine how LRP::FLAG is expressed in cells in order to determine any potential detrimental effects and to perhaps optimize an adaptor that will allow the phosphorylated forms of tau to be specifically targeted.

5.3. LRP::FLAG overexpression affects hTERT and phospho-TERT levels

As telomeres and telomerase have recently been implicated in AD, the limiting factor of telomerase activity, hTERT, was assessed using western blotting. Transfected HEK-293 and SH-SY5Y cells displayed elevated levels of hTERT (Figure 4.6), which is consistent with previous results (Otgaar *et al.,* 2017; Bignoux *et al.,* 2019). Furthermore, the levels of the telomerase active form of hTERT, phospho-TERT (pS824) was assessed using western blotting, as this form is considered to be responsible for the telomere elongation function of telomerase. There was a significant increase in phospho-TERT levels in transfected SH-SY5Y cells (Figure 4.6: B1 and B2). It is known that an increase in hTERT has a corresponding increase in telomerase activity and telomere length (Bodnar *et al.,* 1998; Otgaar *et al.,* 2017; Tomas-Loba *et al.,* 2008). Therefore, the increase in both hTERT and phospho-TERT observed in transfected SH-SY5Y cells is indicative of an increase in telomerase activity and telomere length. Indeed, an increase in telomerase activity has been previously observed in transfected SH-SY5Y cells (Bignoux *et al.,* 2019). Due to the increase seen in telomerase activity, it is likely that there would also be increased telomere elongation (Bodnar *et al.,* 1998; Otgaar *et al.,* 2017; Tomas-Loba *et al.,* 2008). Since neuronal cells affected by $A\beta_{42}$ and tau have critically shortened telomeres (Panossian *et al.,* 2003), this increase in telomere elongation may prevent cell cycle arrest and apoptosis as cells will be able to exit replicative senescence, thereby allowing them to proliferate (Harley and Villeponteau, 1995). Furthermore, since hTERT and phosphorylated tau are mutually exclusive (Spilsbury *et al.,* 2015), a decrease in phosphorylated tau would allow hTERT to persist in neurons. Thereafter, the increased hTERT and phospho-TERT can lead to an in increased telomerase activity and telomere elongation which will allow senescent cells to exit cell arrest. The resultant decrease in apoptosis will further allow hTERT to persist in neurons and protect against the damaging effects of both tau and Aβ (Spilsbury *et al.,* 2015; Wang *et al.,* 2015). Indeed, one of the extra-telomeric functions of hTERT is its ability to protect against ROS (Ahmed *et al.,* 2008; Saretzki, 2009). Since increased ROS results from the cytotoxicity caused by both tau and Aβ, an increase in hTERT can protect against these effects.

However, no significant difference in the levels of phospho-TERT in transfected HEK-293 cells was observed compared to non-transfected cells (Figure 4.6: A1 and A2). Since it is known that HEK-293 cells that overexpress LRP::FLAG have increased telomerase activity (Otgaar *et al.,* 2017), it was expected that they would also have had increased phospho-

TERT. However, embryonic cells have high levels of hTERT and phospho-TERT (Hiyama and Hiyama, 2007; Otgaar *et al.,* 2017). Therefore, it is possible the HEK-293 cells do not necessarily require an increase in phospho-TERT in order for telomerase activity to increase. The increase in hTERT may be able to signal the phospho-TERT that is already present to interact with and elongate telomeres. Alternatively, it is possible phosphorylation of hTERT is time-dependent and is only required when hTERT initially increases or when a high amount of telomerase activity is required. Indeed, following transfection, HEK-293 cells were cultured for at least six weeks in order to obtain a stable transfection. Thus, a transient transfection may show an increase in phospho-TERT following the initial increase in hTERT.

Furthermore, not all hTERT is being converted to phospho-TERT (Table 4.1). Thus, hTERT may be involved in extra-telomeric functions. Of importance to this study is the protective function of hTERT against ROS. Telomerase is able to migrate to the mitochondria when cells are under oxidative stress, and this allows hTERT to interact with mtDNA in order to convey protection (Ahmed *et al.,* 2008; Saretzki, 2009). In AD, there is compromised energy production in the brain due to mitochondrial dysfunction (Atamna and Frey, 2007). This dysfunction can increase ROS production which has been shown to promote the hyperphosphorylation of tau (Melov *et al.,* 2007), 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 (Ebneth *et al.,* 1998). In neurons, mitochondria are distributed to areas of the axon where metabolic demand is high, such as synapses (Treek and Pirsig, 1979; Gotow *et al.,* 1991) and active growth cones or branches (Morris and Hollenbeck, 1993; Ruthel and Hollenbeck, 2003). As a result, ATP depletion can occur in axons and dendrites which can worsen synaptic dysfunction (Mattson and Liu, 2002). However, it is not clear whether oxidative stress or hyperphosphorylation of tau occurs first (Schmitt *et al.,* 2012). Importantly, in AD, the absence of hTERT has been shown to increase the levels of mitochondrial superoxide in neurons (Spilsbury *et al.,* 2015) and truncated tau expressed *in vivo* generates increased oxidative stress over time through an accumulation of ROS (Cente *et al.,* 2006). Furthermore, pathological tau and hTERT have been observed to be mutually exclusive (Spilsbury *et al.,* 2015), suggesting that neurons expressing hTERT may be prevented from developing tau pathology. Alternatively, the development of neurofibrillary tangles may cause a downregulation or redistribution of hTERT. Indeed, it has previously been observed that LRP::FLAG overexpression causes an increase in hTERT on the cell surface, in the

cytoplasm and nucleus (Otgaar *et al.*, 2017; Bignoux *et al.*, 2019). 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-κB (Li and Tergaonkar, 2014). Therefore, the increased nuclear hTERT observed could regulate pro-proliferation and anti-inflammatory genes via these pathways. In AD, this increase could also prevent apoptosis and aid cell survival through the upregulation of these genes. Therefore, an increase in hTERT may prevent cell 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.

5.4. LRP::FLAG overexpression decreases $A\beta_{42}$ and PrP^c levels

To further examine the effect of LRP::FLAG overexpression, tauopathy-related proteins were investigated. First, $A\beta_{42}$ levels were examined using confocal microscopy and an overall decrease in $A\beta_{42}$ expression in all sub-cellular regions was observed (Figure 4.7), confirming previously seen results (Bignoux *et al.*, 2019). It is known that highly neurotoxic Aβ fibrils can induce the hyperphosphorylation of tau by upregulating the kinase GSK3 (Avila, 2006). Thereafter, GSK3 and other kinases can phosphorylate tau and inhibit its binding to microtubules (Cho and Johnson, 2002) leading to the formation of neurofibrillary tangles. The residues examined in this study, T231 and S404, are particularly important in this role (Cho and Johnson, 2002). Therefore, tau hyperphosphorylation is dependent on the extracellular aggregation of $A\beta_{42}$ (Hardy and Selkoe, 2012), and a decrease in $A\beta_{42}$ could aid in the reduction of hyperphosphorylated tau. Indeed, it has previously been seen that LRP::FLAG overexpression specifically reduced extracellular $A\beta_{42}$ shedding (Bignoux *et al.*, 2019). It was postulated that this is due to the protective function of the increased hTERT and telomerase activity (Bignoux *et al.*, 2019). Furthermore, a previous study have shown that upregulation of LRP does not correspond with an upregulation of the mature LRP/LR (Gauczynski *et al.*, 2001). Indeed, it has been suggested that LRP/LR is not produced upon introduction of LRP cDNA into cells due to the necessity for both LRP/LR and LRP (DiGiacomo and Meruelo, 2016). Therefore, it was postulated that the majority of LRP::FLAG remained as immature LRP and did not convert to the mature LRP/LR receptor

form, which is a known receptor for Aβ internalization (Da Costa Dias *et al.*, 2014). Thus, it is the increased cytosolic LRP that functions to increase hTERT levels and telomerase activity. This could occur via an interaction with hTERT (Naidoo *et al.*, 2015) or it is possible that LRP/LR is able to play a role in the translation of hTERT. Therefore, not only does LRP::FLAG overexpression decrease $A\beta_{42}$ shedding and phosphorylation of tau, but it seems to be able to target both functions simultaneously, thereby enforcing a feedback loop that can reduce both amyloid plaque and neurofibrillary tangle formation and thus decrease overall cytotoxicity.

The effect of LRP::FLAG overexpression on PrP^c levels was then examined and a significant decrease in total PrP^c was observed (Figure 4.8). PrP^c is vital in AD as it mediates $A\beta_{42}$-induced synaptoxicity (Laurén *et al.*, 2009) and is considered to be the primary receptor for Aβ. However, its role in tau hyperphosphorylation is poorly understood. It has been suggested that PrP^c has a protective role and is able to downregulate tau expression (Schmitz *et al.*, 2013; Vergara *et al.*, 2015). However, Larson *et al.* (2012) identified a signalling cascade between soluble Aβ and PrP^c which activates Fyn kinase. Activated Fyn kinase is known to cause synaptic and cognitive impairment in transgenic mice (Chin *et al.*, 2005: Robertson *et al.*, 2011). Furthermore, Larson *et al. (*2012) discovered that this PrP^c-dependent activation of Fyn can also cause the missorting and hyperphosphorylation of tau. However, high levels of Aβ are required for this PrP^c-mediated Fyn activation and this may only occur during the later stages of AD (Larson *et al.*, 2012). Therefore, we postulate that PrP^c can switch roles as AD progresses due to the increasing levels of cytotoxic Aβ. Thus, in the current study, the decrease in PrP^c observed may be beneficial in preventing the Aβ-PrP^c signalling cascade in the later stages of AD. However, in the early stages, PrP^c may have a protective role by attenuating tau production (Vergara *et al.*, 2015). Therefore, a decrease in PrP^c in the early stages of AD may have adverse effects. However, at present, it is thought that the physiological process of AD begins many years before diagnosis (Sperling *et al.*, 2011), thus any therapeutic would most likely be used at the later stages of AD. Nevertheless, 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.

5.5. Conclusion

In conclusion, this study has shown that LRP/LR and tau share the same sub-cellular locations and that they directly interact with each other. In addition, LRP::FLAG overexpression caused a decrease in phosphorylated tau, $A\beta_{42}$ and PrP^c, which interact with each other during AD progression. $A\beta_{42}$ binds to PrP^c causing cytotoxicity and increased $A\beta_{42}$ shedding, which results in hyperphosphorylation of tau and the formation of neurofibrillary tangles. Therefore, overexpression of LRP::FLAG is able to affect each of the major proteins in this signalling cascade and thereby decrease the cytotoxic effects of AD. Furthermore, hTERT was increased and thus could provide protection against cytotoxicity through telomere elongation, by reducing ROS production, and by regulating cell survival pathways. Therefore, elevation of LRP levels is a potential therapeutic strategy for the treatment of AD and other tauopathies.

5.6. Future Work

The best way to further this research would be with an *in vivo* study. Tau transgenic mice, such as the P301S strain, could be used to examine the effect of elevating LRP *in vivo*. These mice express mutant human microtubule-associated protein tau (MAPT) causing them to develop neurofibrillary tangle-like inclusions in the neocortex, amygdale, hippocampus, brain stem and spinal cord. Therefore, they exhibit neuronal loss and brain atrophy by eight months. LRP::FLAG could be injected as a plasmid intranasally, to ensure efficient delivery to the brain. Alternatively, the LRP::FLAG sequence could be inserted into a viral vector for delivery. A plasmid would have a low expression rate compared to viral vector, but there is no chance of it integrating into the genome. Therefore, the delivery method would have to be optimized. The plasmid or vector would be under the control of a brain specific promoter. This would enable ectopic expression of LRP::FLAG causing only LRP levels in the brain to be elevated.

Furthermore, both *in vitro* and *in vivo* studies have shown that the anti-LRP/LR specific antibody, IgG1-iS18, can impede neurodegeneration by reducing Aβ levels in AD (Da Costa Dias *et al.,* 2013; Jovanovic *et al.,* 2013; Ferreira *et al.,* 2018). Therefore, it may be beneficial to examine a combinatorial treatment using both LRP::FLAG overexpression and IgG1-iS18 blockade of LRP/LR. This treatment could target more than one aspect of AD, and thereby improve the outcome of the treatment. This could be examined initially in cell culture using

AD cell culture models, such as SH-SY5Y cells. Thereafter, an *in vivo* study could be performed.

Experiments could also be performed to optimize drug design, including mode of administration as well as pharmacodynamics and pharmacokinetics. This would include consideration of any barriers the drug will have to overcome, such as first pass metabolism and the blood brain barrier (BBB). The drug could be encapsulated within a lipophilic complex or nanoparticle that could both protect the drug and allow it to cross the plasma membrane of cells. Additionally, brain-specific ligands could be attached to the active molecule to ensure that the drug does not travel anywhere else in the body. Thereafter, a clinical study could be initiated to determine the efficacy and toxicity of the drug in human patients. This could lead to development of the drug as a therapeutic for AD.

6. References

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Appendix

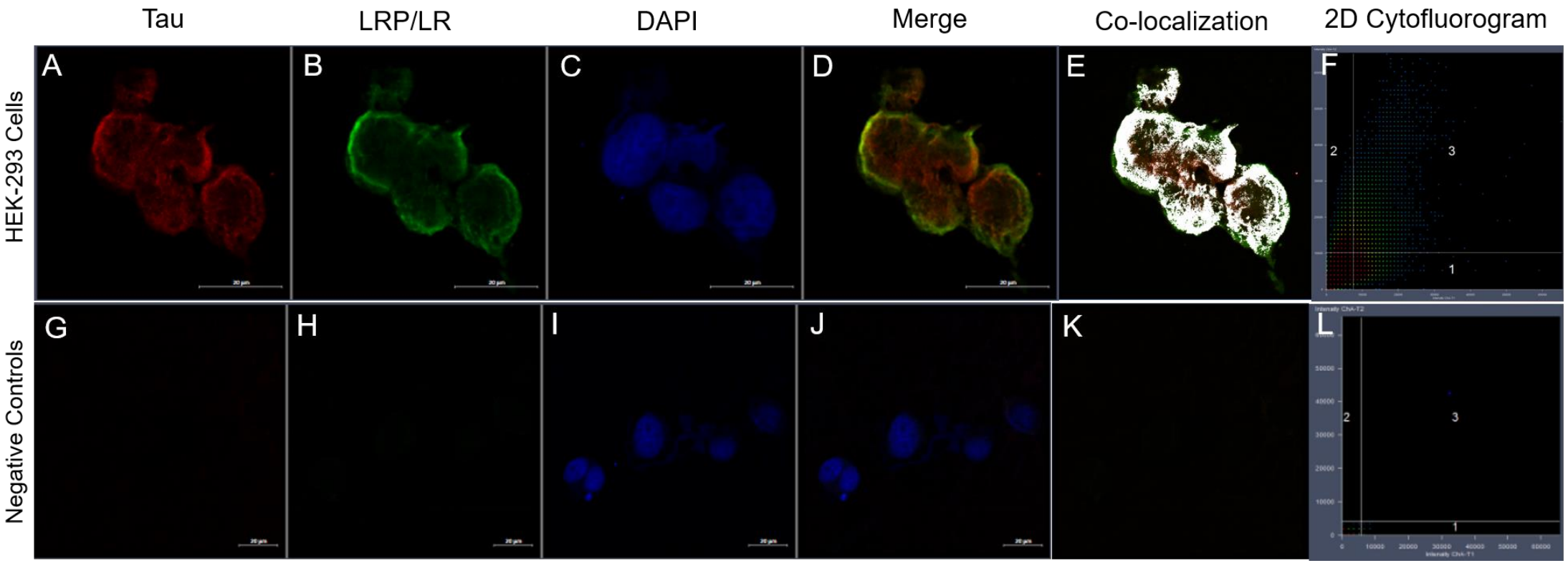


Figure A1: Enlarged LRP/LR and tau localization and co-localization within HEK-293 cells. This figure illustrates the co-localization between LRP/LR (green) and tau (red). A) Endogenous tau levels in HEK-293 cells. Tau localizes to the cytosol. B) Endogenous LRP/LR levels in HEK-293 cells. LRP/LR localizes to the cytosol, nucleus and cell surface. C, I) Nuclei are stained with DAPI (blue). 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). G) CF 647™ only control. H) FITC only control. The merged image (J), co-localization image (K) and 2D cytofluorogram (L) show that there is no co-localization between the negative controls. All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.

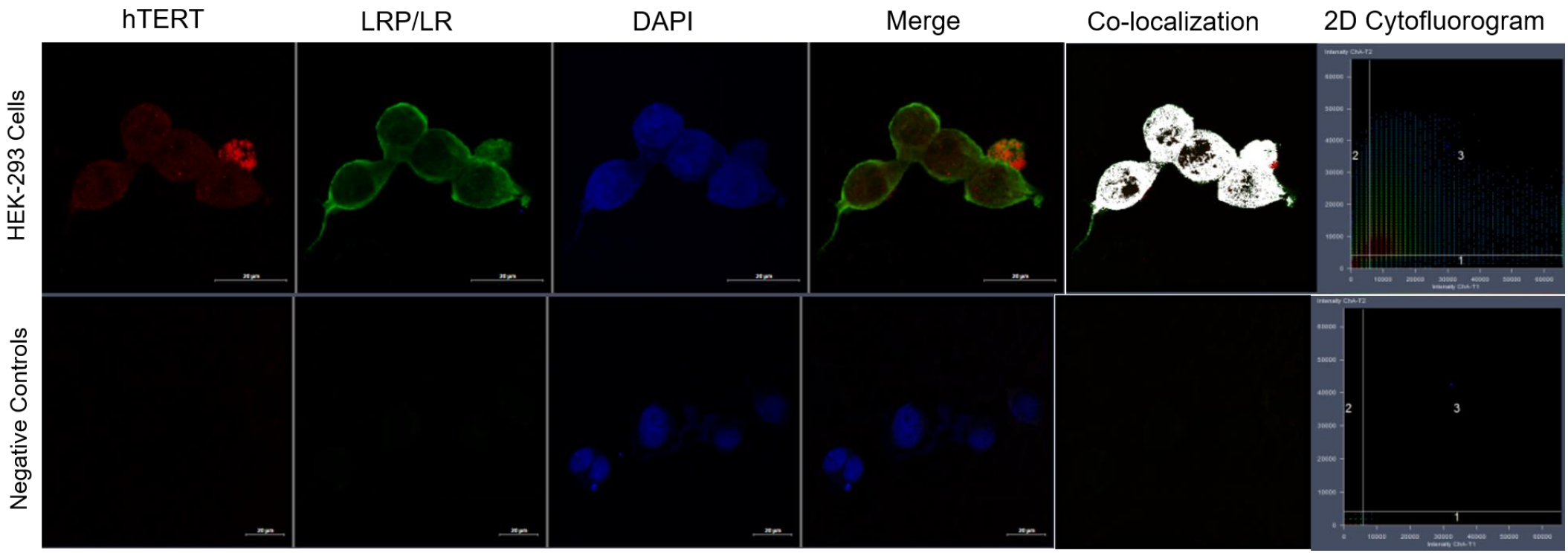
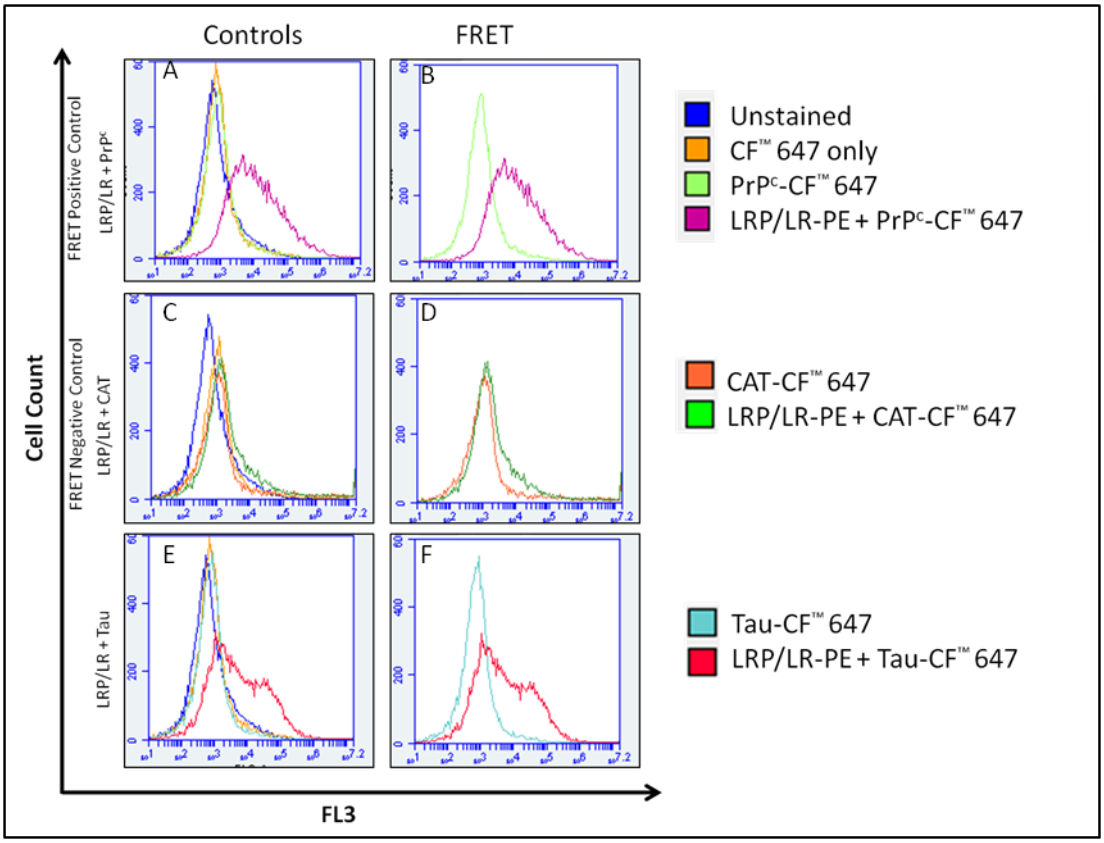


Figure A2: Enlarged LRP/LR and hTERT localization and co-localization within HEK-293 cells. This figure illustrates the co-localization between LRP/LR (green) and hTERT (red). A) Endogenous hTERT levels in HEK-293 cells. hTERT is localized to the cytosol and nucleus. B) Endogenous LRP/LR levels in HEK-293 cells. LRP/LR localizes to the cytosol, nucleus and cell surface. C, I) Nuclei are stained with DAPI (blue). Co-localization occurs between LRP/LR and hTERT in the nucleus and 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). G) CF 647™ only control. H) FITC only control. The merged image (J), co-localization image (K) and 2D cytofluorogram (L) show that there is no co-localization between the negative controls. All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.



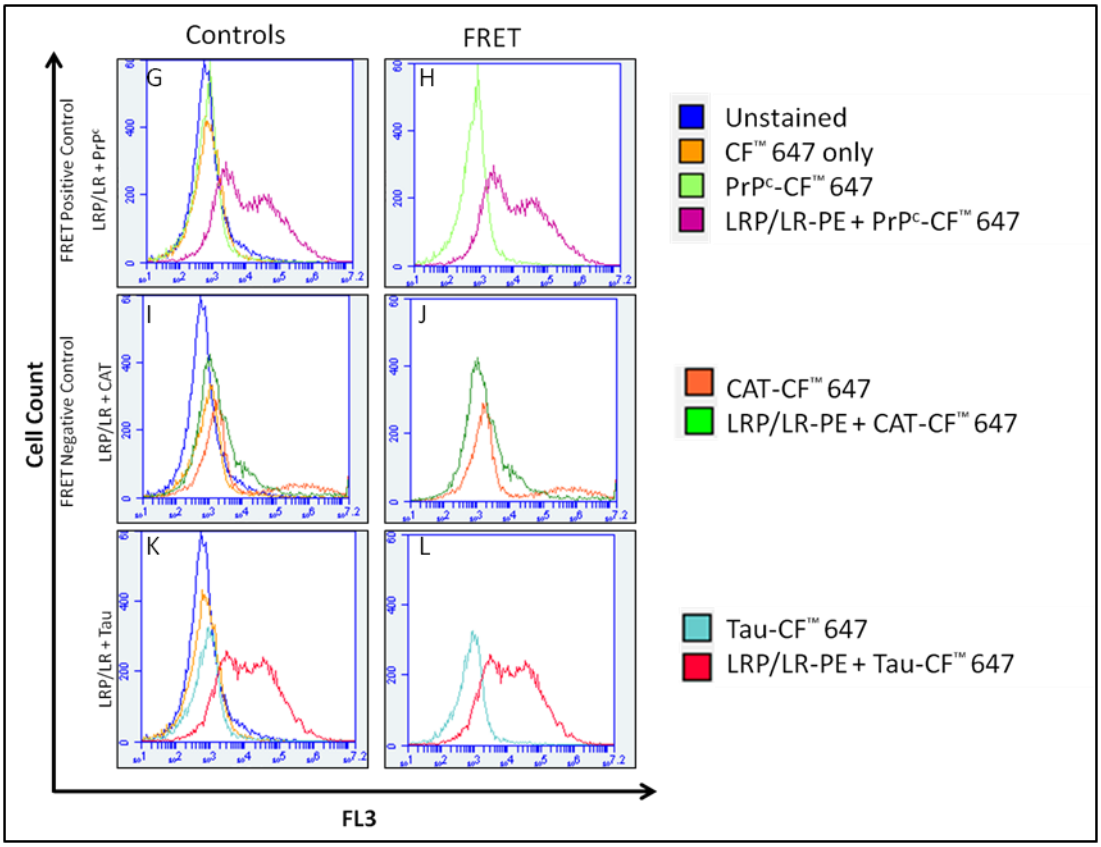


Figure A3: Flow cytometric analysis of FRET between intracellular LRP/LR and tau – Biological replicates. The fluorescence intensity of the unstained HEK-293 cells (dark blue histogram) was superimposed with that of cells labelled with the CF™ 647 secondary antibody only (yellow histogram) as well as with cells in which the proteins of interest; PrP^{C}, CAT and tau, were labelled with CF™ 647 (A, C, E, G, I, K). B, H) Co-labelling of LRP/LR-PE and PrP^{C}-CF™ 647 (positive control) (pink histogram). D, J) Co-labelling of LRP/LR-PE and CAT-CF™ 647 (negative control) (green histogram). F, L) Co-labelling of LRP/LR-PE and Tau-CF™ 647 (red histogram). Each panel is a representative image. Three biological replicates were conducted.

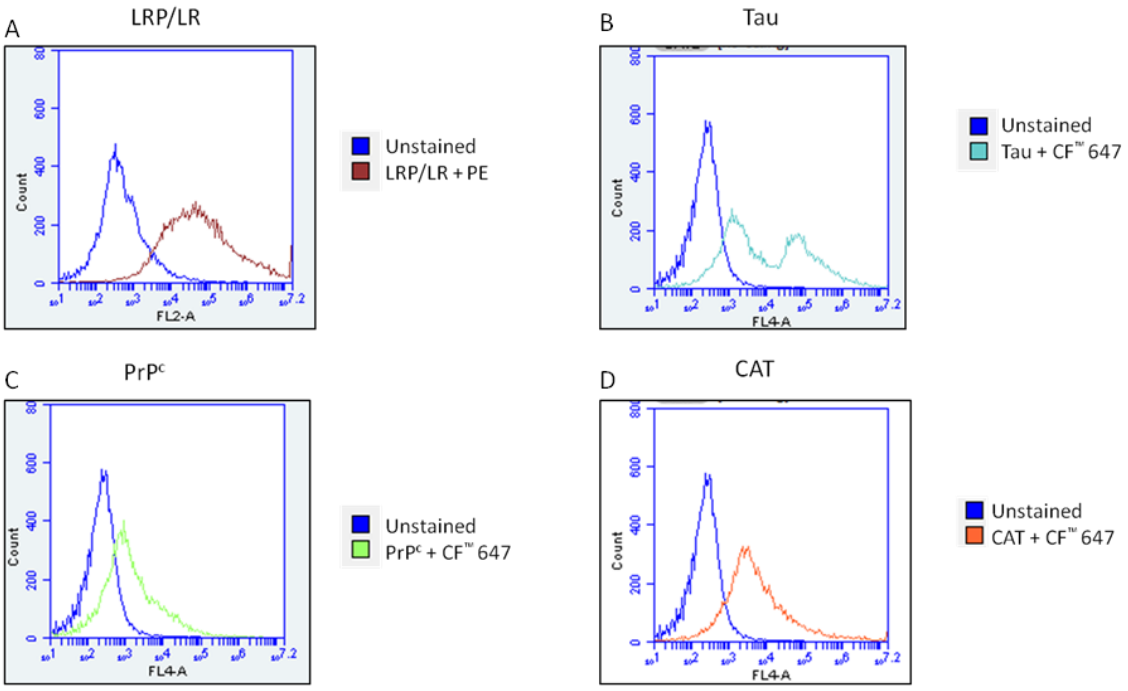
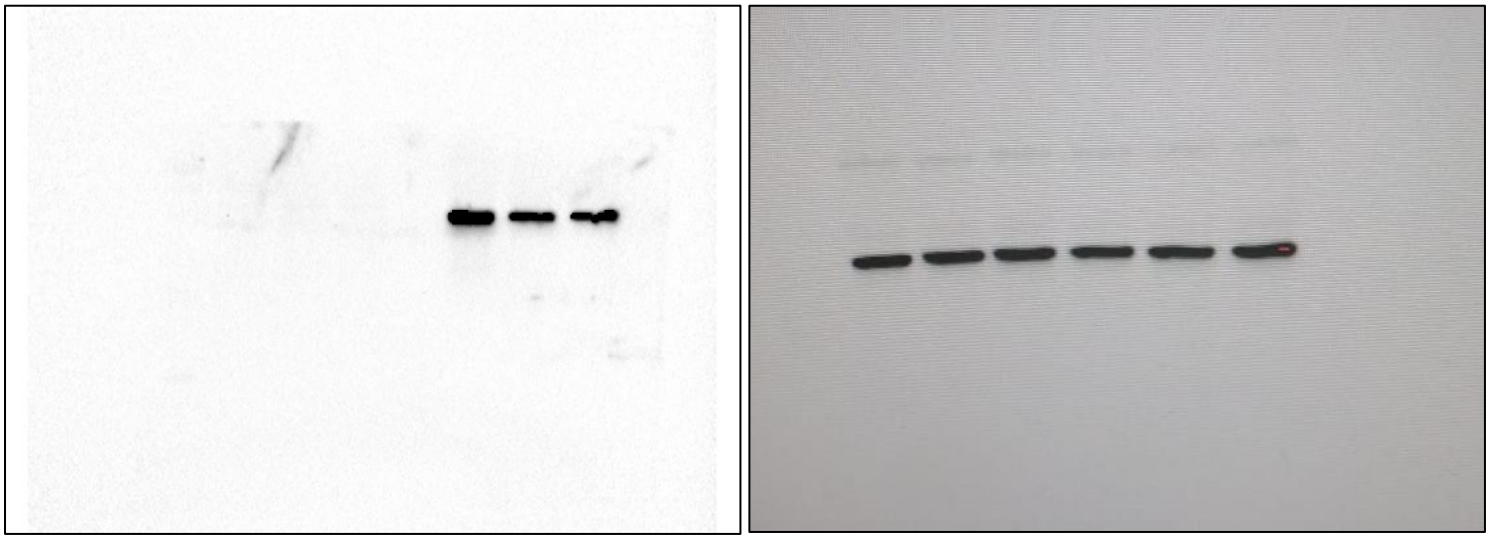


Figure A4: Proteins of interest are successfully labelled during FRET analysis. A) LRP/LR conjugated to PE is successfully detected in the FL2 channel. Tau conjugated to CF™ 647 (B), PrP^{C} conjugated to CF™ 647 (C) and CAT conjugated to CF™ 647 (D) were successfully detected in the FL4 channel.

HEK-293 Cells

FLAG β-Actin



SH-SY5Y Cells

FLAG β-Actin

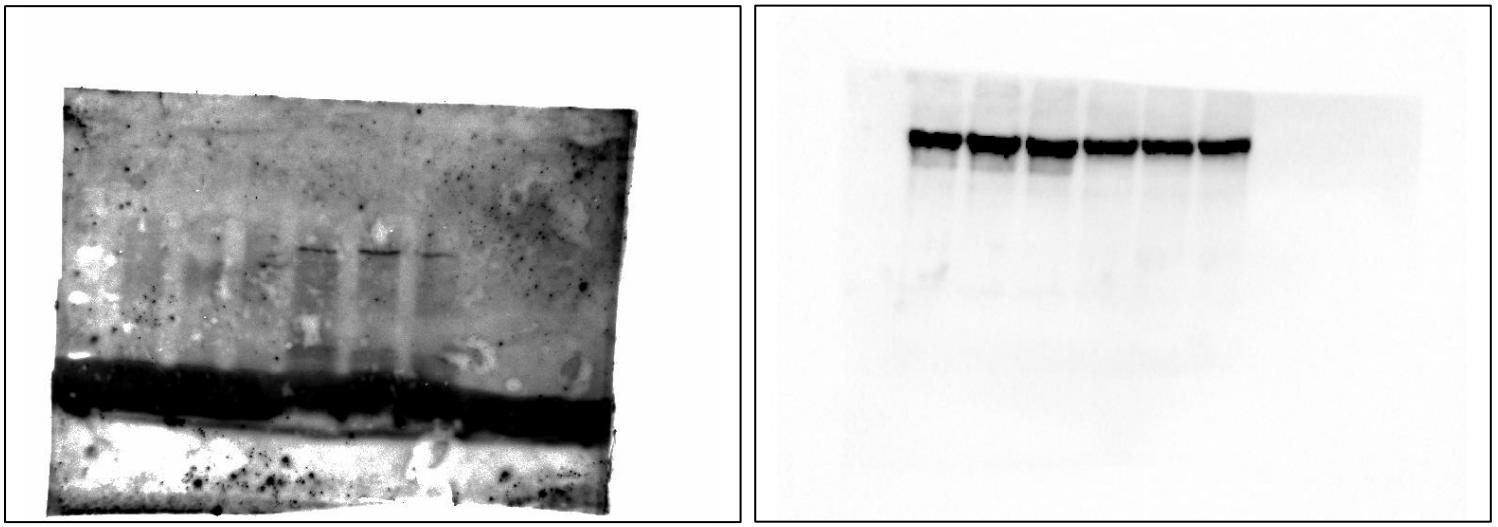
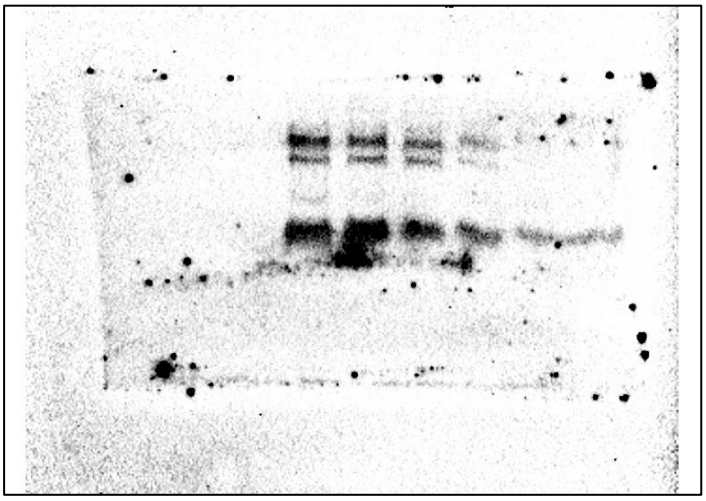


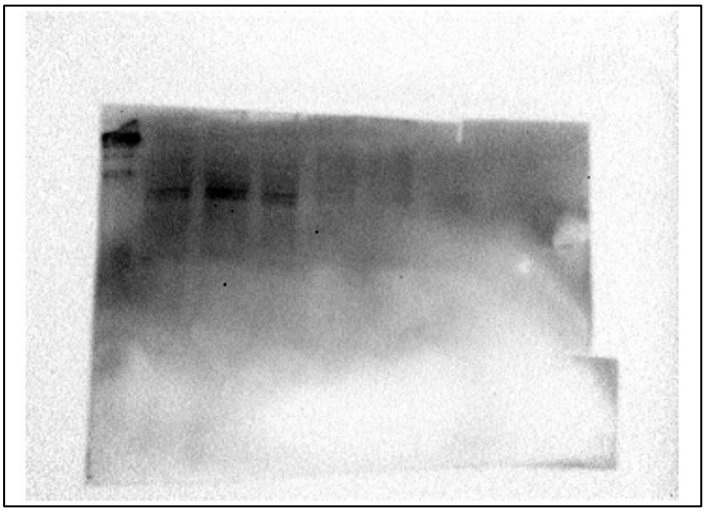
Figure A5: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells transfected with the pCIneo-moLRP-FLAG plasmid – Full Blots. Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells.

HEK-293 Cells

Total Tau



Phosphorylated Tau (pS404)



Phosphorylated Tau (pT231)

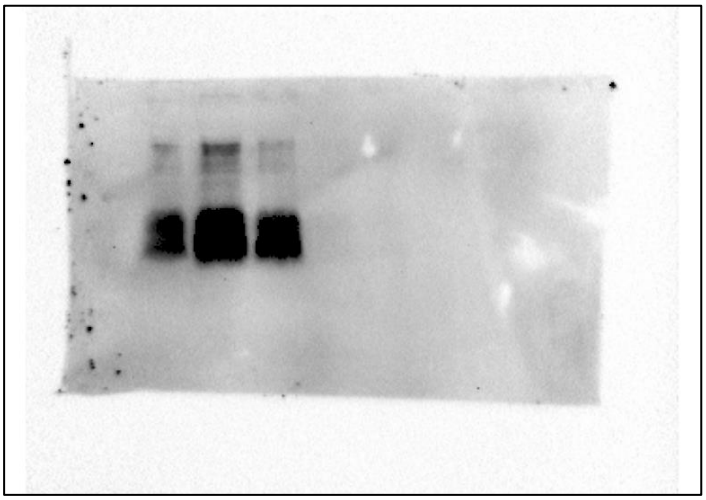
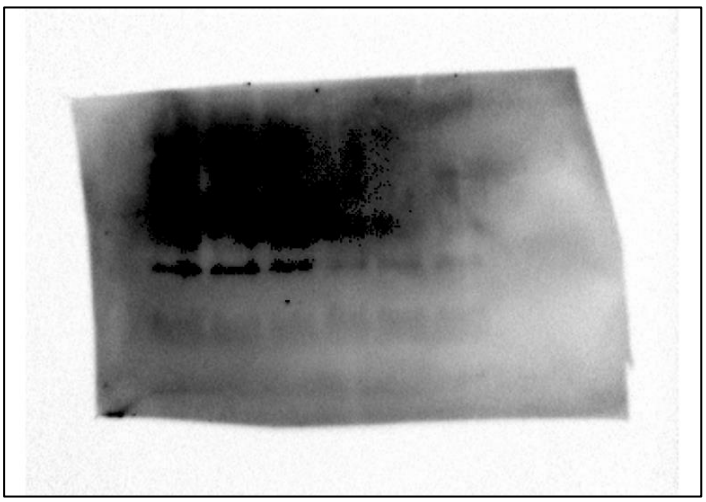


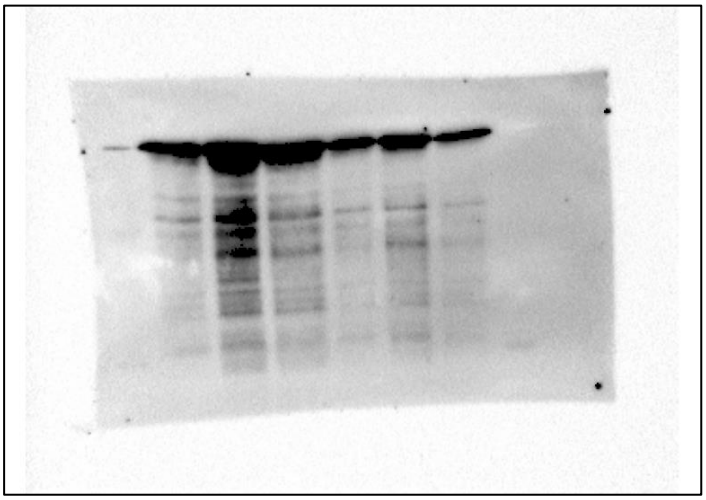
Figure A6: Overexpression of LRP::FLAG in HEK-293 cells decreases levels of total Tau as well phospho-Tau – Full Blots. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells.

SH-SY5Y Cells

Total Tau



Phosphorylated Tau (pS404)



Phosphorylated Tau (pT231)

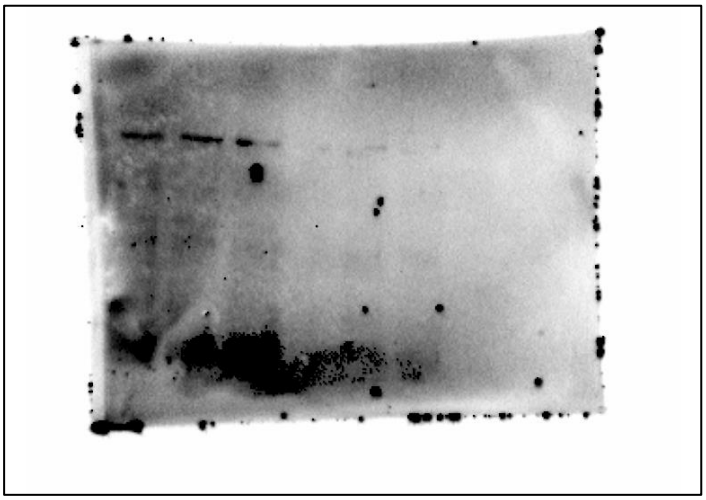


Figure A7: Overexpression of LRP::FLAG in SH-SY5Y cells decreases levels of total Tau as well phospho-Tau – Full Blots. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells.

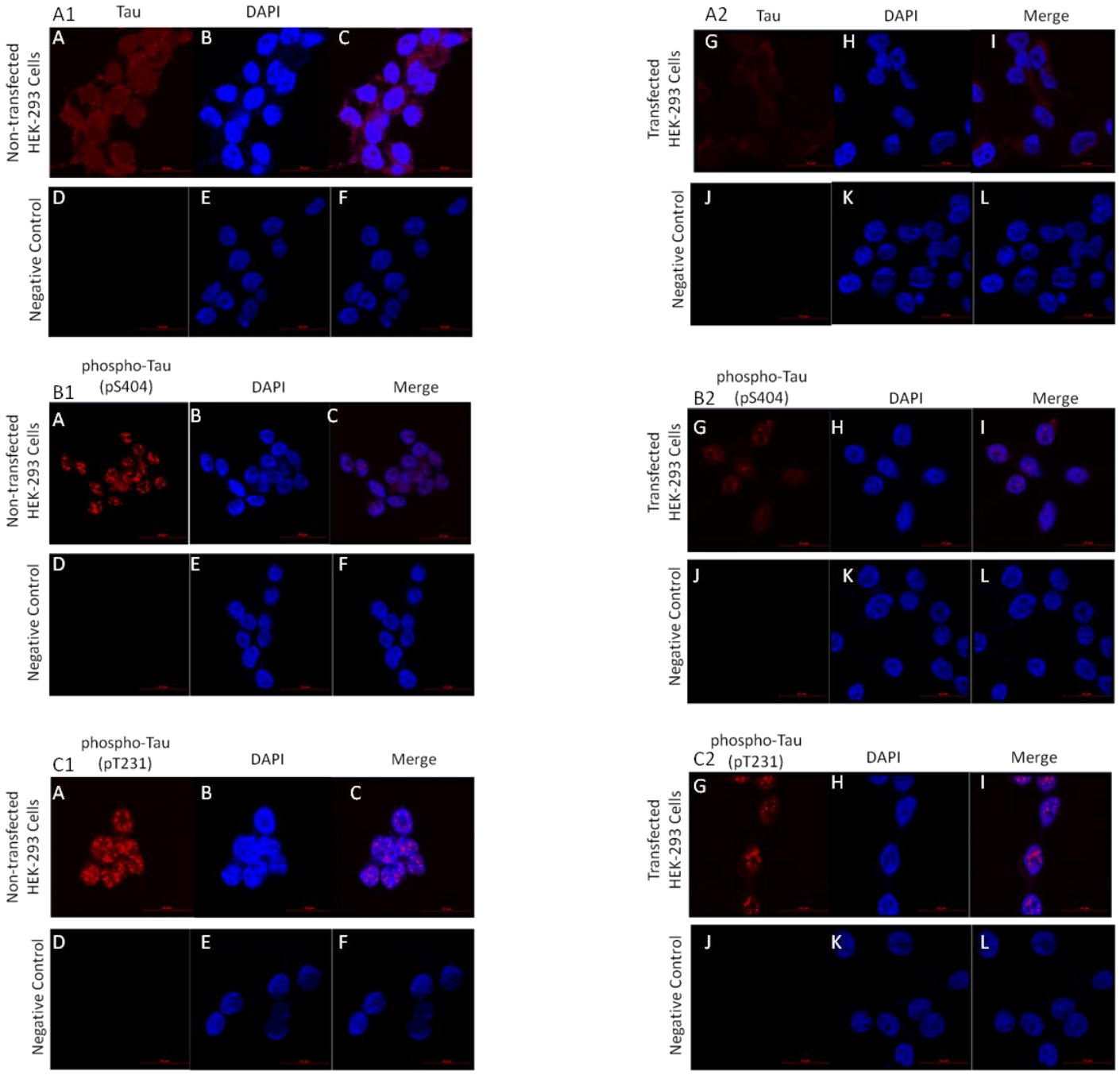
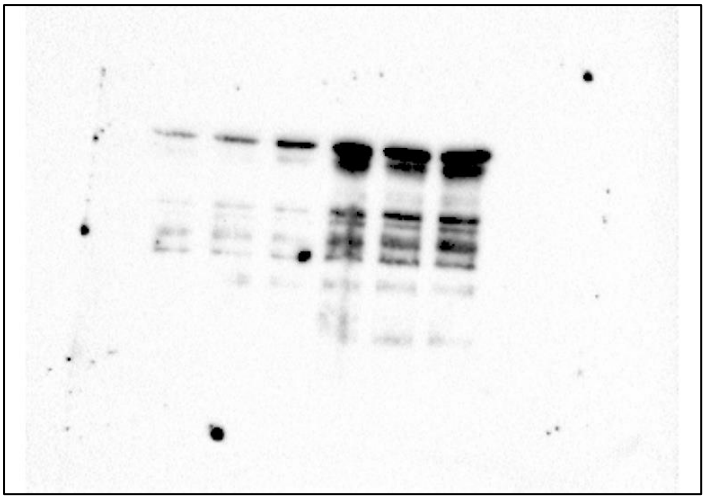


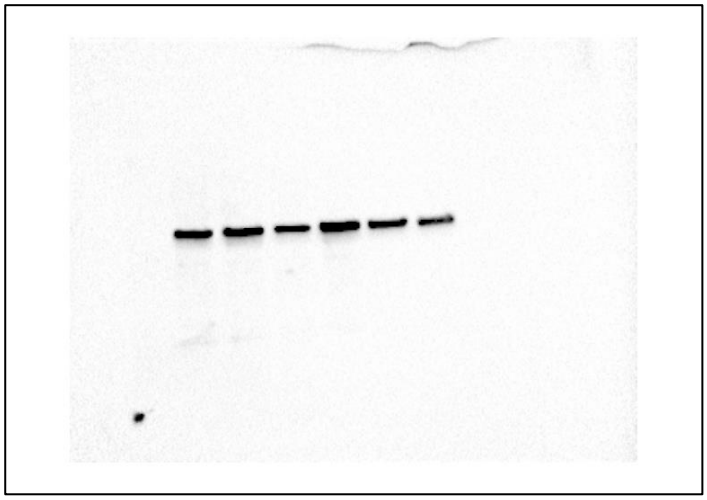
Figure A8: Intracellular expression of Tau and phospho-Tau in transfected and non-transfected HEK-293 cells with negative controls. (A1, A2) A) Endogenous levels of tau. Tau localizes to the cytosol. G) Levels of tau after transfection with pCIneo-moLRP-FLAG plasmid. B, E, H, K) Nuclei are stained with DAPI. C, I) Merge of tau with DAPI staining. D, J) CF 647™ only controls. F, L) Merge of negative control with DAPI staining.
(B1, B2) A) Endogenous levels of phospho-Tau (pS404). phospho-Tau localizes to the nucleus and cytosol. G) Levels of phospho-Tau (pS404) after transfection with pCIneo-moLRP-FLAG plasmid. B, E, H, K) Nuclei are stained with DAPI. C, I) Merge of tau with DAPI staining. D, J) CF 647™ only controls. F, L) Merge of negative control with DAPI staining.
(C1, C2) A) Endogenous levels of phospho-Tau (pT231). phospho-Tau localizes to the nucleus and cytosol. G) Levels of phospho-Tau (pT231) after transfection with pCIneo-moLRP-FLAG plasmid. B, E, H, K) Nuclei are stained with DAPI. C, I) Merge of tau with DAPI staining. D, J) CF 647™ only controls. F, L) Merge of negative control with DAPI staining.
All images were taken with the Zeiss LSM 780 microscope with the addition of Airyscan™ at a 630x magnification. Scale bars represent 20 µm.

HEK-293 Cells

hTERT

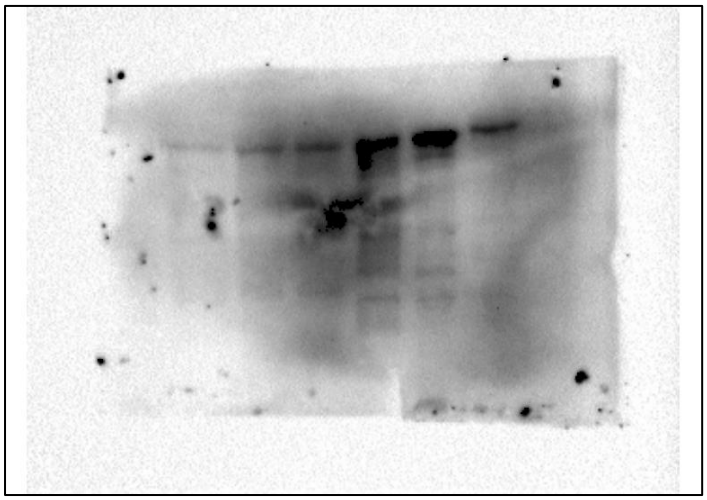


phospho-TERT (pS824)



SH-SY5Y Cells

hTERT



phospho-TERT (pS824)

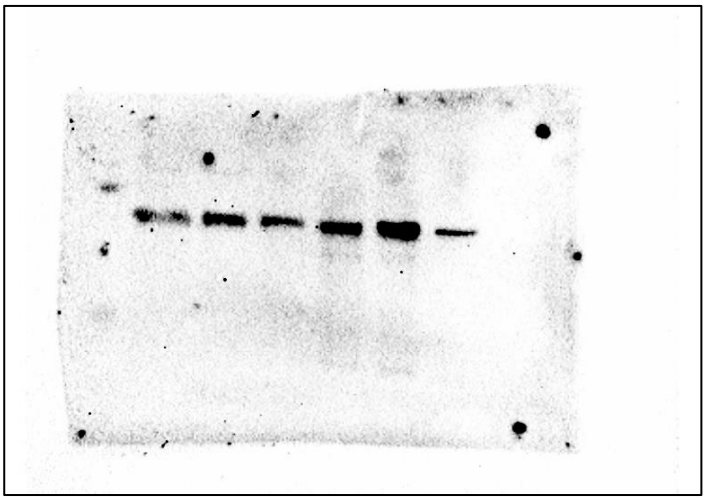


Figure A9: Overexpression of LRP::FLAG increases hTERT levels in HEK-293 and SH-SY5Y cells and phospho-TERT levels in SH-SY5Y cells -Full Blots. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells.

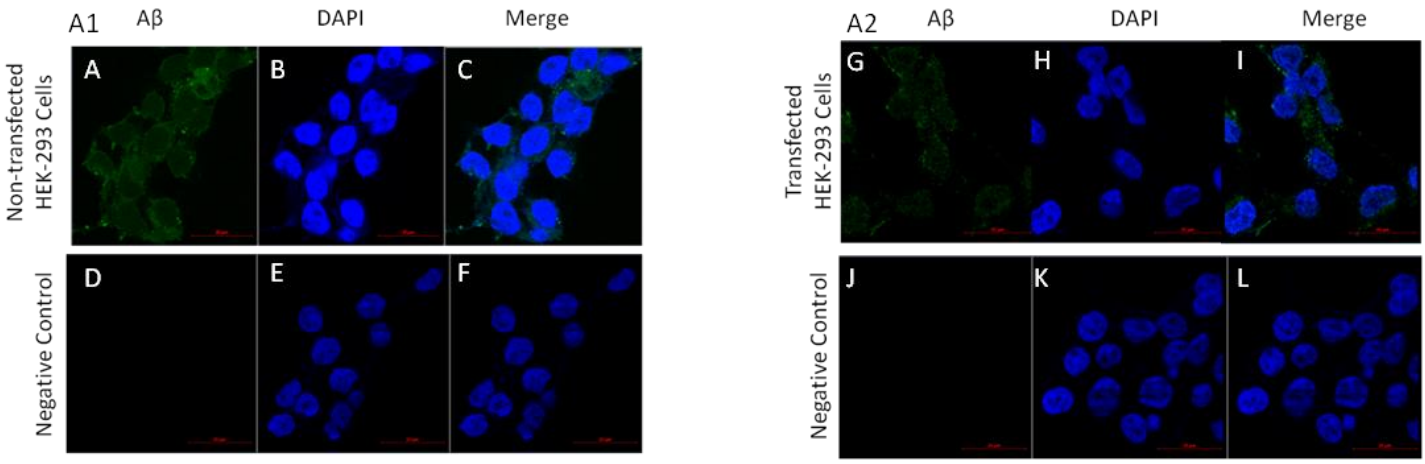
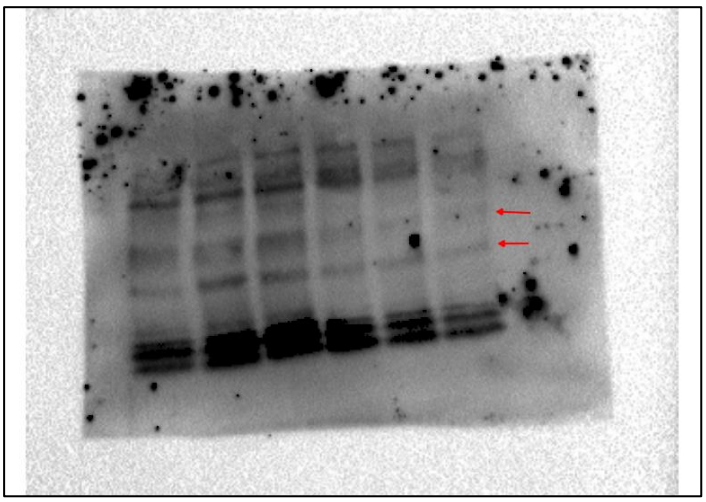


Figure A10: Intracellular expression of $A\beta_{42}$ in transfected and non-transfected HEK-293 cells with negative controls. (A1, A2) A) Endogenous levels of $A\beta_{42}$. $A\beta_{42}$ localizes to the cytosol and cell surface. G) Levels of $A\beta_{42}$ after transfection with pCIneo-moLRP-FLAG plasmid. B, E, H, K) Nuclei are stained with DAPI. C, I) Merge of $A\beta_{42}$ with DAPI staining. D, J) FITC only controls. F, L) Merge of negative control with DAPI staining.

HEK-293 Cells

Total PrP^c



SH-SY5Y Cells

Total PrP^c

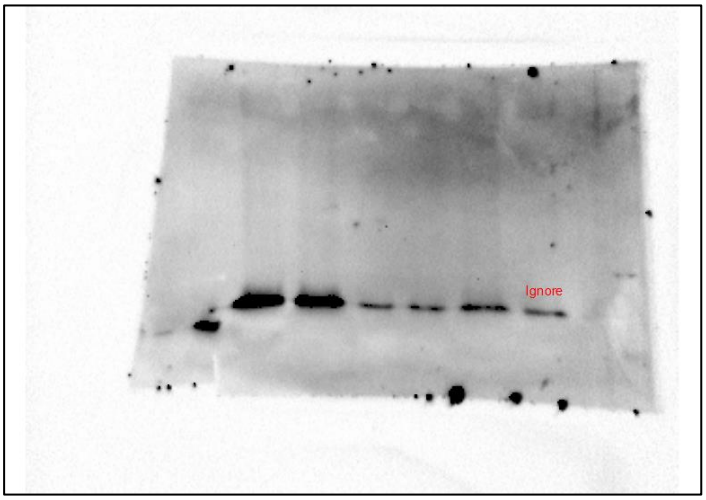


Figure A11: Overexpression of LRP::FLAG in HEK-293 and SH-SY5Y cells decreases PrP^c levels – Full Blots. A1, B1) Lanes 1-3: non-transfected cells. Lanes 4-6: transfected cells. Lane 7: Repeat of transfected SH-SY5Y cells not included in analysis.

Reagents

Table A1: 1X RIPA Buffer

NaCl	150 mM
Triton X-100	1 %
Sodium deoxycholate	0.5 %
SDS	0.1 %
Tris-HCl (pH 8.0)	50 mM
EDTA (pH 8.0)	5 mM

Made up to 100 ml with dH_2O and stored at 4 °C

Table A2: 12% (w/v) SDS-PAGE Gel

Reagent	**12 % Separating Gel**	**5 % Stacking Gel**
30 % Acrylamide/ 0.8 % Bisacrylamide	3.2 ml	0.4 ml
dH_2O	0.8 ml	1.83 ml
2X Tris-HCl/ SDS pH 8.8	4 ml	-
4X Tris-HCl/ SDS pH 6.8	-	0.75 ml
10 % Ammonium Persulfate (APS)	54 µl	30 µl
Tetramethylethylenediamine (TEMED)	10 µl	4.5 µl

Table A3: 30% Acrylamide/ 0.8% Bisacrylamide

Acrylamide	150 g
Bisacrylamide	1.5 g

Made up to 500 ml with dH_2O and stored in the dark at 4 °C

Table A4: 2X Tris-HCl/ SDS pH 8.8

Tris	45.5 g
SDS	1 g

Made up to 500 ml with dH_2O and stored at 4 °C

Table A5: 4X Tris-HCl/ SDS pH 6.8

Tris	30.2 g
SDS	2 g

Made up to 500 ml with dH_2O and stored at 4 °C

Table A6: 1X Electrophoresis Buffer pH 8.8

Tris	3 g
Glycine	14.4 g
SDS	1 g

Made up to 1 l with dH_2O and stored at 4 °C

Table A7: Transfer Buffer pH 8.3

Reagent	**10X**
Tris	30 g
Glycine	144 g

Made up to 1 l with dH_2O and stored at 4 °C

Reagent	**1X**
10X Transfer Buffer	100 ml
Methanol	200 ml
dH_2O	700 ml

Stored at 4 °C

Table A8: 20X Phosphate Buffered Saline (PBS) pH 7.2/7.4

NaCl	160 g
KCl	4 g
Dihydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	28.8 g
Monopotassium phosphate (KH_2PO_4)	4.8 g

Made up to 1 l with dH_2O and stored at room temperature

Cell Line Waiver

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: P V Tobias Building, Room 304, 3rd floor Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-140804-1 04/08/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: **Prof ST Weiss.**

Project title: **Antibodies and shRNA targeting the 37kDa LRP/LR for cancer treatment.**

Reason: This is a laboratory study using commercially available or established cell lines at Wits including USHSY5Y, A375, MDA-MB231, HT-29, LNCaP, WHCO1, HEP G2, HPAC, HUVEC, MCF-7, HeLa and HEK-293 or similar. There are no human participants

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON-JONES
HREC (MEDICAL)
2014 -08- 04
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Anisa Keshav, Zanele Ndlovu.