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2021
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Investigating the possible role of LRP/LR in the pathogenesis of Parkinson's Disease and cancer

By

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DISSERTATION

Submitted in fulfilment of the degree

Master of Science (MSc)

in

BIOCHEMISTRY AND CELL BIOLOGY

in the

FACULTY OF SCIENCE

23rd April 2021

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Co-Supervisor: Prof. Sharon Prince

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Declaration

I, Jessica Burns (1949504), am a student registered for the degree of Master of Science by dissertation (MSc) in the academic year 2019. I hereby declare the following:

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Abstract

Currently, over 10 million people suffer from Parkinson's disease (PD). α-synuclein and parkin are both implicated in PD, where mutations in α-synuclein result in its aggregation. However, the α-synuclein C-terminal domain (CTD) prevents this aggregation. Parkin, an E3 ubiquitin ligase, rescues cells from the effects of α-synuclein aggregation and in PD, mutations in parkin result in its loss-of-function. In addition, parkin has been suggested to be the link between PD and cancer. The 37kDa/67kDa Laminin Receptor Precursor/ high-affinity Laminin Receptor (LRP/LR) is a multifunctional receptor protein that plays a role in the pathogenesis of neurodegenerative disorders as well as cancer. This study aimed to determine the role of LRP/LR in PD and the effect that overexpressing LRP::FLAG has on colorectal cancer. To this end, human embryonic kidney (HEK293) cells and late-stage colorectal cancer cells (DLD-1) were stably transfected with pCIneo-LRP::FLAG and co-localization studies, western blot analysis, MTT cell viability and mitochondrial membrane potential assays were performed. In addition, co-localisation studies were done on neuroblastoma (SH-SY5Y) cells. The results show that LRP/LR and the human telomerase reverse transcriptase (hTERT) co-localise with the PD-related proteins α-synuclein and parkin in SH-SY5Y cells. In addition, LRP/LR and α-synuclein are shown to co-localise in HEK293 non-transfected and LRP::FLAG transfected cells. Furthermore, LRP::FLAG overexpression was shown to increase α-synuclein CTD levels, cell viability and mitochondrial membrane potential in HEK293 cells. Interestingly, LRP::FLAG overexpression in DLD-1 cells decreased cell viability and mitochondrial membrane potential. LRP/LR was also shown to co-localise with parkin in DLD-1 non-transfected and LRP::FLAG transfected cells. However, hTERT and parkin were only found to co-localise in LRP::FLAG transfected cells. Finally, LRP::FLAG was shown to increase parkin levels in DLD-1 cells. In conclusion, this study shows that LRP/LR may play a protective role in PD and that LRP::FLAG overexpression could inhibit the progression of colorectal cancer. Therefore, therapeutic approaches aiming at increasing LRP/LR protein levels may represent a novel alternative for PD and cancer treatment.

Dedication

In loving memory of my grandmothers

Johanna Carolissen

1936 - 2014

Dawn Burns

1942 - 2020

Acknowledgements

I would like to thank Prof. Stefan Weiss, my supervisor, for all the guidance, support and opportunities. Thank you for believing in me and pushing me to be the researcher I am today. I will forever be grateful for being part of your lab and for working with you.

To my co-supervisor Prof Sharon Prince, I would like to thank you for welcoming me into your lab. Thank you for all the advice and time you gave me especially during the last couple of months while I was writing my dissertation. I would like to thank you and all your lab members for all the support, and I hope to have another opportunity to work with you again in the future.

I would also like to give a very big thank you to my parents Claudia Burns and Tony Burns. None of my achievements would be possible without your support and hard work. You have both always encouraged me to be the best possible version of myself and always told me to dream big and to fight for those big dreams. I have learnt so many life lessons from both of you that I will carry till my last day.

To my dear sister Lauren Paioni, you have always been my role model. Thank you for always supporting me and listening to my work stories and for tolerating me when I correct you on scientific topics. Thank you for listening to me when need to vent and for all the advice you give me. Thank you for talking me out of the moments where I just wanted to give up and hide. I would also like to thank Dylan Paioni, my brother, for being so cool when I come over at random times of the day to vent to my sister. Dylan, you have also supported and given me advice throughout my postgraduate studies and for that, I would like to thank you. Of course, I cannot forget my nephew Giorgio Kirk Paioni, you are only 2 but you have been such a blessing. I love building towers for you to knock down and putting out fires in your garden. The fun we have all had together allows me to forget about all the stress even if it is just for a few hours.

To Daniel Pontack, your dedication, hard work and confidence are extremely inspiring. You have supported me from day one with my dreams. Thank you for standing by my side even when I decided to move to another city to carry on with my postgraduate studies. Thank you for being there for me and for pushing me when I was feeling bad about myself or my work. You have helped to get me out of bad spaces just by being present.

To Patronisha Pontack and Brett Pontack, I would like to thank you for your support throughout my studies. Thank you for welcoming me into your home and for all the Sunday lunches and

all the laughs. I do believe your curry and roti was essential to my success. To the rest of the Pontack family, Helena Steenveld and Colin Taylor, I would like to thank you for all the laughs and encouragement. It truly means the world to me.

I would also like to thank my friend Hetal Gopal for her constant encouragement and all the venting sessions. I truly value our friendship and I am undoubtedly blessed to be able to call you my friend.

And last but most definitely not least I would like to thank the Weiss lab members for their help during my project. Thank you for all the conversations and laughs. You have all played a role in helping me become the scientist I am today and for that, I would like to thank you.

Financial aid

Wits Postgraduate Merit Award (2019-2020)

NRF Grant holder-linked Student Support (2019)

NRF Innovations Master's Scholarship (2020)

The South African Medical Research Council (SAMRC) (2019-2020)

Research outputs

Publications:

Original publications:

Vania, L., Morris, G., Otgaar, T.C., Bignoux, M.J., Bernert, M., Burns, J., Gabathuse, A., Singh, E., Ferreira, E., and Weiss, S.F.T. (2019). Patented therapeutic approaches targeting LRP/LR for cancer treatment. *Expert Opinion on Therapeutic Patents,* 29(12):987-1009. http://doi.org/10.1080/13543776.2019.1693543.

Own contributions: assisted with the writing of the manuscript.

Patents:

South African patent PCT/IB20190/60302 Entitled "compounds for use in the treatment of Parkinson's disease" which has been filed in the name of University of the Witwatersrand.https://patentscope.wipo.int/search/en/detail.jsf?docId=WO2020110067&tab=PCTBIBLIO

Inventors: Burns J, Cuttler K, Bignoux MJ, Ferreira E, Weiss SFT

Own contribution: assisted in the design of the study with the help of Dr Eloise Ferreira and Ms Katelyn Cuttler and the generation of data.

Conference attendance

Oral presentations:

Burns, J., Ferreira, E., and Weiss, S.F.T. Investigating the possible role of LRP/LR in the α-synuclein pathogenesis of Parkinson's disease. Life Sciences Imaging Facility Research Day. Johannesburg, South Africa, 24th October 2018.

Burns, J., Prince, S., and Weiss, S.F.T. Investigating the possible role of LRP/LR in the pathogenesis of Parkinson's disease and cancer. UCT HUB-IMBS-Pathology PG research day. Cape Town, South Africa, 26th November 2019.

Poster presentations

Burns, J., Ferreira, E., and Weiss, S.F.T. Investigating the possible role of LRP/LR in the α-synuclein pathogenesis of Parkinson's disease. Poster presentation. Molecular Biosciences Research Thrust (MBRT) Research Day. Johannesburg, South Africa, 29th November 2018.

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List of abbreviations

Aβ	Amyloid-beta
AD	Alzheimer's disease
ALP	Autophagy-lysosomal pathway
APC	Adenomatous polyposis coli
AR-JP	Autosomal recessive juvenile Parkinsonism
BCA	Bicinchoninic acid
BSA	Bovine serum album
CTD	C-terminal domain
CTR	C-terminal ring
DAPI	Diamidino-2-phenylindole
DLD-1	Colorectal adenocarcinoma cell line
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FRET	Fluorescence resonance energy transfer
GBA	Glucocerebrosidase
HEK293	Human embryonic kidney
HSPG	Heparan sulfate proteoglycan
HRP	Horseradish peroxidase

hTERT	Human telomerase reverse transcriptase
hTERC	Human telomerase RNA component
HUVE	Human umbilical vein endothelial
IBR	In between RING domain
IGF	Insulin-like growth factor
JC-1	5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide
LB	Lewy Body
LRP/LR	37kDa Laminin Receptor Precursor/67kDa high-affinity Laminin Receptor
MCC	Mander's co-localisation coefficient
mtDNA	Mitochondrial DNA
MMP	Mitochondrial membrane potential
MPP^+	1-Methyl-4-phenylpyridinium iodide
MPP	Mitochondrial processing peptidase
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	Non-amyloid-β component
NTD	N-terminal domain
PARL	Presenilin-associated rhomboid-like protease
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween-20
PCA	Protocatechuic acid
PCC	Pearson's correlation coefficient
PD	Parkinson's disease

PI4,5P2	Phosphatidylinositol 4,5-bisphosphate
P/S	Penicillin/Streptomycin
PINK1	PTEN-induced putative kinase 1
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH-SY5Y	Neuroblastoma cell line
SN	Substantia nigra pars compacta
SNARE	Soluble NSF attachment protein receptor
SMPD1	Sphingomyelin phosphodiesterase 1
SSA	Sub-Saharan Africa
TOM	Translocase of the outer membrane
TRPV	Transient receptor potential cation channel subfamily V
UbL	Ubiquitin-like domain
UPS	Ubiquitin-proteasome system
WCC	Weighted correlation coefficient

1. Introduction

1.1. Parkinson's disease overview

Parkinson's disease (PD) currently affects 1 % of the population over the age of 60 worldwide and is the most common neurodegenerative disorder affecting motor activity [1]. Due to the scarcity of epidemiological studies on PD in Sub-Saharan Africa (SSA) the prevalence of PD appears relatively low [2]. PD symptoms include slow movement (bradykinesia), rigidity, resting tremor as well as non-motor symptoms including, but not limited to, sleep disturbances and depression [3]. However, due to the lack of knowledge on PD in SSA, these symptoms are often confused as normal ageing symptoms [2].

Due to the complex nature of PD, no disease-modifying treatments are available and thus only palliative treatments are administered, the most common and effective being levodopa [4]. However, levodopa is associated with several adverse side effects including dyskinesia, confusion, hallucinations, and sleep disturbance [4]. Other palliative treatment options that are used for the treatment of PD-motor related symptoms include but are not limited to anti-cholinergic, amantadine as well as dopamine agonists [4]. Furthermore, non-pharmacological palliative treatments include functional stereotaxic neurosurgery, dietary alterations as well as physiotherapy [5]. The risk of developing PD has been shown to be lowered by anti-inflammatory drugs as inflammation is a hallmark of PD [6]. Therefore, non-palliative disease-modifying therapies with little to no side effects are required for the treatment of PD.

1.2. Parkinson's disease pathology

PD pathology can mainly be described by the degradation of dopamine-producing neurons in the substantia nigra (SN) (**Fig 1**) which results in the motor and sleep disturbances seen in PD [7]. The exact cause of dopaminergic neuron degeneration is thought to be caused by neuroinflammation, oxidative stress, mitochondrial dysfunction, and genetic mutations, however, due to the complexity of the disease, it is not fully understood [8]. Another key pathological hallmark of PD is the presence of Lewy bodies (LB) (**Fig 2**) which are intraneuronal inclusions that comprise over 90 different proteins with α-synuclein being the main component [9]. Since LBs are present in all PD patients they are used as a biomarker for neuronal degeneration [10].

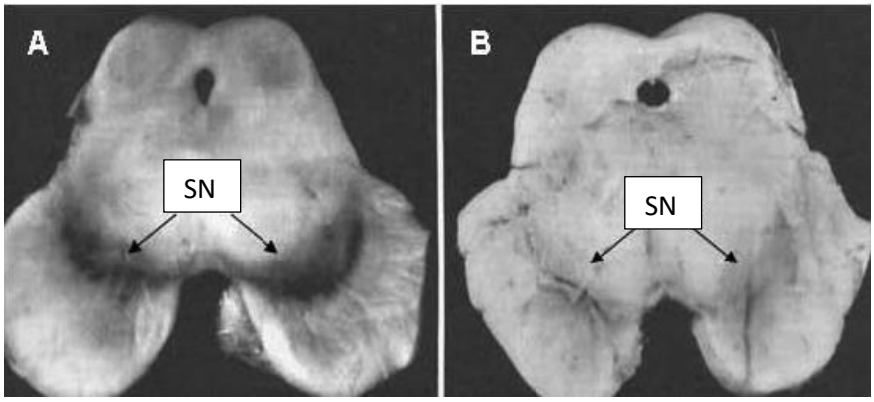

Figure 1: Substantia nigra pars compacta (SN) in healthy and PD patients. SN of a healthy patient (A) and SN of patient with PD (B) where depigmentation is evident indicating advanced PD [216].

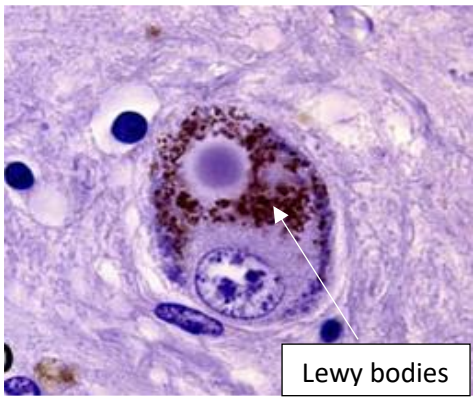

Figure 2: Intraneuronal Lewy bodies present in PD patient [214]**.**

Although over 90% of PD cases occur sporadically, genetics can play a role in PD [11]. Indeed, mutations in, among others, the *SNCA* and *PARK2* genes which encode α-synuclein, the main component of LBs, and parkin respectively cause familial cases of PD [12]. Mutations in *PARK2* result in the loss-of-function of parkin, an E3 ubiquitin ligase responsible for the degradation of proteins [13]. One such protein is the O-glycosylated form of α-synuclein, which forms toxic aggregates [13] and studies have shown that overexpression of parkin prevents α-synuclein induced cytotoxicity [14].

1.2.1. α-synuclein in Parkinson's disease

As the name suggests, α-synuclein is predominantly located in the presynaptic terminals and the nuclear envelope within neuronal cells [15]. It is composed of 140 amino acid residues and is made of three main domains two of which plays an important role in PD (**Fig 3**). The N-terminal domain (NTD) (residues 1-60) contains KTK lipid-binding repeats that adopt a helical structure in the presence of lipids [16]. The non-amyloid-β component (NAC) (residues 61-95)

is responsible for the aggregation of α-synuclein as this region has been shown to change from a randomly coiled structure to a highly hydrophilic structure that is prone to aggregation [17]. Finally, the highly negatively charged C-terminal domain (CTD) (residue 95-140) plays an important anti-aggregation role by interacting with the NAC domain [18]. Studies suggest that α-synuclein plays role in neurotransmission by interacting with soluble NSF attachment protein receptor (SNARE) complexes located at the synapses of neurons [19]. SNARE complexes are involved in neurotransmission and regulate the release of dopamine [19]. Indeed, it was reported that mice overexpressing either full length or truncated α-synuclein show a redistribution of SNARE proteins at the synapse which leads to a decrease in exocytosis and dopamine release [19]. Another study showed that the CTD of α-synuclein binds synaptobrevin, a protein that plays an important role in the assembly of the SNARE complex [20]. Simultaneously, the NTD of α-synuclein binds phospholipids to promote SNARE complex assembly [20]. A more recent study has shown that α-synuclein also plays a role in the elongation of main axons as well as branches generated from the main axon, enhancing axonal arborization [21]. This study also shows that α-synuclein is able to regulate membrane levels of phosphatidylinositol 4,5-bisphosphate (PI4,5P2) allowing it to enhance axonal outgrowth [21]. Furthermore, a recent study demonstrated that α-synuclein and S129-phospho-synuclein bind DNA and could be involved in DNA damage repair [22]. Indeed, this study demonstrated that α-synuclein knockout mice had increased DNA double-stranded breaks that were reversed by the reintroduction of α-synuclein [22]. The authors suggest that α-synuclein aggregation in PD results in a decrease in nuclear α-synuclein levels, and thus increased double-stranded breaks leading to the activation of programmed cell death [22].

In PD, mutations in and or duplications of *SNCA* result in the formation of α-synuclein fibrils [23]. Increased levels of phosphorylated α-synuclein have also been detected in the brains of PD patients [24]. Indeed, phosphorylation of α-synuclein has been shown to be associated with its accelerated aggregation [25,26]. Furthermore, in PD mutations in lysosomal hydrolases such as sphingomyelin phosphodiesterase 1 (*SMPD1*) and glucocerebrosidase (GBA) result in dysfunctional lysosomal clearing and thus aggregation of α-synuclein [27-29]. In addition, variants of the *TMEM175* gene, which encodes the transmembrane endolysosomal potassium channel that regulates lysosomal function, have also been associated with α-synuclein accumulation as well as an increased risk for PD [30]. The ubiquitin-proteasome system (UPS) together with the autophagy-lysosomal pathway (ALP) also play an important role α-synuclein

degradation, therefore dysregulation of these systems results in the aggregation of α-synuclein [24]. Taken together, the above information highlights α-synuclein as an important target in PD research not only for a better understanding of the disease but also for therapeutic options.

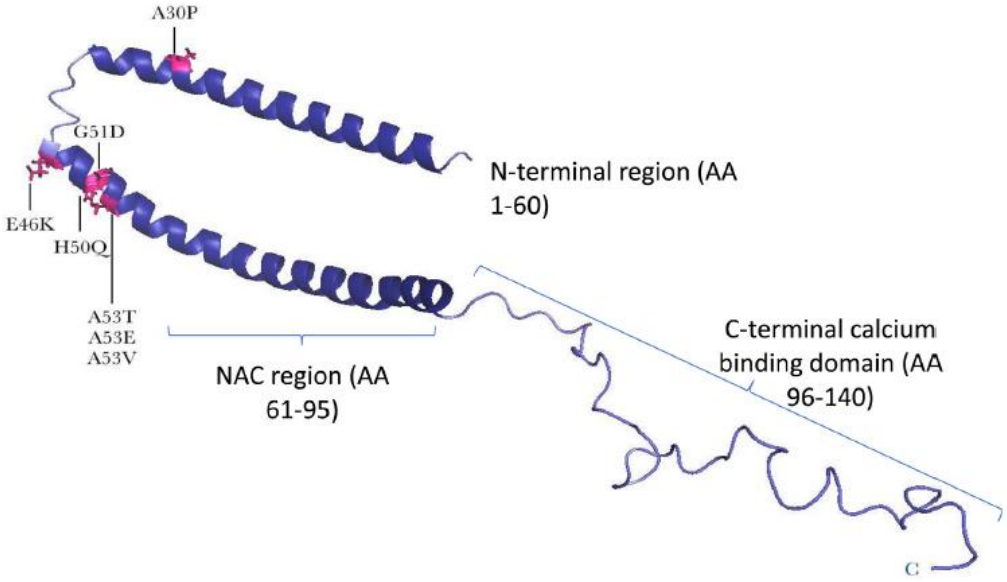

Figure 3: Structure of a-synuclein indicating location of the three main domains. Mutations commonly associated with PD are depicted in pink [215].

1.2.2. Parkin in Parkinson's disease

Another protein implicated in PD is the 52kDa E3 ubiquitin ligase, parkin. It plays a role in the degradation of proteins [31] and is made up of three core domains. At the NTD of parkin lies a ubiquitin-like (UbL) domain (**Fig 4**) [32]. The UbL domain is followed by the RING0, RING1, in between ring (IBR), repressor element of parkin and finally the RING2 located at the CTD of parkin [32,33]. Parkin has many neuroprotective roles including protection against dopamine cytotoxicity and manganese-induced cell death [34]. However, parkin is known more for its involvement in mitochondrial processes. By ubiquitinating mitochondrial associated proteins, parkin can inhibit fusion while promoting mitochondrial fission, biogenesis, transport as well as mitochondrial-endoplasmic reticulum interactions. Furthermore, parkin acts together with the phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1) protein to regulate mitochondrial integrity by promoting mitophagy [35]. PINK1 is located at the mitochondrial surface where it detects mitochondrial dysfunction and recruit's parkin from the cytoplasm to the mitochondria [35]. Following translocation from the cytoplasm to the mitochondria, parkin will induce mitophagy [36]. In addition, parkin also plays a role in cellular

signalling processes by activating the NF-KB pathway under stress conditions [37] . Parkin has also been shown to be important for maintaining the mTOR complex 1 during mitochondrial stress [38].

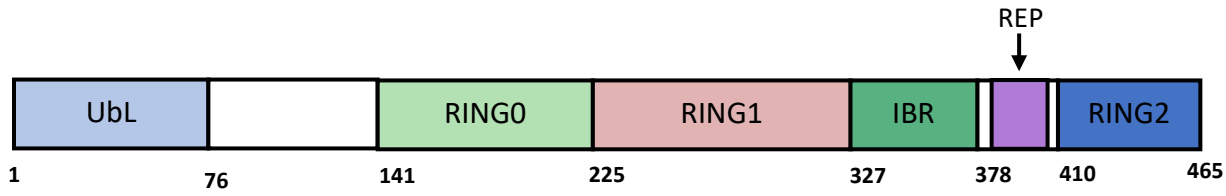

Figure 4: 2D structure of parkin colour coded highlighting the different domains [31].

Furthermore, autosomal recessive juvenile Parkinsonism (AR-JP) is mainly caused by the loss of function of the protein as a result of mutations in *PARK2* [39]. Although parkin ubiquitinates the outer mitochondrial membrane to induce mitophagy, it is also involved in the ubiquitination of cytosolic proteins such as O-glycosylated forms of α-synuclein [13,40]. Therefore, mutations that lead to the loss of function of parkin are also associated with the aggregation of α-synuclein [13]. Moreover, overexpression of parkin has been shown to rescue cells from the cytotoxicity of α-synuclein [41]. Therefore, parkin is an important protein that plays several neuroprotective roles two of which are the regulation of dysfunctional mitochondria as well as preventing α-synuclein aggregation.

1.3. Mitochondrial dysfunction in Parkinson's disease

One of the main contributing factors of dopaminergic neuronal death in the SN is mitochondrial dysfunction [42]. It was first linked to PD after drug abusers using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) went on to develop Parkinson-like symptoms [43]. Once it has crossed the blood-brain barrier MPTP is converted to 1-methyl-4-phenylpyridinium (MPP^+) which disrupts complex I of the electron transport chain activity resulting in a decrease in the production of ATP and ultimately neurodegeneration in the SN [44]. Another study has shown that post-mortem samples from patients suffering from PD also have a deficiency of both mitochondrial complex I and complex II [45]. Another study found that mitochondrial complex I deficiency was also accompanied by the presence of α-synuclein aggregates [46]. Furthermore, fluctuations in mitochondrial DNA (mtDNA) copy number can be used as an indicator of mitochondrial dysfunction, increased ROS production as well as mitochondrial genome modifications and can, therefore, be used as a biomarker for the early onset of diseases

such as certain cancer types as well as PD [47]. Indeed, a decrease in wild-type mtDNA copy number was seen in patients suffering from PD when compared to healthy patients [48]. A recent study revealed that siRNA-mediated knockdown of parkin resulted in a decrease in mitochondrial turnover [49,50].

Studies have shown that parkin and α-synuclein can be translocated to the mitochondria where they will induce degradation of dysfunctional mitochondria [51]. Mutations in α-synuclein have been associated with the accumulation of mitochondria [52] as well as mitochondrial morphology abnormalities [53]. It has also been proposed that α-synuclein is translocated into the mitochondria where it interacts with complex I to inhibit its activity [54]. A study has also shown that α-synuclein binds to the translocase of the outer membrane 20 (TOM20) receptor and prevents the interaction of TOM20 with TOM22 which impairs the import of mitochondrial proteins [55]. In the same study, blocking the import of mitochondrial proteins led to a reduction in mitochondrial membrane potential (MMP) and mitochondrial respiration as well as an increase in reactive oxygen species (ROS) [55]. In addition, α-synuclein has been shown to interact with the ATP synthase, complex V in the electron transport chain, which, ultimately results in changes in mitochondrial morphology, increased oxidative stress and death of neurons [56]. Transgenic mice overexpressing α-synuclein also show an increase in mitochondrial abnormalities [52]. It is therefore clear that increased levels of α-synuclein plays a significant role in mitochondrial dysfunction. On the other hand, parkin is responsible for the induction of mitophagy and therefore the loss-of-function of parkin leads to deficiencies in mitophagy [57]. Interestingly, dopaminergic neurons in the SN are particularly vulnerable to deficiencies in mitophagy due to the increased bioenergetic stress of these neurons [58]. Moreover, parkin knockout fruit flies have been shown to have increased mitochondrial impairments [59]. In the absence of parkin, a decrease is seen in the capacity of the mitochondrial electron transport chain which, ultimately results in the death of dopaminergic neurons [60]. Furthermore, α-synuclein has also been implicated in mitochondrial network fragmentation and overexpression of parkin is able to rescue the cells from the adverse effects of α-synuclein on mitochondrial dynamics [61].

1.4. Linking Parkinson's disease and cancer

Early epidemiological studies showed that the cancer risk is lower in patients suffering from PD [62,63]. However, recent epidemiological studies have shown that although this is true for

most cancer types, PD patients have a higher risk of developing certain cancer types such as melanoma, thyroid gland cancer, breast cancer as well as brain tumours [64]. At first glance, the mechanisms underpinning PD and cancer appear fundamentally different. For example, whereas cancer involves the uncontrolled growth of cells, PD involves the death of dopaminergic neurons [65]. However, genes that are compromised in PD including α-synuclein and parkin have been suggested to be implicated in the progression of cancer [66]. Overexpression of α-synuclein has been shown to increase proliferation rates as well as the number of S-phase cells through increasing cyclin B levels [67]. On the other hand, parkin has been shown to have a more tumor suppressing role in cancer by increasing the sensitivity of hepatocarcinoma cells to apoptotic inducers [68]. The authors suggested that under cellular stress parkin plays a role in delaying cell cycle progression. Another study showed that re-expressing parkin significantly reduced tumour volumes of lung carcinoma cells, in nude mice [69].

1.5. Colorectal cancer

In 2018, cancer caused around 9.6m deaths worldwide and 18.1m cancer patients were recorded annually [70] and are, therefore, the leading cause of death in the 21st century [70]. The third most common cancer worldwide in terms of incidence rate is colorectal cancer [70]. In terms of mortality colorectal cancer is ranked second with 862 000 deaths recorded in 2018 [71]. In South Africa, colorectal cancer is the fourth most common cancer in terms of incidence rates and the sixth most common in terms of mortality [70].

Cancer displays key hallmarks that can be used for insight into the disease and to develop therapeutics that treat the disease [72]. The main hallmarks include replicative immortality, evasion of growth suppressors, evasion of apoptosis, promotion of proliferation, activation of invasion and metastasis, and induction of angiogenesis [73]. Although these six hallmarks are not the only hallmarks of cancer, they are the most important as well as the most studied and play a vital role in the initiation, promotion, and progression of cancer (**Fig 5**). For example, the highly regulated form of programmed cell death, apoptosis, is initiated by both extracellular and intracellular signals [74]. It is responsible for the elimination of unwanted cells and is crucial in the development and homeostasis [75]. Apoptosis serves as an important barrier to cancer and mutations that result in the inhibition of apoptosis allow cells to thrive and form cancer [75].

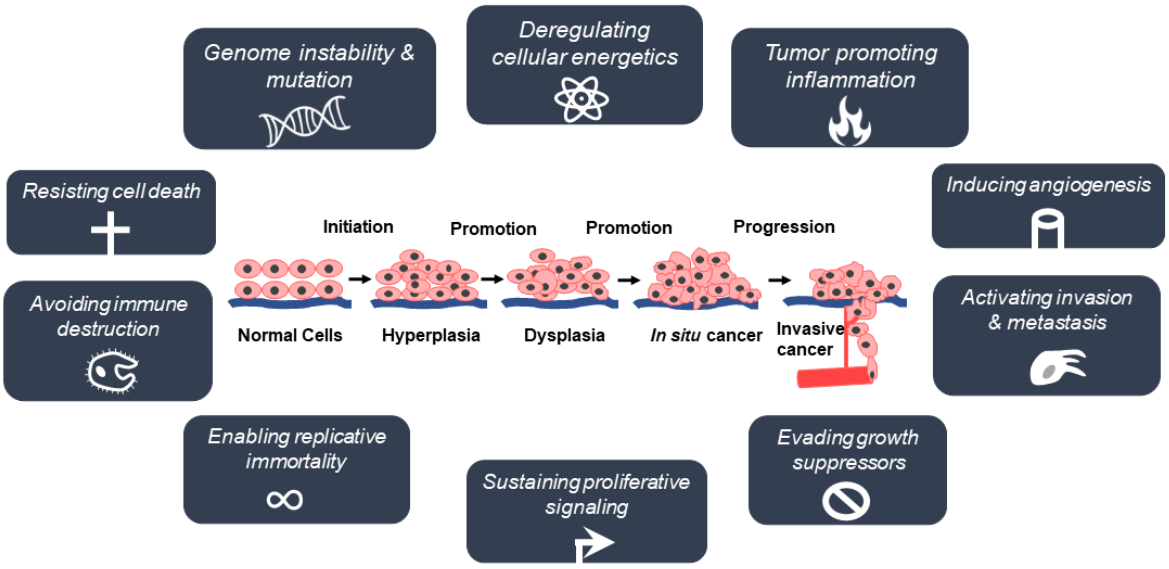

Figure 5: Hallmarks of cancer and stages of cancer. Hallmarks displayed by cancers that aid in the initiation, promotion, and progression of cancer [65].

1.5.1. Parkin in cancer and colorectal cancer

Although studies have shown that the risk for cancer is higher in some cancer types, colorectal cancer falls part of the group of cancers that displayed a lower risk in PD patients. A nested case-control study showed an inverse relationship between PD and colorectal cancer [76]. Furthermore, a meta-analysis performed on 11 cohort studies and 2 case-control studies revealed a 21% decrease in the risk of developing colorectal cancer in PD patients in the western population [77]. However, recent evidence suggests that the PD-related protein parkin plays an important role as a tumour suppressor. For example, gene alterations and expression differences in parkin have been detected in several cancers including colorectal cancer [78]. Indeed, studies have shown that the overexpression of parkin in several cancer cell lines including breast cancer, colon cancer and glioblastoma inhibits cell growth whereas knockdown of parkin promotes tumorigenic properties such as cell growth in pancreatic cancer *in vitro and in vivo* [78]. The tumour suppressor role of parkin is thought to occur through its ability to regulate the cell cycle because the overexpression of parkin correlated with an increase in cyclin-dependent kinase 6 and a decrease in breast cancer cell proliferation, and a decrease in cyclin E and proliferation in glioblastomas, lung, and colorectal cancers [79,80]. Parkin has also been shown to induce apoptosis by causing the depolarisation of mitochondria followed by the ubiquitination of the anti-apoptotic protein Mcl-1 [81]. Moreover, studies have

shown that parkin plays a role in other hallmarks of cancer including metastasis, metabolic reprogramming, angiogenesis, and tumor-promoting inflammation among others [82,83].

Parkin has been found to play a significant tumour suppressor role in colorectal cancer. For example, parkin expression levels are lower in localized colorectal tumours when compared to adjacent tissue in patients suffering from colorectal cancer [84]. Furthermore, increased expression of parkin in advanced colorectal cancers correlated with an increased chance of survival of patients [84] and decreased parkin expression is associated with the deficiency of adenomatous polyposis coli (APC) resulting in the progression of tumours [85]. A cascade of events caused by the loss of the APC gene has been found to cause malignant transformation in the large bowel [86]. APC is a tumour suppressor and plays multiple functions by participating in cell adhesion and migration, chromosome segregation, microtubule assembly and signal transduction. Its more well-known tumour suppressor role is to regulate β-catenin, blocking the Wnt signalling pathway from increasing cell-cycle related proteins that promote carcinogenesis [87-89]. It has been suggested that initial APC suppression influences parkin expression and although a study has demonstrated a correlation between APC and parkin expression they could not confirm the mechanism of this correlation [84]. Another study has shown that increased polyp multiplicity and progression of intestinal adenoma development was also observed in *PARK2* heterozygous knockout and APC^{min} interbred mice [85].

1.6. LRP/LR

One of the proteins implicated in both cancer and neurodegenerative diseases is the 37kDa/67kDa Laminin Receptor Precursor/ high-affinity Laminin Receptor (LRP/LR). The 67kDa LR share sequence homology with the 37 kDa LRP, it was therefore hypothesized that the 67 kDa LR could arise from homodimerization of the 37 kDa LRP [90]. However, this theory has since been replaced with the idea that the 37kDa isoform undergoes post-translational modifications, such as the addition of heparin sulphates, acetylation or sumoylation, to become the 67kDa LR [91,92]. LRP/LR has several functions with its most well-known function involving its role as a cell surface receptor [93]. In this regard, LRP/LR has a high affinity to the laminin-1 protein and binds with it on the cell surface where it mediates adhesion and migration [92,94]. The ability of LRP/LR to bind to laminin-1 has also been associated with axonal development and guidance, neuronal migration and neuronal development [95,96]. LRP/LR has also been found to bind the prion protein PrP^{c}, the antibody IgG and (heparan sulfate proteoglycans) HSPGs (**Fig 6**). In addition, LRP/LR maintains the

structure of the nucleus through the interaction with histones [97] and is crucial for the maturation of the 40s ribosome [98]. LRP/LR also plays a role in the regulation of cell growth, differentiation, adhesion, and translation [99] and LRP::FLAG overexpression increases telomerase activity [100,101].

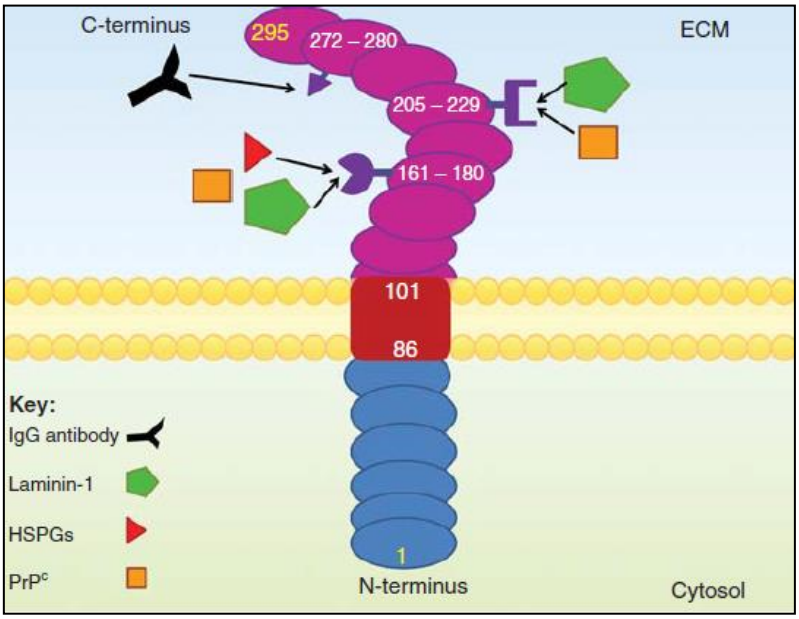

Figure 6: Structure of and molecules interacting with LRP/LR and their respective binding sites [110].

The 67kDa LR was initially discovered in cancers cells [94] and both the 37kDa LRP and 67kDa LR was later found to be implicated in cancer metastasis [102]. It was also shown that surface LRP/LR levels are increased in several cancers including, but not limited to, cancers of the lung, cervix, colon, prostate and pancreas as well as neuroblastoma [102,103]. The increase in LRP/LR levels enhanced invasion, adhesion, tumor angiogenesis, and evasion of apoptosis [102-106]. Studies demonstrate that the interaction between LRP/LR and laminin-1 promotes both invasion and adhesion, while treatment with the anti-LRP antibody significantly reduced invasion and adhesion in several cancer cell types including, but not limited to, lung, colorectal and prostate cancer cells [102,103,106,107]. A recent study also implicated LRP/LR has also been implicated in angiogenesis by mediating cellular attachment and inducing matrix metalloproteases including type IV collagenase which leads to the release of pro-angiogenic factors in human umbilical vein endothelial (HUVE) cells [105]. This study also showed that the anti-LRP antibody, W3, blocked angiogenesis [105]. In addition, LRP/LR has been found

to impede apoptosis and knockdown of LRP/LR using siRNA technology-induced apoptosis by increasing the pro-apoptotic protein, caspase 3 in lung and cervical cancer cells [108]. Another study showed that LRP/LR mediates telomerase activity in breast cancer cell lines which were reduced when LRP/LR was knocked down using siRNA technology [109].

In addition to cancer, LRP/LR plays a vital role in many infections and diseases through its cell surface receptor function [110]. Not only does LRP/LR act as a receptor for viruses and bacteria but it also binds to the non-infectious prion protein PrP^{c} as well as the infectious form of the prion protein PrP^{Sc} aiding in the internalisation of both proteins [111,112]. Scrapie infected mice and hamsters also display increased levels of LRP/LR and blocking LRP/LR using the anti-LRP antibody, W3, significantly reduced PrP^{Sc} propagation [111,113]. LRP/LR plays a similar role in Alzheimer's disease (AD) where it binds amyloid β (Aβ) and aids in its internalisation [114]. Aβ shedding and internalisation has been found to be prevented by the anti-LRP antibody, IgG1-iS18, or downregulating LRP/LR with the use of shRNA [114-116]. AD mouse models treated with the IgG1-iS18 antibody also showed a decrease in both the insoluble and soluble forms of Aβ in the brain as well as a reduction of amyloid plaques in the hippocampus and displayed improved cognitive functions and memory [117]. More recent studies have shown that LRP::FLAG overexpression decreased Aβ-induced cytotoxicity through the upregulation of telomerase activity [100]. Furthermore, LRP/LR was shown to interact with tau and the LRP::FLAG overexpression reduces phosphorylated tau *in vitro* possibly through increased human telomerase reverse transcriptase (hTERT) levels [118].

1.7. Telomerase in diseases

LRP/LR has recently been shown to interact with the telomerase, ribonucleoprotein, that maintains telomere length and thus genomic stability [119]. The RNA-dependent DNA-polymerase reverse transcriptase activity of telomerase is responsible for the adding telomeric repeat, TTAGGG, at the ends of linear chromosomes [120]. Telomerase activity remains high during embryonic development in most tissue types and reaches peak activity at embryonic day 13 after which its activity declines and remains low until day 3 postnatal [121]. During embryonic brain development, telomerase activity remains high until the differentiation from neuronal stem cells to astrocytes and neurons [121]. The telomerase reverse transcriptase (hTERT) and the telomerase RNA component (hTERC)Telomerase are the two key subunits of the telomerase protein complex [122] (**Fig 7**). hTERT in the telomerase protein complex is the rate-limiting factor as telomerase activity has been shown to be dependent on hTERT levels

[123-125]. Interestingly, the two main subunits of telomerase have been found to have vastly different half-lives where hTERC can remain in cells for up to five days, hTERT is only present for up to 24 hours [123,126]. Furthermore, hTERT expression has been shown to be dependent on cell type and tissue microenvironment [127]. While telomerase activity is low in white blood cells and stem cells, high levels of telomerase activity are found in reproductive organs [125].

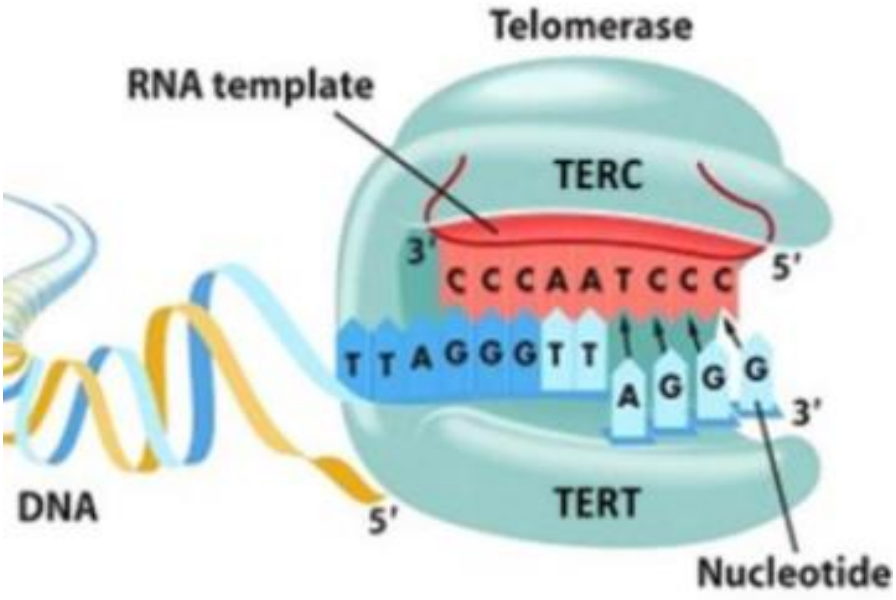

Figure 7: The telomerase protein complexes two main subunits: hTERT and HTERC. Telomerase is responsible for the adding telomeric repeat, TTAGGG, at the ends of linear chromosomes. The hTERC subunit is read by the hTERT subunit which adds the TTAGGG repeats to replenish telomere ends

1.7.1. Extra-telomeric functions of hTERT

Over and above telomere maintenance the hTERT subunit of the telomerase complex has been shown to have extra telomeric roles that include the regulation of cell migration, proliferation, and adhesion [127]. Another example of its extra telomeric roles includes the interaction of hTERT with mtDNA to protect it from bromide-induced damage [128,129]. Furthermore, hTERT also protects mitochondria from ROS and the over expression of hTERT decreases ROS and increases mitochondrial membrane potential (MMP) in human lung fibroblasts [128,129]. The overexpression of TERT has also been shown to improve mitochondrial function and to reduce mtDNA damage [128]. An increase in hTERT also inhibitsp53-dependent apoptosis and has been associated with Bcl-2 overexpression, an anti-apoptotic protein, to inhibit apoptosis [130,131]. Another extra-telomeric function of hTERT involves DNA repair. Indeed, hTERT interacts with the DNA replication-associated protein primase to promote DNA repair [132].

1.7.2. Telomerase and hTERT in cancer

As seen above hTERT plays a role in several cellular processes and its deregulation has been implicated in several diseases including diabetes, cardiovascular diseases, neurodegenerative disorders, and cancer. The role of telomerase activity and hTERT in cancer is well-known, with about 90% of all cancers exhibiting increased telomerase activity [133]. This increase in telomerase activity in several cancer types including melanoma, bladder, thyroid, and glioblastomas results in part from mutations in the TERT promoter that increase telomerase expression [134-136]. These TERT promotor mutations have also been associated with increased invasive potential in thyroid cancer, therefore, highlighting the role of hTERT in cancer [135]. Functionally, an increase in telomerase activity results in the extension of telomeres, preventing cells from entering senescence an important barrier to cancer [127]. Furthermore, telomere dysfunction and increased telomerase activity are associated with resistance to chemotherapy and cancer recurrence [137]. hTERT has also been shown to play a role in cancer progression independent of its telomerase activity by regulating apoptosis [138]. Indeed, studies have shown that overexpression of hTERT makes cells more resistant to apoptosis and siRNA mediated knockout of hTERT promotes the mitochondrial cell death pathway by activating the proapoptotic protein Bax [139-141].

The effects of telomerase activity and hTERT in cancer have been shown to be exacerbated by LRP/LR. Indeed, studies have shown that LRP/LR and hTERT interact in the perinuclear compartment and on the cell surface of breast cancer cells and telomerase activity is enhanced by LRP/LR [100,101,109]. Furthermore, siRNA mediated LRP/LR knockdown results in decreased telomerase activity [109]. Taken together, increased telomerase activity promotes the survival of cancer cells and is associated with chemotherapy resistance and an increased risk for tumour recurrence in cancer. Therefore, targeting LRP/LR in cancer could lead to a decrease in telomerase activity and thus decreased aggressiveness of cancer as well as sensitising cancer to chemotherapy.

1.7.3 Telomerase and hTERT in neurodegenerative diseases

Recent studies have shown that in neurodegenerative disorders including Alzheimer's disease (AD), increased telomerase activity plays a neuroprotective role. Indeed, the telomere length in neuronal cells is shorter in AD patients in comparison to control patients [142,143]. This has been attributed to the decrease in telomerase activity caused by Aβ oligomers binding to the DNA-RNA template complex [142]. Several studies have demonstrated a neuroprotective role

for hTERT in AD. Indeed, the overexpression of hTERT was also able to rescue cells from Aβ-induced apoptosis and protect cells from Aβ plaques [100,117,118,144]. hTERT has also been shown to protect neurons from tau-induced oxidative stress [145]. More recently, investigators have taken advantage of the relationship between LRP/LR and hTERT and used LRP/LR as a target to increase telomerase activity and hTERT in AD. Recent studies have shown that LRP::FLAG overexpression increases hTERT levels and as a result telomerase activity in a non-oncogenic fashion and rescued cells from Aβ-mediated cytotoxicity [100,101]. Taken together, hTERT and telomerase activity plays a crucial role in the protection against AD and although telomere shortening has not been observed in PD patients [146], hTERT could still play a role in PD through its mitochondrial extra-telomeric functions. This increase in telomerase activity prevents senescence pathways and promotes proliferation indicating that hTERT plays a vital role in anti-ageing [101].

1.8. Scope

LRP/LR is implicated in neurodegenerative diseases like prion disorder and AD but its possible role in PD has not yet been investigated. Indeed, studies have shown that LRP/LR is a receptor for both PrP^{c} and the infectious PrP^{Sc} aiding in the internalisation of both proteins and that these interactions can be prevented by the anti-LR antibody W3 [147]. Furthermore, LRP/LR also aids in the internalisation of Aβ which can be prevented by the anti-LRP antibody IgG1-iS18 [114-116]. Several studies have shown that α-synuclein aggregates can be exported to extracellular spaces and then be taken up by neighbouring cells, therefore, spreading like prion-like aggregates [148-153]. Given that LRP/LR has been found to not only act as a receptor for the infectious prion protein PrP^{Sc} [111] but also Aβ [114], LRP/LR could, therefore, act as a receptor for α-synuclein. Moreover, overexpression of LRP::FLAG was able to protect cells from the effects of amyloid plaques as well as hyperphosphorylated tau by increasing hTERT levels [100,117,118]. Importantly, LRP/LR is overexpressed in several cancers and plays a vital role in tumour progression [102,103,105,107,154]. In addition, blocking LRP/LR using the IgG1-iS18 antibody or knockdown of LRP/LR using siRNA was able to prevent tumour progression [102,103,105,107]. These studies provide evidence that LRP/LR, like parkin, plays opposing roles in neurodegenerative disorders and cancer.

This study determines the effects of overexpressing LRP::FLAG on the pathogenesis of PD and colorectal cancer. It provides insight into the possible role that LRP/LR plays in both these diseases and has important implications for improved therapeutics for PD and cancer.

2. Aims and objectives

2.1. Aim 1 and objectives: To assess whether LRP/LR plays a role in Parkinson's Disease

2.1.1 To overexpress LRP::FLAG in human embryonic kidney (HEK293) cells

2.1.2 To determine whether LRP/LR, α-synuclein, parkin and hTERT co-localise in HEK293 and neuroblastoma (SY-SY5Y) cells

2.1.3 To determine whether LRP::FLAG overexpression affects α-synuclein levels in HEK293 cells

2.1.4 To investigate the effect of overexpression of LRP::FLAG on cell viability and mitochondrial dysfunction in MPP+ treated HEK293 cells

2.2. Aim 2 and objectives: To assess the effect of LRP::FLAG overexpression on colorectal cancer

2.2.1 To overexpress LRP::FLAG in colorectal adenocarcinoma (DLD-1) cells

2.2.2 To determine whether LRP/LR, hTERT and parkin co-localise in DLD-1 cells

2.2.3 To assess the effects of overexpression of LRP::FLAG on cell viability and mitochondrial dysfunction in DLD-1 cells

3. Methods

3.1. Cell culture

Cell lines that are commonly used for PD research and that were used for this study are the Human Embryonic Kidney (HEK293) and the human neuroblastoma (SH-SY5Y) cell line [155]. The SH-SY5Y cells have been and are to this day extensively used in PD research as they produce and release catecholamines [156]. However, these cells can be difficult to maintain, therefore, HEK293 were used as they are easy to transfect and are also commonly used in PD research [156]. The HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hyclone), while the SH-SY5Y cells were cultured in a mixture of DMEM and Ham's F12 (1:1). The colorectal adenocarcinoma (DLD-1) cell line has used the effect of overexpressing LRP::FLAG in colorectal cancer and was cultured in RPMI. All media was

supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) that provided the cells with essential nutrients, including growth factors as well as to prevent bacterial and fungal growth, respectively. All cells were cultured in T75 flasks and were grown in a humidified incubator at 37˚C and 5% CO_2 that provided conditions that mimic *in vivo* conditions. Media was replaced as needed until cells reached 70-80% confluency, where they were passaged. Cells were passaged by washing cells with 5ml of 1X phosphate-buffered saline (PBS) (**Table S7**) followed by the addition of 2ml of Trypsin-EDTA for 2-5 minutes until all cells were detached from the flask. Once all cells were detached 8mls of cell culture media was added to deactivate the trypsin-EDTA. Thereafter, cells were harvested for further downstream experiments.

3.2. Cell Transfections

To determine the effect of LRP/LR in PD cells were transfected to overexpress LRP::FLAG. In addition, to determine the effect overexpressing LRP/LR would have on colorectal cancer, cells were transfected with LRP::FLAG. Non-transfected HEK293, SH-SY5Y and DLD-1 cells were used as a control. In this study, transfection was performed to stably overexpress LRP::FLAG by using the DNA construct, pCIneo-moLRP::FLAG [157]. Transfections were performed in a 3mm dish once HEK293, SH-SY5Y and DLD-1 cells reached 50-60% confluency. The Clontech Xfect^{TM} transfection protocol was followed, briefly, 5µg pCIneo-moLRP::FLAG plasmid DNA was added to 100µl Xfect Reaction Buffer, following this 1.5µl Xfect Polymer was added. The solution was incubated for 10 minutes, to allow the formation of nanoparticle complexes. The nanoparticle complexes containing the plasmid DNA was then added to the subconfluent cells in a dropwise manner. The cells were incubated for 48 hours at 37˚C and 5% CO2 in a humidified incubator to allow uptake of the nanoparticle complexes containing the plasmid DNA by the cells. Thereafter, all media was replaced with fresh complete growth media. The pCIneo-moLRP::FLAG plasmid has been characterised by Vana and Weiss, 2006 and contains the neomycin resistance gene, which confers resistance to the geneticin antibiotic. Therefore, 800ng/ml geneticin was used as a selective treatment for the first week and thereafter, cells were treated with 400ng/ml whenever media was replaced, to maintain the transfected cell population.

3.3. Confocal microscopy

Confocal microscopy is a visualisation tool that is used to determine the localisation of fluorescently labelled proteins of interest. Confocal microscopy was used in this study to determine whether co-localization occurs between LRP/LR, α-Synuclein, parkin and hTERT in HEK293, SH-SY5Y and DLD-1 transfected and non-transfected cells. Confocal microscopy was also used to determine the relative expression levels of the above-mentioned proteins. Cells were seeded at a density of 1.2 x 10^5 onto a coverslip (Labocare – 18X18 mm; 0.19 mm thick) and allowed to attach overnight. Gentle shaking was used for all subsequent steps. Thereafter, the media was removed, and the cells were washed with 1ml of 1X PBS for 2 minutes, followed by fixing with 1ml of 4% formaldehyde for 20 minutes at room temperature. All subsequent washes were performed using 1ml of 1X PBS for 2 minutes. Once the cells were fixed, two washes were performed as above and permeabilised using 1ml of 0.1% of Triton X-100 for 30 minutes. The cells were then washed thrice and blocked with 1ml of 0.5% Bovine Serum Albumin (BSA) in 1 X PBS for 30 minutes. Blocking is essential as it coats the membrane where no protein has been bound. This reduces the background signal by preventing the non-specific binding of the antibodies to the membrane. The primary antibody (**Table S1**) was diluted in 0.5% BSA and 500µl was added to the cells followed by overnight incubation at 4˚C. The cells were then washed thrice, and the secondary antibody (**Table S1**) was diluted in 0.5% BSA and 500µl was added to the cells followed by incubation for one hour at room temperature (RT). Thereafter, the cells were washed thrice as above and 1ml of 0.1µg/ml diamidino-2-phenylindole (DAPI) was added to stain the nucleus of the cells. The coverslips were then incubated in the dark at RT for 5 minutes. Thereafter, four washes were performed and then the coverslips were mounted on a glass microscope slide with Sigma Fluoromount (Sigma). The samples were then stored at 4˚C until visualisation was performed using the Zeiss LSM 880 confocal microscope (Zeiss, Oberkochen, Germany). All Confocal experiments were performed with secondary antibody only controls. The Zen 3.4 software was used to image, process and quantify all co-localisation experiments.

3.4. Protein extraction and Bicinchoninic acid (BCA) assay

Cell lysates were prepared, and proteins quantified by performing the bicinchoninic acid (BCA) assay before further analysis by western blotting. This assay determines the protein concentration by focusing on Cu^{2+} ions. Cu^{+} is formed when the Cu^{2+} is reduced by the peptide bonds in proteins. The Cu^{+} ions then form a complex with the BCA which results in a purple

product that can be measured by absorbance. Cell lysates were prepared by harvesting cells as stated in 3.1. Once cells were harvested in 15ml tubes the cells were centrifuged at 5000 rpm for 10 minutes. The supernatant was removed and depending on the size of the pellet 300-500µl of 1 X Radioimmunoprecipitation assay (RIPA) (**Table S3**) buffer was added. The pellet was then resuspended in the RIPA buffer and incubated for 15 minutes at 4 ˚C followed by centrifugation at 5000rpm for 10 minutes to separate the proteins from cell debris.

The BCA assay was then performed to quantify the protein concentration of the cell lysates. A BSA protein standard curve of known concentrations (0, 0.025, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5 and 2.0mg/ml) were prepared and 25µl of each standard was added in triplicate in a 96-well plate. The protein cell lysate was diluted (1/5) in MilliQ water and 25µl was added in triplicate to the 96-well plate. The BCA solution was then made up to 1:50 copper sulphate ($CuSO_4$): BCA. Thereafter, 200µl of the BCA solution was added to each well containing BSA standard and lysate. The 96-well plate was then incubated at 37 ˚C for 30 minutes. Thereafter, the Multiskan® GO ELISA plate reader (Thermo Scientific, Massachusetts, USA) was used to read the absorbance at 562nm. A standard curve was then constructed and was used to calculate the protein concentrations. Protein samples were then standardised to 2mg/ml for western blot analysis.

3.5. SDS PAGE and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to separate the prepared protein samples according to molecular weight prior to western blotting. SDS binds to the proteins causing the proteins to denature and gain a net negative charge. The overall net charge will be proportional to the protein of interest's molecular weight. The migration allows the proteins to pass through pores according to their molecular weight. Furthermore, western blotting is used for the immunological detection of proteins by using labelled antibodies. This procedure was performed to confirms LRP::FLAG overexpression in HEK293 cells. In addition, it was performed to assess the effect of overexpressing LRP::FLAG on LRP/LR and α-Synuclein, protein levels in HEK293 transfected and non-transfected cell lines.

Following protein extraction, the protein samples were made up with Blue Loading Buffer (New England Biolabs) with 40mM of dithiothreitol to obtain a final concentration of 35µg (LRP and FLAG) and 20µg (α-synuclein). Protein samples were heated for 5 minutes at 95 ˚C and were loaded onto the gel along with the protein samples a PageRuler Prestained Protein

Ladder (Thermo Fisher Scientific, Massachusetts, USA). The samples were resolved on a 12% (w/v) polyacrylamide gel (**Table S4**) 120V using the Mini Protean gel system (Bio-Rad, California, USA) in 1X electrophoresis buffer (**Table S5**). Electrophoresis was stopped when the dye front reached the bottom of the gel. Thereafter, 6 filter papers were soaked in 1X Transfer buffer (**Table S6**) and the polyvinylidene difluoride (PVDF) membrane was cut to sized and activated in methanol for 5 minutes. The stacking gel was then cut off from the gel and three filer papers were placed on the Trans-Blot Turbo Transfer System (BioRad) followed by the PVDF membrane and then the gel. Finally, the remaining three filter papers were placed on top, and the system was closed and set to transfer at 1A and 25V for 30 minutes. Thereafter, the membranes were blocked with 5% skim milk in PBS with 0.1% TWEEN 20 (PBST) for 1 hour at RT with gentle agitation to prevent the non-specific binding of antibodies. Due to the low molecular weight of α-Synuclein the protein can be easily washed off the membrane leading to low or no signal, to overcome this the membranes were fixed before blocking with 0.4% formaldehyde for 30 minutes. The membranes were then incubated with the respective primary antibody diluted in 5% skim milk in PBST overnight at 4ºC with gentle agitation. Following incubation overnight in primary antibody, the membranes were washed five times with PBST for 5 minutes per wash. The membranes were then incubated with the appropriate secondary antibody diluted in 5% skim milk in PBST for one hour at RT. The membranes were then washed five times in PBST for 5 minutes followed by visualisation with Clarity™ Western ECL Blotting Substrate (Bio-Rad) and the ChemiDoc™ Imaging System (Bio-Rad). The Image Lab 5.1 software (Bio-Rad) was used to perform densitometric analysis and all values were normalised against the loading control, β-actin.

3.6. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The effects of treatment of cells with a specific substance or protein on cell viability can be determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [158]. MTT is a tetrazolium salt that is yellow and is converted to purple formazan crystals by the mitochondrial enzyme succinate dehydrogenase (Fig. 8) [159]. The succinate dehydrogenase is only active in viable cells and therefore only viable cells will convert MTT to formazan [159]. The MTT assay was chosen over other cell viability assays, such as the ATP

assay, the resazurin reduction assay and the protease viability marker assay, due to its high throughput screening.

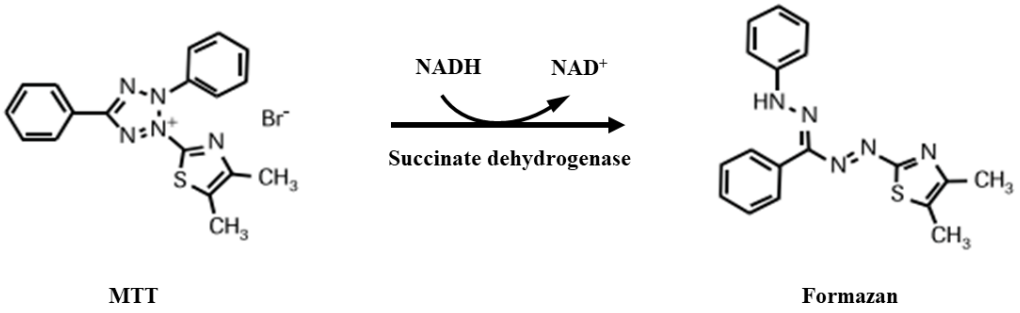

Figure 8: MTT structure and formula.

For this study, HEK293 and DLD-1 transfected, and non-transfected cells were seeded into a 24-well plate at 2 x10^4 cells per well. The cells were then incubated overnight to allow reattachment in a humidified incubator at 37ºC. HEK293 cells were treated with 500µM and 750µM MPP^+, a neurotoxin that is used in PD related studies to induce a PD-like state [160]. The known apoptotic inducer, protocatechuic acid (PCA) was used as the positive control. Following treatment, the cells were incubated for 48 hours in a humidified incubator at 37ºC to determine the effects of LRP::FLAG on cell viability. With regards to the DLD-1 cells, cells were seeded and incubated for 48 hours in a humidified incubator at 37ºC. Following incubation, 1mg/ml MTT was added to each well and incubated for 3 hours at 37˚C to allow the formation of formazan crystals. Thereafter, the remaining MTT was discarded, and the formazan crystals were dissolved by the addition of dimethyl sulfoxide (DMSO). The absorbance was measured at 570nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. High absorbance is indicative of a high number of viable cells.

3.7. Mitochondrial membrane potential assay

The Mitochondrial Staining Kit (Sigma: CS0390-1KT) was used to determine the effects of overexpressing LRP::FLAG on mitochondrial dysfunction in HEK293 and DLD-1 cells. The kit makes use of the cationic, lipophilic dye component 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide (JC-1) to detect changes in the MMP. In normal healthy cells, JC-1 dye accumulates within the mitochondrial matrix. The JC-1 Dye will form red fluorescent aggregates within the mitochondrial matrix, however, when the MMP is affected the JC-1 dye is unable to accumulate in the mitochondrial matrix. The dye then disperses in the cytoplasm of cells resulting in green fluorescence. Studies have shown that

cells undergoing apoptosis show a significant loss of MMP [161] as mitochondrial permeability pores open during apoptosis [162]. Therefore, this assay does not only provide information about mitochondrial health but can also be used to detect apoptosis. This can be detected by flow cytometry or visually by fluorescent microscopy. For this study fluorescent microscopy was used to visualize the stained mitochondria.

The procedure was carried out according to the manufacturer's protocol. Briefly, cells were seeded into a 6-well plate at 3 x 10^5 cells per well. The cells were then incubated overnight to allow reattachment in a humidified incubator at 37ºC. Following overnight incubation, a JC-1 staining solution was prepared by diluting 25µl of the 200X stock solution in 4mls of Milli Q water. The mixture was then mixed by inversion and incubated at RT for 2 minutes. Following incubation 1ml of JC-1 5X staining buffer was added and mixed by inversion. For the positive control, 1µl of 1mg/ml valinomycin was added to the staining solution. Thereafter, 5ml of complete cell culture media was added to the staining solution. The cell culture media was then removed from the cells and 1.92ml of the JC-1 staining solution (0.2ml per cm^2) was added to each well and incubated in a humidified incubator at 37ºC for 20 minutes. Following incubation, the JC-1 staining solution was removed from the cells and the cells were washed twice with complete cell culture media. The stained cells were then viewed with the EVOS Floid imaging system (Thermo Fisher Scientific, Massachusetts, USA). JC-1 monomers were viewed in the green channel and JC-1 aggregates were viewed under the red channel and images were overlayed using the FLoid Cell Imaging Station Software to form a merged image.

3.8. Statistical analysis

All experiments were performed with 3 biological repeats and statistical evaluation was then performed on all data that was collected. A student's *t*-test was performed and a confidence interval of 95% was used. If the *p*-value was less than 0.05 the data was considered significant and if the *p*-value was more than 0.05 the data was considered non-significant.

4. Results

4.1. LRP/LR and hTERT co-localise with PD-related proteins in SH-SY5Y cells

The relationship between LRP/LR and hTERT has been well defined with studies showing that hTERT levels can be increased by LRP::FLAG overexpression [101,118]. hTERT was also

found to promote PINK1/parkin-mediated mitophagy by preventing PINK1 degradation [163]. Therefore, this study also investigated whether hTERT interacts with α-synuclein and parkin. To determine whether LRP/LR plays a role in PD, it was important to establish whether LRP/LR interacts with PD-related proteins. This was done by examining the subcellular localisation as well as the co-localisation between LRP/LR and parkin, α-synuclein as well as hTERT in the SH-SY5Y neuroblastoma cells by confocal microscopy. Fluorescence microscopy is often used by researchers to study protein-protein interactions, although it does not provide a definite answer it can infer an interaction [164]. To confirm whether two proteins co-localise, the merged, co-localisation and 2D cytofluorogram panels were analysed. In addition, Mander's co-localisation coefficient (MCC), weighted correlation co-efficient (WCC) and Pearson's correlation coefficient (PCC) were obtained from the Zen 2.3 software (Zeiss). The MCC, WCC and PCC were used to measure the proportion of one protein that co-localises with another protein and vice versa. The MCC value indicates the relative number of co-localising pixels where 1 indicates all pixels co-localise and 0 no pixels co-localise. The WCC value indicates the intensities in the co-localisation channel (third quadrant of the 2D cytofluorogram) in comparison to all pixels above the threshold, where 1 indicates all pixels co-localise and 0 indicates no pixels co-localise. Finally, the PCC indicates the linear dependence between the two proteins in the region of co-localisation, where +1 indicates complete co-localisation and -1 indicates complete exclusion.

Indeed, co-localisation can be seen between LRP/LR and α-synuclein in the cytoplasm and to a lesser extent in the nucleus (**Fig 9a**). Co-localisation was also seen between LRP/LR and parkin in the cytoplasm (**Fig 10a**). However, MCC, WCC and PCC values indicate that a higher proportion co-localises with a smaller proportion of parkin (**Fig 10a G**). In addition, confocal microscopy was performed to confirm co-localisation of LRP/LR with hTERT in SH-SY5Y cells [100]. Indeed, co-localisation was seen between LRP/LR and hTERT in the cytoplasm (**Fig 9b**). Interestingly, increased hTERT levels have been shown to increase α-synuclein degradation [165], therefore, co-localisation was analysed between these two proteins. Indeed, co-localisation was seen between hTERT and α-synuclein in the cytoplasm and the nucleus (**Fig 9c**). Moreover, co-localisation was analysed between parkin and hTERT due to the roles that both proteins play in the regulation of dysfunctional mitochondrial regulation. Indeed, parkin and hTERT were found to co-localise in the cytoplasm (**Fig 10b**). However, MCC, WCC and PCC values indicate that a lower proportion of hTERT co-localises with a higher proportion of parkin (**Fig 10b N**). Therefore, LRP/LR could play a role in PD through the

possible interaction with α-synuclein and parkin as well as an indirect role through the interaction of hTERT with α-synuclein and parkin.

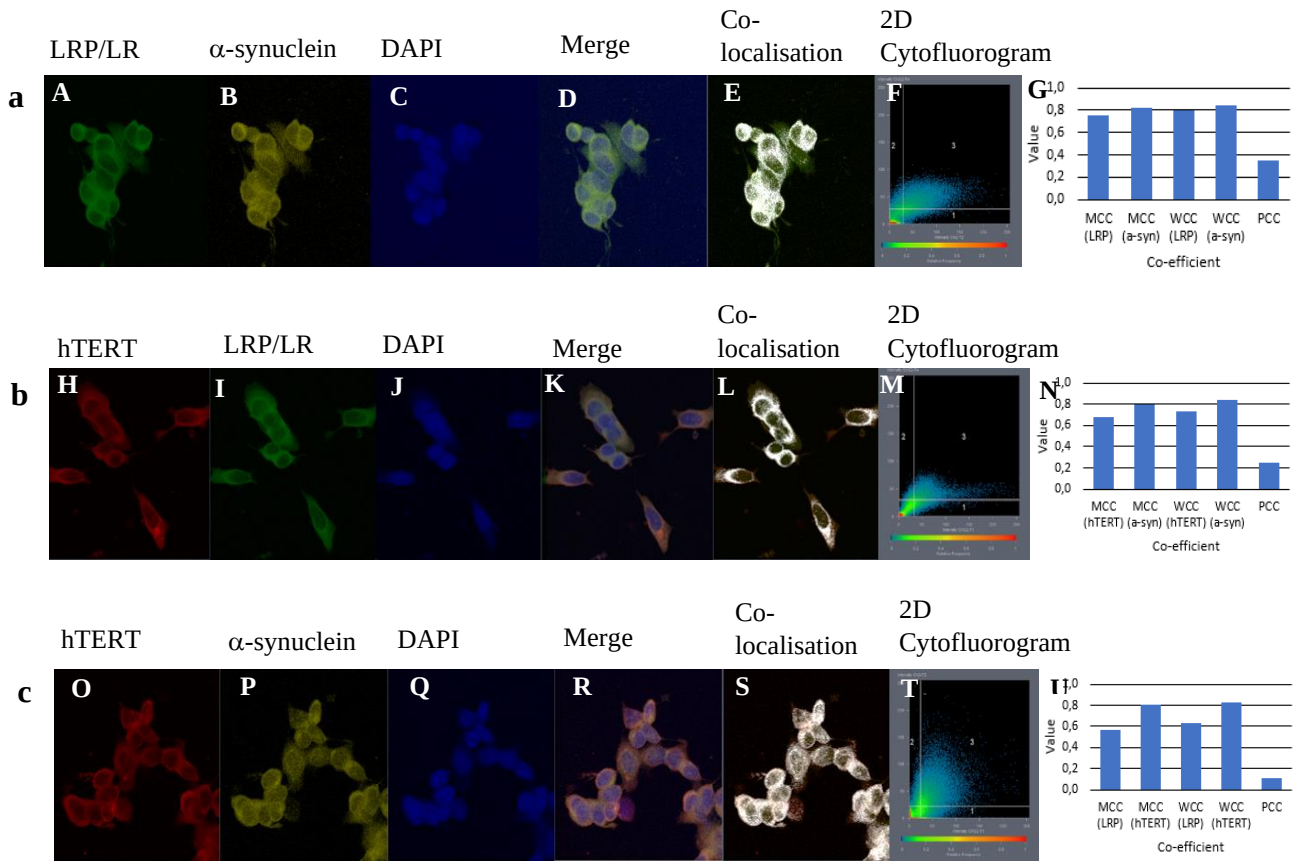

Figure 9: LRP/LR, α-synuclein and hTERT co-localisation in SH-SY5Y cells. Intracellular localisation and co-localisation between a) LRP/LR (green) and α-synuclein (yellow), b) hTERT (red) and LRP/LR (green) as well as c) hTERT and α-synuclein. LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (A and I). α-synuclein was detected using an anti-α-synuclein primary antibody and alexa flour 647 conjugated secondary antibody (B and P). Finally, hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (H and O). Nuclei were stained blue with DAPI (C, J and Q). Co-localisation between hTERT and α-synuclein; hTERT and LRP/LR as well as LRP/LR and α-synuclein is shown in the merged images (D, K and R). Co-localisation is confirmed by the white in the co-localisation images (E, L and S) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F, M and T). Furthermore, co-localisation between LRP/LR and α-synuclein in b) HEK293 and HEK293 Transfected cells, was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss) (G, N and U). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

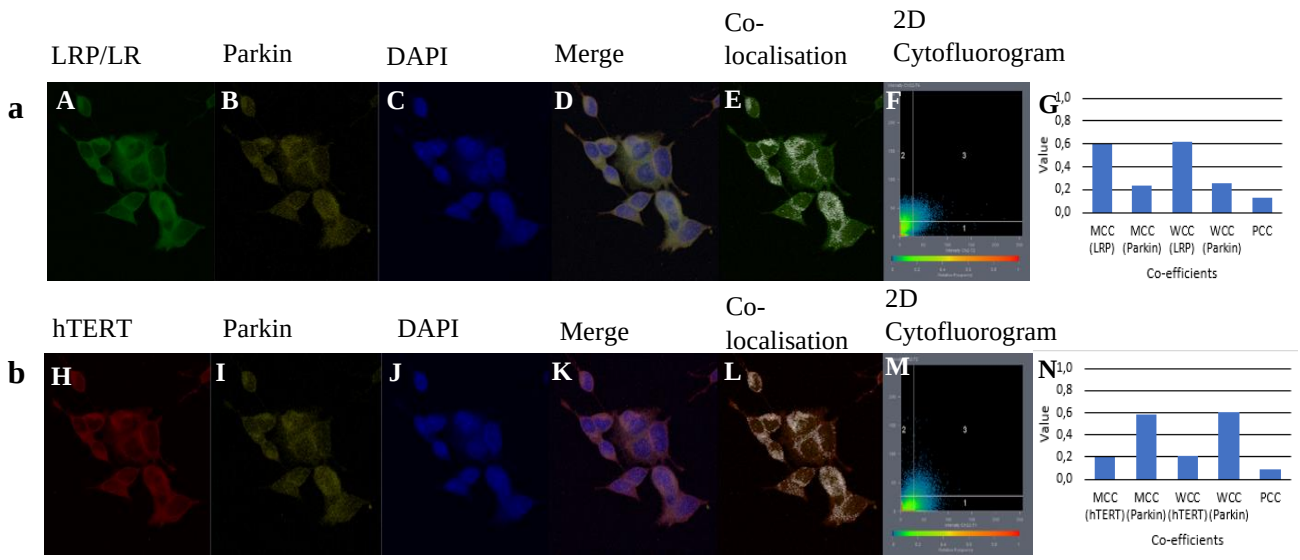

Figure 10: hTERT, LRP/LR and parkin co-localisation in SH-SY5Y cells. Intracellular localisation and co-localisation between a) LRP/LR (green) and parkin (yellow) as well as b) hTERT (red) and parkin. LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (A). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (B and I). Finally, hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (H). Nuclei were stained blue with DAPI (C and J). Co-localisation between hTERT and parkin as well as LRP/LR and parkin is shown in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between was quantified the Mander's (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficient using the Zen 2.3 software (Zeiss) (G and N). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

4.2. Confirmation of LRP::FLAG overexpression in HEK293 cells

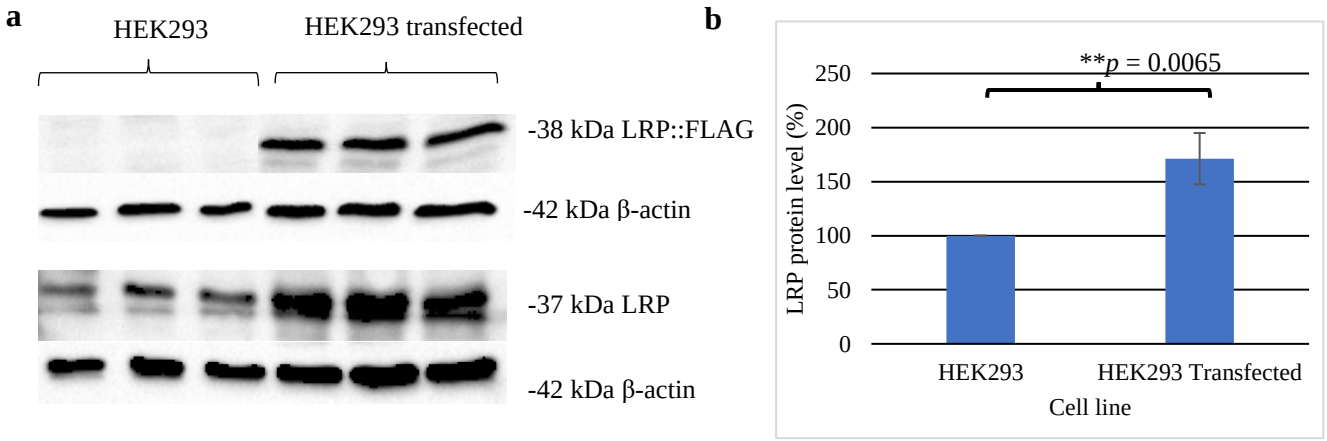

Figure 11: Transfection of HEK293 with pCIneo-LRP::FLAG plasmid was successful. Western blot analysis performed on pCIneo-LRP::FLAG transfected and non-transfected HEK293 cells (a). Endogenous LRP/LR and LRP::FLAG was detected using the anti-LRP, IgG1-iS18 and anti-Flag primary antibodies and HRP-conjugated secondary antibodies. β-actin was detected using a HRP coupled primary antibody. Transfection of HEK293 cells with the pCIneo-LRP::FLAG plasmid resulted in LRP::FLAG overexpression as well as an increase of total LRP protein levels. Densitometric and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 71.24% increase of total LRP levels (b). Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

The next set of experiments investigated the role that LRP/LR plays in regulating α-synuclein levels and MMP, which are both linked to PD-related pathogenesis. This was done by stably transfecting the human embryonic kidney (HEK293) cells with the pCIneo-moLRP-FLAG expression plasmid and determining the impact of expressing LRP::FLAG on α-synuclein protein levels, cell viability and finally mitochondrial membrane potential. Due to the difficulty to transfect the SH-SY5Y cells in this study HEK293 cells were selected for these experiments since they express neuronal markers [166] and because they are easily transfectable [155]. Briefly, 48 hours after the cells were transfected they were subjected to geneticin selection and drug-resistant cells were pooled and analysed as described below.

To determine whether the HEK293 cells were successfully transfected and what the effect of overexpressing LRP::FLAG would have on total LRP/LR levels western blotting was performed with antibodies to FLAG and LRP respectively (**Fig 11**). The results show that the transfected HEK293 cells did indeed express LRP::FLAG and that this led to increased LRP/LR levels (**Fig 11a**). Furthermore, statistical analysis confirmed total LRP levels increased significantly by 71.24% in LRP::FLAG transfected cells (**Fig 11a**).

4.3. LRP/LR and α-synuclein co-localise in HEK293 cells

To determine whether overexpression of LRP/LR influences α-synuclein protein levels and its co-localization with LRP/LR, confocal microscopy in non-transfected and LRP::FLAG transfected HEK293 cells was performed with the antibodies as indicated in **Figure 12**.

The results confirmed the western blot data in Figure 3 that show that LRP::FLAG was successfully overexpressed in the transfected but not non-transfected HEK293 cells (**Fig 12a J**) and that this led to an increase in total levels of LRP/LR in the transfected cells (**Fig 12a I**). Importantly, while α-synuclein was detectable in non-transfected cells (**Fig 12a A**) it was expressed at much higher levels in LRP::FLAG transfected cells (**Fig 12a H**). In addition, confocal microscopy indicates that the increase in LRP/LR and α-synuclein levels can be seen in all the cells. As expected, α-synuclein co-localised with LRP/LR in the cytoplasm of both non-transfected (**Fig 12a E and F**) and transfected HEK293 cells (**Fig 12a L and M**). Furthermore, overexpression of LRP::FLAG in HEK293 cells resulted in an increase in the co-localisation strength as shown by the CC, WCC and PCC (compare **Fig 12b** and **12c**). Therefore, LRP::FLAG overexpression increases α-synuclein levels throughout all cells and

thus a more intense co-localisation can be seen when compared to the non-transfected cells. Therefore, LRP/LR could play a role in PD through the interaction with α-synuclein.

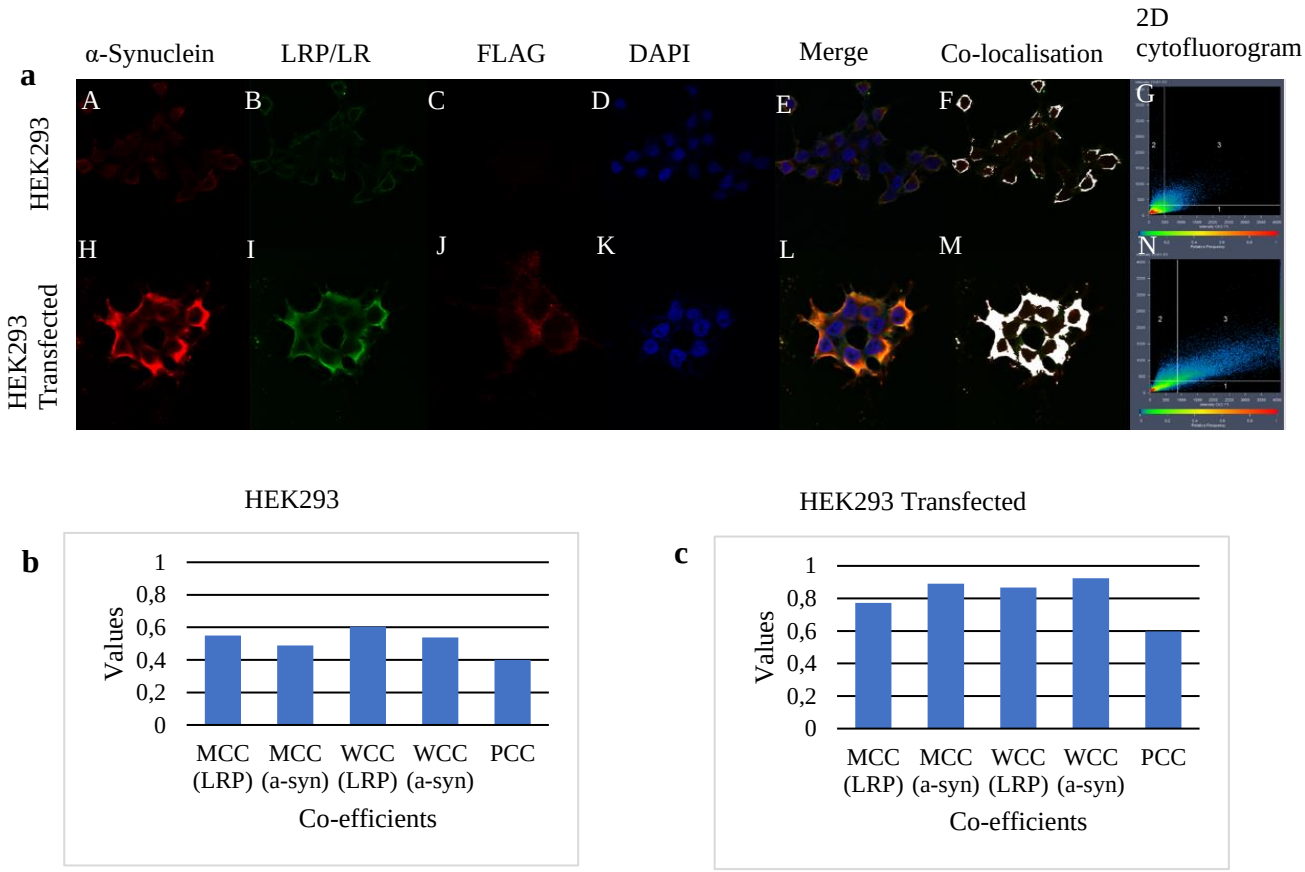

Figure 12: LRP/LR and α-synuclein co-localisation in HEK293 cells. Intracellular localisation and co-localisation between α-synuclein (red) and LRP/LR (green) (a). α-synuclein was detected using an anti-α-synuclein primary antibody and alexa flour 647 conjugated secondary antibody (A and H). LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B and I). LRP::FLAG was detected by using a Cy3 conjugated primary antibody (C and J). Finally, nuclei were stained blue with DAPI (D and K). Co-localisation between α-synuclein and LRP/LR and parkin is shown in the merged images (E and L). Co-localisation is confirmed by the white in the co-localisation images (F and M) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (G and N). Furthermore, co-localisation between LRP/LR and a-synuclein in b) HEK293 and HEK293 Transfected cells, was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (Life Sciences Imaging facility, WITS).

4.4. LRP::FLAG overexpression affects α-synuclein protein in HEK293 cells.

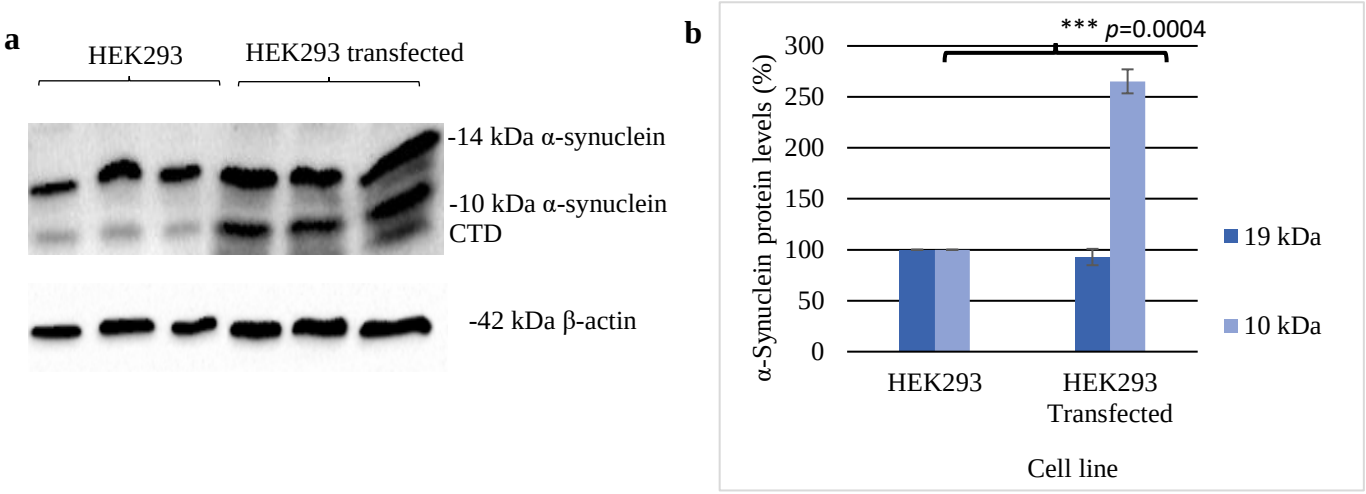

Figure 13: LRP::FLAG overexpression increases the α-synuclein C-terminal domain (CTD). Western blot analysis performed on pCIneo-LRP::FLAG transfected and non-transfected HEK293 cells. Total levels of full length (FL) - and C-terminal domain- α-synuclein were detected using an anti-α-synuclein primary antibody and HRP-conjugated secondary antibody. β-actin was used as a loading control and detected with an HRP coupled primary antibody. Transfection of HEK293 cells with the pCIneo-LRP::FLAG plasmid resulted in an increase of the 10 kDa α-synuclein CTD (a). Densitometric and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 165.22% increase of the α-synuclein CTD (b). Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

To confirm that α-synuclein expression increases in LRP::FLAG overexpressing HEK293 cells, western blot analysis was performed (**Fig 13**). The results reveal that overexpression of LRP::FLAG did not affect the 14kDa full-length α-synuclein, however, there was a significant increase of 165.22% in the a band located at the 10kDa mark (**Fig 13a, b**). The anti-α-synuclein primary antibody used was raised against CTD of α-synuclein (amino acid sequence 111-132) indicating that this band could represent the CTD. This, together with the confocal microscopy results, suggest that LRP/LR increases the 10kDa CTD of the PD-related protein α-synuclein.

4.5. LRP::FLAG overexpression of increases cell viability in MPP$^+$ treated HEK293 cells

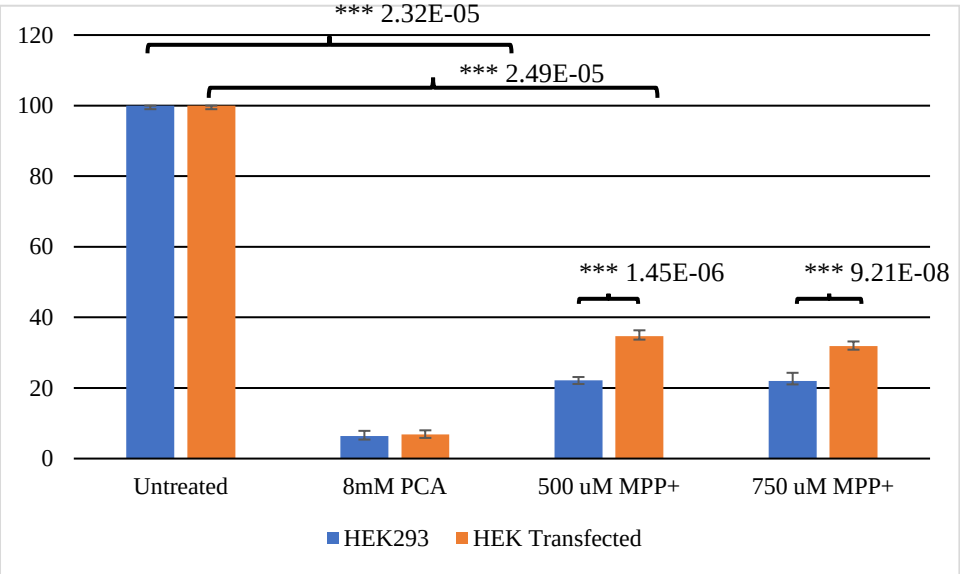

Figure 14: LRP::FLAG overexpression increases cell viability of cells treated with MPP$^+$. MTT assay assessing cell viability of HEK293 non-transfected and LRP::FLAG transfected cells treated with 500µM and 750µM MPP$^+$ for 48 hours. Untreated HEK293 non-transfected and transfected cells were set to 100% and PCA was used as a positive control. Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

As seen above LRP/LR interacts with α-synuclein, parkin as well as hTERT in the SH-SY5Y cell line and overexpressing LRP::FLAG in HEK293 cells increases the CTD of α-synuclein. These results suggest that LRP/LR could play a role in PD, however, it was not clear whether it is playing a role in the progression or impediment of the disease. Therefore, an MTT cell viability assay was performed to determine whether LRP/LR affects the viability of non-transfected and LRP::FLAG transfected HEK293 cells treated with the PD inducer MPP$^+$ and 8mM PCA was used as a positive control. The results revealed that, compared to the non-transfected HEK293 cells, 500µM and 750µM MPP$^+$ treated LRP::FLAG transfected HEK293 cells were significantly more viable (**Fig 14**). Indeed, LRP::FLAG overexpressing HEK293 cells treated with 500µM and 750µM MPP$^+$ had a 12.58% and 9.83% increase in cell viability respectively. Therefore, the overexpression of LRP::FLAG increases cell viability in cells treated with the PD inducer MPP$^+$. This suggests that LRP::FLAG overexpression rescues cells from the cytotoxic effects of MPP$^+$ and that LRP/LR could play a protective role in PD.

4.6 Overexpression of LRP::FLAG increases mitochondrial membrane potential of HEK293 cells treated with MPP+

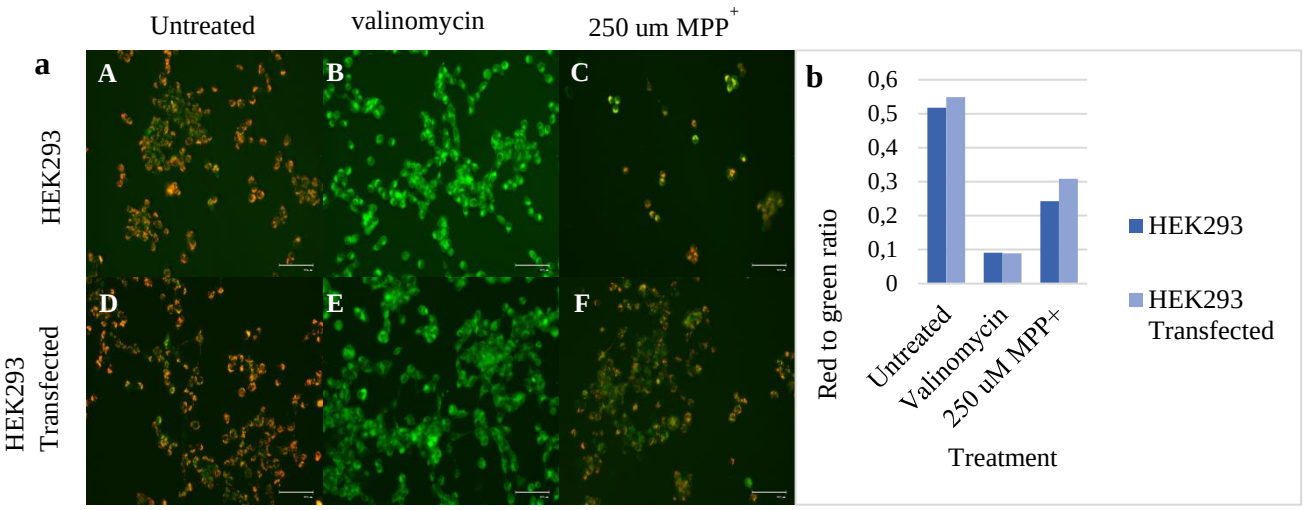

Figure 15: LRP::FLAG overexpression increases the mitochondrial membrane potential of cells treated with MPP+. a) Mitochondrial membrane potential was analysed using the JC-1 fluorescent dye. In the cytoplasm the JC-1 dye exist as monomers (green), however, by means of the mitochondrial membrane potential the JC-1 dye migrates into the mitochondria where it forms aggregates (red). Untreated non-transfected and trnasfected HEK293 cells (A and D) where compared to cells treated with 250 uM MPP+ (C and F). 0.2ug/ml valinomycin was used as a positive control. b) Quantitative analysis of the JC-1 dye red to green ratio. All images were taken at 100X magnification using the Zeiss Evos microscope (WITS).

Based on the results shown in Fig 14, it was hypothesised that LRP::FLAG rescues HEK293 cells from MPP+ by increasing their mitochondrial membrane potential. To test this, the mitochondrial membrane potential of non-transfected and LRP::FLAG transfected HEK293 cells treated with 250µM MPP+ was analysed using the mitochondrial staining assay and treatment with 0.2µg/ml valinomycin for one hour was used as a positive control. Briefly, the JC-1 dye was used to analyse the mitochondrial membrane potential, which reflects mitochondrial health as well as early apoptosis. In healthy cells, the JC-1 dye forms aggregates in the mitochondrial matrix where it is seen as red fluorescence signal. However, when the mitochondrial membrane potential is reduced the JC-1 dye fails to form aggregates in the mitochondrial membrane and thus remains in the cytoplasm in a monomeric form where it is visible as a green, fluorescent signal. Furthermore, to quantify these results the red to green (R/G) ratio was calculated, where the higher the ratio the higher the polarization of the mitochondrial membrane. The results revealed that treatment with the positive control, valinomycin, resulted in a complete loss of mitochondrial membrane potential of both non-transfected and LRP::FLAG transfected HEK293 cells (**Fig 15a B and E**). Furthermore,

compared to non-transfected cells, LRP::FLAG transfected cells treated with 250µM MPP^+ showed a higher level of mitochondrial membrane potential (compare **Fig 15a C and F**). Indeed, overexpression of LRP::FLAG increased the R/G ratio from 0.24 to 0.30 (**Fig 15b**). These results together with the MTT results show that overexpression of LRP::FLAG could be playing a protective role in PD possibly through the protection of mitochondria and thus blocking apoptosis. This protection could possibly come from the well-established relationship between hTERT and LRP/LR. Indeed, hTERT has recently been shown to play a role in the PINK1-parkin mediated mitophagy and could explain the protection we see when overexpressing LRP::FLAG.

4.7. LRP/LR and hTERT co-localise in DLD-1 colorectal cancer cells

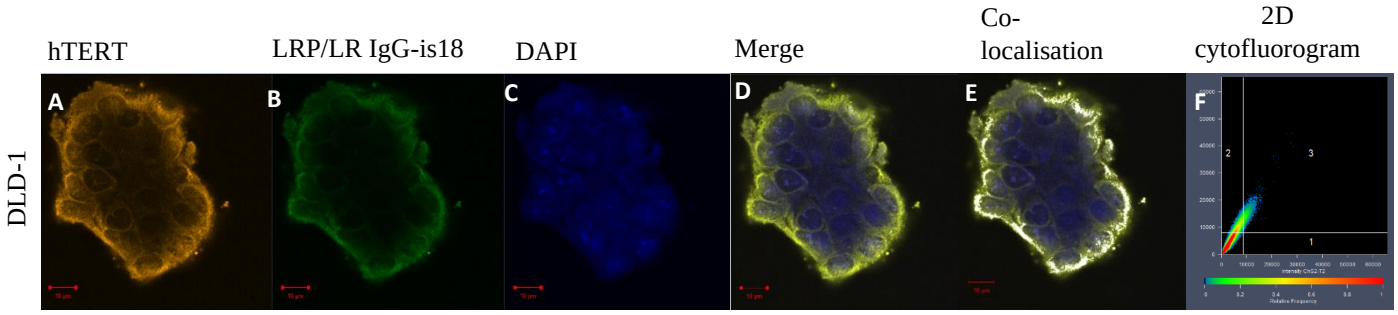

Figure 16: LRP/LR AND hTERT co-localise in DLD-1 cells. Intracellular localisation and co-localisation between a) hTERT (yellow) and LRP/LR (green). hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (A). LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B). Nuclei were stained blue with DAPI (C). Co-localisation between hTERT and LRP/LR is shown by yellow fluorescence in the merged images (D). Co-localisation is confirmed by the white in the co-localisation images (E) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

The role of LRP/LR in cancer has been well established. Indeed, LRP/LR has been found to play a role in the adhesion of tumour cells, angiogenesis as well as the evasion of apoptosis [104]. To this end, this study specifically focuses on the role of LRP/LR in colorectal cancer. Interestingly, studies have shown that telomerase activity is increased in 90% of malignant tumours [133]. In addition, increased hTERT levels have also been shown to inhibit apoptosis [139,140]. Moreover, LRP/LR has also been shown to co-localise with hTERT in the perinuclear compartment and the cytoplasm of breast cancer cells, therefore, mediating telomerase activity [109]. As a start, confocal microscopy was performed on the DLD-1 colorectal cancer cells to determine the subcellular localisation of endogenous LRP/LR and hTERT and to determine if these two proteins co-localise. The results show that hTERT and LRP/LR co-localise in the DLD-1 colorectal cancer cells as can be seen by the merged, co-

localisation and 2D cytofluorogram panels (**Fig 16 D-F**). It is, however, important to note that DLD-1 cells grow in colonies which resulted in poor penetration of antibodies in cells located at the centre of these colonies. These results provided an initial indication that hTERT and LRP/LR do indeed co-localise in DLD-1 cells but due to technical problems, the subcellular localisation of the two proteins, as well as their co-localisation, could not be accurately determined for single cells.

4.8. Establishment of LRP::FLAG overexpressing DLD-1 colorectal cancer cells

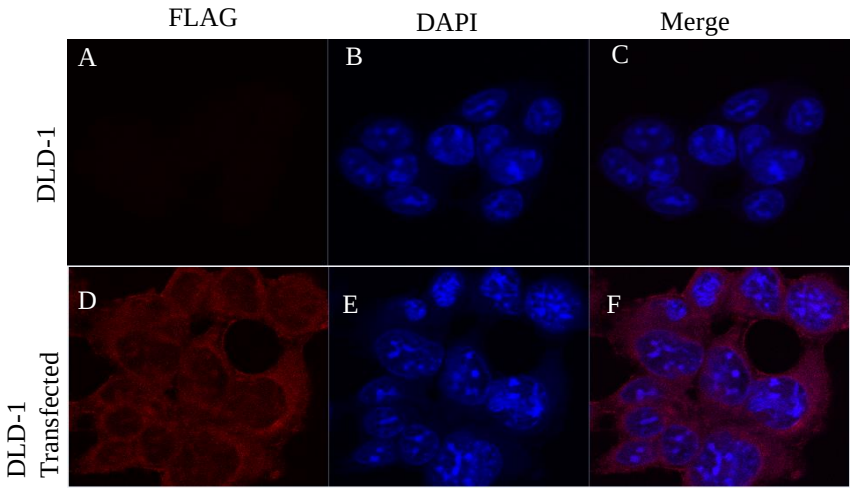

Figure 17: LRP::FLAG overexpression localisation and confirmatin in DLD-1 cells. Confirmation and localisation of LRP::FLAG (red) in DLD-1 cells. LRP::FLAG was detected by using a Cy3 conjugated primary antibody (D). Nuclei were stained with blue with DAPI (B and E). All images were taken at 630X magnification using the Zeiss LSM780 microscope (Confocal and Light Microscope Imaging Facility, UCT).

A recent study showed that LRP/LR plays a role in colorectal cancer by impeding apoptosis [107]. The authors show that siRNA mediated knockdown of LRP was able to induce apoptosis in both early and late-stage colorectal cancer cells [107]. The current study, therefore, investigated whether overexpressing LRP/LR would impede or promote the progression of colorectal cancer. Briefly, the pCIneo-moLRP-FLAG plasmid was stably transfected in DLD-1 cells and successfully transfected was analysed by confocal microscopy with an antibody to FLAG (**Fig 17**). Confocal microscopy was chosen over western blotting because DLD-1 transfected cells proliferated slowly and therefore to obtain sufficient protein for western blotting was time-consuming. As expected, the results revealed an absence of fluorescence in

non-transfected DLD-1 cells (**Fig 17 A**) and red fluorescence, indicating LRP::FLAG, in transfected cells (**Fig 17 D**). LRP::FLAG expression was particularly visible in the perinuclear compartment and cytoplasm.

4.9. Overexpression of LRP::FLAG decreases cell viability of DLD-1 cells

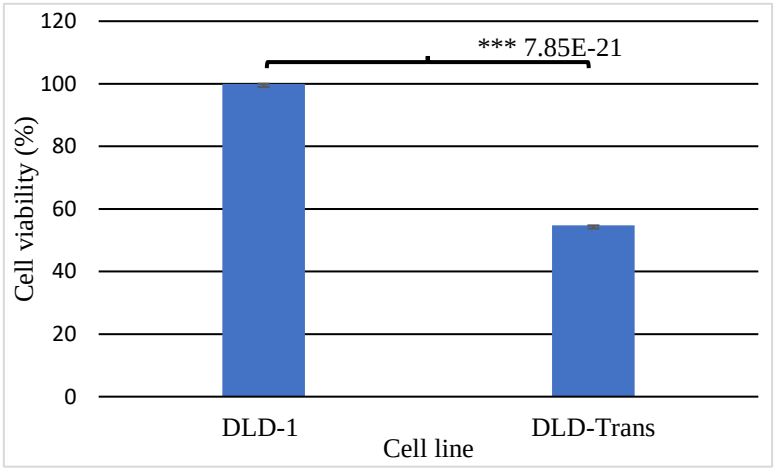

Figure 18: Overexpression of LRP::FLAG decreases cell viability of DLD-1 cells. MTT assay assessing the cell viability of DLD-1 non-transfected and transfected cells. Non-transfected cells were set to 100%. Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

Several studies have shown that LRP/LR is overexpressed in cancer and knockdown or blockage of LRP/LR induces apoptosis. However, it is not clear whether overexpressing LRP/LR would result in further progression of the disease. Therefore, the cell viability of non-transfected and LRP::FLAG transfected DLD-1 cells was assessed using the MTT assay. The results revealed that, compared to the non-transfected DLD-1 cells, LRP::FLAG transfected DLD-1 cells were significantly less viable (**Fig 18**). Indeed, DLD-1 cells overexpressing LRP::FLAG had a 45.27% decrease in cell viability which suggest that LRP::FLAG overexpression exhibit anti-cancer activity.

4.10. Overexpression of LRP::FLAG decreases mitochondrial membrane potential of DLD-1 cells

To explore the possible anti-cancer activity of overexpressing LRP::FLAG in colorectal cancer, the mitochondrial membrane potential of non-transfected and LRP::FLAG transfected DLD-1 cells was analysed using the mitochondrial staining assay. Compared to non-transfected cells,

LRP::FLAG transfected DLD-1 cells showed a lower level of mitochondrial membrane potential (**Fig 19a** compare **A and B**). Indeed, overexpression of LRP::FLAG decreased the R/G ratio from 0.33 to 0.17 (**Fig 19b**). Furthermore, treatment with 0.2µg/ml valinomycin for one hour resulted in a complete loss of mitochondrial membrane potential in non-transfected and LRP::FLAG transfected DLD-1 cells (**Fig 19a and b**). A decrease in MMP in cancer cells is an indicator of mitochondrial apoptosis that leads to the release of mitochondrial apoptogens initiating the caspase cascade ultimately resulting in programmed cell death [167,168]. Together the results from the MTT and mitochondrial staining assays show that overexpression of LRP::FLAG can inhibit colorectal cancer cell viability by inducing mitochondria dysfunction and apoptosis.

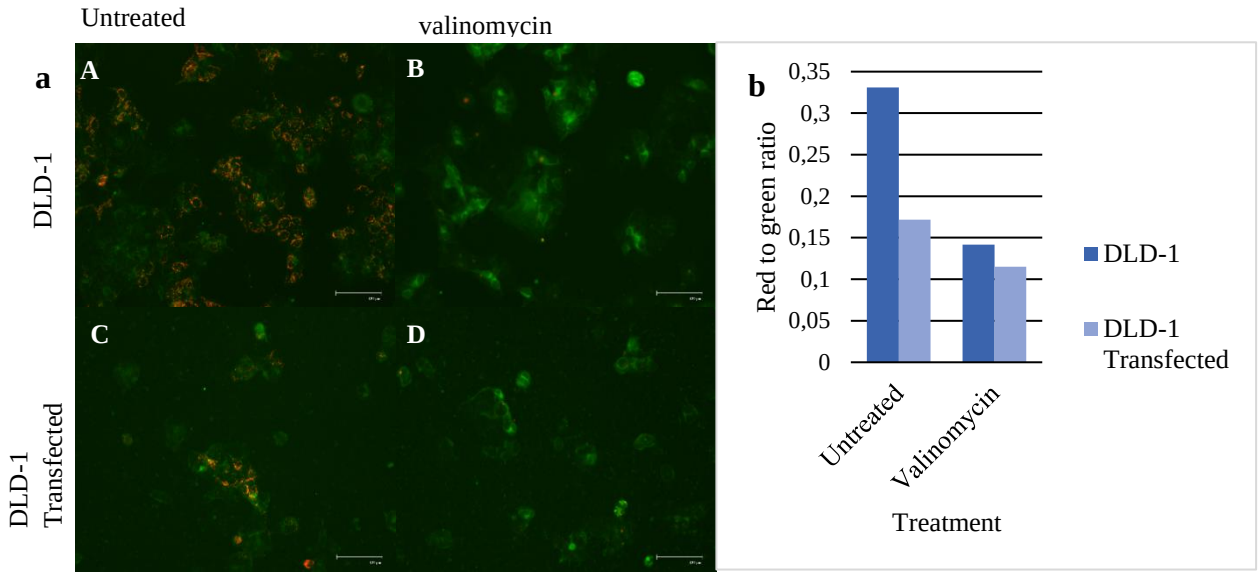

Figure 19: LRP::FLAG overexpression decreases the mitochondrial membrane potential of cells overexpressing LRP::FLAG. Mitochondrial membrane potential was analysed using the JC-1 fluorescent dye. In the cytoplasm the JC-1 dye exist as monomers (green), however, by means of the mitochondrial membrane potential the JC-1 dye migrates into the mitochondria where it forms aggregates (red). Non-transfected DLD-1 cells (A) where compared to LRP::FLAG overexpressing DLD-1 cells (C). 0.2ug/ml valinomycin was used as a positive control. All images were taken at 100X magnification using the Zeiss Evos microscope.

4.11. LRP/LR, parkin and hTERT co-localise in DLD-1 cells

The above results indicate that overexpressing LRP::FLAG could be playing an important role in the impediment of colorectal cancer. Furthermore, cancer and PD have recently been linked through several studies. For example, the PD-related protein, parkin, was recently demonstrated to function as a tumour suppressor in several cancers [68,69,82,169]. The current study aimed to determine whether LRP/LR could be the link between the two diseases and hypothesised that LRP/LR could be interacting with

parkin to inhibit the progression of colorectal cancer. Confocal microscopy was therefore performed to determine whether LRP/LR and parkin co-localise. The results revealed that parkin and LRP/LR do indeed co-localise in the cytoplasm of both non-transfected and transfected DLD-1 cells (**Fig 20** see merged, co-localisation and 2D cytofluorogram panels). Furthermore, MCC, WCC and PCC values indicate that a higher proportion of parkin co-localises with a smaller proportion of LRP/LR in LRP::FLAG transfected DLD-1 cells (**Fig 20 G**). Given the well-established relationship between LRP/LR and hTERT, the possibility was considered that parkin may also interact with hTERT. Interestingly, whereas no co-localisation was observed between hTERT and parkin in non-transfected DLD-1 cells (**Fig 21 A-G**), the two proteins co-localised in the cytoplasm and nucleus of LRP::FLAG transfected DLD-1 cells (**Fig 21 H-N** see merged, co-localisation and 2D cytofluorogram panels). These findings indicate that endogenous and transfected LRP/LR and parkin could interact in DLD-1 cells, but that hTERT and parkin only interact when LRP::FLAG was overexpressed in DLD-1 cells.

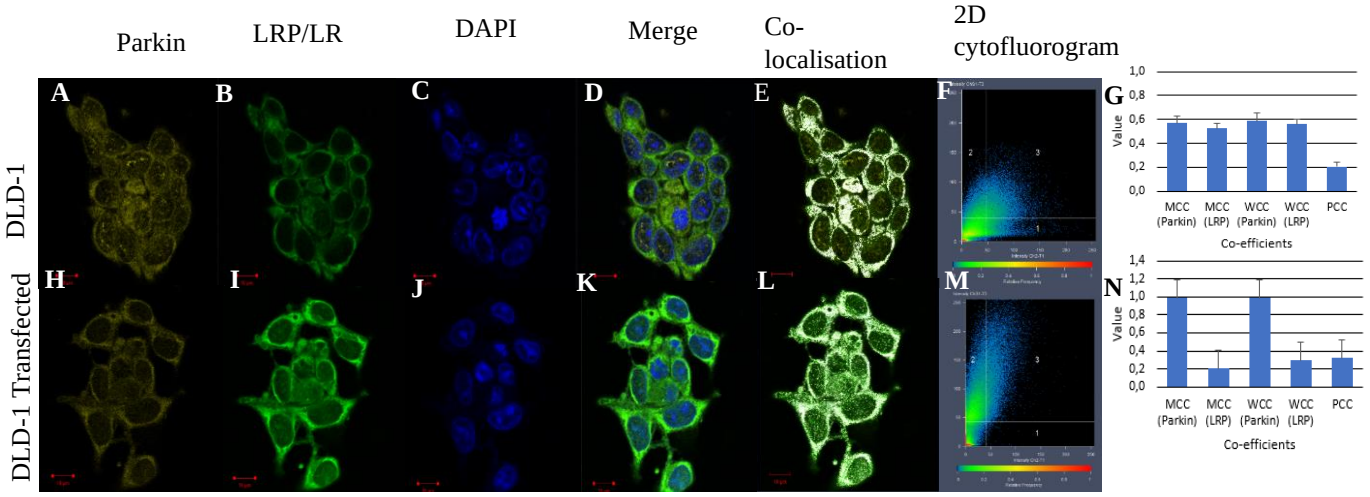

Figure 20: Parkin and LRP/LR co-localisation in DLD-1 cells. Intracellular localisation and co-localisation between parkin (yellow) and LRP/LR (green). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (A and H). LRP/LR was detected by the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B and I). Finally, nuclei were stained blue with DAPI (C and J). Co-localisation between parkin and LRP/LR is shown in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between parkin and LRP/LR in DLD-1 (A-G) and DLD-1 Transfected cells (H-N), was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT).

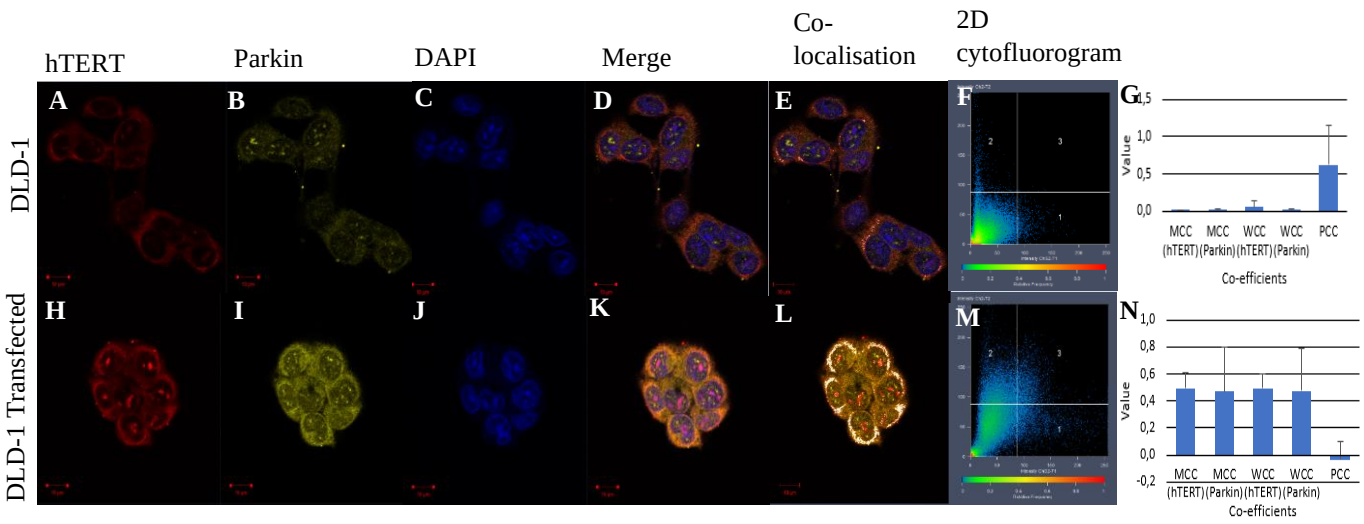

Figure 21: hTERT and Parkin co-localisation in DLD-1 cells. Intracellular localisation and co-localisation between hTERT (red) and parkin (yellow). hTERT was detected by using the anti-hTERT primary antibody and a Cy3 conjugated secondary antibody (A and H). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (B and I). Finally, nuclei were stained blue with DAPI (C and J). Co-localisation between hTERT and parkin is shown by yellow fluorescence in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between hTERT and parkin in DLD-1 (A-G) and DLD-1 Transfected cells (H-N), was quantified the Mander's (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT).

4.12. LRP::FLAG overexpression effects Parkin expression and localisation in DLD-1 cells

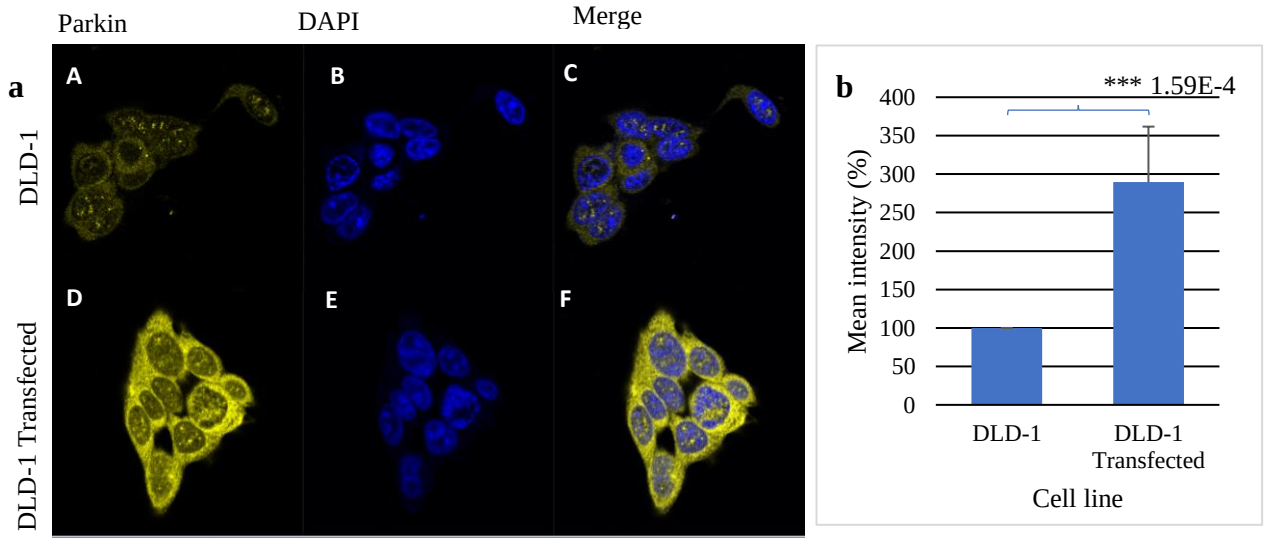

Figure 22: Parkin overexpression and localisation in DLD-1 cells. a) Expression and localisation of parkin (yellow) in DLD-1 cells. Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (A and D). Nuclei were stained with blue with DAPI (B and E). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT). b) Intensity measurements and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 189.71% increase of parkin fluorescence intensity. Standard deviation is depicted by the error bars. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

Finally, to further investigate the effect of overexpressing LRP::FLAG on colorectal cancer, confocal microscopy was used to analyse relative parkin expression in both non-transfected and LRP::FLAG transfected DLD-1 cells (**Fig 22a**). The results revealed that, while parkin was detectable in non-transfected cells (**Fig 22a A**) it was expressed at much higher levels in LRP::FLAG transfected cells (**Fig 22a D**). Fluorescence intensity measurements revealed a significant increase of 189.71% was seen of the fluorescent signal of parkin indicating that parkin levels increased (**Fig 22b**). In addition, parkin is localised in the cytoplasm and nucleus of both non-transfected and LRP::FLAG transfected DLD-1 cells (**Fig 22a**). These results indicate that overexpression of LRP::FLAG increases parkin levels in DLD-1 cells.

5. Discussion

5.1. Parkinson's disease study

The role of LRP/LR in AD and prion diseases has been extensively reported on [92,100,110,112,117,118,170]. However, the role of LRP/LR in PD has yet to be investigated. One study showed that LR levels increase upon MPP^+ treatment [171]. Furthermore, blocking LR with an anti-LR antibody or treatment with the LR inhibiter, YIGSR significantly increased microglia activation and dopamine neurotoxicity [171]. This study also found that treatment with exogenous laminin, induced microglia and dopamine neurotoxicity which was further attenuated by blocking LR with the anti-LR antibody [171]. However, follow up studies investigating LRP/LR in PD have yet to be performed.

The interaction between LRP/LR and hTERT has recently been shown to play a vital role in both ageing and AD [101,118]. Indeed, recent studies have shown that hTERT levels and telomerase activity can be increased, in a non-oncogenic fashion, by LRP::FLAG overexpression which can also decrease Aβ-mediated cytotoxicity [100,101]. Although the role of hTERT in PD is poorly understood a recent study has shown that increased hTERT levels correlated with a decrease in α-synuclein levels and improved motor functions in a PD-mouse model [165]. It was, therefore, hypothesised that LRP/LR and hTERT could play a role in PD. To assess this the current study analysed the interaction of LRP/LR and hTERT with the PD-related proteins; α-synuclein and parkin. Thereafter, the α-synuclein protein levels were analysed following transfection of HEK293 cells with the pCIneo-moLRP-FLAG expression

plasmid. Finally, the effect of LRP::FLAG overexpression on cell viability and MMP was assessed.

5.1.1. LRP/LR and hTERT co-localise with PD-related proteins

Due to the lack of studies investigating the role of LRP/LR on PD pathology, the first step was to determine whether LRP/LR interacts with the PD-related proteins, α-synuclein and parkin. To assess this, SH-SY5Y cells were used for initial co-localisation analysis since these cells are frequently used *in vitro* neuronal models for their expression of neuronal markers [155]. Furthermore, the relationship between LRP/LR and hTERT has been well defined with studies showing that hTERT levels can be increased by LRP::FLAG overexpression [101,118]. hTERT was also found to promote PINK1/parkin-mediated mitophagy by preventing PINK1 degradation [163]. Therefore, this study also investigated whether hTERT interacts with α-synuclein and parkin. To determine whether an interaction exists between these proteins, confocal microscopy was performed, and the subcellular localisation of the proteins was analysed.

Indeed, LRP/LR and α-synuclein was found to co-localise in the cytoplasm and to a lesser extent in the nucleus (**Fig 9a**). Studies have shown that α-synuclein can be detected in human plasma as well as cerebrospinal fluid, indicating that α-synuclein could enter cells from an extracellular space [172]. This study along with several other studies point out the prion-like behaviour of α-synuclein where α-synuclein aggregates can be exported to extracellular spaces and then be taken up by neighbouring cells [148-153]. Given that LRP/LR has been found to not only act as a receptor for the infectious prion protein PrP^{Sc} [111] but also Aβ [114], LRP/LR could, therefore, act as a receptor for α-synuclein. Furthermore, the NTD of 37kDa LRP and members of the HSP70 family have high sequence similarities [173]. HSP70 has been shown to promote α-synuclein degradation through the HSP70-mediated ALS and the UPS systems [174] and could explain the possible interaction seen between LRP/LR and α-synuclein. However, fluorescence resonance energy transfer (FRET) microscopy imaging would provide information on whether the interaction between these two proteins is indirect or direct. In addition, LRP/LR was found to co-localise with parkin in the cytoplasm (**Fig 10a**). HSP70 has also shown to be a binding partner for parkin [175] and due to the high sequence similarities between the NTD of the 37kDa LRP and HSP70, this could also explain the co-localisation seen between the two proteins.

The relationship between LRP/LR and hTERT has been described in several studies where knockdown of LRP/LR by siRNA reduces telomerase activity as well as hTERT protein levels [109]. Furthermore, LRP::FLAG overexpression increases telomerase activity and hTERT levels [101]. Therefore, confocal microscopy was performed to confirm co-localisation between LRP/LR and hTERT in SH-SY5Y cells [100]. As expected LRP/LR and hTERT co-localised in the cytoplasm (**Fig 9b**). hTERT has been shown to have many neuroprotective roles [127] including protecting cells from Aβ plaques as well as tau-induced oxidative stress [100,117,118,145]. To determine whether hTERT plays a role in PD, confocal microscopy was performed and co-localisation of hTERT with α-synuclein and parkin was analysed. Indeed, hTERT and α-synuclein co-localised in the cytoplasm as well as in the nucleus (**Fig 9c**). The relationship between hTERT and α-synuclein is not fully understood, however, a recent *in vivo* study has shown that increasing hTERT levels by using telomerase activators improved motor function in a PD-mouse model [165]. Furthermore, these mice have decreased ROS levels as well as total, phosphorylated, and aggregated α-synuclein levels in the neocortex, CA1 and CA3 regions of the hippocampus [165]. This study also showed that following treatment with telomerase activators the autophagy markers LC-3 and p62 decreased indicating that these mice have enhanced autophagic vesicle clearing, indicating increased α-synuclein clearance.

In addition, hTERT and parkin were found to co-localise (**Fig 10b**). hTERT has recently been shown to play a role in the PINK1-parkin mediated regulation of mitochondria by promoting mitophagy [163]. PINK1 accumulates on the outer mitochondrial membrane upon onset of mitochondrial depolarisation where it phosphorylates ubiquitin as well as parkin, thereby recruiting and activating parkin and ubiquitin [176,177]. Parkin then ubiquitinates mitochondrial proteins, triggering mitophagy [178]. A recent study has found that hTERT binds to the mitochondrial processing peptidase β catalytic subunit (MPPβ) [163]. MPPβ binds directly to PINK1 and triggers PINK1 cleavage [179]. One study has shown that even modest decreases in MPP levels dramatically affect PINK1 turnover. Indeed, MPP siRNA mediated knockdown results in an accumulation of PINK1 on the outer mitochondrial membrane. When hTERT binds to MPPβ PINK1 cleavage is blocked and thus promotes mitophagy [163,179]. Therefore, the co-localisation seen in this study could be due to an indirect interaction of hTERT and parkin through MPP and PINK1. These results indicate that LRP/LR could play a role in PD by promoting α-synuclein degradation through the increase of hTERT levels.

5.1.2. LRP/LR and α-synuclein co-localise and overexpression of LRP::FLAG increases total LRP/LR and α-synuclein the CTD levels

To further investigate the role of LRP/LR in PD, the HEK293 cell line was used as this cell line expresses neuronal markers [166], is easy to transfect and is frequently used as a PD cell culture model [155,180]. Briefly, HEK293 cells were stably transfected using the pCIneo-moLRP-FLAG expression plasmid to increase total LRP levels in HEK293 cells (**Fig 11**). Thereafter, confocal microscopy was performed to confirm co-localisation between α-synuclein and LRP/LR in HEK293 cells and to determine the effects of LRP::FLAG overexpression on this interaction. As expected, LRP/LR and α-synuclein co-localise in HEK293 cells and LRP::FLAG overexpression increased LRP/LR and α-synuclein levels (**Fig 12**). Furthermore, the co-localisation signal between LRP/LR and α-synuclein strengthened in LRP::FLAG transfected HEK293 cells (**Fig 12)**. These findings provide further evidence that LRP/LR and α-synuclein interact and provides insight into the role of LRP/LR in the α-synuclein pathogenesis of PD.

To further investigate the interaction seen between LRP/LR and α-synuclein levels and to confirm the increase in α-synuclein levels western blotting was performed. Western blot analysis revealed no significant change in the full-length 14kDa α-synuclein levels (**Fig 13a and b**). However, an increase in the α-synuclein CTD was observed in LRP::FLAG transfected HEK293 cells (**Fig 13a and b**). This could suggest that due to the high sequence similarity seen in the NTD of LRP to HSP70, LRP promotes the degradation of α-synuclein through the UPS and ALP systems. The UPS system is mainly responsible for the degradation of α-synuclein, however, large oligomeric forms can only be degraded by the ALP system [181]. A recent study has shown that monoubiquitination of α-synuclein promotes degradation through the UPS system, while deubiquitination targets the protein to the ALS system [182]. Therefore, to assess the hypothesis that LRP promotes degradation of α-synuclein through the UPS and ALP systems these ubiquitin sites would need to be analysed. Alternatively, LRP::FLAG overexpression could be resulting in the truncation of the NTD. Studies have shown that, NTD truncations have either no effect on or slightly reduces α-synuclein aggregation [183,184]. However, to confirm this, it would be vital to determine the identity of the band occurring at the 10 kDa mark through mass spectrometry.

5.1.3. LRP::FLAG overexpressing rescues cells from MPP+-induced cytotoxicity

Indeed, LRP::FLAG overexpression increases the α-synuclein CTD, however, it was unclear whether this is cytotoxic or beneficial to the cells. An increase in the α-synuclein CTD could be as a result of cleavage or truncation. Indeed, studies have shown that truncation of the α-synuclein CTD promotes aggregation, which is toxic to cells [185]. To investigate whether the increase in the α-synuclein CTD seen in LRP::FLAG transfected cells is cytotoxic, cell viability and MMP was analysed in non-transfected and LRP::FLAG transfected HEK293 cells treated with MPP^+. Indeed, compared to non-transfected cells, LRP::FLAG transfected cells treated with 500uM and 750uM MPP+ exhibited higher cell viability (**Fig 14**). In addition, cells were treated with 250uM MPP+ for 24 hours and the MMP was analysed. As expected, LRP::FLAG overexpression increased the MMP of HEK293 cells (**Fig 15**). Therefore, overexpression of LRP::FLAG could promote α-synuclein degradation by the UPS and ALS systems ultimately preventing cytotoxicity caused by aggregation of α-synuclein. Parkin has been shown to aid in the degradation of α-synuclein. Intriguingly, our research group has also shown that parkin levels increase following overexpression of LRP::FLAG [186]. Therefore, LRP could be decreasing α-synuclein cytotoxicity by promoting parkin-mediated degradation of the protein. On the other hand, LRP::FLAG overexpression increases hTERT levels as well as telomerase activity [101,118]. This increase in hTERT can impede cellular ageing and protect cells from Aβ cytotoxicity [100,101]. A recent study has also shown that hTERT inhibits the degradation of PINK1 [163]. Upon mitochondrial stress, PINK1 accumulates on the outer mitochondrial membrane where it recruits and activates parkin to regulate mitochondrial homeostasis [176,177]. PINK1 is highly regulated by the MPP and presenilin-associated rhomboid-like protease (PARL), which binds directly to PINK1 resulting in cleavage of the protein [187]. Additionally, hTERT has recently been found to bind to MPPβ, thus preventing PINK1 cleavage [163]. This results in the accumulation of full-length PINK1, therefore allowing for the activation of parkin and ubiquitin. Once parkin has been recruited it ubiquitinates the mitochondria and induces mitophagy [163].

5.2. Colorectal cancer study

The second part of this study aimed to look at the effect that overexpressing LRP::FLAG would have on colorectal cells. Indeed, the relationship between LRP/LR and cancer has been extensively researched and was observed to play a vital role in the progression of cancer.

LRP/LR is implicated in the key hallmarks of cancer including metastasis, angiogenesis, and the evasion of apoptosis [104]. It has been found that LRP/LR is overexpressed in several cancer types including colorectal cancer [104]. LRP/LR plays a role in several physiological processes including, but not limited to, cell cycle regulation [92], cell adhesion, cell migration [188] and the maintenance of cell viability [189]. Cancerous cells that overexpress LRP/LR can therefore manipulate these roles to promote tumourigenesis. As a result, researchers have investigated possible ways of targeting LRP/LR to either downregulate or block the function of the protein to inhibit tumourigenesis [104]. Indeed, studies have shown that siRNA mediated knockdown of LRP/LR has been successful in reducing the viability of several cancer cell lines including, but not limited to, breast [106], lung [108], melanoma [154] and colorectal cancer cells [107] Furthermore, blocking of LRP/LR with anti-LRP/LR antibodies significantly reduced invasion and adhesion of cancer cells [102,103,106,107,154]. Although the role of LRP/LR in cancer has been extensively studied, the effect that overexpressing LRP/LR would have in cancer has not yet been investigated. Therefore, the current study investigated the effect that overexpressing LRP::FLAG has on colorectal cancer.

In addition, this study investigated the link between PD and cancer. Interestingly, PD-related proteins have been also been implicated in cancer [66]. One such protein is the E3 ubiquitin ligase, parkin. Indeed, parkin has been shown to function as a tumour suppressor in several types of cancer including colorectal cancer [69,169,190]. Moreover, if LRP/LR can increase parkin levels in colorectal cancer this could provide a possible link for PD and cancer, therefore, addressing two diseases with one treatment. Therefore, this study investigated the effect of overexpressing LRP::FLAG in colorectal cancer. Firstly, cell viability and MMP were analysed in LRP::FLAG transfected DLD-1 cells and compared to non-transfected cells. Thereafter, the interaction of LRP/LR and hTERT with the PD-related protein and possible tumour suppressor, parkin, was analysed. Finally, the effect of LRP::FLAG overexpression on parkin protein levels was assessed.

5.2.1. LRP/LR and hTERT co-localise in colorectal cancer cells

Indeed, LRP/LR interacts with the catalytic subunit of telomerase, hTERT [101,109,118]. Interestingly, studies have also shown that 85-90% of malignant tumours display increased telomerase activity [133,137]. hTERT has also been implicated in the regulation of apoptosis, independently of its role in telomerase activity and telomere maintenance [104]. Previous studies have shown that LRP/LR and hTERT co-localise in the perinuclear compartments as

well as the cell surface [100,101,109]. Furthermore, co-immunoprecipitation studies have also confirmed that LRP/LR and hTERT interact [109]. Therefore, confocal microscopy was performed to confirm co-localisation between LRP/LR and hTERT in DLD-1 cells. As expected, LRP/LR and hTERT co-localise in DLD-1 cells (**Fig 16**). However, DLD-1 cells grow in clusters thus resulting in poor penetration of antibodies in cells located at the centre of these clusters. As a result, the subcellular localisation, as well as their co-localisation, could not be accurately determined for single cells. To overcome this, sufficient resuspending of the cells was performed to ensure individual cells were seeded onto the coverslip. This was taken into consideration for future confocal microscopy experiments performed on the DLD-1 cells.

Interestingly, studies have shown that siRNA-mediated knockdown of LRP/LR reduced telomerase activity in breast cancer [109] and more recently, overexpression of LRP::FLAG increased telomerase activity [101]. In addition, increased hTERT levels have also been associated with the inhibition of apoptosis [139], while knockdown of hTERT with siRNA activated the Bax, a pro-apoptotic protein, thus activating mitochondrial cell death [140,141]. Therefore, these results reveal that LRP/LR does indeed interact in the colorectal cells. However, to determine the subcellular localisation and single-cell co-localisation this experiment would need to be repeated with the above-mentioned alterations.

5.2.2. Overexpressing LRP::FLAG decreases cell viability and MMP of colorectal cancer cells

To investigate the effect that overexpressing LRP/LR has on colorectal cancer, DLD-1 cells were stably transfected using the pCIneo-moLRP-FLAG expression plasmid to increase total LRP levels. LRP::FLAG transfection was confirmed using confocal microscopy (**Fig 17**). Thereafter, the MTT cell viability assay was performed to assess the effect of overexpressing LRP::FLAG on DLD-1 cells. Indeed, compared to non-transfected cells, LRP::FLAG transfected cells had lower cell viability (**Fig 18**). To further investigate the effect of LRP::FLAG overexpression on DLD-1 cells, the MMP was analysed. The results indicate that compared to non-transfected cells, LRP::FLAG transfected DLD-1 cells had a lower MMP (**Fig 19**). During apoptosis, several key events occur in the mitochondria, one being a significant decrease in the MMP [161]. Therefore, the decrease in MMP following LRP::FLAG overexpression could be inducing apoptosis in DLD-1 cells, which could explain the decrease in cell viability seen in these cells.

These results are interesting as one would expect that overexpressing LRP::FLAG would increase cell viability as both LRP/LR and hTERT have not only been shown to be overexpressed in several cancers but this overexpression has been associated with an increase in tumourigenesis [104]. However, a recent study has shown that mice overexpressing LRP::FLAG had increased p53 levels [191]. The nuclear transcription factor, p53, is responsible for the activation of several genes that are involved in apoptosis, senescence, and cell cycle arrest [192]. Once cellular stress such as DNA damage, hypoxia and oncogene activation has been detected, p53 is translocated to the nucleus where it is activated. Interestingly, the extent of cellular stress determines the fate of cells, whereby low levels of cellular stress results in cell cycle arrest and DNA repair, high levels of cellular stress result in the induction of apoptosis [193]. Moreover, p53 is one of the most well-known tumour suppressors that promotes the removal of developing cancer cells. p53 is regulated by the E3 ubiquitin ligase, MDM2, which is responsible for keeping p53 levels low during normal conditions. Indeed, p53 overexpressing mice have also been shown to be more resistant to developing spontaneous tumours [194,195]. Indeed, loss-of-function mutations of p53 have been detected in over 50% of human cancers and p53 has therefore been classified as a Knudson-type tumour [193]. Therefore, the decrease in cell viability seen in LRP::FLAG overexpressing DLD-1 cells possibly as a result of elevated p53 levels. However, p53 mRNA and protein levels would need to be analysed in future studies by qPCR and western blotting, respectively.

In addition, mice overexpressing LRP::FLAG have been shown to have elevated levels of the anti-ageing proteins, SIRT1 and klotho [191]. Interestingly, SIRT1, a NAD-dependent deacetylase, has been found to act as a tumour suppressor in some cancer types, including breast cancer and colorectal cancer [196]. Indeed, $APC^{min/+}$ mice overexpressing SIRT1 had reduced colorectal cancer formation [197]. This study also found that SIRT1 deacetylates β-catenin, therefore reducing cell proliferation [197]. Moreover, Klotho is essential for the fibroblast growth factor (FGF) 23-FGF receptor (FGFR) interaction [198]. Additionally, klotho is responsible for regulating the transient receptor potential cation channel subfamily V (TRPV) member 5 calcium channel and it can also inhibit the insulin-like growth factor (IGF)-1 pathway [199,200]. Furthermore, klotho functions as a tumour suppressor in several cancer types [199,201-204]. Moreover, klotho has been found to be down-regulated in colorectal cancer and overexpression of klotho in colorectal cancer cells inhibits cell growth [205-207]. In addition, a more recent study has shown that klotho inhibits the proliferation of colorectal

cancer cells *in vitro* and significantly slows down the growth of tumours in mice [208]. Therefore, if overexpression of LRP::FLAG is able to increase the tumour suppressors p53, SIRT1 and klotho it could be used as a powerful therapeutic for the treatment of colorectal cancer. However, p53, SIRT1 and klotho levels and the effect that overexpressing LRP::FLAG has on these proteins in colorectal cancer should be investigated further by analysing mRNA and protein levels by qPCR and western blotting, respectively.

5.2.3. LRP/LR and hTERT co-localise with the PD-related protein, parkin

Although the risk for developing cancer is lower for PD patients, the opposite trend is seen for some cancers [63,64]. Furthermore, PD-related proteins have recently been shown to play vital roles both in the progression and impediment of cancer, thus explaining the link between the two diseases [66]. In PD, parkin is responsible for inducing mitophagy as well as ubiquitination of cytosolic proteins which include α-synuclein [13,41]. However, recent studies have shown that the PD-related protein, parkin, also functions as a tumour suppressor [78]. Therefore, the current study investigated whether LRP/LR and hTERT could interact with and possibly increase the expression of parkin levels in colorectal cancer cells. To investigate this, confocal microscopy was initially performed to determine whether LRP/LR and hTERT interact with the PD-related protein, parkin. Indeed, LRP/LR and parkin co-localise in the cytoplasm of both non-transfected and LRP::FLAG transfected DLD-1 cells (**Fig 20**). Interestingly, a study has shown that overexpression of parkin results in a reduction of LRP/LR in human cervical cancer cells [209]. Although no follow-up studies were published these results indicate a possible interaction between LRP/LR and parkin. As stated above, LRP/LR plays a vital role in the progression of cancer by promoting metastasis, angiogenesis, preventing apoptosis and promoting tumourigenesis further by increasing hTERT expression [104]. Therefore, if parkin can aid in the degradation or downregulation of LRP/LR, it can serve as a powerful tumour suppressor for many cancer types where LRP/LR expression is increased. However, it is unclear how overexpressing LRP/LR would affect this interaction. These results indicate that LRP/LR and parkin do indeed co-localise in LRP::FLAG overexpressing cells. Once co-localisation between LRP/LR and parkin was established, co-localisation between hTERT and parkin was analysed. Interestingly, although hTERT and parkin do not co-localise in non-transfected DLD-1 cells (**Fig 21 A-G**), co-localisation was observed in LRP::FLAG transfected DLD-1 cells in the cytoplasm and the nucleus (**Fig 21 H-N**). This was the first study to investigate the interaction between hTERT and parkin. Indeed, hTERT has recently been shown to play a role upstream of parkin, by regulating the degradation of PINK1 [163]. PINK1

recruits and activates parkin and ubiquitin to regulate mitochondrial homeostasis [176,177]. Furthermore, hTERT has recently been shown to bind to MPPβ, , therefore, preventing the cleavage of PINK1 and as a result, preventing mitophagy [163]. Therefore, hTERT plays a protective role in the regulation of dysfunctional mitochondria. This is important as mitochondrial dysfunction has been associated with cancer progression and therefore, clearing dysfunctional mitochondria could be vital for the inhibition of tumourigenesis.

5.2.4. Overexpressing LRP::FLAG increases parkin protein levels in colorectal cancer cells

Indeed, overexpression of LRP::FLAG in DLD-1 cells resulted in a decrease in cell viability and mitochondrial membrane potential. Furthermore, co-localisation was seen between LRP/LR and parkin in non-transfected and LRP::FLAG transfected DLD-1 cells, while co-localisation between hTERT and parkin was only seen in DLD-1 cells LRP::FLAG transfected. These results indicate that overexpressing LRP::FLAG in DLD-1 cells could be inhibiting tumourigenesis possibly through the tumour suppressor parkin. Therefore, to determine whether parkin is a key player in these cells, parkin protein levels were examined. As expected, parkin levels were significantly increased in LRP::FLAG transfected cells (**Fig 22**). Indeed, parkin has been shown to function as a neuroprotector in PD [78,210], however, it has also recently been shown to be a promising tumour suppressor [69,169,190]. Expression differences and gene alterations of the PARK2 gene have been detected in several tumour biopsies and cell lines [69,169,211,212]. Parkin has been associated with the inhibition of proliferation by ubiquitinating cyclin E, thus inducing G1/S cell cycle arrest [80,213]. Parkin also plays a role in sensitizing HeLa cells to apoptosis by PINK1-dependant polyubiquitination of the anti-apoptotic protein Mcl-1, leading to its degradation and thus, promoting apoptosis [81]. In addition to proliferation and apoptosis, parkin also plays a role in other hallmarks of cancer including metastasis, metabolic reprogramming, angiogenesis, and tumour-promoting inflammation, among others [78,83].

These results are promising as a recent study has shown that a third of colorectal cancers lack the PARK2 gene [85]. Furthermore, hypermethylation of the PARK2 promoter, as well as a decrease in parkin mRNA levels, have also been demonstrated in colorectal cancer primary tumours [85]. Another study demonstrated that 42% of human colorectal cancer tissues displayed alternatively spliced isoforms of parkin with various deletions [213]. In addition, wild type parkin was also found to induce cyclin E degradation, thus inhibiting proliferation

[213]. Moreover, PARK2 haploinsufficiency and APC gene mutations work together to increase polyp multiplicity and accelerate the progression of adenomas [85]. Interestingly, parkin expression was also found to correlate positively with the survival of patients with advanced colorectal cancer [84]. Therefore, the overexpression of LRP::FLAG results in the increase in parkin protein levels which could be a promising therapeutic treatment for colorectal cancer that could ultimately lead to the decrease in tumourigenesis of these cells.

6. Conclusions

This study demonstrated for the first time, that LRP/LR plays a role in PD *in vitro*. Here, an interaction was established between LRP/LR and hTERT with the PD-related proteins, α-synuclein and parkin, in the neuroblastoma (SH-SY5Y) cells. Once these relationships were established, human embryonic kidney (HEK293) cells were transfected with the pCIneo-moLRP-FLAG expression plasmid to increase total LRP levels. The relationship between LPR/LR and α-synuclein was then further analysed in HEK293 non-transfected and LRP::FLAG transfected cells. Indeed, LRP/LR and α-synuclein co-localised in both non-transfected and LRP::FLAG transfected HEK293 cells. In addition, overexpression of LRP::FLAG resulted in an increase in α-synuclein CTD. Finally, to assess whether the increase in α-synuclein CTD is cytotoxic or due to the degradation of α-synuclein the cell viability and MMP of MPP^+ treated cells were analysed. Interestingly, overexpression of LRP::FLAG resulted in an increase in both cell viability and MMP, possibly indicating that LRP/LR rescues cells from MPP^+ induced cytotoxicity and that the increase in α-synuclein CTD could be as a result of degradation.

Furthermore, this study also looked at the effect that overexpressing LRP::FLAG would have on colorectal cancer *in vitro*. Here, the well-established interaction between LRP/LR and hTERT was confirmed in colorectal carcinoma (DLD-1) cells. Thereafter, DLD-1 cells were transfected with the pCIneo-moLRP-FLAG expression plasmid to increase total LRP levels. To assess the effect that overexpressing LRP::FLAG would have in colorectal cancer cells, the cell viability and MMP were analysed. Intriguingly, LRP::FLAG overexpression significantly decreased cell viability and MMP. These results indicate that overexpression of LRP::FLAG could be impeding the progression of tumorigenesis. Thereafter, an interaction between LRP/LR and hTERT with the PD-related protein parkin was established. Finally, to determine whether the tumour suppressor parkin could be causing the decrease seen in the cell viability

and MMP, parkin protein levels were analysed and were found to be significantly increased in LRP::FLAG transfected DLD-1 cells. Therefore, these results indicated that the overexpression of LRP::FLAG could be impeding the progression of colorectal cancer *in vitro* through the increased parkin protein levels.

In conclusion, this study showed that LRP/LR plays a protective role in PD, likely through the degradation of α-synuclein and by increasing cell viability and the MMP of cells treated with the PD-inducer, MPP^+. Furthermore, this study also showed that the overexpression of LRP::FLAG impedes tumourigenesis of colorectal cancer possibly through elevated parkin protein levels. These novel results show the effect that overexpressing LRP::FLAG has in both PD and colorectal cancer. In addition, this study also provides a possible link between PD and cancer through LRP/LR and could result in one therapeutic option for both diseases. However, more research would need to be performed to determine the mechanism behind the protection offered by LRP::FLAG seen in PD and the tumour impediment caused by LRP::FLAG seen in colorectal cancer *in vitro* models. Ultimately, LRP/LR presents as a promising target for the treatment of both PD and colorectal cancer and may provide a crucial disease-modifying therapeutic treatment for both diseases.

6.1. Future work

Taken together, this study revealed that overexpressing LRP::FLAG could be a potential therapeutic target for PD and colorectal cancer. However, both diseases are complex and multifaceted and therefore more research would need to be performed to determine the exact mechanism of action. Indeed, the relationship between LRP/LR, α-synuclein and parkin seen in this study, would need to be further analysed. Therefore, future studies will assess whether LRP/LR is a receptor for α-synuclein and whether an increase in parkin levels, following LRP::FLAG transfection, aids in the degradation of α-synuclein. It is also important to note that oxidative stress and mitochondrial dysfunction plays a major role in neuronal cell death, and it is important to establish the role that LRP/LR and hTERT plays in PD. To assess this, the mitochondrial membrane potential and ROS levels will be analysed in LRP::FLAG overexpressing cells. Furthermore, several other proteins have also been affected in PD, and therefore transcriptomic and proteomic analysis would provide a great deal of information on gene expression as well as PD-related pathways affected by LRP/LR. Once the role of LRP/LR in PD is better understood *in vitro*, an *in vivo* study would need to be performed. A PD mouse model will be used and tests such as the forced swimming, rotarod and the step-down inhibitory

tests will analyse the effect of LRP::FLAG overexpression on motor symptoms. An *in vivo* study would provide insight into whether overexpressing LRP::FLAG would also treat the motor and nonmotor symptoms of PD. Moreover, LRP/LR has been implicated in cancer and has been known to aid in the progression of several cancer types. Therefore, is it surprising that overexpression of LRP::FLAG could be impeding tumourigenesis. Therefore, the effect of LRP::FLAG overexpression on key hallmarks of cancer, such as migration, invasion, and adhesion, will need to be analysed. An *in vivo* study will also provide further insight into the effect of overexpressing LRP::FLAG on tumour growth.

7. References

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8. Appendix

Table S1: List of primary and secondary antibodies used for western blotting

Target protein	Primary antibody	Dilution	Secondary antibody	Dilution
LRP::FLAG	Murine anti-FLAG® M2, (Sigma F3165)	1:4000	anti-murine IgG-HRP (Sigma A4416)	1:2500
LRP/LR	Human anti-LRP/LR IgG-iS18, (Affimed)	1:6500	anti-human IgG-HRP, (abcam 6858)	1:6500
α-synuclein	Rabbit anti-α-synuclein (Sigma S3062)	1:1000	anti-rabbit IgG-HRP, (Cell Signaling Technology® 7074S)	1:2500
β-actin	Murine anti-β-actin-peroxidase, (Sigma A3854)	1:5000	-	-

Table S2: List of primary and secondary antibodies used for confocal microscopy

Target protein	Primary antibody	Dilution	Secondary antibody	Dilution
LRP::FLAG	Anti-FLAG® M2 –Cy3 (Sigma A9594)	1:200	-	-
LRP/LR	Human anti-LRP/LR IgG-iS18, (Affimed)	1:200	anti-human IgG-FITC (abcam 6854)	1:500
α-synuclein	Rabbit anti-α-synuclein (Sigma S3062)	1:200	Anti-rabbit IgG CF™647 (Sigma SAB4600393)	1:500
Parkin	Murine anti-parkin (ThermoFisher 390900)	1:100	Anti-mouse Cy™3 AffiniPure F(ab')₂ Fragment	1:500
hTERT	Rabbit anti-hTERT, (abcam 183105)	1:200	Anti-rabbit IgG CF™647 (Sigma SAB4600393)	1:500
hTERT	Mouse anti-hTERT, (ThermoFisher MA5-16033)	1:200	Anti-mouse Cy™3 AffiniPure F(ab')₂ Fragment	1:500

Table S3: List of reagents for 1x RIPA lysis buffer

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

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

Table S4: List of reagents for 12% SDS-PAGE gel

Reagent	12% Separating	5% Stacking
dH_2O (ml)	2.15	1.83
40% Acrylamide (ml)	1.5	0.313
Resolving buffer (ml)	1.25	-
Stacking buffer (ml)	-	0.313
10% SDS (µl)	50	25
10% APS (µl)	50	25
TEMED (µl)	2	2.5
Total volume per gel (ml)	5	2.5

Table S5: List of reagents for 1X electrophoresis tank buffer (pH 8.3)

Tris	3g
Glycine	14.4g
SDS	1g

Made up to 1 L with dH_2O and stored at 4˚C.

Table S6: List of reagents for transfer buffer

Tris	30g
Glycine	144g

Made up to 1 L with dH_2O and stored at 4˚C.

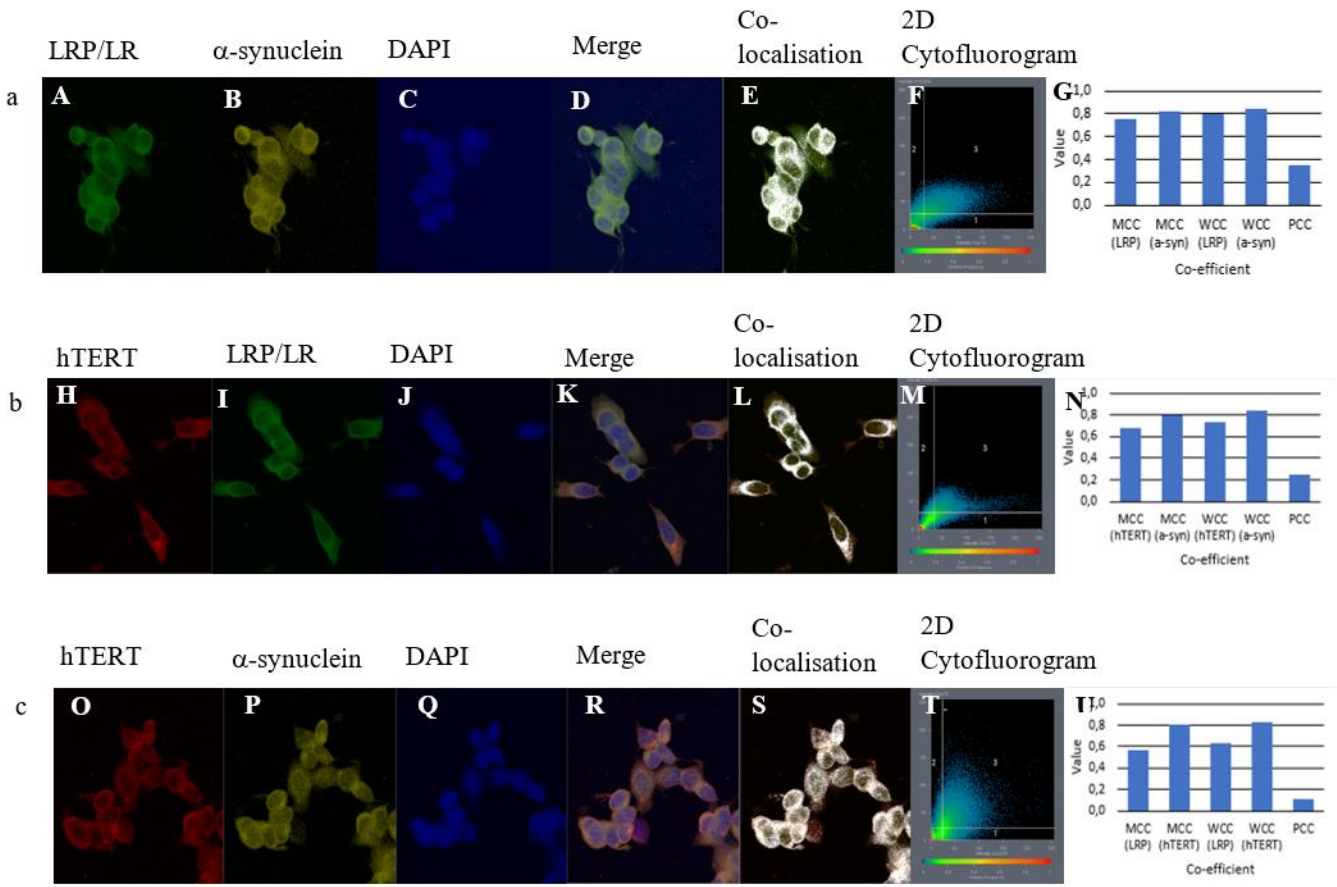
10X Transfer buffer	100ml
Methanol	200ml
dH_2O	700ml

Stored at 4˚C.

Table S7: List of reagents for phosphate buffered saline (PBS) (7.2/7.4)

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

Made up to 1 L with dH_2O and stored at room temperature.


LRP/LR
α-synuclein
DAPI
Merge
Co-localisation
2D Cytofluorogram
a
A
B
C
D
E
F
G
1,0
0,8
0,6
0,4
0,2
0,0
Value
MCC (LRP)
MCC (a-syn)
WCC (LRP)
WCC (a-syn)
PCC
Co-efficient
hTERT
LRP/LR
DAPI
Merge
Co-localisation
2D Cytofluorogram
b
H
I
J
K
L
M
N
MCC (hTERT)
MCC (a-syn)
WCC (hTERT)
WCC (a-syn)
hTERT
α-synuclein
DAPI
Merge
Co-localisation
2D Cytofluorogram
c
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T
MCC (LRP)
MCC (hTERT)
WCC (LRP)
WCC (hTERT)

Figure 23: LRP/LR, α-synuclein and hTERT co-localisation in SH-SY5Y cells. Intracellular localisation and co-localisation between a) LRP/LR (green) and α-synuclein (yellow), b) hTERT (red) and LRP/LR (green) as well as c) hTERT and α-synuclein. LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (A and I). α-synuclein was detected using an anti-α-synuclein primary antibody and alexa flour 647 conjugated secondary antibody (B and P). Finally, hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (H and O). Nuclei were stained blue with DAPI (C, J and Q). Co-localisation between hTERT and α-synuclein; hTERT and LRP/LR as well as LRP/LR and α-synuclein is shown in the merged images (D, K and R). Co-localisation is confirmed by the white in the co-localisation images (E, L and S) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F, M and T). Furthermore, co-localisation between LRP/LR and α-synuclein in b) HEK293 and HEK293 Transfected cells, was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss) (G, N and U). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

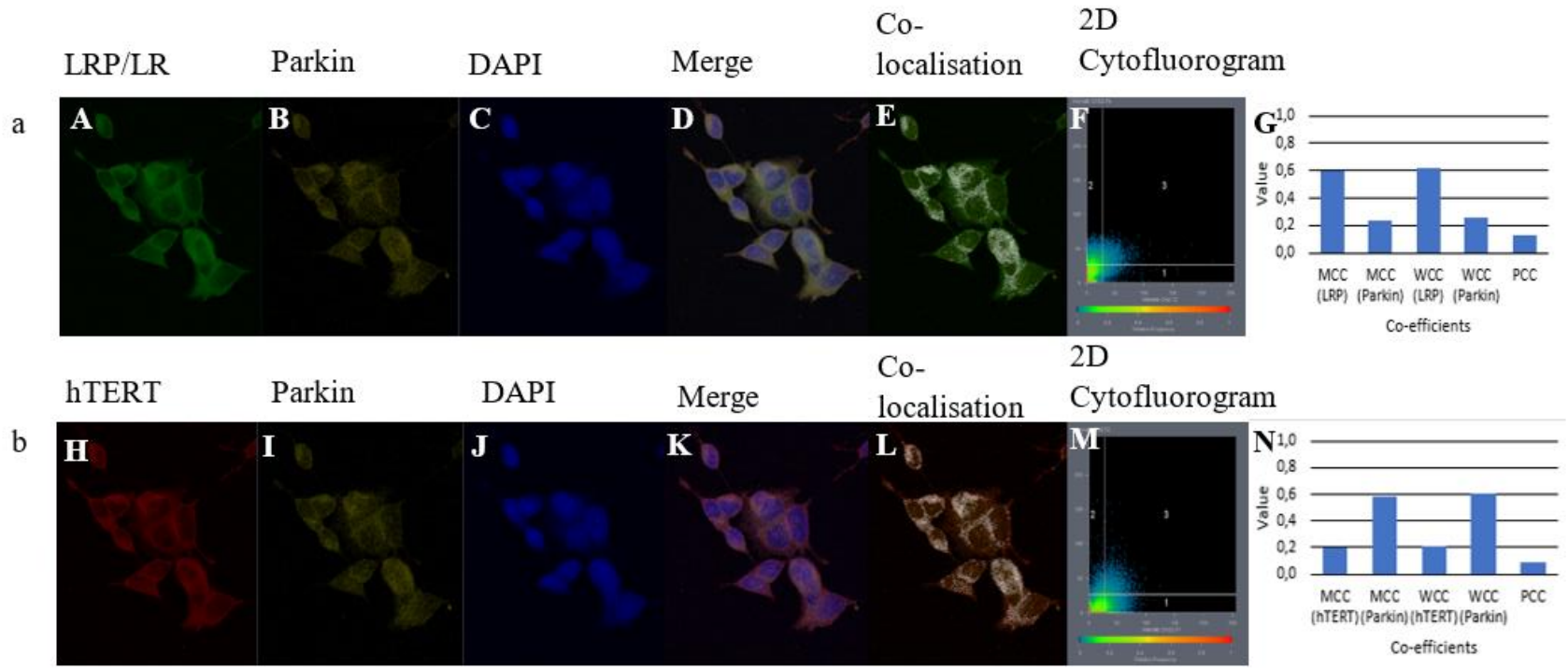

Figure 24: hTERT, LRP/LR and parkin co-localisation in SH-SY5Y cells. Intracellular localisation and co-localisation between a) LRP/LR (green) and parkin (yellow) as well as b) hTERT (red) and parkin. LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (A). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (B and I). Finally, hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (H). Nuclei were stained blue with DAPI (C and J). Co-localisation between hTERT and parkin as well as LRP/LR and parkin is shown in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between was quantified the Mander's (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficient using the Zen 2.3 software (Zeiss) (G and N). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

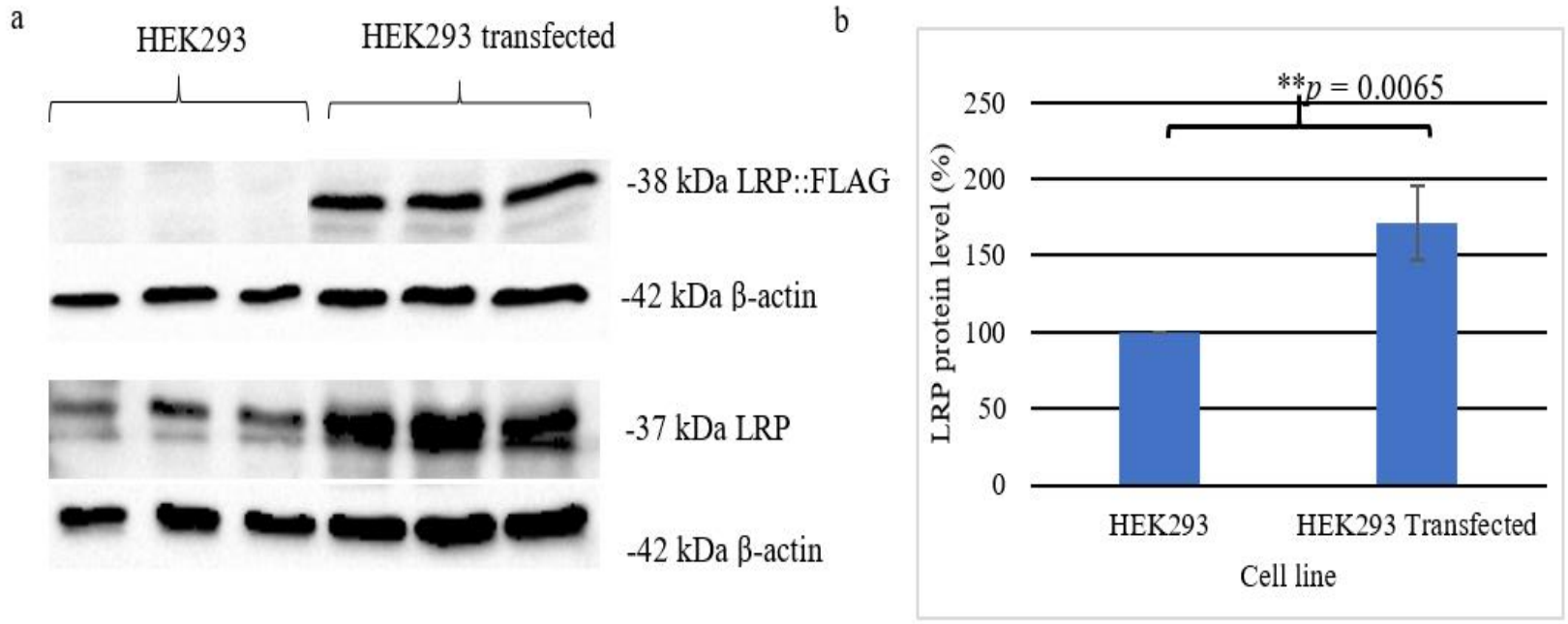

Figure 25: Transfection of HEK293 with pCIneo-LRP::FLAG plasmid was successful. Western blot analysis performed on pCIneo-LRP::FLAG transfected and non-transfected HEK293 cells (a). Endogenous LRP/LR and LRP::FLAG was detected using the anti-LRP, IgG1-iS18 and anti-Flag primary antibodies and HRP-conjugated secondary antibodies. β-actin was detected using a HRP coupled primary antibody. Transfection of HEK293 cells with the pCIneo-LRP::FLAG plasmid resulted in LRP::FLAG overexpression as well as an increase of total LRP protein levels. Densitometric and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 71.24% increase of total LRP levels (b). Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

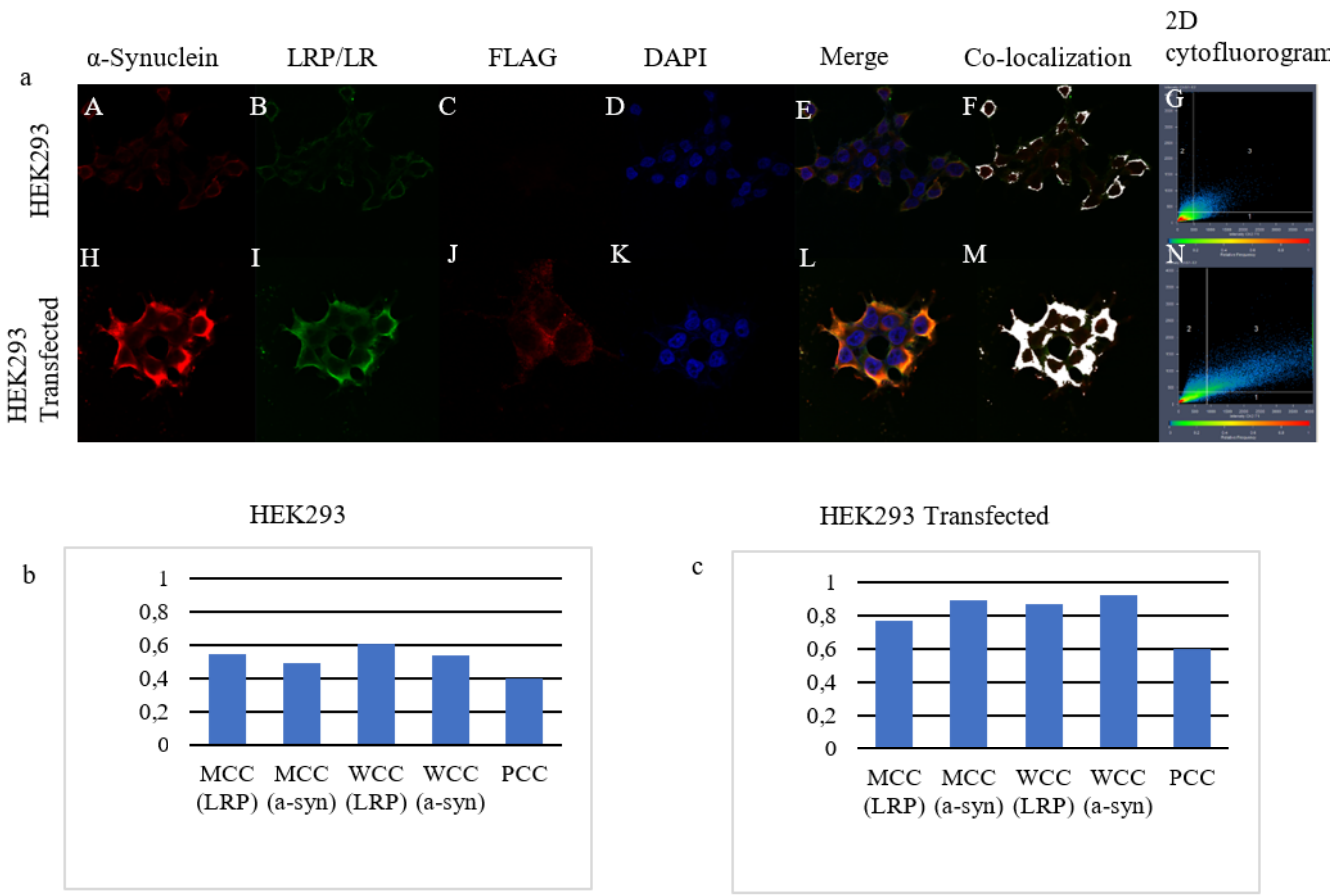

Figure 26: LRP/LR and α-synuclein co-localisation in HEK293 cells. Intracellular localisation and co-localisation between α-synuclein (red) and LRP/LR (green) (a). α-synuclein was detected using an anti-α-synuclein primary antibody and alexa flour 647 conjugated secondary antibody (A and H). LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B and I). LRP::FLAG was detected by using a Cy3 conjugated primary antibody (C and J). Finally, nuclei were stained blue with DAPI (D and K). Co-localisation between α-synuclein and LRP/LR and parkin is shown in the merged images (E and L). Co-localisation is confirmed by the white in the co-localisation images (F and M) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (G and N). Furthermore, co-localisation between LRP/LR and a-synuclein in b) HEK293 and HEK293 Transfected cells, was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (Life Sciences Imaging facility, WITS).

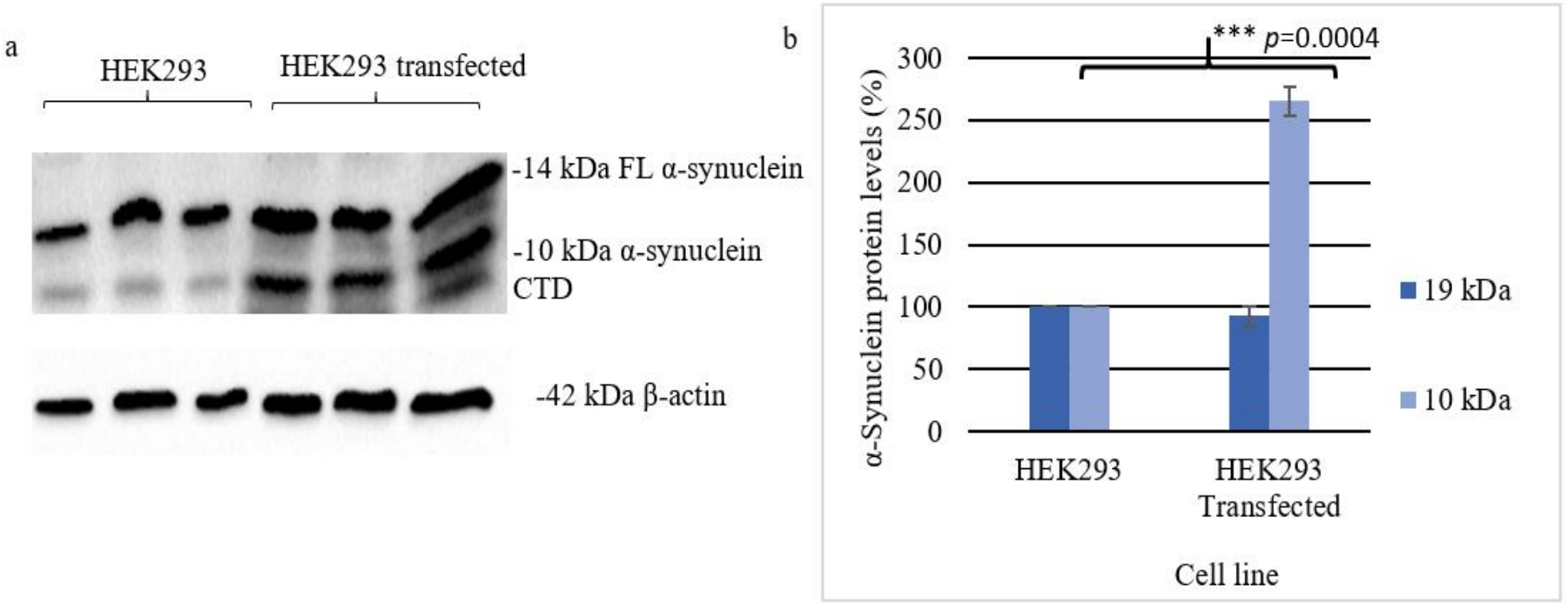

Figure 27: LRP::FLAG overexpression increases the α-synuclein C-terminal domain (CTD). Western blot analysis performed on pCIneo-LRP::FLAG transfected and non-transfected HEK293 cells. Total levels of full length (FL) - and C-terminal domain- α-synuclein were detected using an anti-α-synuclein primary antibody and HRP-conjugated secondary antibody. β-actin was used as a loading control and detected with an HRP coupled primary antibody. Transfection of HEK293 cells with the pCIneo-LRP::FLAG plasmid resulted in an increase of the 10 kDa α-synuclein CTD (a). Densitometric and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 165.22% increase of the α-synuclein CTD (b). Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

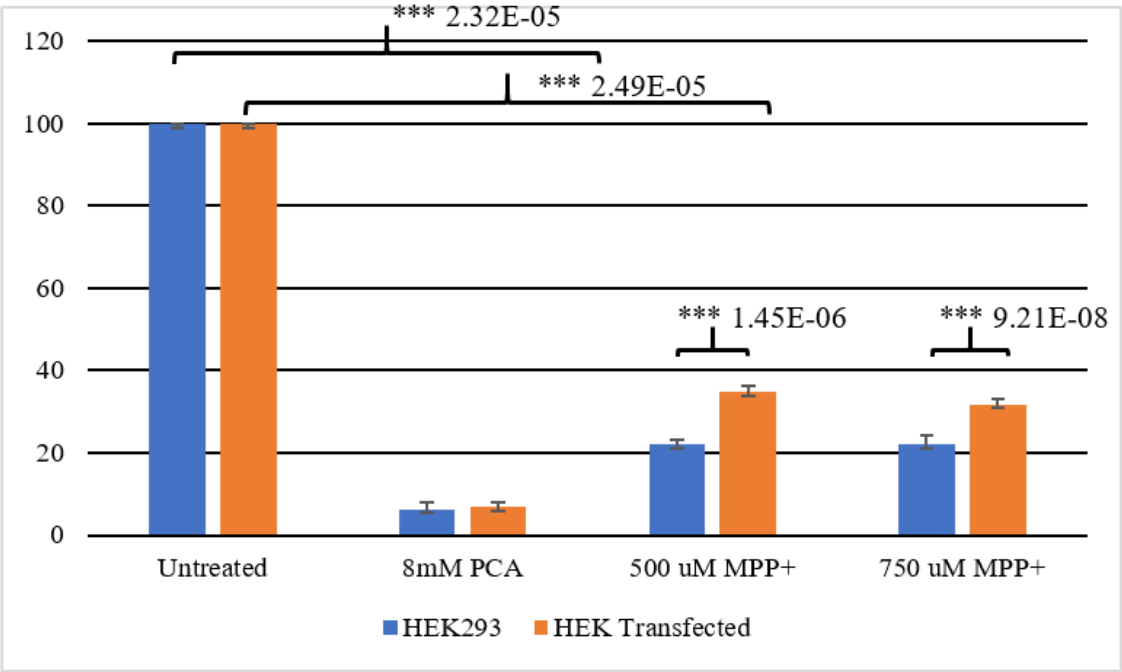

Figure 28: LRP::FLAG overexpression increases cell viability of cells treated with MPP^+. MTT assay assessing cell viability of HEK293 non-transfected and LRP::FLAG transfected cells treated with 500µM and 750µM MPP^+ for 48 hours. Untreated HEK293 non-transfected and transfected cells were set to 100% and PCA was used as a positive control. Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

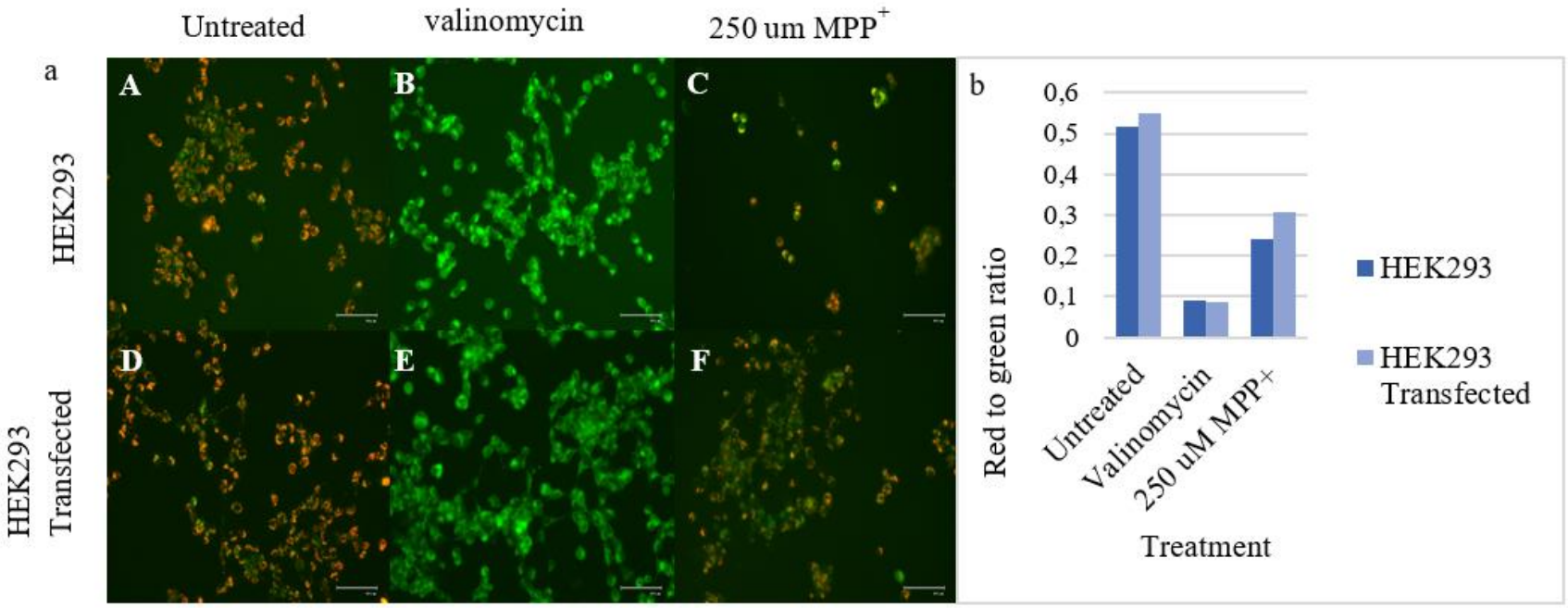

Figure 29: LRP::FLAG overexpression increases the mitochondrial membrane potential of cells treated with MPP^+. a) Mitochondrial membrane potential was analysed using the JC-1 fluorescent dye. In the cytoplasm the JC-1 dye exist as monomers (green), however, by means of the mitochondrial membrane potential the JC-1 dye migrates into the mitochondria where it forms aggregates (red). Untreated non-transfected and trnasfected HEK293 cells (A and D) where compared to cells treated with 250 uM MPP^+ (C and F). 0.2 ug/ml valinomycin was used as a positive control. b) Quantitative analysis of the JC-1 dye red to green ratio. All images were taken at 100X magnification using the Zeiss Evos microscope (WITS).

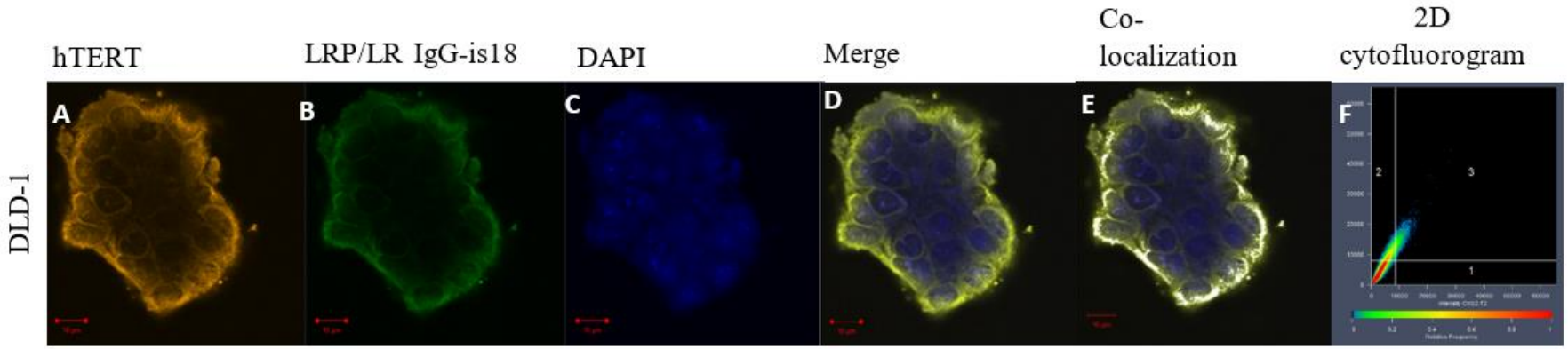

Figure 30: LRP/LR AND hTERT co-localise in DLD-1 cells. Intracellular localisation and co-localisation between a) hTERT (yellow) and LRP/LR (green). hTERT was detected by using an anti-hTERT primary antibody and Cy3 conjugated secondary antibody (A). LRP/LR was detected by using the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B). Nuclei were stained blue with DAPI (C). Co-localisation between hTERT and LRP/LR is shown by yellow fluorescence in the merged images (D). Co-localisation is confirmed by the white in the co-localisation images (E) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F). All images were taken at 630X magnification using the Zeiss LSM880 microscope (Confocal and Light Microscope Imaging Facility, UCT).

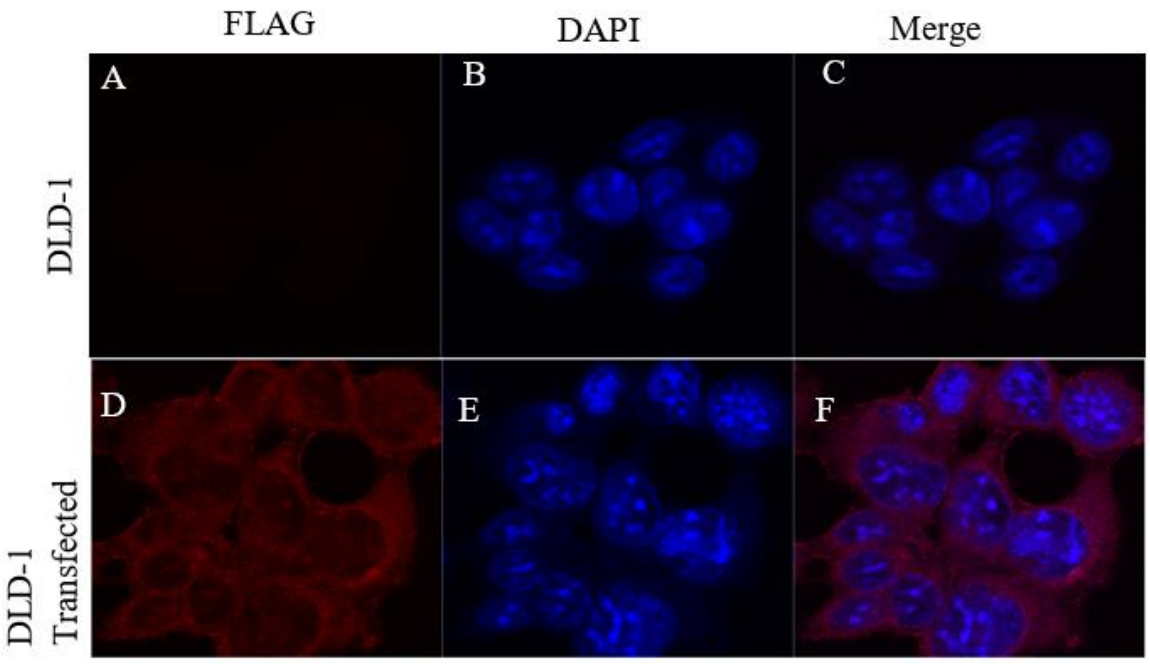

Figure 31: LRP::FLAG overexpression localisation and confirmatin in DLD-1 cells. Confirmation and localisation of LRP::FLAG (red) in DLD-1 cells. LRP::FLAG was detected by using a Cy3 conjugated primary antibody (D). Nuclei were stained with blue with DAPI (B and E). All images were taken at 630X magnification using the Zeiss LSM780 microscope (Confocal and Light Microscope Imaging Facility, UCT).

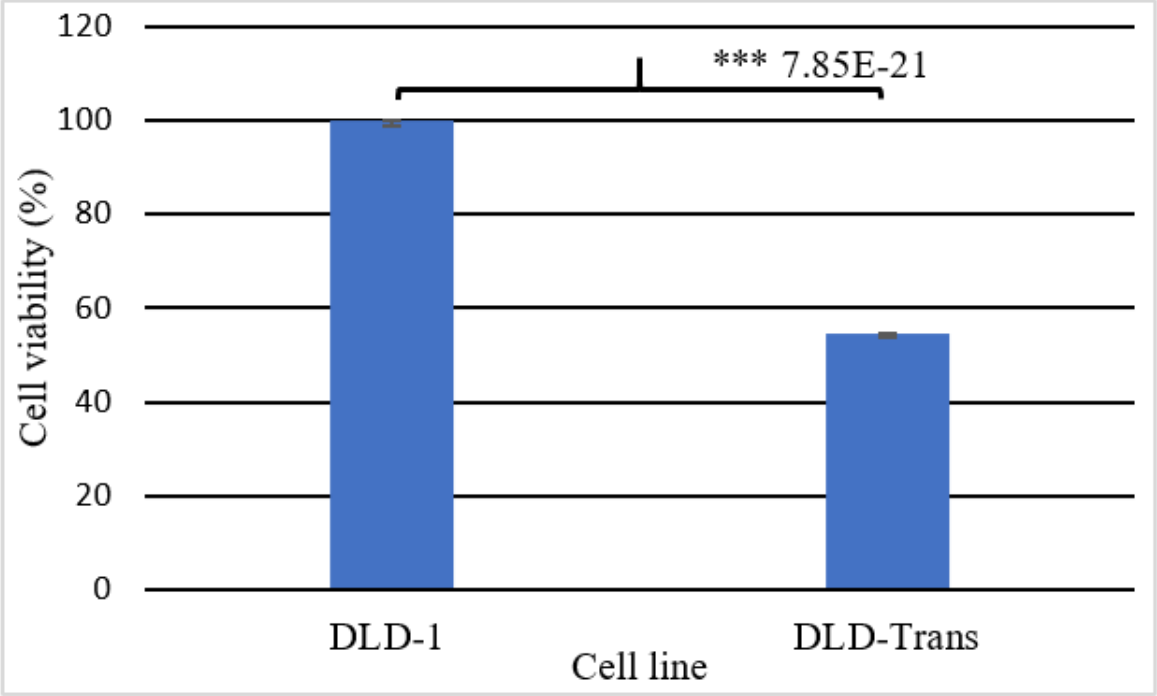

Figure 32: Overexpression of LRP::FLAG decreases cell viability of DLD-1 cells. MTT assay assessing the cell viability of DLD-1 non-transfected and transfected cells. Non-transfected cells were set to 100%. Standard deviation is depicted by the error bars, n= 3 biological repeats. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.

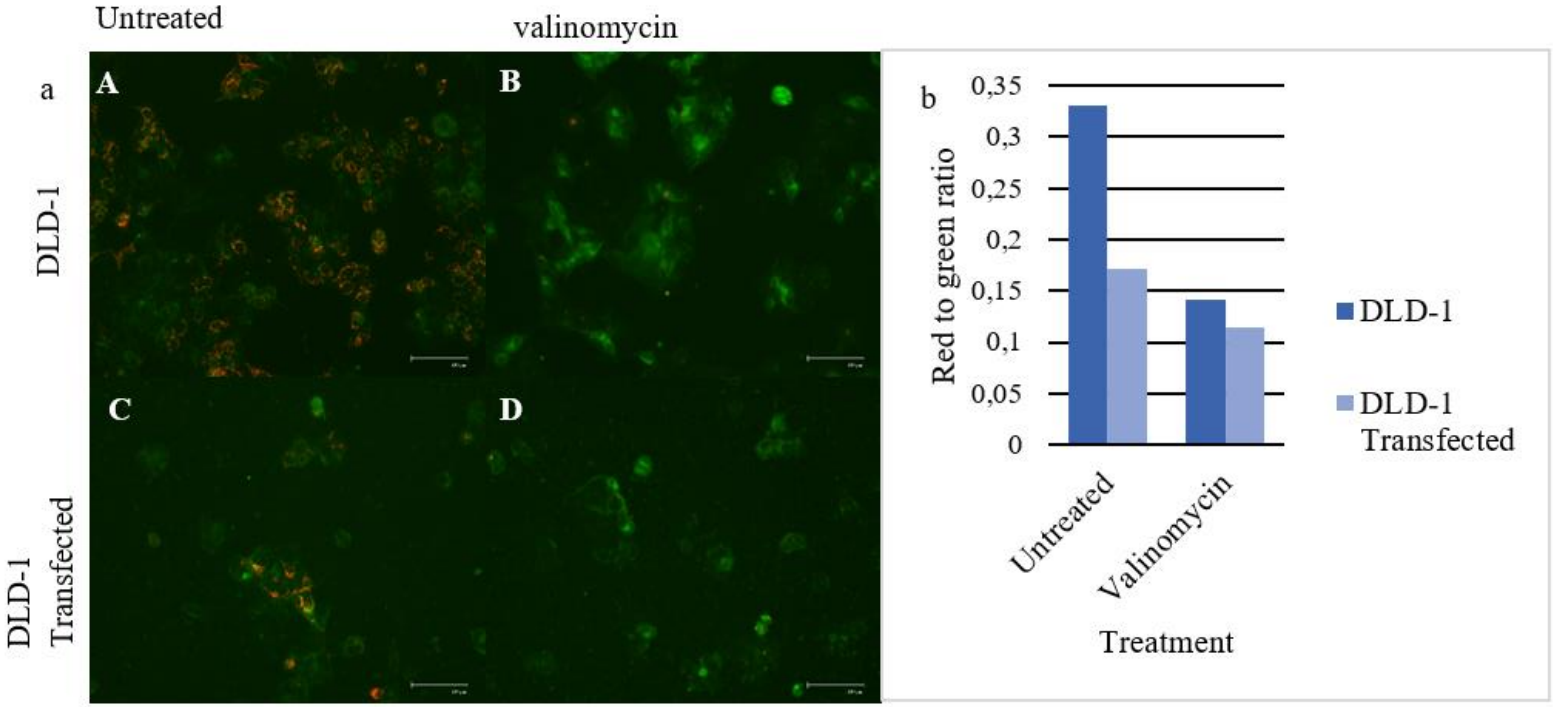

Figure 33: LRP::FLAG overexpression decreases the mitochondrial membrane potential of cells overexpressing LRP::FLAG. Mitochondrial membrane potential was analysed using the JC-1 fluorescent dye. In the cytoplasm the JC-1 dye exist as monomers (green), however, by means of the mitochondrial membrane potential the JC-1 dye migrates into the mitochondria where it forms aggregates (red). Non-transfected DLD-1 cells (A) where compared to LRP::FLAG overexpressing DLD-1 cells (C). 0.2 ug/ml valinomycin was used as a positive control. All images were taken at 100X magnification using the Zeiss Evos microscope.

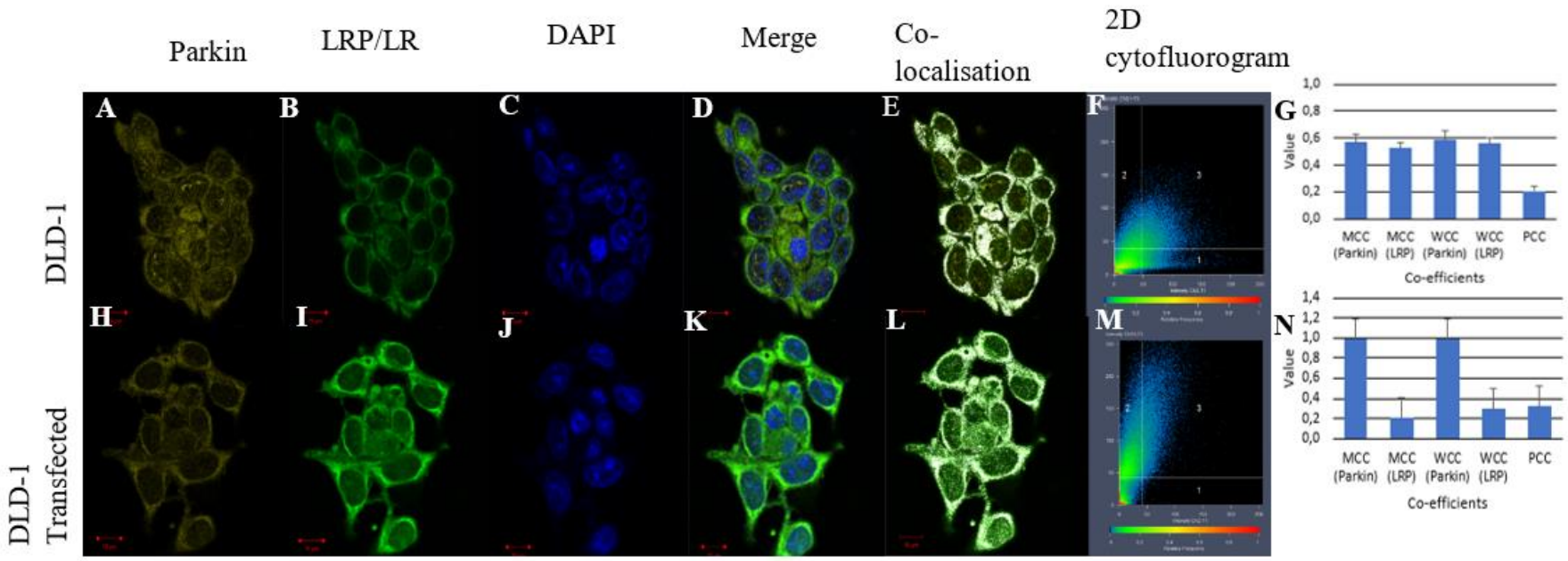

Figure 34: Parkin and LRP/LR co-localisation in DLD-1 cells. Intracellular localisation and co-localisation between parkin (yellow) and LRP/LR (green). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (A and H). LRP/LR was detected by the anti-LRP primary antibody, IgG1-iS18, and a FITC conjugated secondary antibody (B and I). Finally, nuclei were stained blue with DAPI (C and J). Co-localisation between parkin and LRP/LR is shown in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between parkin and LRP/LR in DLD-1 (A-G) and DLD-1 Transfected cells (H-N), was quantified the Manders (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT).

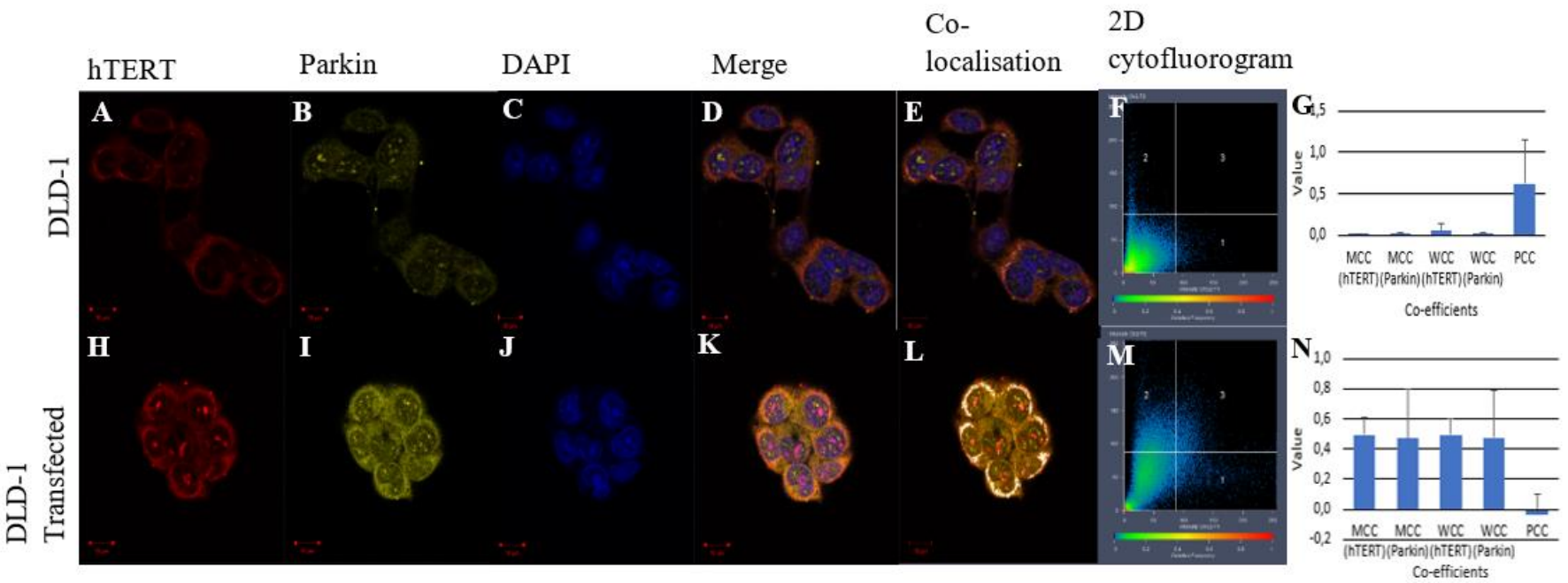

Figure 35: hTERT and Parkin co-localisation in DLD-1 cells. Intracellular localisation and co-localisation between hTERT (red) and parkin (yellow). hTERT was detected by using the anti-hTERT primary antibody and a Cy3 conjugated secondary antibody (A and H). Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (B and I). Finally, nuclei were stained blue with DAPI (C and J). Co-localisation between hTERT and parkin is shown by yellow fluorescence in the merged images (D and K). Co-localisation is confirmed by the white in the co-localisation images (E and L) as well as fluorescence signal in the third quadrant of the 2D cytofluorogram (F and M). Furthermore, co-localisation between hTERT and parkin in DLD-1 (A-G) and DLD-1 Transfected cells (H-N), was quantified the Mander's (MCC), Weighted (WCC) and Pearson's (PCC) correlation co-efficients using the Zen 2.3 software (Zeiss). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT).

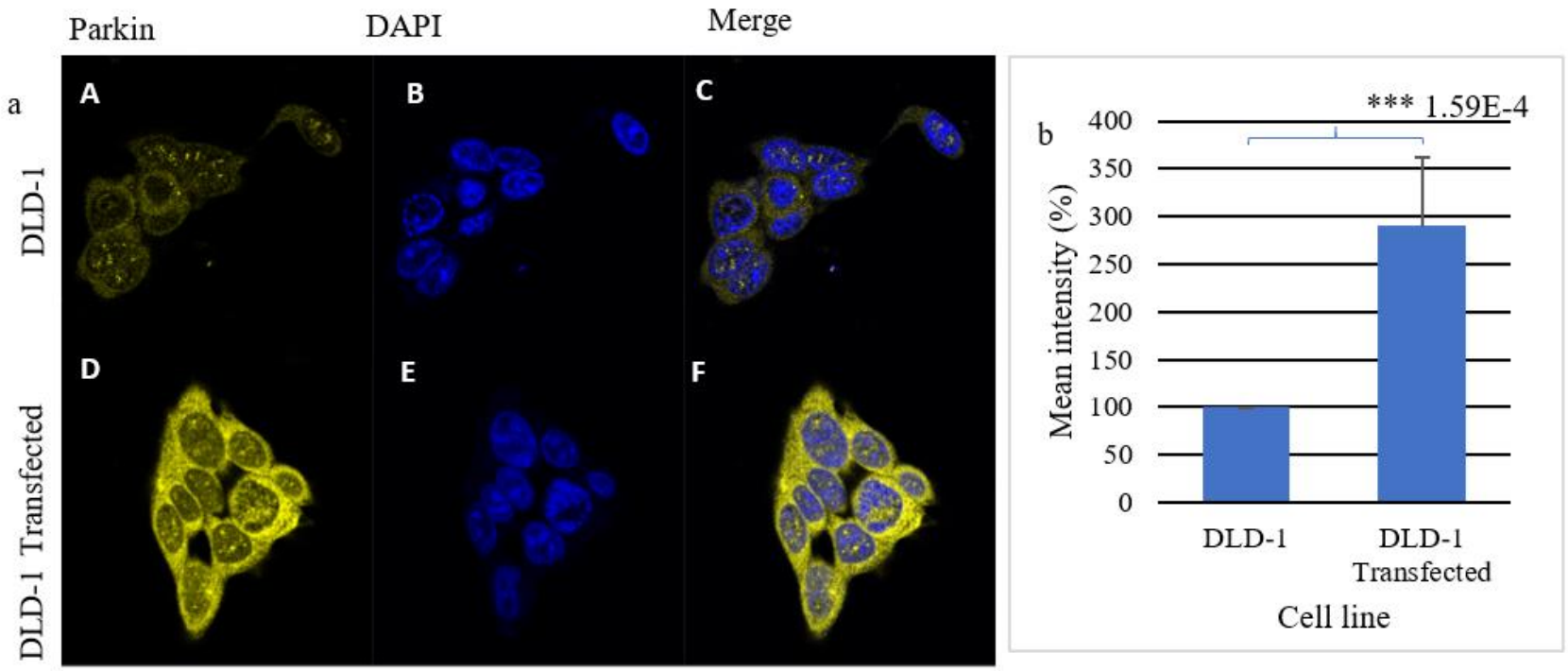

Figure 36: Parkin overexpression and localisation in DLD-1 cells. a) Expression and localisation of parkin (yellow) in DLD-1 cells. Parkin was detected using an anti-parkin primary antibody and alexa flour 647 conjugated secondary antibody (A and D). Nuclei were stained with blue with DAPI (B and E). All images were taken at 630X magnification using the Zeiss LSM780 microscope (UCT). b) Intensity measurements and statistical analysis using the student's t-test revealed that pCIneo-LRP::FLAG transfections resulted in a 189.71% increase of parkin fluorescence intensity. Standard deviation is depicted by the error bars. *p< 0.05, **p< 0.01, ***p< 0.001; Student's *t*-test.