UNIVERSIDADE DO ALGARVE

Characterisation of *PRKRA* and *WDR45* gene function, involved in Parkinson's disease.

Marie Catherine Bordone

Dissertação realizada sob a orientação de Patrick Lewis, PhD
& Inês Araújo, PhD

Mestrado em Ciências Biomédicas
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"Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída."

"I declare that I am the author of this work, which is original and unpublished. Authors and works consulted are properly cited in the text and included in the list of references."

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(Marie Catherine Bordone)

“Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.”

Albert Einstein

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ABSTRACT

The *PRKRA* gene is situated on human chromosome 2p. It plays an important role in the regulation of gene expression in interferon–treated and virus–infected animal cells and is also implicated in the control of cell growth, proliferation and differentiation. The downstream target of this activation is a stress response protein involved in the Protein Kinase R (PKR) signalling pathway that mobilizes somatic cell death programs. It has been shown that aggregates of phosphorylated PKR are increased in the hippocampus of patients with Parkinson's disease. The P222L mutation in this gene is associated to dystonia-parkinsonism syndrome (DYT16) although the mechanism underlying this disorder is not yet understood.

BPAN (Beta propeller associatied neurodegeneration) disorder is caused by mutations in the *WDR45* gene, which results in loss of function, and presents with some dystonia-parkinsonism features. This gene is situated on chromosome X (Xp11.23) and the mutations that occur are *de novo*, suggesting an atypical X-linked pattern disorder. WDR45 (WIPI4) belongs to the WD-40 family and is characterised by a seven-bladed β structural shape. Its ortholog in yeast is the *Atg18*, known to be involved in autophagy, so it is thought that WDR45 acts in the early steps of the autophagy cascade as a regulator of the ATG9A marked vesicles that transiently localize to the autophagosome formation site and induce autophagosome formation. *WDR45* mutations primarily affect the brain, despite expression of the gene in several human tissues, suggesting that autophagy plays an important role in the brain. To date, *WDR45* loss of function has been thought to cause impairments in autophagy, leading to a neurodevelopment and neurodegenerative phenotype.

The main goals of this thesis, were to analyse if the PKR pathway was altered by overexpressing *PRKRA* wild-type and mutated in the HT1080 cell line and to investigate the impact of overexpressing *WDR45* in H4, HEK and SHSY5 cell lines, in order to possibly provide insights with regard to the mechanisms that are underlie BPAN, DYT16 and Parkinson's disease. These goals were performed by western blots and analyzing well known hallmarks of autophagy such as LC3 and p62. Immunocytochemistry analysis was also performed to investigate the localisation of WDR45 within the cell as well if the autophagy was induced, in standard and induced autophagy conditions.

KEYWORDS

DYT16 (dystonia 16); PRKRA (protein kinase, interferon inducible double stranded RNA dependent activator); P222L mutation; WDR45; autophagy impairments; BPAN (Beta propeller associated Neurodegeneration); cell lines HT1080, H4, HEK and SHSY5; LC3 and p62 autophagy hallmarks; Parkinson's disease.

RESUMO

Esta dissertação visa caracterizar os genes *PRKRA* e *WDR45* para perceber melhor a patologia da doença de Parkinson. A doença de Parkinson é a segunda doença neurodegenerativa mais comum no mundo, com uma incidência que vai aumentando com o aumento da idade. É também uma doença onde a maior parte dos casos são idiopáticos sendo cada vez mais necessário descobrir os mecanismos subjacentes à doença.

A distonia 16 (DYT16) é caracterizada por uma distonia secundária generalizada com um início precoce. Os sintomas desta doença aparecem normalmente por volta dos 20 anos de idade e começam por distonia num braço ou numa perna, progredindo gradualmente para o tronco e o pescoço. Apresenta igualmente características parkinsonianas, como por exemplo tremores. Tais similaridades podem sugerir que haja uma correlação a nível bioquímico ou anatómico que ligue a DYT16 à doença de Parkinson.

A distonia 16 é devida a uma mutação, P222L, no gene *PRKRA* (proteína kinase, interferão induzido por ativador dependente de RNA dupla cadeia). Este gene encontra-se situado no cromossoma 2p e desempenha um papel importante na regulação da expressão génica em células animais tratadas com interferões ou infetadas com vírus. Sabe-se que se encontra implicado no controlo do crescimento, proliferação e diferenciação celular. O seu alvo "*downstream*" é o PKR (proteína kinase R), uma proteína envolvida na resposta ao *stress*, que se encontra envolvida na via que mobiliza a morte celular de células somáticas. Já foram observados níveis elevados de agregados de PKR no hipocampus de pacientes com a doença de Parkinson. No entanto, os mecanismos subjacentes à distonia 16 não se encontram ainda bem caracterizados.

A doença BPAN (Neurodegeneração associada à hélice beta) é devida a mutações associadas ao gene *WDR45* que se encontra situado no cromossoma X (Xp11.23). As mutações envolvidas neste gene resultam em perda de função génica e são mutações que ocorrem *de novo*, sugerindo um modelo atípico de herança ligado ao cromossoma X. Esta doença é caracterizada por um desenvolvimento retardado na infância com ganhos motores e cognitivos baixos até à adolescência ou à vida adulta onde ocorre de seguida um início abruto de demência progressiva e distonia-

parkinsonismo. Devido a estes fenótipos, pensa-se que também poderá haver uma correlação entre os mecanismos bioquímicos da BPAN com a doença de Parkinson.

A proteína WDR45 (também conhecido por WIPI4) pertence à família WD-40 e é composta por uma estrutura heptagonal laminar β. O seu ortólogo génico em leveduras é o gene *Atg18*, conhecido por estar envolvido na autofagia. Devido a esta relação pensa-se que WDR45 também se encontra envolvido no início da cascada da autofagia como regulador das vesiculas marcadas com ATG9A que se encontram localizadas transientemente no local da formação dos autofagossomas e que poderá induzir a formação de autofagossomas. Sabe-se também que apesar do gene encontrar-se expresso em vários tecidos do corpo humano (como por exemplo o musculo esquelético), as mutações do *WDR45* afetam primariamente o cérebro, sugerindo que desempenha um papel fundamental no cérebro. Até à data, sabe-se que a perda de função do gene *WDR45* leva a deficiências no mecanismo da autofagia dando origem a fenótipos neurodegenerativos.

Tendo em conta que o processo da autofagia se encontra envolvido com os genes *PRKRA* e *WDR45*, os objetivos desta tese consistiram em sobre expressar o gene *PRKRA* com e sem a mutação P222L na linha celular HT1080 e observar as mudanças na via PKR; em sobre expressar o gene *WDR45* nas linhas celulares H4, SHSY5 e HEK, induzir condições de *stress* nas células e observar as alterações causadas com a ajuda de marcadores de autofagia (tais como o LC3 e o p62). Tais procedimentos seriam necessários para tentar criar uma correlação entre o mecanismo da autofagia e os mecanismos que estão subjacentes às doenças DYT16, BPAN e Parkinson.

Após ter sobre expressado na linha celular HT1080, com sucesso, a estirpe selvagem e a estirpe mutada do gene *PRKRA*, os alvos "downstream" como: o nível total de PKR e o nível de PKR fosforilado, o nível total do eIF2α (fator de iniciação 2 eucariótico α) total e fosforilado, foram analisados através da técnica Western blot. Nesta linha celular, nenhum dos alvos "*downstream*" da via PKR se encontrou afetado pelos altos níveis de PRKRA celular tanto de estirpe selvagem como de estirpe mutada. Estes resultados podem ser devido ao facto do PRKRA endógeno (que é igual ao da estirpe selvagem) ter resgatado o fenótipo da estirpe mutada sobre expressa. O facto da linha celular HT1080 ser derivada de um fibrossarcoma poderá também ter comprometido os resultados tendo em conta que as suas vias celulares se encontrariam

bastante alteradas para se registar qualquer alteração com a sobre expressão do *PRKRA*. No entanto, pode também ser devido ao facto de para se registar alterações na via do PKR, as células têm que ser submetidas a situações de *stress* como por exemplo retirar os nutrientes das células.

Quanto à sobre expressão do gene *WDR45*, foi feita com sucesso apenas nas linhas celulares H4 e HEK. Foi analisado, por um ensaio MTT, a viabilidade das células após a transfecção feita com o auxílio de PEI onde se observou que transfectar o plasmídeo WDR45 juntamento com o PEI era extremamente tóxico para as células. Esta análise sugere que para a linha celular SHSY5, o método de transfecção escolhido para trasnfetar WDR45 era demasiado tóxico para a sua viabilidade.

Em seguida, o processo de autofagia foi analisado por Western blot, através do marcador LC3, nas linhas celulares H4 e HEK transfectadas e comparado entre as condições normais e as condições de retiro completo de nutrientes. Nenhuma alteração na conversão de LC3-I para LC3-II (indicador de ocorrência de autofagia) foi observada, o que pode sugerir que WDR45 é capaz de substituir LC3, fazendo parte de uma via paralela para autofagia. Após estes resultados, foi feito uma análise imunocitoquimica apenas na linha celular H4 (por ser derivada de um neuroglioma, isto é, células cerebrais) com o marcador de autofagia p62 e também para o WDR45-Flag para averiguar a localização celular do WDR45. Tal como para a análise por Western blot, foi igualmente feita uma comparação entre as condições normais e as condições de retiro completo de nutrientes. A coloração para p62 encontrava-se aumentada nas células que tinham sido transfectadas com WDR45 e retirados os nutrientes comparando com as células que tinham apenas sido tratadas com PEI. Esta observação pode ser devido ao facto de p62 estar a tentar eliminar o excesso de WDR45 ou simplesmente corroborar os resultados obtidos pela análise MTT, isto é a transfeção com o plasmídeo WDR45 ser extremamente tóxica para as células, induzindo assim morte celular. O ensaio imunocitoquímico também demonstrou, através do marcador WDR45-Flag, que WDR45 encontra-se dentro das vesiculas autofágicas juntamente com p62 e que também pode localizar-se dentro do núcleo das células. No entanto, através desta ultima observação não se pode concluir que esta seja a verdadeira localização de WDR45 pois pode ser devida à sobre expressão do WDR45.Para tentar averiguar a situação, foi feita uma análise da sequência do plasmídeo utilizado através do programa MultiLoc que tem a capacidade de predizer a localização subcelular da proteína. No entanto, após correr o

programa, a predição da localização não era para o núcleo da célula mas sim para o complexo de Golgi. Como para o PRKRA, não se pode esquecer que todos estes testes foram feitos em linhas celulares que já possuem muitas vias celulares alteradas, o que pode falsear os resultados comparando ao que pode ocorrer na vida real.

Os dados obtidos nesta tese demonstram que ainda há muito por investigar para perceber os mecanismos que se encontram subjacentes a estes dois genes e por consequência para a doença de Parkinson.

PALAVRAS-CHAVE

DYT16 (distonia 16); PRKRA (proteína kinase, interferão induzido por ativador dependente de RNA dupla cadeia); mutações P222L; WDR45; BPAN (Neurodegeneração associada à hélice beta);autofagia; linhas celulares HT1080, H4, HEK e SHSY5; marcadores de autofagia LC3 e p62; Parkinson.

ABBREVIATION LIST

A

AC – Adenylate cyclase

AD – Alzheimer's disease

ALS – Amyotrophic lateral sclerosis

AMBRA – Activating molecule in beclin-1 regulated autophagy

AMPK – AMP activated protein kinase

Atg – Autophagy related proteins

B

BPAN – β-propeller protein associated neurodegeneration

cAMP – Cyclic adenosine monophosphate

C

CP – Ceruplasmin gene

CMA – Chaperone mediated autophagy

COMT – Catechol-O-metyltransferase

D

DBS – Deep brain stimulation

dsRBD – dsRNA domains

DYT16 – Dystonia 16

E

eIF2α – Eukaryotic initation factor 2

Epac – Guanine nucleotide exchange factor

ERK1/2 – Extracellular signal regulated kinase 1/ 2

F

FAHN – Fatty acid hydroxylase associated with neurodegeneration

FOXO – Forkhead box O

FTD3 – Frontal temporal dementia

G

GAP – GTPase activating protein

GBA – Glucocerebrosidase

GPi – Globus pallidus

GSK3 – Glycogen synthase kinase 3

GWAS – Genome wide association study

H

HD – Huntington disease

HLA – Human leukocyte antigen

I

IGF1 – Insulin growth factor 1

IP3 – Inositol 1,6,5 – trisphosphate

J

JnK1 – Jun N-terminal kinase 1

L

LRRK2 – Leucine reach repeat kinase 2

M

MAPT – Microtubule associated tau

MAO-B – Monoamine oxidase B

Met-tRNA – Methionine ribonucleic acid of interference

MPAN – Mitochondrial membrane protein associated neurodegeneration

MRI – Magnetic ressonance imaging

MT – Mutated

MTT –3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mTOR – Mammalian target of rapamycin

N

NBIA – Neurodegeneration brain iron accumulation

P

PAS – Phagophore assembly site(s)

PD – Parkinson's disease

PEI – Polyethylenimine

PET – Positron emission tomographic

PINK1 – PTEN induced kinase 1

PI3K – Phosphoinositide 3 kinase

PIP3 – Phosphatidylinositol-3,4,5 triphosphate

PKAN – Pantothenate kinase associated with neurodegeneration

PkB – Protein kinase B

PKCα – Protein kinase Cα

PKR – Protein activator of the interferon induced protein kinase

PLAN – Phospholipase A2 Group associated with neurodegeneration

PLCε – Phospholipase Cε

PRKRA – Protein kinase, interferon inducible double stranded RNA dependent activator

R

Rheb – Ras homolog enriched in brain

RSK1 – Ribosomal S6 kinase 1

S

S6K1 – Ribosomal S6 kinase 1

SGK1 – Serum and glucocorticoid induced protein kinase 1

SN – Substancia ningra

SNCA – α-synuclein

STN – Subthalamic nucleus

T

TSC1/2 – Tuberous Sclerosis ½

U

ULK51 – Unc51 like autophagy activating kinase 1

UPS – Ubiquitin proteosome system

UVRAG – Ultra-violet radiation resistance associated gene

V

Vim – Ventral intermediate nucleus

Vps34 – Regulatory class III PI3 kinase complex

W

WT – Wild-type

4E-BP1 – Eukaryotic initiation factor 4E binding protein

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I. INTRODUCTION

1. Parkinson's disease

1.1 Incidence of Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting over 10 million people around the world [1]. In Europe, there are more than 500 000 people with the disease [2]. The incidence of PD rises with increasing age, from 17.4/100 000 person years, between 50 and 59 years old, to 93.1/100 000 person years, between 70 and 79 years old with a 1,5% of lifetime risk of developing the disease [3]. The most frequent age of onset is in the sixth decade of life with a mean duration of 15 years from the diagnosis until death [3].

1.2 Definition of Parkinson's disease

In 1817, PD was first described by James Parkinson as the shaking palsy, reflecting the fact that his patients were presenting with "*involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured*" [4]. Depigmentation of the *substantia nigra* (due to a progressive and selective loss of dopaminergic neurons) and the presence of Lewy bodies (which are intracellular aggregations of proteins and lipids) are characteristics of PD [3]. The clinical manifestation of PD such as slowness of movement (known as bradykinesia), resting tremor, muscular rigidity and postural instability are the consequence of the deregulation of the motor circuits due to the loss of dopamine within the striatum [5].

1.3 Genetics and Parkinson's

This disease can be caused by an inherited mutation or be idiopathic (without known cause) [1],[6]. Although the majority of PD cases have an unknown aetiology, the remaining 5-10% have a strong genetic component that can help to understand the molecular pathogenesis' underlying of the PD in general [1],[2],[6]. To date, 16 monogenic genes (denominated *PARK* genes) and loci known to cause

PD through familial inheritance have been identified [1]. The most common, as autosomal dominant genes involved in the disease, are the α-synuclein gene (*SNCA*), leucine-rich repeat kinase 2 (*LRRK2*) and as the autosomal recessive genes there are Parkin (*PARK2*), PTEN induced kinase 1 (*PINK1*) and DJ1 (*PARK7*) [1], [5], [6] (Table 1). More recently, genome wide association studies (GWAS) performed and reported many more genes whose variation contributes to susceptibility for idiopathic PD, for example the microtubule associated tau (*MAPT*), Glucocerebrosidade (*GBA*) and the human leukocyte antigen (*HLA*) loci [1], [2], [5]–[7].

Table 1- Genes involved in PD with autosomal dominant (AD) or autossomal recessive (AR) inheritance.

Gene	Types of mutation	Mendelian inheritance
SNCA	Triplication Duplication Point mutations	AD
LRRK2	Point mutations	AD
GBA	Point mutations	AD
PINK 1	Point mutations	AR
PARK 2	Deletion	AR
DJ1	Point mutations	AR

The *SNCA* mutation was the first locus to be described for PD. Point mutations were the first observed in that gene: A53T and E46K [2]. The duplication or a triplication of the gene mutations were found after and have a dose dependent effect on the disease severity [5]–[7]. If a duplication is present, the onset of the disease will be in the 5^{th} or 6^{th} decade of life and if a triplication is present, the disease will start in the 4^{th} decade of life [2].

Point mutations in *LRRK2* lead to a kinase gain of function or reduction in GTPase function with a toxic impact on cells [7]. Mutations in *LRRK2* are much more common than those in *SNCA* and has a frequency of 1.5% in sporadic cases of PD and 4% in familial cases, in the European population [2].

The *GBA* mutations are a loss of function of a gene that is normally responsible for hydrolysing the β-glycosidic linkage of glucosyleceramide (an ubiquitous sphingolipid) responsible for producing glucose and ceramide and that is present in the plasma membrane of mammalian cells [6]. They are considered as a risk factor for the development of PD, but the mechanism by which the mutations exert their effects is still unknown.

PINK1 is located on the mitochondria and may exert a protecting effect on the cell that is disrupted when there is a mutation, leading to an increased susceptibility to cellular stress [6]. That is, when *PINK1* is mutated, the mitochondria are enlarged and have christae fragmentation leading to the apoptosis of the cell.

When the *PARK2* gene (also known as Parkin) is mutated, there is a inactivation of the gene leading to a reduction of ubiquitin proteasome system (UPS) mediated degradation of its substrates [5]. It was also shown that Parkin has a protective function in mitochondria, where it enhances mitochondrial gene transcription and so, when mutated, causes an increase in mitophagy (autophagy of mitochondria) [5]. However the mechanism is still not clear. Mutations in this gene are the principal cause of juvenile and early onset recessive Parkinsonism.

It is known that DJ1, in response to oxidative stress, is translocated to the mitochondria preventing this process to sensitize the cell to toxic cell damage but the specific role of the protein is not known [5], [7]. So, when there is a mutation on this gene, the cell is damaged by the oxidative stress liberated from the mitochondria. Similar to PINK1 and Parkin, it also involved in juvenile and early onset recessive Parkinsonism.

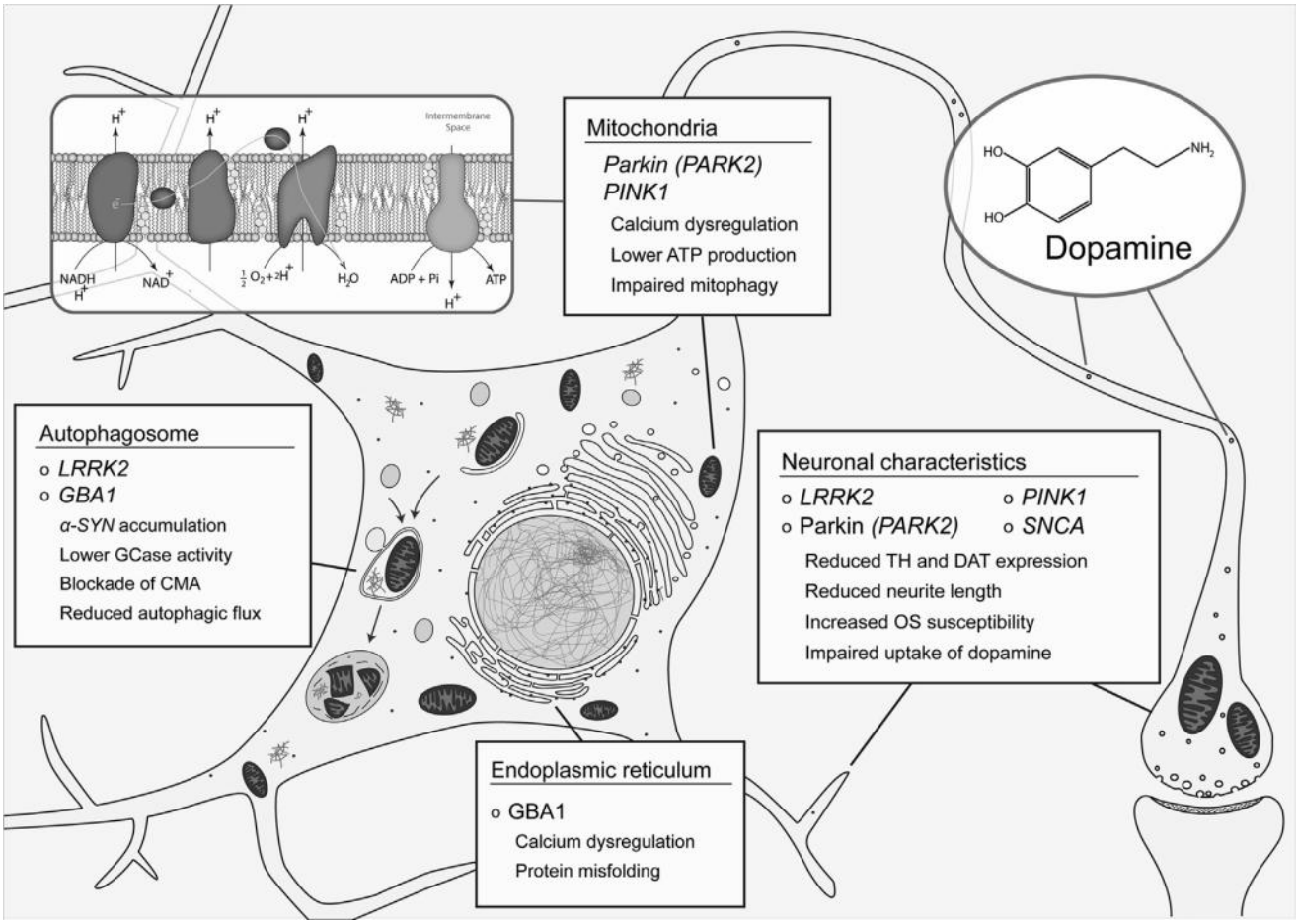

Figure 1.1 – Mutations associated with Parkinson's disease.
Scheme which summarises the ways in which mutations to key PD-associated genes LRRK2, GBA1, SNCA, PINK1 and PARK2, can cause pathway dysfunction, and ultimately cell death.
Adapted from [1].

As it can be observed, it seems that the most common genes involved with PD (*SNCA*, *GBA* and *LRRK2*) can cause accumulation of toxic compounds within the cells. These accumulations will difficult the discharging of cellular toxic compounds which may lead to the fact that the underlying cause of PD is involved with autophagy processes [8].

1.4 Diagnosis, symptoms and therapeutic of Parkinson's disease

The diagnosis of PD is mainly due by observing the patient: if he or she has an essential asymmetric tremor in one member of the body (usually the fingers or the foot), difficulties on writing, among others [3],[8]. However, the diagnosis can be supported by PET analysis or MRI [9]. It is also important to underline that a definitive diagnosis of PD is post mortem because only then it can be assessed if the

patient presented with Lewy bodies and had loss of dopaminergic neurons, two mandatory features of Parkinson's disease [9].

1.4.1 Dopamine therapy

The main clinical symptoms in this disease are due to a dopaminergic deficiency. The motor features that are always noticed in patients diagnosed with PD are: tremor, rigidity and bradykinesia. They are caused by a loss of dopamine in the posterior putamen and in the motor circuits [8]. Nevertheless, a number of other clinical symptoms (non-motor symptoms) such as sensory symptoms (e.g: pain and tingling), hyposmia, sleep alterations, depression, anxiety, neuro-endocrinal problems and abnormal executive, working-related functions, co-exist with the more prominent motor symptoms [8], [10]. At a molecular level, most of the brains with PD present with nigrostriatal dopaminergic degeneration, as mentioned before. That is, neuron loss in the *pars compacta* of the *susbtantia nigra* (SN), particularly in the ventrolateral tier of neurons in this area [11].

No curative treatment exists for PD. Some medication already exists to help the motor symptoms that can be incapacitating. The replacement of dopamine by using the dopamine precursor L-dopa is the most used therapy [10]. This treatment can, for some years, alleviate the motor symptoms and allows a less incapacitating life [11]. However, not all the patients with parkinsonism respond to this treatment and some have severe secondary effects (fluctuations in motor response and dyskinesia are the most common) [10]. Besides L-dopa, and to try to minimize the secondary effects, it is also used dopamine modulators that act directly in dopamine receptors as monoamine oxidase B (MAO-B) inhibitors or catechol-O-metyltransferase (COMT) inhibitors [10],[12]. The MAO-B inhibitors block the central dopamine oxidative metabolism, increasing synaptic dopamine levels and the COMT inhibitors block peripheral metabolism causing an increase in the elimination half-life and bioavailability of L-dopa [10]. However, none of these dopamine agonists present a better benefit in the antiparkisonian features than L-doppa.

1.4.2 Therapy based on surgery

Another therapy that has proved to benefit many cases is deep brain stimulation (DBS) [13]. As the disease progresses, dopamine drugs become less effective and their use is associated with an increasing of a number of adverse effects [14], [15]. The most common problems are the appearance of motor fluctuations and dyskinesias or drug-induced involuntary movement [14]. This therapy is, for now, considered by patients with late stage of PD and with severe disabilities despite the medication consumed [15].

It is known that neuronal degeneration and transmitter deficiencies that are associated with PD can produce important disruptions in the correct function of neuronal circuits [14]. Thus, parkinsonian state is characterized by pathological neuronal activity in the thalamus, the internal segment of the *globus pallidus* (GPi) and the *subthalamic nucleus* (STN), which are included in the motor system. The chronic electrical stimulation can suppress this abnormal activity in patients with PD.

There are three main surgical targets that are used for treating PD: the ventral intermediate nucleus of the thalams (Vim), GPi and STN [14]. The Vim target is used to treat the tremor symptom as GPi and STN are used to treat much more symptoms like bradykinesia, tremor, rigidity , drug-induced involuntary movements, and postural and gait disturbances [14], [16].

However, to apply this therapy, surgery is required and so there are complications that can occur.

It is not clear yet how DBS functions but it seems a good alternative to patients with severe drug-related symptoms [14], [15]. In the future, when the surgery will become more reliable and technically easier, it could be done earlier in patients to prevent complications related to continuous drug use.

2. Young onset dystonia parkinsonism (DYT 16)

Dystonia is a heterogeneous group of movement disorders, characterized by sustained involuntary contractions of agonist and antagonist muscles leading to abnormal postures [17]–[20]. Its aetiology is complex and the underlying cause is still not clear. However it seems to reflect a dysfunction in central nervous system regions that control movement [20].

It is the third most common disorder after parkinsonism and tremor: early onset dystonia has a prevalence of 20-50 cases per million and late onset has a prevalence of around 100-200 cases per million [17], [21].

The several types of dystonia are classified by their somatic distribution of symptoms (i.e. focal, segmental or generalized), aetiology (primary – no brain degeneration or secondary – with brain degeneration) and age of the disease onset [20]. There are 20 subtypes of dystonia (DYT1-DYT20) that include the pure dystonias, the dystonias plus syndromes where other manifestations are also present (like parkinsonism) and the paroxysmal dyskinesias where dystonia can be an additional feature[20].

In part of this thesis, the focus will be on DYT 16 – Young onset dystonia parkinsonism. It is an early onset generalized secondary dystonia with a recessive pattern of inheritance due to a missense mutation -P222L- in the *PRKRA* (protein kinase, interferon inducible double stranded RNA dependent activator) gene [17]–[19], [22]. However, it is still not clear if the P222L mutation is a loss or gain of function [17]. The symptoms usually appear before the age of 20 and start with dystonia in an arm or leg progressing to the trunk and neck [17]–[20]. It can also present with laryngeal dystonia causing speech defects and present parkinsonism features. The patients with DYT16 disorder do not respond to pharmacological treatment including Levodopa and high doses of anticholinergics and are not sensitive to alcohol as some patients with other subtypes of dystonia such as DYT11 [19].

The fact that in young onset Parkinson's disease (PD) dystonia is a common feature and that in some dystonia cases (like DYT16) some parkinsonism features

(e.g.: tremor) are present, suggest that there may be a common biochemical or anatomical link between PD and dystonia [18]. These facts suggest that it is important to study in more detail dystonia disorders since they may share light on some aspects of PD.

2.1 Genetics of DYT16

As mentioned before, the genetic cause for DYT 16 is a mutation c. 665 C>T (P222L) in the *PRKRA* gene [18]. The gene is situated on the human chromosome 2p and it is composed by 17 exons [23]. It codes for PRKRA (also known as PACT), a protein activator of the interferon-induced protein kinase (PKR) which mediates antiviral action of interferon and it is involved in growth regulation, cellular signal transduction, differentiation and apoptosis [24].

2.2 PKR mechanism

PKR is a double stranded RNA dependent protein kinase that is present at low constitutive levels in the cell and it is synthesize to a latent state (i.e.: when PKR is synthesized it is in an inactive form) [24]. It plays a critical role as a central component of the interferon antiviral defence pathway [25].

PKR is composed of a kinase domain shared by other eiF2α kinase and by two dsRNA domains (dsRBD) which regulate its activity and it is located at the ribosome [25],[26]. It is activated when dsRNA cellular, viral or synthetic (with more than 30 nucleotides) enters the cell and binds to the dsRBD domains of PKR [25]–[28]. Once bound to these domains, it induces a conformational change in PKR (dimerization) that releases it from its latent state by inactivating its inhibition [25]. When activated, it will undergo into autophosphorylation and will affect its downstream pathway [24], [27]. PKR can also be activated besides the entry of dsRNA in the cells. Endoplasmic reticulum stress, serum deprivation, Ca^{2+} overload, or hydrogen peroxide toxicity can also activate PKR [28]. Thus, some stress signals can stimulate PACT that will activate PKR without needing the presence of dsRNA. It was also suggest that it is PACT which is recruiting the dsRNA in virus infected cells to PKR [24].

The α subunit of the eukaryotic initiation factor (eIF2α) is the best studied physiological substrate of active PKR [29]. eIF2 is a heterodimer with 3 subunits: α, β, and γ, and mediates the binding of Met-tRNA$_i$ (Methyonil -Ribonucleic acid of interference) to the 40 S ribosome in a GTP dependent manner [29]–[31]. In order to participate in a round of translation initiation, the GDP has to be exchanged for a GTP. This conversion, eIF2-GDP to the active eIF2-GTP form is catalyzed by eIF2B [31]. When eIF2α is phosphorylated, it increases its affinity for eIF2B-sequestering it in an inactive complex with phosphorylated eIF2 and GDP [29], [31]. Since the eIF2B as a greater affinity for phosphorylated *versus* non phosphorylated eIF2, it will not be available to catalyze nucleotide exchange on non-phosphorylated eIF2 [24],[30],[32]. However, because eIF2B is present at lower molar concentrations in the cells comparing with eIF2, once eIF2 phosphorylated exceeds the amount of eIF2B, the rate of nucleotide exchange falls lowering the availability of eIF2-GTP creating a slow initiation of the polypeptide chain [31].

Since PKR can phosphorylate eIF2α which is responsible for stopping translation and even leads to apoptosis, it is clear that PKR is one of the key regulators in translation control [28], [32]. However, phosphorylation of eIF2α can also be activated by other stimuli than PKR such as heat shock, serum deprivation, exposure to heavy metal ions, viral infection, glucose and aminoacid starvation [24], [28].

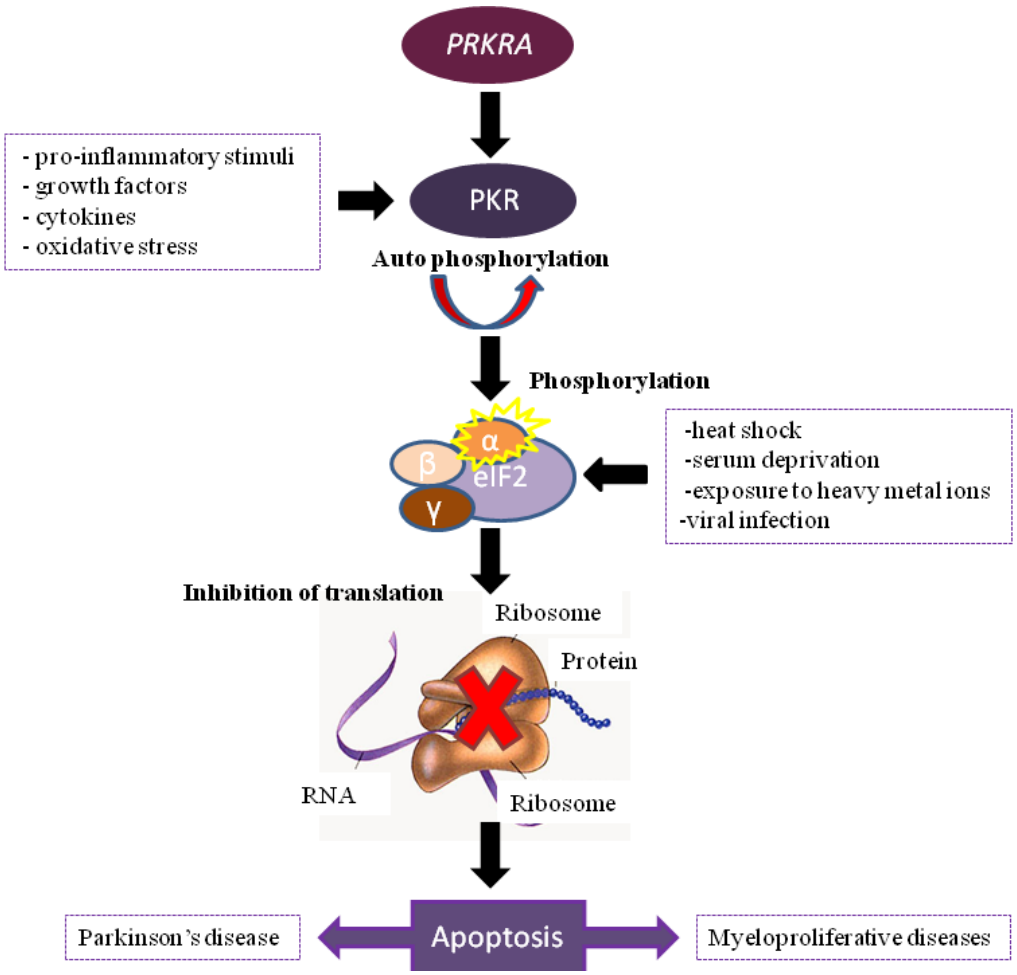

Figure 1.2- The PKR signalling pathway.

PRKRA activates PKR that become active by auto-phosphorylation. However, growth factors, cytokines, pro-inflammatory stimuli and oxidative stress can also induce the activation of PKR. Once activated, it phosphhoryaltes eIF2α that will inhibit translation. eIF2α can also be activated by other processes like heat chock, serum deprivation, exposure to heavy metal ions and viral infection. When the translation is inhibited, it will cause cell apoptosis that can lead to Parkinson's disease or Myeloproliferative diseases.

Thus, it was also found a strong induction of PKR phosphorylated in several neurodegenerative diseases such as in the hippocampal neurons of Parkinson's brains [28],[32],[33].

3. Neurodegeneration with brain iron accumulation (NBIA) disorders

The regulation of iron metabolism is very important for health since low or increased levels of iron can lead to disease [34]. Furthermore, iron is vital for different types of brain's cell functions such as mitochondrial function, phospholipid metabolism, DNA synthesis and repair, neurotransmitter synthesis and myelination [35]. With increasing age, iron accumulates mostly in microglia and astrocytes (cells that are responsible for brain protection), in the *globus pallidus* and in the *substantia nigra pars reticulata* [34], [36]. It has been hypothesized that this brain iron accumulation is due to an age dependent decrease of function of the blood brain barrier or even triggered by apoptotic cascades and/or cellular damage [34]. A variety of genes have been linked to disturbed brain iron metabolism and that may cause NBIA.

3.1 Neurodegeneration with Brain Iron Accumulation (NBIA)

NBIA is a group of inherited neurologic disorders where the iron accumulates in the basal ganglia (mainly the *globus pallidus*) and *substantia nigra*, causing progressive dystonia, neuropsychiatric abnormalities, spasticity, optic atrophy or retinal degeneration and parkinsonism [37], [38], [39].

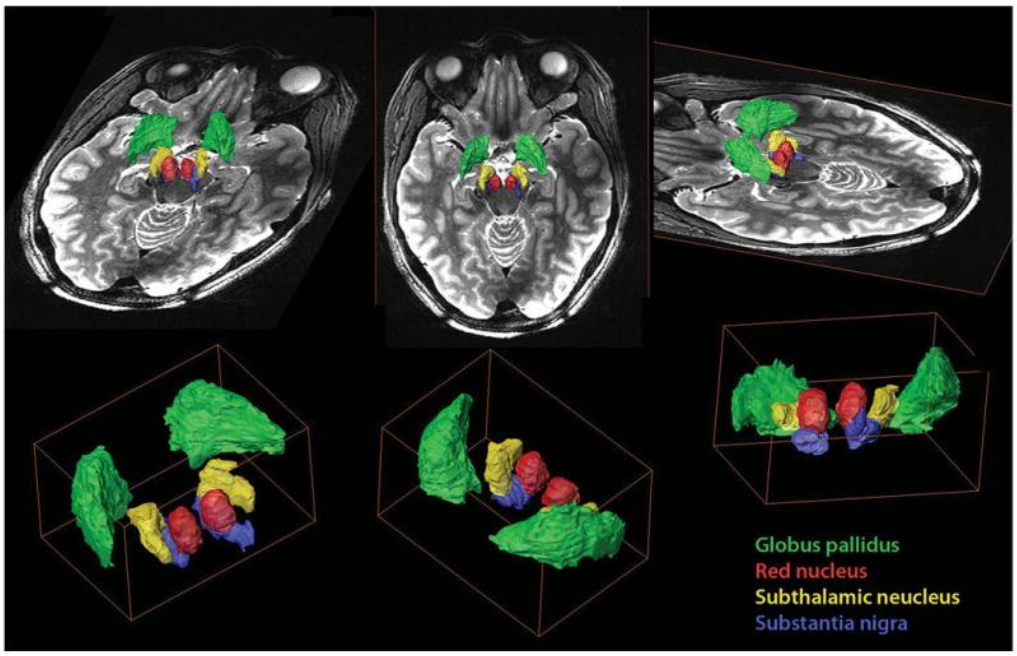

Figure 1.3 - Localization of *globus pallidus*, *substantia nigra*, red nucleus and subthalamic neucleus.

Image taken from [76].

At a pathologic level, NBIA brains present with iron deposition, neuronal loss, gliosis, loss of myelin and the presence of widely disseminated rounded structures termed spheroids that are identified as swollen axons [40]. Some of NBIA cases also showed Lewy bodies and Lewy neurites, suggesting that NBIA disorders also belongs to α-synucleinopathies. Some cases presented accumulation of tau in addition [40].

3.2 Types of NBIA

Nine genes: *PANK2*, *PLA2G6*, *C19orf12*, *FA2H*,*ATP13A2*, *FTL*, *CP*, *DCAF17* and *WDR45*, are found associated to NBIA disorders [34]. However, 40% of NBIA disorders are idiopathic [37]. The age of onset varies since it can appear early in the childhood (before 10 years old) and have a slow or quick progression, or appear between 10 and 18 years old, normally with a slow progression, or even in adulthood also with a slow progression [40].

The four most common subtypes of NBIA disorders are:

- PKAN, also known as NBIA type I, responsible for 35-50% of NBIA cases, with a world-wide prevalence of 1: 100 000 [34], [41]. It has two forms: the classic form that is characterized by an early onset and rapid progression, and an atypical form that as a late onset and a slower progression[41] . The genetic mutations associated with PKAN, is a loss of function of PANK2 gene[40].
- PLAN, the second mutation most common in NBIA disorders (also known as NBIA type II or PARK14 (Parkinson disease 14)), being responsible for 30% of the cases [37]. The genetic mutations associated with PLAN are found in the PLA2G6 gene. As with PKAN, it seems to have an age dependent phenotype [37], [38].
- MPAN, responsible for 6-10% of NBIA cases with a prevalence of 1 in 20 NBIA cases worldwide, where the mutation associated is a deletion in the gene C19orf12 that causes early truncation of protein and it is very common in Western Europe population, mainly in Polish [37], [38] ,[60]. It is a disease that also has two onsets: one in early childhood and another in adulthood [42].

- BPAN, responsible for 1% - 2% of NBIA disorders [37],[42], also known as SENDA (Static Encephalopathy with Neurodegeneration in Adulthood). It is characterized by an early onset spastic paraplegia and mental retardation that remains stable until early adulthood (in general between 20 and 30 years old) when a sudden onset and fast deterioration of dystonia-parkinsonism develop [34], [43], [44].

The other subtypes such as:

- Fatty acid hydroxylase associated with neurodegeneration (FAHN) - disorder due to a mutation in *FAH2* gene [45].;
- Kufor-Rakeb syndrome - due to a loss of function mutation in *ATP13A2* gene, also known as PARK9 [34],[45];
- Neuroferritinopathy – due to mutations involved with the *FTL* (ferritin light chain) gene, found in approximately 80% of cases [37];
- Aceruloplasminemia - characterized by mutations in the *Ceruplasmin* gene (*CP*) [34];
- Woodhouse-Sakati syndrome – due to mutation in the *DCAF17* gene [37];

are considered as rare NBIA disorder.

For the purpose of this thesis, BPAN disorder will be the only one discussed in more detail.

3.2.1 Beta- Propeller Protein Associated Neurodegeneration – BPAN

As mentioned above, the principal phenotype of BPAN disease is a global development delay during childhood with slow cognitive and motor gains that remain static until adolescence or adulthood where the patients experience a relative abrupt onset of progressive dementia and dystonia-parkinsonism [37], [46]. However other features can be seen in BPAN patients such as sleep disorders, eye movement abnormalities, epilepsy signs, frontal release and dysautonomia [34], [42].

On MRI, the brains of patients with WDR45 mutations present a T1 hyperintensity of the cerebral peduncleus and *substantia nigra* as well a T2 hypointensity of *substantia nigra* and the *globus pallidus*. Thus, BPAN is the only

NBIA disorder that presents a bigger hypointensity in the *substantia nigra* than in the *globus pallidus* [37], [42], [44], [47], [48].

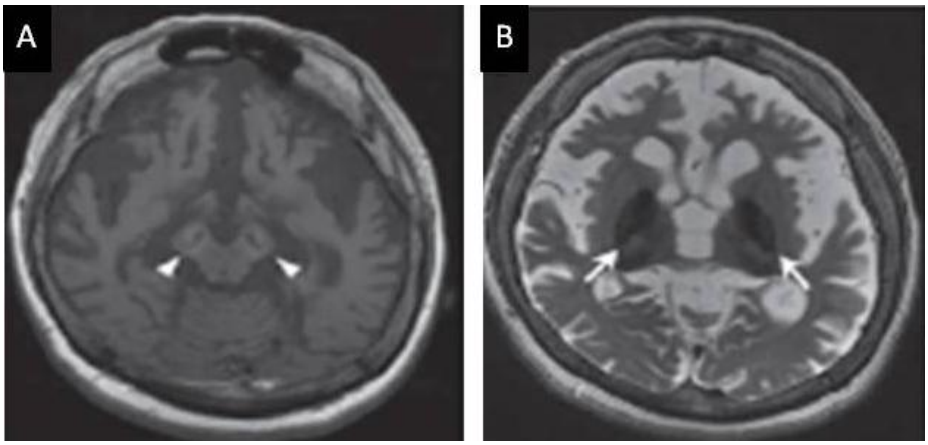

Figure 1.4 – Example of a brain MRI with WDR45 mutation.

It is a 33 years old brain with WDR45 mutation, presenting a T1 hyperintensity in the susbtantia nigra with a central band of T1 hypointensity (A – arrow heads) and a T2 hypointensity in the globus pallidus (B - arrows). Cerebral atrophy can also be observed.

Image adapted from [48].

Although there are no autopsy reports for WDR45 mutation brains, in 1987, there was a description of three patients who suggested had BPAN [42]. In these cases, iron deposition and cortical atrophy where found as axonal spheroids widespread with a big amount in the *globus pallidus* and *substantia nigra* [42]. Neurofribillar tangles were also found into the cortex, deep grey nuclei, hippocampus and brain stem and Lewy bodies were observed in one of the patients [42].

The function of WDR45 gene will be discussed in the next chapter.

3.3 Model of Inheritance

Seven in nine types of NBIA are inherited in an autosomal recessive manner [34], [37], [38]. The other two types are inherited in a different way:

BPAN – All the known cases have only one occurrence in the family, with a majority of females, being considered an inheritance by an X-linked dominant manner. This kind of pattern suggests that they are *de novo* mutations and suggest that they are lethal to most males.

Neuroferritinopathy – it is inherited by an autosomal dominant manner.

3.4 Treatment methods

There is no cure for all these subtypes of NBIA. The only treatments that can be administrated are palliative in order to reduce the pain or treat some symptoms [37]. The most used are [34], [37], [49]:

- Pharmacologic treatment for spasticity and seizures;
- Oral baclofen administration for treating severe dystonia;
- Botulinum toxin for treating with focal dystonia;
- L-DOPA treatment especially for the disorders which present parkinsonism features;
- Deep brain stimulation for treating dystonia with increasing frequency;
- Psychiatric treatment for those with a later-onset and with neuropsychiatric symptoms;

As NBIA progresses, a lot of individuals present episodes of dystonia that can last for days or weeks, raising the risk for fractures without apparent trauma [37].

3.5 Therapies under investigation

Some therapies are being investigated to reduce some symptoms and/or try to be a cure for some of the NBIA disorders (especially PKAN disorder since it represents 35% to 50% of NBIA cases) [37], [49].

Deep brain stimulation (DBS) – Nowadays it is used as a frequent treatment for primary dystonia and now it is being used to attempt to treat the secondary dystonia seen in NBIA disorders. The PKAN patients are the population who has been studied in more detail but the results are controversial since some studies present benefices (like improvements in speech, writing, walking and global measures of motor skills) 6 to 42 months after treatment and others suggest a decreased benefit. However this method doesn't halter the neurodegeneration process [37], [49].

Baclofen – It has been investigated to deliver baclofen in an intraventricular way. However the study needs more critical mass and needs to determine the optimal dose and efficacy for the NBIA disorders. The fact of administrate intraventricular

baclofen will allow a better treatment of dystonia upper body and facial and may result in a higher concentrations over the cortex than when administrate by oral [37].

Iron chelation – Deferripone is a drug that is able to cross the blood brain barrier and remove intracellular iron. However, this study it is still in clinical trials in US and Italy [37], [49].

4. WDR45

4.1 Gene localization and its inheritance pattern

The WDR45 gene is located on the X chromosome and it is localized at Xp11.23 [48]. It is involved in BPAN disease, a subtype of NBIA disorders as seen in the previous chapter [34]. Until now, all the mutations found in the WDR45 gene, responsible for the BPAN disease, are loss-of-function or nonssense mutations [46].

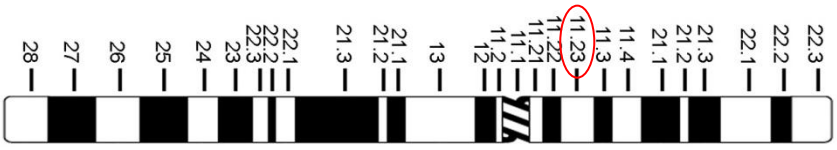

Figure 1.5 – Ideogram of X chromosome.

It is a representation of the X chromosome with the localization of the gene WDR45 (red circle).

The inheritance pattern of WDR45 gene is not clear. However, it was suggested that the mutations in WDR45 follow an atypical pattern of X-linked disorder [50]. This is based on the fact that all the cases reported are from individuals with no family history of NBIA and most WDR45 variants are nonsense mutations that arise *de novo* [50]. Besides, most of the patients with BPAN disorder are female, supporting an X-linked dominant pattern of disease and suggesting that WDR45 mutations are lethal in most males [46]. However, three males were reported with the same phenotype and could not be distinguish from affected females [46]. These facts suggest that during embryogenesis germline mutations can occur in hemizygous males resulting in males that carry a variable mutation leading to either exceptional severe or relatively mild BPAN symptoms [46], [50], [42]. The timing when somatic mutations occur during embryogenesis will determine phenotypic severity [46]. For example, females harbouring mutations in the germ cells, have a

probability to have 50% of female offspring with BPAN and 50% of male offspring non viable [46].

4.2 Gene function

WDR45 is a homologue of Atg18 in yeast, known to be involved in autophagy [44]. Based upon this, WDR45 is thought to act at the very beginning of the autophagy cascade as a regulator of the distribution of vesicles marked with ATG9A, which transiently localize to the autophagosome formation site and induce autophagosome formation [48], [43]. It codes for a protein with 360 aminoacids (Gene Database).

4.3 Protein WDR45

WDR45 belongs to the WD-40 family, also known as WIPI β propellers family (large family of molecules with repeating units containing a conserved core of 40+ aminoacids that terminate with tryptophan-aspartic acid(WD)) [50],[42],[44],[51]. It is a seven-bladed β structural protein family that provides a scaffold which facilitates protein-protein interactions in the formation of multimeric protein complexes [50],[42]. Within the β propeller domain, the core is composed by Phe-Arg-Arg-Gly domain that binds to phosphoinositoides [42].

The WDR45 protein (also known as WIPI4) is presen in several human tissues, with highest expression in skeletal muscles [48]. However, the BPAN phenotypes seem to be limited to the brain which could suggest that autophagy could be more important in neurons. It also suggests that the other WIPI homologues could balance the deficiency in WIPI4 in a cell type dependent manner meaning that the relative contribution of WIPI4 among WIPI factors may be high in neurons [48].

Until now, the presumption is that defects in the protein WDR45 leading to its loss of function causes an impairment in autophagy that will lead to a neurodevelopment and neurodegenerative phenotype, being the proof that autophagy is indeed associated with neurodegeneration [50], [52]. However the underlying mechanism it is still not clear as the question how increased autophagy relates to iron accumulation [50].

4.4 Diseases associated with impairments in WDR45

Beside BPAN disorder, WDR45 mutations have been found involved in other diseases such as [51],[53]:

- Pancreatic cancer (WIPI4 was down regulated) [54]
- Kidney cancer (WIPI4 was down regulated) [54]
- Rett syndrome

Rett syndrome is a neurodevelopmental disorder characterized by loss of acquired purposeful hand skill and language regression, stereotypic hand movements and gait abnormalities [53]. In some patients with BPAN, some features very similar to the Rett syndrome were observed [53].

5. Autophagy and Neurodegenerative diseases

5.1 Different types of autophagy

The word autophagy comes from the Greek *auto phagos* which means self eating [1],[2]. It refers to any catabolic process that involves the delivery of a cytoplasmic cargo to the lysosome [57],[58]. Basal autophagy has important homeostatic functions in cells, involved in the degradation of proteins, protein aggregates and organelles [59],[8].

To date, three autophagic pathways have been identified: macroautophagy, microautophagy and chaperone mediated autophagy (CMA) [8], [56], [57], [59]. They all differ with their physiological functions and the way they deliver their cargo to the lysosome [56]. Macroautophagy is the principal regulated catabolic mechanism most used in eukaryotic cells to degrade long-lived proteins and organelles. A double membrane structure elongates engulfing a portion of cytoplasm forming a vesicle named autophagosome that will finally fuse with the lysosome where the lysosomal hydrolases will degrade the cytosolic contents [2],[57],[5]. Microautophagy results in a portion of the cytoplasm that is directly capturated into lysosomes trough invagination of the lysosomal membrane. As for the CMA, it is a selective transport of cytosolic proteins that present a pentapeptide motif related to

KERFQ, across the lysosomal membrane via the lysosomal membrane receptor LAMP-2A and the chaperone hsc70 [1],[4],[5],[8].

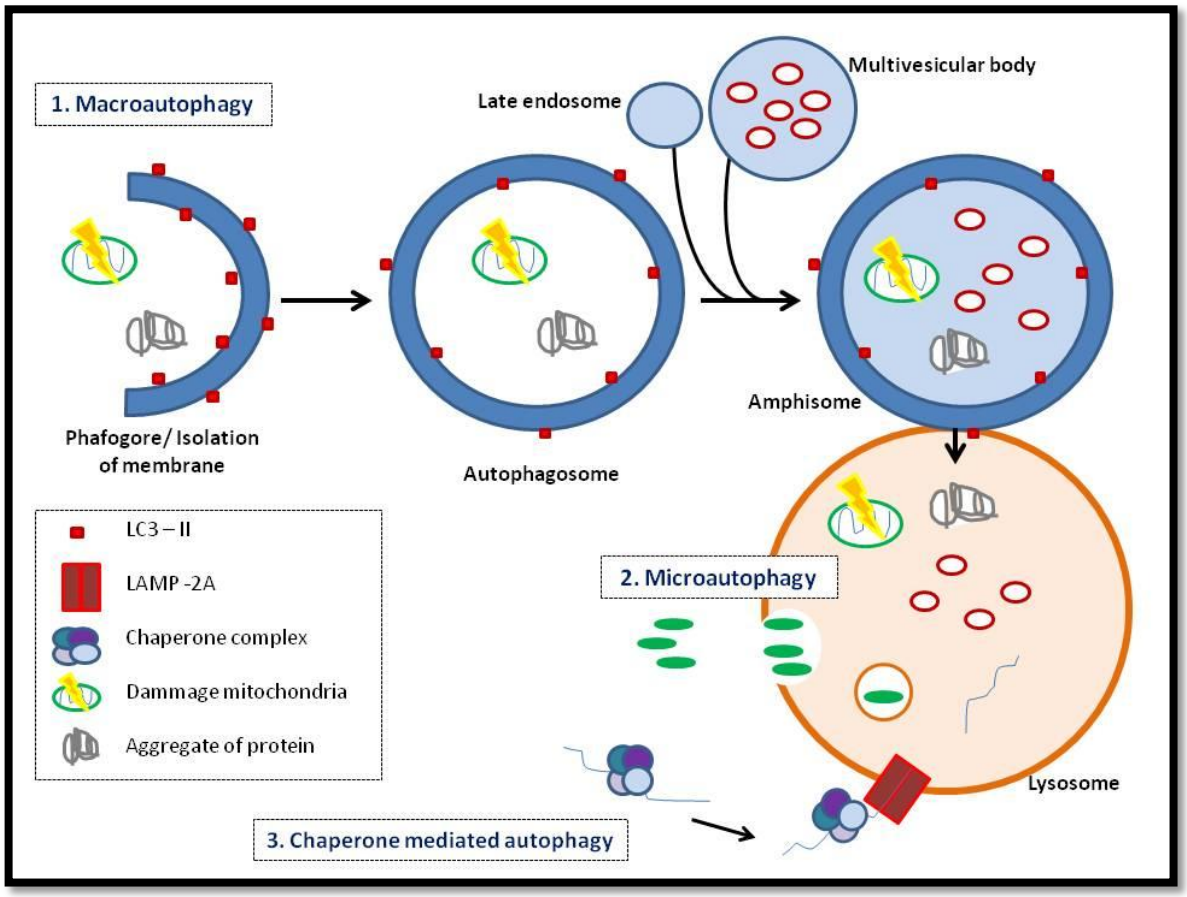

Figure 1.6 – Three different types of autophagy.

There is three different types of autophagy in the cell: macroautophagy (1) where the cytosolic components are delivered to the lysosome by vesicles; microautophagy (2) where the lysosomes capture small volumes of cytosol, and chaperone-mediated autophagy (CMA) (3), where the soluble substrates that present a specific chaperone complex are translocated into the lysosome through the LAMP-2A lysosomal receptor.
There are a lot of maturarions steps in the macroautophagy system:
firstable, a portion of cytoplasm is surrounded by a phagophore .Then, the phagophore closes to structure an autophagosome that will then fuse and form an amphisome by the fusion of the late endosomes and multivesicular bodies. Finally, the fusion of the amphisome with a lysosome will shape an autolysosome where the cytosolic cargo will be degraded by lysosomal hydrolases. LC3-II is a protein that is in and out of the surface of autophagic membranes and can be used as a histological marker of autophagy vacuoles.

Adapted from [58].

5.2 Macroautophagy process

Since it is the most used catabolic process in the cell, macroautophagy is often referred to as simply autophagy and this term will be applied for the remainder of this thesis.

Autophagy plays an essential function in the turnover of long-lived proteins and organelles, as well as in the degradation of protein aggregates [2],[5]. It can be quickly upregulated when: cells need to produce intracellular nutrients and energy (for example during high bioenergetic demands or under starvation conditions, following growth factor withdrawal); when they are preparing to go through structural changes as in during developmental transitions or when they need to clear themselves of damaging cytoplasmic components as in oxidative stress conditions, protein aggregate accumulation, or infection [56],[60].

As mentioned before, the autophagy process consists of several steps: autophagosome formation, elongation, maturation and fusion[8], [59], [60]. More than sixteen autophagy related proteins (Atg) are need for the formation of the double-membrane vesicle [60],[61]. The *ATG* genes are conserved from simple eukaryotes (such as yeasts) to mammals, highlighting their critical importance to cellular function.

The site of autophagosome complex formation is not yet clear in humans but it occurs at the phagophore assembly site(s) (PAS) in yeasts. ATG18, a β-propeller protein formed by seven WDR40 repeats, was shown to be recruited to the PAS and binds to ATG2 [60]. This protein is also important for general vesicle homeostasis and even endosomal functions. For the initiation of autophagosome formation, the *de novo* creation of an initiation complex consisting of ULK (Unc-51 like autophagy activating kinase 1) in association with ATG1, ATG13, ATG17 and ATG9, regulatory class III PI3 kinase complex (Vps34) with beclin-1 (also known as ATG6) and ATG5-ATG12-ATG16 is required [5],[6],[55],[57],[62],[63].

The ATG1-ATG13 complex recruits ATG9, which is crucial for the initial formation of the autophagosome membrane [8]. Then, depending on the interaction partners of PI3 kinase – beclin-1 complex the autophagosome will continue developing or not. If the complex interacts with *UVRAG* (Ultra-violet Radiation Resistance associated gene) associated with AMBRA (Activating molecule in beclin-1regulated autophagy) and ATG14, the autophagy is promoted [8]. Although, if the PI3 kinase – beclin-1 complex interacts with *UVRAG* associated with RUBICON (RUN domain and cystein rich domain containing) or with the anti-apoptotic proteins Bcl-2 and Bcl-X_L the autophagosome formation will lead to autophagy repression [5],[6],[10],[55],[57],[62].

For the elongation step, after autophagy stimulation has occurred, beclin-1 is released from Bcl2 at the endoplasmic reticulum, giving rise to the formation of a complex UVRAG/AMBRA that will trigger the ATG5-ATG12-ATG16 multimeric complex formation mediated by ATG7 and ATG10 [59],[8],[62]. After the new membrane is formed, LC3-II, a cleaved and lipidated product by ATG4 from LC3-I (ATG8), is inserted into the both side of the membrane by ATG9 of the ULK complex. When the autophagosome is complete, the LC3-II on the exterior surface of the membrane is released and then recycled [55], [59].

The final step (maturation and fusion), occurs when the autophagosome with the cargo to be degraded is trafficked by a dynein-dependent manner along microtubules to the lysosomes for the lysosomal membrane proteins (LAMP1/2 and Rab7, a member of RAB family GTPases and vesicular proteins, class III Vps, SNARE and ESCRT) allows the fusion [5],[6],[9]. The lysosomes are normally located at the microtubule organising center (MTOC) located near the nucleus [2],[6]. Once fused, these vesicles are called autophagolysosomes. However, there is a transient state before the fusion (named amphisome), that provides the low pH necessary for the optimal activity of lysosomal proteases [56].

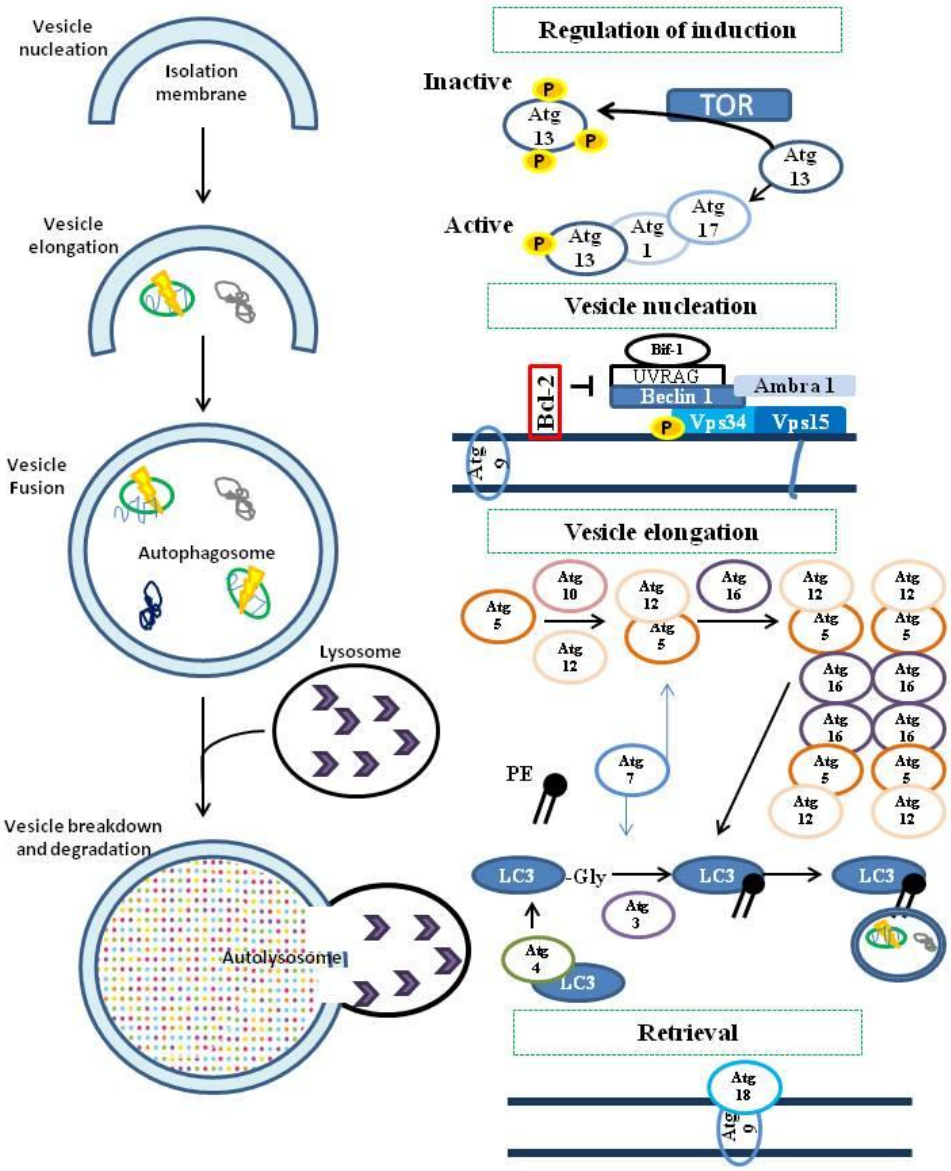

Figure 1.7 – Molecular pathways of autophagy.

Distinct stages are required during autophagy. The vesicle passes through several steps such as nucleation, elongation and completion forming an autophagosome. Then the double membrane of the autophagosome will fuse with the lysosome yielding an autolysosome where the autophagosome inner membrane will suffer lyses as the contents inside of the autolysosome. All these steps occur at a basal level and are regulated by several different signalling pathways. The Atg proteins form distinct complexes that function in different stages of autophagy.

Adapted from [56].

5.3 Regulation of autophagy

The autophagosome formation can be regulated by two different mechanisms: mammalian target of rapamycin (mTOR) dependent pathway or mTOR independent pathway.

5.3.1 mTOR dependent pathway

The mTOR protein is a serine-threonine kinase belonging to the phosphoinositide 3-kinase (PI3K-) related kinase family, with a weight of 289 kD [65]. It is also highly conserved through evolution.

mTOR complexes

The mTOR complex is a master negative regulator of autophagy that acts as a sensor of nutrient signals, growth factors and energy status [59], [65]. In neurons, switching between the inactivation and activation of mTOR complex acts to promote protein synthesis instead of autophagy and is important for several neuronal function such as synaptic plasticity, myelination and dendritic arborisation [63]. mTOR can adapt two forms: complex 1 (mTORC1) and complex 2 (mTORC2) that have different upstream inputs and downstream outputs as well as different sensitivities to rapamycin [66].

The upstream key regulator of mTORC1 is a heterodimer composed of tuberous sclerosis 1 (TSC1 - hamartin) and TSC2 (tuberin) [66] . Its function is to be a GTPase activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. The mTORC1 kinase activity is strongly stimulated when the GTP-bound form of Rheb directly interacts with it. So TSC1/TSC2 can also negatively regulate mTORC1 by converting Rheb into its inactive GDP-bound [66], [67].

Little is known about mTORC2 compared with mTORC1 complex. However, it is known that mTORC2 regulates the cytoskeletal organization, cell survival and metabolism by phosphorylating and activating AGC kinases such as SGK1 (serum- and glucocorticoid-induced protein kinase 1) and PKCα (protein kinase Cα) [66].

When the cell is under nutrient-rich conditions, mTORC1 suppresses autophagy by interacting directly with the ULK complex and mediates phosphorylation-dependent inhibition of the kinase activity of ULK1 and Atg13 [2],[5],[63]. However, if the cell is under starvation conditions (or treated with rapamycin – inhibitor of mTORC1), the mTORC1 mediation of the phosphorylation of Atg13 and ULK1 is inhibited [2],[5],[63]. This will then lead to dephosphorylation-dependent activation of ULK complex which triggers the autophagy process.

mTORC2 is insensitive to nutrients variations but does respond to growth factors like insulin trough a not completely defined mechanism that requires PI3K. One

hypothesis is that the major role of mTORC2 is to fulfil maximal activity to Akt by phosphorylating it on Ser473, in its hydrophobic motif [67].

5.3.1.2 PI3/Akt and Ras pathways and mTOR

The PI3/Akt pathway can also interact through the mTOR complex and is involved in inhibition of autophagy [2],[5],[61], in cell growth, survival, metabolism and proliferation [67]. When growth factors or insulin growth factor 1 (IGF1) bind to the cell surface receptors, it activates the PI3K and Ras pathways [59], [66]. Once PI3K is activated, it catalyses the production of phosphatidylinositol-3,4,5 – triphosphate (PIP3) at the plasma membrane which will increase the membrane recruitment of Akt/PKB and its activator PDK1, causing the activation of Akt by phosphorylation. The following effector kinases of the PI3K and Ras pathways: protein kinase B (Akt/PKB), ribosomal S6 kinase (RSK1),), and extracellular-signal-regulated kinase 1/2 (ERK1/2directly phosphorylate the TSC1/TSC2 complex inactivating it and therefore activate mTORC1 [66]. Note that Akt can also activate mTORC1 complex independently of the TSC1/TSC2 heterodimer by leading to the dissociation from raptor of PRAS40, an mTORC1 inhibitor. Thus, the activation of Akt proceeds in the phosphorylation of a several number of other proteins including FOXO (forhead box O) and GSK3 (glycogen synthase kinase 3) that are involved in metabolism and cell survival respectively. It will also activate downstream targets of mTORC1: S6K1 (ribosomal S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E-binding protein 1) which are a part of the translation machinery. The PI3/Akt pathway can be also triggered by using pharmaceutical compounds such as 3-methyladenine (which will target the autophagosome formation) or bafilomycin A1, chloroquine and lithium (which will target the fusion of the autophagosome with the lysosome) [56]. However these compounds are not specific for these targets.

5.3.1.3 AMPK pathway and mTOR

AMPK (AMP- activated protein kinase) is another kinase that can regulate autophagy through mTOR. When cells have a deficit of energy, resulting in an elevation in the ratio of AMP/ATP, there is a concomitant activation of AMPK that results in an inhibition of mTOR signalling promoting autophagy [59],[64]. This is drieven by phosphorylation of the TSC2 complex and an increase in its GAP activity toward Rheb or by interacting directly with mTORC1, phosphorylating raptor causing the inhibition of mTORC1 [66].

5.3.1.4 MAPKK pathway and mTOR

The Ras-Raf-Mek-Erk pathway is involved in several different cellular processes, for example in the development of neurons and glia [68]. The Raf family of protein kinases activates MAPKK (dual-specific protein kinase, also known as MEK) by phosphorylation, which in turn activates the extracellular regulated kinases 1 and 2 (ERK1 and ERK2), also by phosphorylation. ERK1 and ERK2 can also be activated by a range of other signals, for example: growth factors, cytokines, chemokines and oxidative stress.

Activated ERK1 and ERK2 are responsible for controlling gene expression important for the regulation of cell proliferation and differentiation. Under pathological condition, its activation could result in apoptosis [68].

This pathway also interacts with mTOR. When ERK1/2 are phosphorylated, they interact with Raptor, a protein involved in mTORC1, by phosphorylating it leading to the activation of mTORC1 and signalling to its downstream substrates like 4-eBP1[69].

5.3.1.5 Other signals/molecules acting via mTOR

Several cellular stress signals as low concentrations of growth factors, specific amino acids, ATP hypoxia, some types of protein aggregates and endoplasmic reticulum stress, can suppress the mTOR complex leading to the activation of UNC-51-like kinase (ULK) complex that will trigger the activation of autophagy [63],[64].

Other molecules have also been reported as being able to regulate autophagy through mTOR for example: p53, which can regulate it positively or negatively depending on localization; Bcl-2 or Bcl-XL which when bound to to Beclin 1 inhibits autophagy; or even the Jun N-terminal kinase 1 (Jnk1 - activated under starvation conditions) that phosphorylates Bcl-2 ending the interaction with Beclin-1 and therefore promoting autophagy [59],[61].

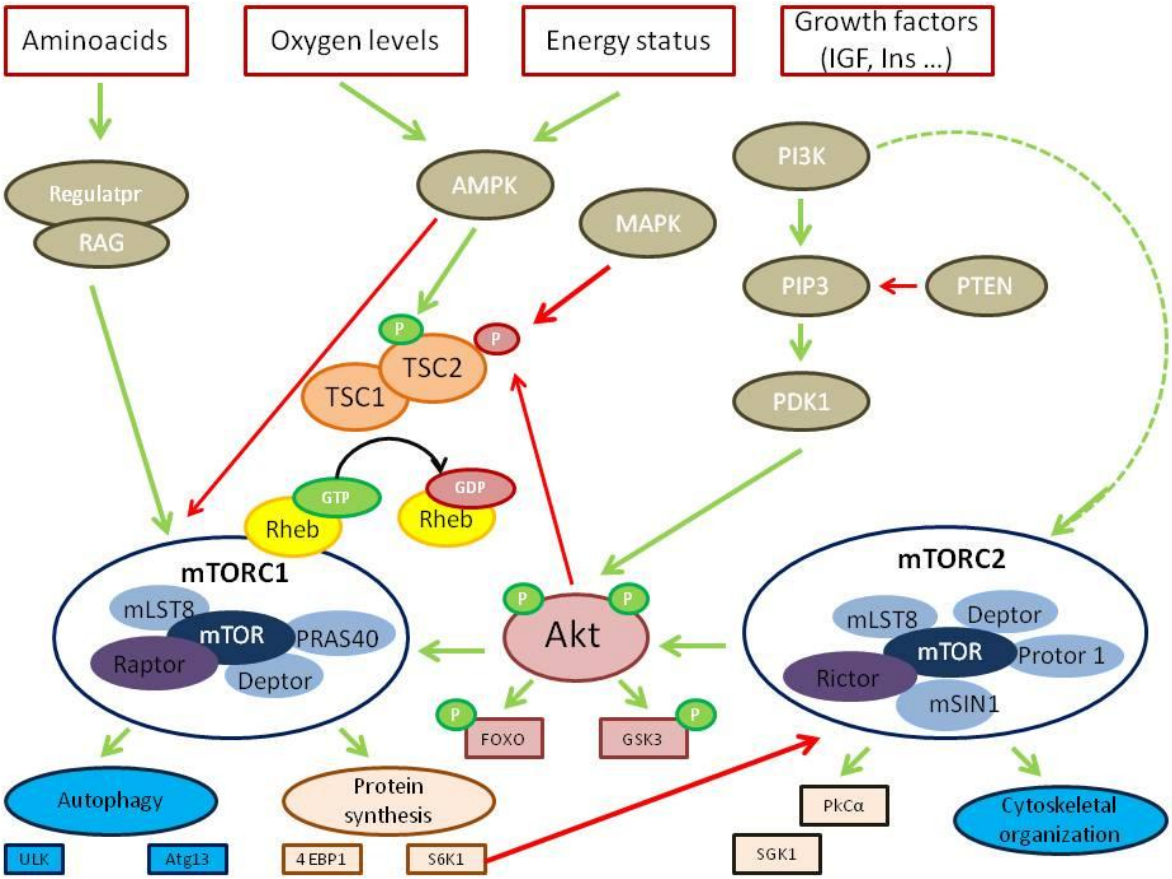

Figure 1.8 – A simplified model of the mTOR dependent-pathway.

The mTORC1 complex receive signals from amino acids, oxygen levels, growth factors and energy status to regulate numerous cellular functions involved in proliferation and cell growth like lipid synthesis, adipogenesis, mitochondrial and glycolytic metabolism, autophagy and protein synthesis.
In contrast with mTORC1, mTORC2 is mainly regulated by growth factors, and, in addition to Akt, it also regulates for example SGK1, PKC*α* and cytoskeletal organization. (red arrows: inhibition; green arrows: activation).

Atg13, autophagy 13; Deptor, DEP domain-containing mTOR-interacting protein; FOXO, forkhead box O; Ins, insulin; mLST8, mammalian lethal with sec-13 protein 8; mSIN1, mammalian stress-activated protein kinase-interaction protein 1; PRAS40, proline-rich Akt substrate of 40 kDa; Protor1, protein observed with rictor 1; ULK1, unc51-like kinase 1.
Adapted from [67].

5.3.2 mTOR independent pathway

There are some signalling pathways that can regulate autophagy independent of mTOR. For example, intracellular inositol and IP_3 (inositol 1,4,5-trisphosphate) levels negatively regulate autophagy, leading to inhibition of this process. Conversely, the reduction of free inositol and IP_3 levels by inhibition of inositol monophosphatase (IMPases) leads to an upregulation of autophagy [59], [64].

Another example is the cAMP (cyclic Adenosine Monophosphate) pathway. cAMP activates Epac (guanine nucleotide exchange factor) that will activate Rap2B, which in turn activate phospholipase Cε (PLCε) leading to the production of IP_3 [59]. This acts to mediate the release of Ca^{2+} from ER stores. The increasing intracytosolic levels of Ca^{2+} activates calpains (a family of Ca^{2+} - dependent cysteine proteases) and blocks autophagy through Gsα that is activated after calpain undergoes cleavage. This will increase AC (adenylate cyclase) activity, which in turn increases cAMP levels. Overall, it will create a potential cyclic pathway where calpains regulate autophagy through Gsα. That is, when intracellular cAMP levels are increased, the autophagy is inhibited.

Finally, it has been reported that reactive oxygen species (ROS) can modulate autophagy [59]. When cells are under starvation conditions, there is an increase in ROS in a PI3K dependent manner. The mechanism underlying this is not yet clear.

5.4 Neurodegenerative diseases and autophagy

Several human neurodegenerative diseases share the same pathological feature: the formation of aggregates within the cytoplasm in neurons and other cell types. We have the case of Alzheimer's disease (AD) where β – amyloid protein is deposited in the cytoplasm in amyloid plaques and tau protein causes neurofibrillary tangles due to its intracellular aggregation; - Parkinson's disease (PD) where the aggregates are composed of α-synuclein and Huntington's disease (HD) where polyglutamine expansions are the aggregated species [59]. Intriguingly, autophagosome accumulation has also been reported in the brains of patients with these disorders [56].

The knockout of essential genes for autophagy causes lethality soon after birth on mice revealing the fact that autophagy is very important for cell survival [59],[70]. Indeed, in post-mitotic cells such as neurons, the recycling of proteins and organelles is very important for cellular quality control. These results suggest that autophagy may play an important role in the pathogenesis of these neurodegenerative diseases since it is a key mechanism responsible for the clearance of aggregate - prone proteins that are toxic to cells [56].

As the focus of this thesis is on PD, this section will concentrate on this disorder and how it relates to autophagy. As mentioned before, the formation of Lewy bodies containing α-synuclein in dopaminergic neurons in the *substantia nigra* is a hallmark of PD as well as neuronal losses in this area [59],[70]. It is well known that wild type α-synuclein is degraded by both the UPS (ubiquitin proteosome system) and autophagy, especially *via* CMA (chaperone mediated autophagy) and that its mutant forms have a toxic gain of function that bind and block CMA receptors [8]. This causes a reduction in function of this degradation process, leading to an accumulation of more aggregated protein and a positive feedback loop.

Similar to the overexpression of the wild type form of α-synuclein, the mutant forms also cause aggregation of α-synuclein and production of oligomers [8]. These oligomers can then interact with lipid membranes and interfere with their normal function leading to mitochondrial damage and fragmentation as well lysosomal and proteosomal dysfunction. The accumulation of α-synuclein can also lead to inhibition of autophagy [71].

Some genes implicated in PD have already been shown to be involved with autophagy. The PINK1-parkin pathway when mutated causes aberrant mitophagy (autophagy of mitochondria). PINK1 can also interact with beclin-1 causing an elevation of basal and starvation-induced autophagy [5],[6],[70]. Mutation in DJ1, also involved in PD, cause mitochondrial dysfunction leading to elevated levels of ROS and consequent decrease of lysosomal-autophagic degradation [70]. Aberrant accumulation of autophagosomes in PD are thought to be due to defects in the autophagic clearance apparatus that can be linked to an alteration in the microtubule network driven by mitochondrial dysfunction. Mutations in LRRK2, the most frequently mutated gene in Mendelian PD, may cause an impairment in the

autophagy-lysosomal pathway [70]. Thus, specific defects in some steps of autophagy or even in the cargo recognition may be sufficient to cause PD [8].

Table 2 – Regulation of autophagy in proteinopathies

Adapted from [70].

Regulation of autophagy in proteinopathies		
Disease	**Mutant protein**	**Autophagy activity**
Alzheimer	PS1 -	Inhibition Induction
Parkinson	α-synuclein LRRK2 PINK1 Parkin DJ-1	Inhibition Inhibition Inhibition Inhibition Induction
Huntington	Huntingtin	Inhibition
ALS (Amyotrophic Lateral Sclerosis)	Dynein Dynactin ESCRT-III Fg4	Inhibition Unknown Inhibition Inhibition
FTD3 (Frontal Temporal Dementia)	ESCRT-III	Inhibition

Autophagy is an attractive pathway for therapeutic intervention in neurodegenerative disease. Some evidence suggests that in certain models for AD, PD and even HD, increasing autophagy can lead to an improvement in their phenotype leading to the suggestion that autophagy could be a valid therapeutical target for these disorders [8],[70].

Despite extensive research a number of mechanisms linking to autophagy and PD are not yet clear. One of the most important questions remains unanswered: is inhibition of autophagy the cause of PD or a consequence of it? Other diseases which share some symptoms similar to PD (such as NBIA – Neurodegeneration with brain iron accumulation and DYT 16 -Dystonia 16) may help to understand the true link between autophagy and PD since they present some underlying mechanisms that are similar to those in PD.

II. MATERIALS & METHODS

2.1 Cell lines

For the purpose of these experiments, the cell lines used were derived from Human Embryonic Kidney 293 (HEK-293), Human Neuroglioma (H4), Human Neuroblastoma (SH-SY5Y) and Human Fibrosarcoma (HT-1080). They were obtained from the American Tissue Cell Culture (ATCC), a global bioresource center (HEK-293(ATCC® CRL-1573™); H4 (ATCC® HTB-148™); SH-SY5Y (ATCC® CRL-2266™); HT-1080 (ATCC® CCL-121™)).

The cells were cultured in cell medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate, (+) 10% of FBS from GIBCO) in 75 cm^2 cell culture flaks with canted neck (CORNING, tissue cultured treated, nonpyrogenic, polystyrene, sterile). They were passaged at 90% confluence by washing once with PBS (GIBCO, DPBS (1x) Dulbecco's Phosphate Buffered Saline, (-) $CaCl_2$, (-) $MgCl_2$) and adding 2 ml of trypsin (GIBCO, TrypLE™ Express (1x), Stable trypsin replacement enzyme, (+) Phenol Red) per T-Flask, for 10 minutes in the incubator (37ºC).

2.2 Plasmid transformation and purification

The PRKRA plasmids wild-type (WT) and mutated (MT) were obtained from Sonja Scholz, National Institute on Aging. The WDR45 plasmid was obtained from GeneCopoeia (EX-Z3254-M11) (see Appendix I, to assess the plasmid construct).

The plasmids were transformed via heat shock protocol (Invitrogen, One Shot® TOP10 Chemically Competent *E. coli*) into *E.coli* bacterial cells (TOP 10 competent cells, Invitrogen). They then were grown on agar medium (SIGMA) in 10 cm dishes containing ampicillin (1:1000; SIGMA, ready made solution, 100mg/mL, 0,2 µm filtered) over-night. Once the colonies were formed in the dishes, it was chosen one colony with a sterile tip and then put the tip with the chosen colony in a PETG Flask with baffled bottom with vented closure (VWR) with LB medium (SIGMA) and ampicilin (1:1000 concentration; SIGMA, ready made solution, 100mg/mL, 0,2 µm filtered). The flasks were let shaken in an incubator (37ºC) over-night at 220 rpm.

The transformed cells were then purified by maxi-prep using EndoFree Plasmid Maxi kit (10) (Qiagen) according to manufacturer's instructions. However, it was used high speed maxi coloumns (Qiagen) instead of the ones provided with the kit.

Once the DNA was obtained, its concentration was assessed in the machine Nanodrop 2000 (Thermo Scientific) as well as its purification degree (see Appendix II for assessing the quality of the DNA obtained).

2.3 Transient transfection and cell lysates

As mentioned before, the different types of cells were cultured in cell medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate, (+) 10% of FBS from GIBCO). The transfection method used was by polyethylenimine (PEI) (SIGMA). Briefly, the medium was removed from cells and the cell layers were washed 1 time with DPBS (GIBCO, DPBS (1x) Dulbecco's phosphate buffered saline, (-) $CaCl_2$, (-) $MgCl_2$) prior to incubation with trypsin (GIBCO, TrypLE^{TM} Express (1x), Stable trypsin replacement enzyme, (+) Phenol Red) for 10 minutes in the incubator (37ºC). For carry out the transfection, 10 µg of plasmids were incubated with 20 µl of PEI along with 1 ml of transfection medium (cell medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate) without FBS) for 20/30 minutes at room temperature and then plated in 10 cm tissue culture dishes (BD Falcon). Detached cells were used to re-seed the T-flasks and plated out in the 10 cm dishes previously covered with the plasmid of interest with PEI. 48h post-transfection, cells were washed 2 times with PBS (GIBCO) on ice and also lysed on ice in 400 µl (for each plate) of RIPA lysis buffer (SIGMA), 10% of protease inhibitor (100x) (Thermo Scientific), 10% 0.5M EDTA (Thermo Scientific) and 10% of phosphatase inhibitor (100x) (Thermo Scientific). Then the cell lysates were frozen in -20ºC for future use.

2.4 BCA (Bicinchoninic Acid Protein) assay

Before every western blot performed, the samples were submitted to a BCA assay. This experiment was performed with the Pierce BCA® protein assay kit (Thermo Scientific, product reference 23228). The results were then read at 540 nm, in the Infinte F50 machine (TECAN).

2.5 Western blot

Before starting the Western blot the cell lysates were defrosted on ice and then rotated at 4ºC for 30 min. After, the cell lysates were clarified by centrifugation at 5000_g for 10 minutes at 4ºC, and the resultant supernatant taken and kept for further use. 100 µl of the supernatant of cell lysates were prepared by adding 33 µl of loading buffer composed by NuPAGE LDS sample buffer (4x) (Invitrogen) with 10% of 2-mercaptoethanol (ALDRICH SIGMA). The ladder used for each western blot performed was a sharp pre-stained protein standard with a range from 3.5 to 260 kDa (Novex). The proteins were then separated on NuPAGE 4-12% Bis-Tris Gel (1 mm x 10 well; Novex) at 180V for 45 minutes in running buffer containing - 950 ml of ultra pure water and 50 ml of NuPAGE MOPS SDS running buffer (20x) (Invitrogen). After that, gels were transferred onto PVDF membranes (Millipore Immobilon®-P, pore size: 0.45 µm) at 25V over-night in transfer buffer containing 10% of methanol (VWR Chemicals), 10% of Tris-Glycine buffer (10x) (SIGMA) and 80% ultra pure water. Membranes were blocked in PBS-T (PBS (GIBCO) with 0.05% of Tween®20 (SIGMA ALDRICH)) and 10% of milk powder during 1 hour. They were then probed according to the protein in study, i.e. for confirm the presence of PRKRA transfected WT and MT in cells, the membranes were probed with primary anti- PRKRA antibody (Abcam, ab56444) at 1:1000 concentration for 90 minutes. For assessing the presence of PKR and PKR phosphorylated the membranes were probed with primary anti-PKR (Abcam, ab58301) and anti-PKR phosphorylated (Abcam, ab38036) at 1:1000 concentration for 90 minutes. For eIF2α and eIF2α phosphorylated the concentrarion and the exposition time were the same but the membranes were probed with primary anti- eIF2α (Abcam, ab5369) and primary anti -eIF2α phosphorylated (Abcam, ab32157). For assessing the presence of the WDR45 transfected, the membranes were probed with primary anti- WDR45-FLAG (SIGMA) at 1:1000 concentration for 90 minutes. For LC3 the time of exposure was the same but the concentration used was 1:500 and the anti-LC3 (NOVUS Biologicals, NB100-22220) antibody was used as the primary antibody. As for the β-actin, the membranes were probed with primary monoclonal anti-actin antibody (SIGMA, A3853-200UL) at a 1:2500 dilution for 90 minutes. After the probing with the primary antibody, the membranes were then washed (3x 10 min)

with PBS-T and probed with the secondary antibody. For PRKRA, PKR phosphorylated and eIF2α phosphorylated it was used secondary anti- Rabbit IgG (whole molecule) peroxidise antibody (SIGMA, A0545-1ML) at 1:2000 concentration during 60 minutes. For PKR, eIF2α, WDR45-FLAG, LC3 and β- actin it was used the secondary anti-Mouse IgG (Fab-specific) – peroxidise antibody (SIGMA, A3682-1ML) at 1:2000 concentration during 60 minutes. Membranes were then washed with PBS-T (3x 10 minutes) and treated with 800 µl - with the Pierce® ECL Western Blotting substrate kit (Thermo Scientific) during 1 minute prior to exposure in the luminescent image analyser (ImageQuant LAS 4000 mini, GE Health Care Life Sciences, version 1.2).

2.6 Starvation condition

24h post transfection, the cells were washed once with PBS (GIBCO, DPBS (1x) Dulbecco's Phosphate Buffered Saline, (-) $CaCl_2$, (-) $MgCl_2$) and grown in medium without FBS for 16h (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate). Then, the medium without FBS was replaced by Earle's balanced salt solution (SIGMA E2888) for 2h. After that, lysis of the cells was performed.

2.7 Toxicity assay by MTT (Thiazolyl Blue Tetrazolium Blue)

A MTT assay was performed for H4 cells with and without transfection of WDR45 plasmid. The 250 mg bottle of MTT (SIGMA, M2128) was dissolute in 50 ml of water and then was aliquoted in 1ml tubes (5mg/ml) and frozen.

In a 96- well- plate (CORNING), the different conditions were plated in 32 wells each with 100 µl/ well of cell medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate, (+) 10% of FBS from GIBCO).

24h before the MTT assay was performed, the cell medium was changed by 100 µl of cell medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate) with 1% FBS (GIBCO) for slowing down the cell growth. Then, in each well, 15 µl of MTT was added. The 96-well-plate was incubated for 3 hours, at 37ºC. After the incubation time, the MTT was carefully removed to avoid the aspiration of crystals. In order to solubilise the crystal, it was

added 100 µl of DMSO (SIGMA Aldrich). After the solubilisation process, the absorbance was accessed at 570 nm.

2.8 Immunocytochemistry assay

In a 24-well-plate (with cover slips in each well previously auto-claved), the H4 cells were plated in triplicates and were transfected withWDR45 or just treated with PEI (with the same protocol mentioned above). After that, they were submitted to different conditions that is non-starved, starved and treated with Torin in triplicates.

33 hours after carrying the transfection, the untreated condition's medium was changed with medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate) containing 10 % of FBS (GIBCO), the starved condition's medium was changed with medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate) without FBS and the Torin treated's condition medium was changed with medium (GIBCO, DMEM Dulbecco's modified eagle medium 1x, 4.5g/L of D-Glucose, (+) pyruvate) containing 100nM torin-1 for mTORc1 and c2 inhibition (Cayman Chemicals, CAY10997). 16 hours after this step, the starved condition's medium was changed with Earle's balanced salt solution (SIGMA E2888) for 2h.

After the 2 hours needed to induce starvation, the medium was removed from all wells and washed one time with 500 µl of PBS (GIBCO, DPBS (1x) Dulbecco's Phosphate Buffered Saline, (-) $CaCl_2$, (-) $MgCl_2$). The PBS was then removed and replaced by 300 µl of 4% of paraformaldehyde (PFA) (Polysciences, Inc., 270547) in PBS and let incubate at room temperature for 15 min. Next, the PFA was removed and the wells were washed 3 times with PBS. The cover slips were then fished out and put in a self-made staining device and one drop of PBS was added to each cover slip in order to avoid the cells to dry and proceed to the staining.

The PBS was removed from each cover slip by bending it in the staining device. 50 µl of blocking solution (PBS + 15% goat serum (VECTOR laboratories S-1000) + 0.1% Triton (SIGMA Aldrich, T8787)) was added in each cover slip and let incubate for 30 min at room temperature. After, the blocking solution was removed and 50 µl of the follow primary antibodies were added:

- FLAG (anti-rabbit) (SIGMA Aldrich) that was diluted 1:2000 in (PBS + 10% of goat serum + 0.1% Triton);
- p62 (anti-mouse) (BD Transduction Laboratories, 610833) that as diluted 1:400 in (PBS + 10% of goat serum + 0.1% Triton);
- FLAG (anti-mouse) (SIGMA) that was diluted 1:2000 in (PBS + 10% of goat serum + 0.1% Triton);

The primary antibodies were incubated over-night, at 4ºC, in a wet chamber.

Next, the primary antibodies were removed and the cover slips were washed 3 times with a drop of PBS. Then, 50 µl of secondary were added in each cover slip:

- Alexa Fluor® 488 goat- anti-rabbit (Life Technologies, A11008) diluted 1:750 in (PBS + 10% of goat serum + 0.1% Triton);
- Alexa Fluor® 568 goat – anti-mouse (Life Technologies, A21124) diluted 1:750 in (PBS + 10% of goat serum + 0.1% Triton);
- Alexa Fluor® 633 phalloidin (Life Technologies, A 22284) diluted 1:300 in (PBS + 10% of goat serum + 0.1% Triton);

The secondary antibodies were incubated for 2 hours at room temperature.

Then, the secondary antibodies were removed and the cover slips were washed 3 times with one drop of PBS. After, they were incubated for 2 minutes with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride) (SIGMA, D9542) diluted 1:2000 in (PBS + 10% of goat serum + 0.1% Triton). Then, the cover slips were washed 3 times with PBS.

After that, one drop of 10 µl of mounting medium (Southern Biotech, 0100-01) was placed on glass sides and the cover-slips were gently placed on top of the drop of mounting medium (side with cells facing down, in direct contact with the glue). During 48 hours they were let dry at room temperature, in the dark, and store at 4ºC.

The images of the cells were then accessed by confocal analysis in the Zeiss LSM 710 confocal microscope and processed by the Zen 2009 software.

III. RESULTS

PRKRA

3.1 PRKRA transfected in HT1080 cells

As mentioned in the INTRODUCTION, section 2.1, it is known that PRKRA is involved in DYT16. However, it is still not clear if the mutation is or not a loss of function mutation.

In order to investigate any differences between PRKRA wild-type (WT) and mutated (MT- c. 665 C>T), HT1080 cells were transfected with a plasmid containing PRKRA WT or MT. This transfection caused an over-expression of the amount of PRKRA in the HT1080 cells.

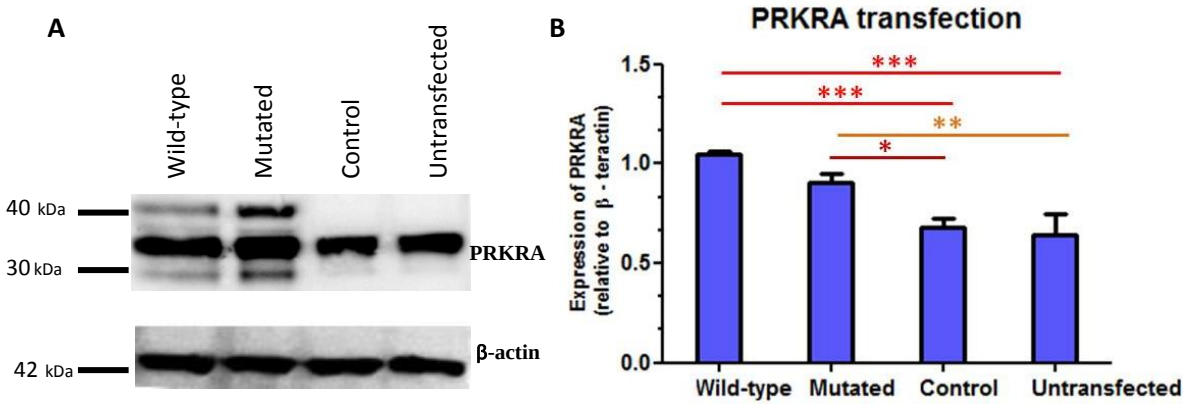

Figure 3.1- PRKRA transfected in HT1080 cells.

(A) Western blot analysis showing transfection of PRKRA wild-type (WT) and mutated (MT) and the β-actin expression in HT1080 cells. The control condition stands for HT1080 cells treated only with the transfection reagent (PEI) and the last condition stands for untransfected (Unt.). **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the PRKRA amount within HT1080 cells when transfected with PRKRA wild-type (WT), PRKRA mutated (MT), treated only with the transfection reagent –PEI (CT) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**)and extremely statistically significant when $P < 0.001$ (***).The results are represented by their mean and standard deviation.

HT1080 cells express PRKRA at an endogenous level since in the control (CT) and untransfected condition, PRKRA expression can be detected (34 kDa band). This cell line was successfully transfected with both WT and MT plasmids of PRKRA. Since there is an over-expression of PRKRA in the WT and MT conditions, there are

immunoreactive bands larger and smaller upstream and downstream of the PRKRA band that can be due to a precursor form of PRKRA protein or due to a glycosylation, phosphorylation or ubiquitination of the protein. These characteristics could be responsible for the protein run at an apparently higher or lower molecular mass within the gel.

3.1.2 Levels of non phosphorylated and phosphorylated PKR are not affected by overexpression of PRKRA

As previously described, PRKRA protein causes the auto-phosphorylation of PKR since it is his downstream target following binding and conformational change.

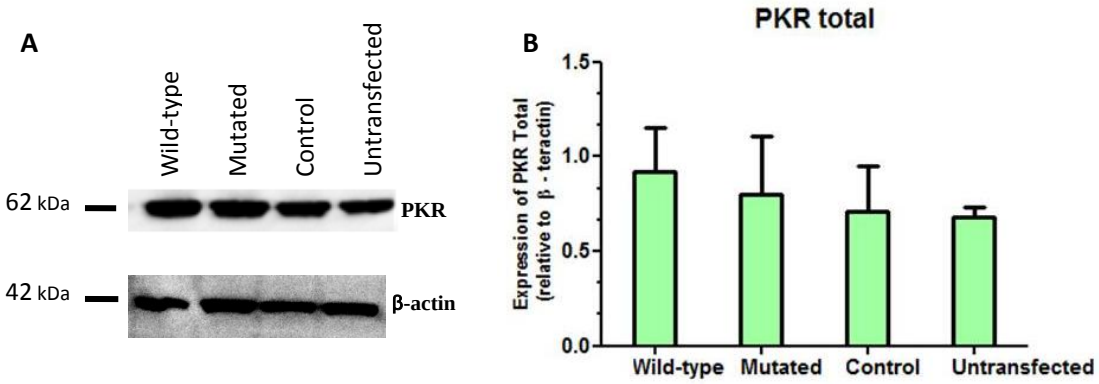

Figure 3.2- Levels of non-phosphorylated PKR are not affected by PRKRA overexpression.

(A) Western blot analysis showing PKR and β-actin expression in wild-type (WT) and mutated (MT) conditions in HT1080 cells. The control condition stands for HT1080 cells treated only with the transfection reagent (PEI) and the last condition stands for untransfected (Unt.). **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the PKR levels within HT1080 cells when transfected with PRKRA wild-type (WT), PRKRA mutated (MT), treated only with the transfection reagent –PEI (CT) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$ (***).The results are represented by their mean and standard deviation.

After overexpressing WT and MT PRKRA condition, the levels of total PKR were accessed. The levels of PKR in the different conditions did not seem to suffer a significant alteration, meaning that the levels of total PKR were not affected by the two different conditions.

Before these results, there was the hypothesis that levels of PKR phosphorylated could be altered since PRKRA activates PKR by leading to its auto-phosphorylation. The levels of PKR phosphorylated were then accessed.

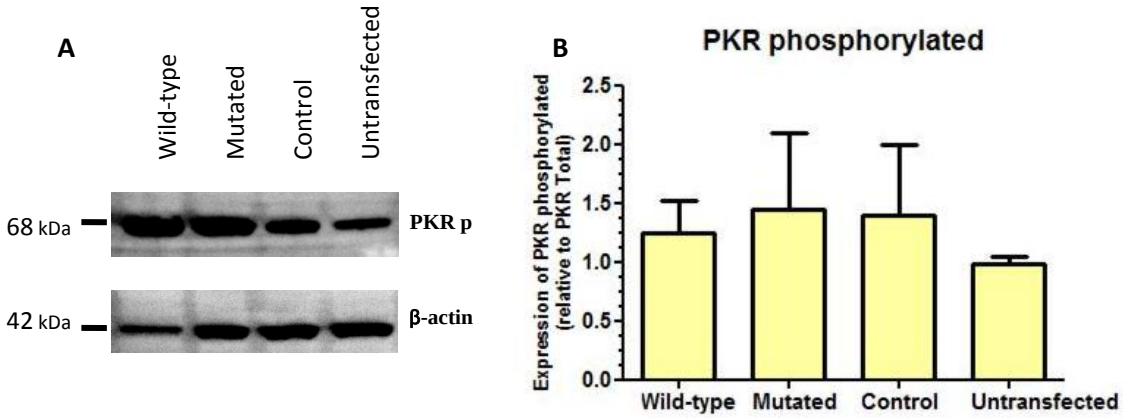

Figure 3.3- Levels of phosphorylated PKR are not affected by PRKRA overexpression.

(A) Western blot analysis showing PKR phosphorylated and β-actin expression in wild-type (WT) and mutated (MT) conditions in HT1080 cells. The control condition stands for HT1080 cells treated only with the transfection reagent (PEI) and the last condition stands for untransfected (Unt.). **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the PKR phosphorylated levels within HT1080 cells when transfected with PRKRA wild-type (WT), PRKRA mutated (MT), treated only with the transfection reagent –PEI (CT) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$ (***).The results are represented by their mean and standard deviation.

The levels of PKR phosphorylated are not significant altered by the over-expression of PRKRA WT or MT. However, it seems that in condition treated with PEI, especially the MT condition, the level of phosphorylation of PKR is a little bit high. This could be due to the fact that PRKRA MT raises a little the auto-phosphorylation of PKR leading to apoptosis or that there is a trend towards increase phosphorylation in all the PEI treated cells, so it could represent an increase in cell stress due to transfection. Also, since the cells have normal PRKRA at endogenous levels, over-expressing the mutant form can lead them to apoptosis to get read of the mutant PRKRA.

3.1.3 Levels of non- phosphorylated and phosphorylated eIF2α are unchanged with the overexpression of PRKRA.

Beside the results obtained before, the levels of eIF2α were assessed since it is the downstream of PKR phosphorylated and although their levels were not changed maybe it activates a stronger response in the eIF2α levels.

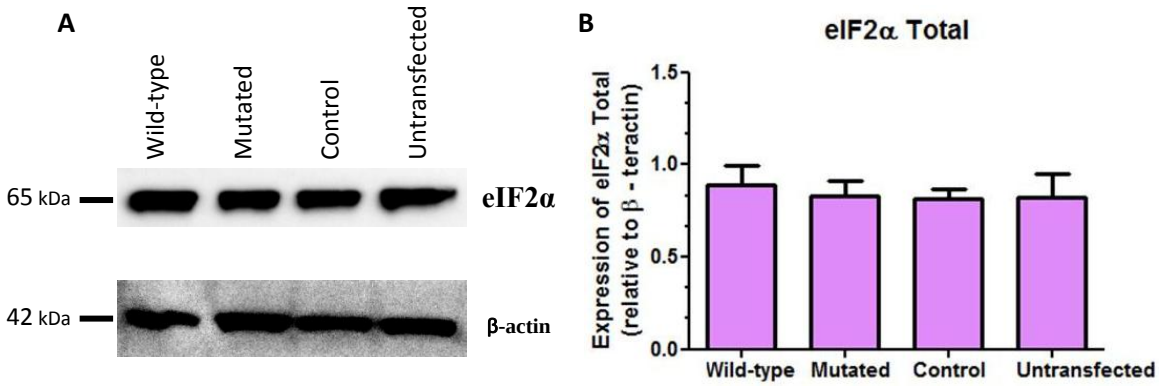

Figure 3.4- Expression of non-phosphorylated eIF2α are unchanged by PRKRA overexpression.

(A) Western blot analysis showing eIF2α and β-actin expression in wild-type (WT) and mutated (MT) conditions in HT1080 cells. The control condition stands for HT1080 cells treated only with the transfection reagent (PEI) and the last condition stands for untransfected (Unt.). **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the eIF2α levels within HT1080 cells when transfected with PRKRA wild-type (WT), PRKRA mutated (MT), treated only with the transfection reagent –PEI (CT) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$ (***).The results are represented by their mean and standard deviation.

The eIF2α levels were not altered between the different conditions which do not support the hypothesis of a stronger induction in the expression of eI2 α. However, as mentioned in the introduction section 2.2, is the eIF2α phosphorylated which is responsible for the inhibition of the translation machinery leading to apoptosis. Although the eIF2α were not altered maybe there was more eIF2α phosphorylated in the WT and/or MT conditions. So, the eIF2α phosphorylated levels were assessed.

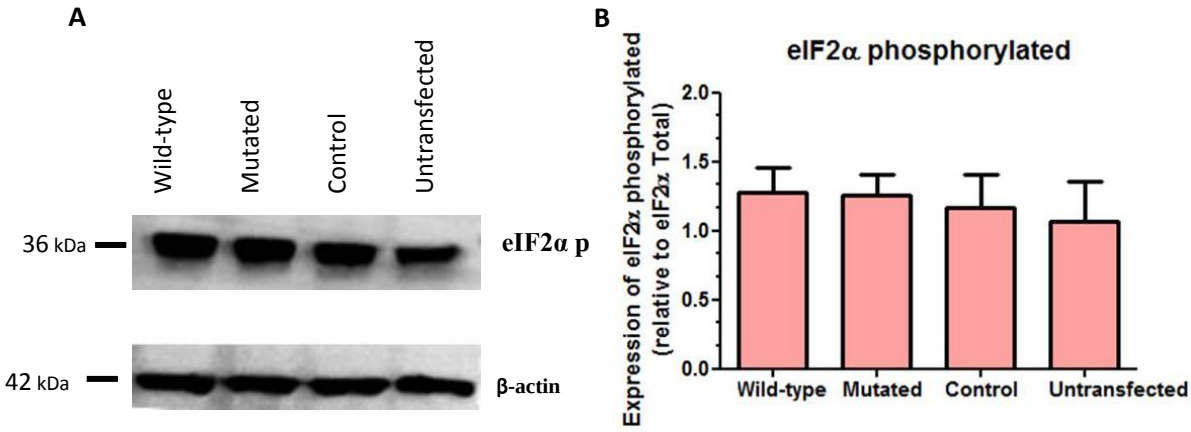

Figure 3.5- Expression of phosphorylated eIF2α are unchanged by PRKRA overexpression.

(A) Western blot analysis showing eIF2α phosphorylated and β-actin expression in wild-type (WT) and mutated (MT) conditions in HT1080 cells. The control condition stands for HT1080 cells treated only with the transfection reagent (PEI) and the last condition stands for untransfected (Unt.). **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the eIF2α phosphorylated levels within HT1080 cells when transfected with PRKRA wild-type (WT), PRKRA mutated (MT), treated only with the transfection reagent –PEI (CT) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$ (***).The results are represented by their mean and standard error.

Once again the levels of eIF2α phosphorylated did not change between the different conditions. Based on these data, overexpressing PRKRA in its WT or MT form does not have a major impact on the inhibition of translation machinery.

WDR45

3.2 Expression of WDR45 in HEK and H4 cells

Since at the moment there is no validated antibody available for detecting endogenous levels of WDR45, it was established that the best option would be overexpressing WDR45 by a plasmid with the FLAG epitope in three cell types: HEK, H4 and SH-SY5. However, the results obtained with SH-SY5 were negative and so will not be presented in this thesis.

HEK and H4 were successfully transfected with the plasmid WDR45-FLAG as it can be seen in the following Western blot (figure 3.6).

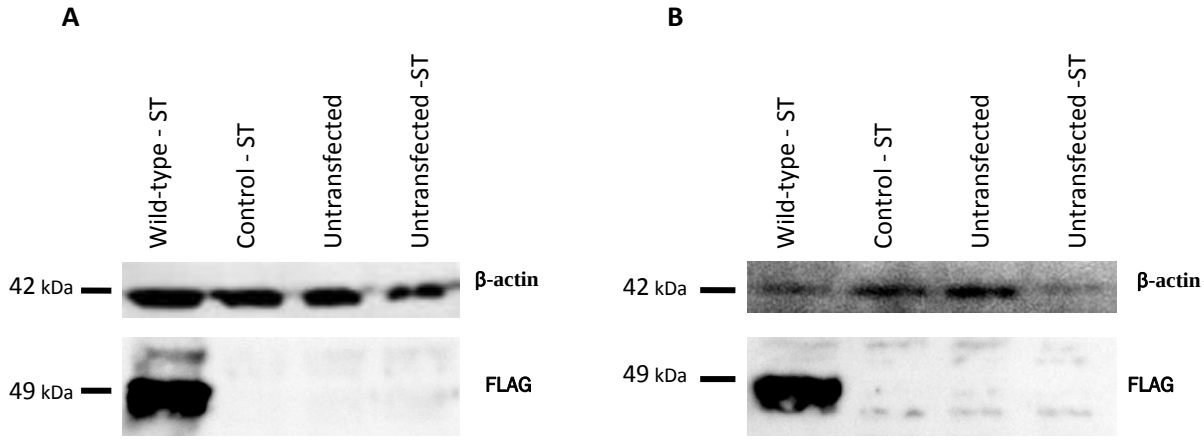

Figure 3.6- WDR45-Flag is expressed in HEK and H4 cells.

(A) Western blot analysis showing successfully transfection of WDR45 plasmid with Flag epitope and β-actin in HEK cells.The control condition stands for HEK cells treated only with the transfection reagent (PEI) and ST stands for the conditions which were submitted to starvation. **(B)** Western blot analysis showing successfully transfection of WDR45 plasmid with Flag epitope and β-actin in H4 cells. The control condition stands for H4 cells treated only with the transfection reagent (PEI) and ST stands for the conditions which were submitted to starvation.

Since WDR45 has been implicated in autophagy, after transfecting HEK and H4 cell lines, the autophagy process was induced in the cells by using the starvation protocol (ST) (MATERIAL & METHODS section). Only the untransfected condition was not submitted to this protocol.

In order to access if autophagy was induced, the levels of LC3-I and LC3-II were accessed. As explained in the INTRODUCTION section 5.2, if LC3-II levels

(normalised with β-actin) are increased compared with LC3-I levels (normalised with β-actin) it means that autophagy is active.

3.2.1 LC3-I and LC3-II expression levels were unaffected in HEK and H4 cells in starved and not starved conditions

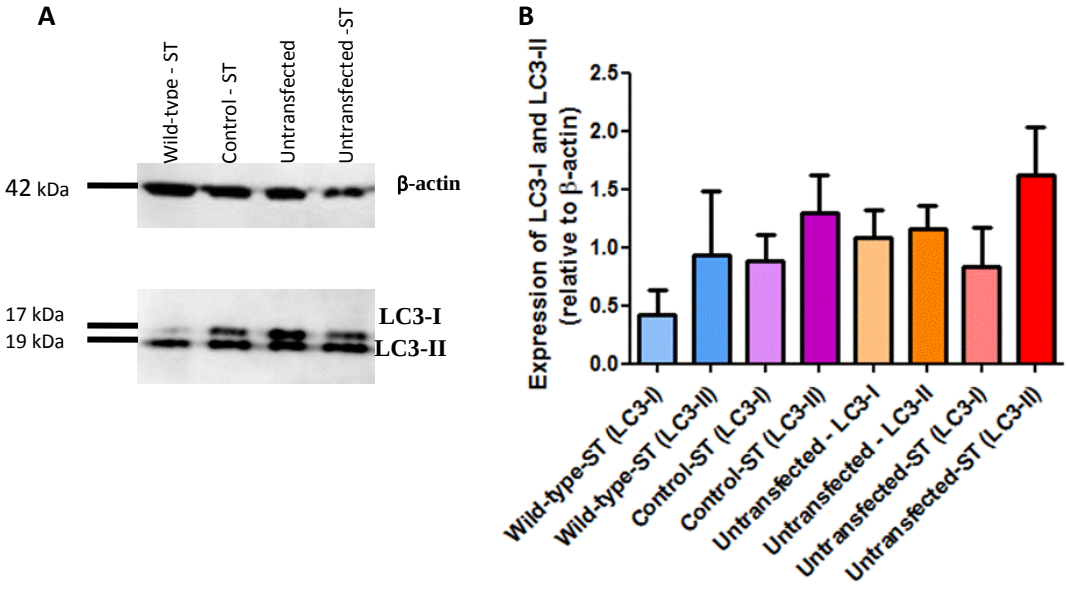

Figure 3.7- Expression of LC3-I and LC3-II in HEK cells were unaffected in starvation and non-starvation condition.

(A) Western blot analysis showing LC3-I and II and β-actin expression in wild-type-ST (WT), Control-ST (CT), untransfected-ST (Unt.) conditions submitted to starvation (ST) in HEK cells. The control condition stands for HEK cells treated only with the transfection reagent (PEI) and the untransfected (Unt.) was not submitted to starvation . **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the LC3-I and II levels within HEK cells when submitted to starvation (ST) and transfected with WDR45 (wild-type-ST), treated only with the transfection reagent –PEI (Control-ST) and not transfected (untransfected-ST). The only condition not submitted to starvation was untransfected. The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$. (***).The results are represented by their mean and standard error.

As it can be seen in the graph, although there is a trend towards a conversion of LC3-I to LC3-II, this does not reach significance. These results also show that overexpressing WDR45 in HEK cells does not have a major impact in autophagy since the levels of LC3-II are not so different between conditions.

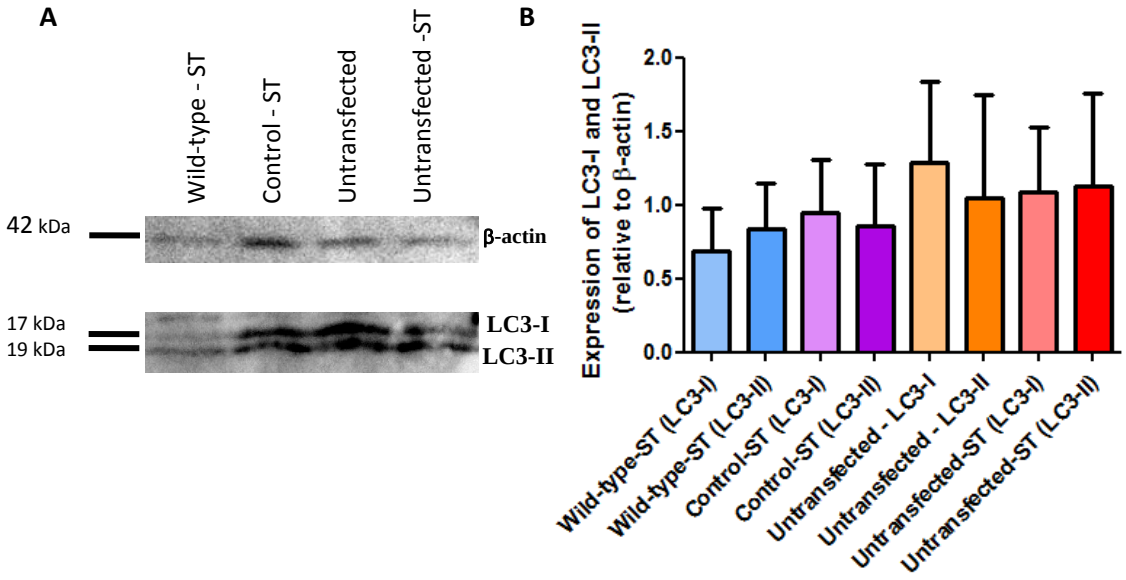

Figure 3.8- Expression of LC3-I and LC3-II in H4 cells were unaffected in starvation and non-starvation condition.

(A) Western blot analysis showing LC3-I and II and β-actin expression in wild-type-ST (WT), Control-ST (CT), untransfected-ST (Unt.) conditions submitted to starvation (ST) in H4 cells. The control condition stands for H4 cells treated only with the transfection reagent (PEI) and the untransfected (Unt.) was not submitted to starvation . **(B)** Analysis by Bonferroni's test – ANOVA (n=3) of the LC3-I and II levels within H4 cells when submitted to starvation (ST) and transfected with WDR45 (wild-type-ST), treated only with the transfection reagent –PEI (Control-ST) and not transfected (untransfected-ST). The only condition not submitted to starvation was untransfected. The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$. (***).The results are represented by their mean and standard error.

For H4 cells, the results are similar to those observed in HEK cells. There is a conversion of LC3-I to LC3-II but it does not seem to have a big difference between the conditions. Also, over-expressing WDR45 in H4 cells does not seem to have such a big impact in autophagy.

However, after realizing that it would be necessary to have more conditions in order to access if autophagy was or was not compromised, the experiment was repeated. In this replicate, only H4 cells were used since they are derived from cells originating in the brain and the results are similar of the ones found in HEK cells.

3.2.2 LC3-I and LC3-II expression levels in H4 cells do not suffer a major impact in starved and not starved conditions

It was established that wild-type, control and untransfected conditions had to be all non starved (basal conditions) and starved in order to test if over-expressing WDR45 would affect considerably the autophagy process.

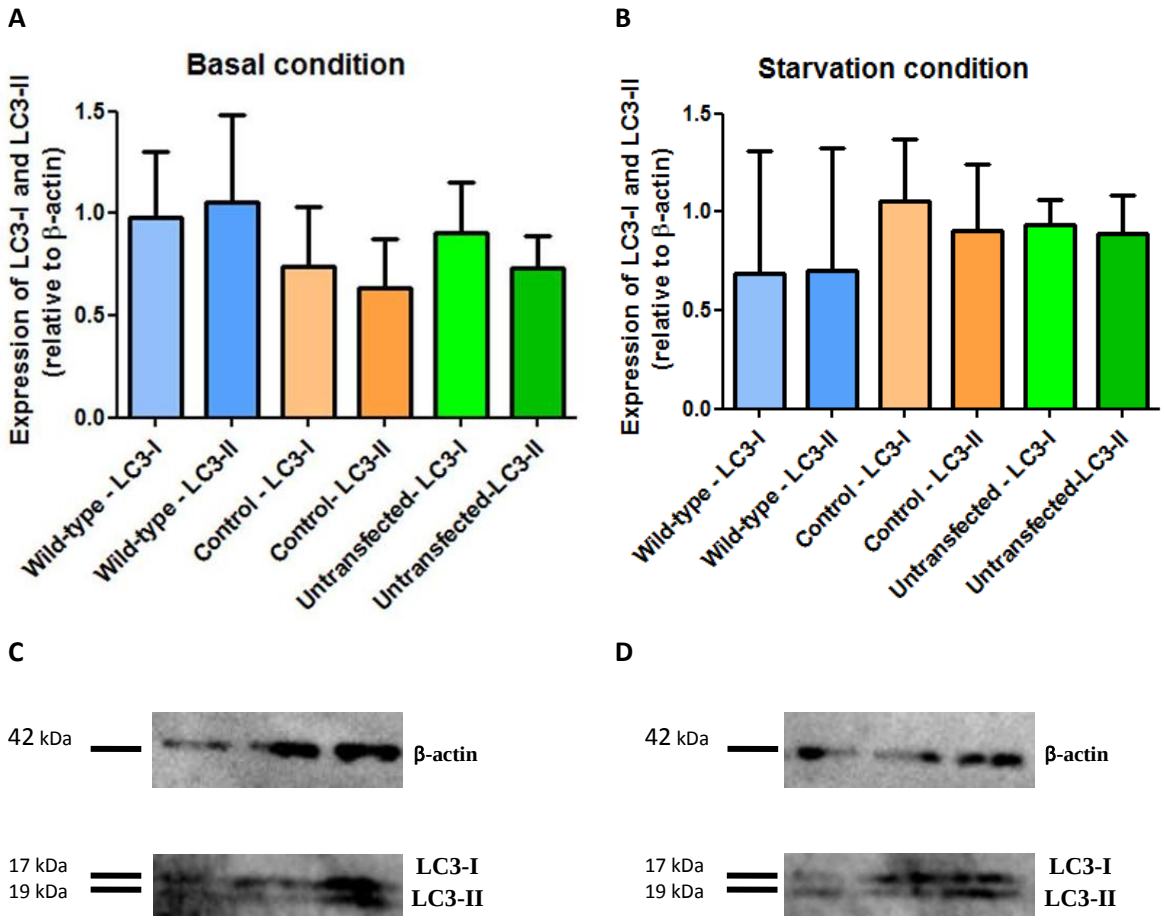

Figure 3.9- LC3-I and LC3-II expression levels in H4 cells do not suffer a major impact in starved and not starved conditions after WDR45 over expression.

(A-B) Analysis by Bonferroni's test – ANOVA (n=3) of the LC3-I and II levels within H4 cells in a basal and starvation conditions, respectively, transfected with WDR45 (wild-type), treated only with the transfection reagent –PEI (Control) and not transfected (untransfected). The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$. (***).The results are represented by their mean and its standard error. (C-D) Western blot analysis showing LC3-I and II and β-actin expression in wild-type(WT), Control (CT), untransfected (Unt.) conditions in a steady and starvation condition, respectively, in H4 cells.

Once again, expression of WDR45 does not have a major impact on the conversion of LC3-I to LC3-II when transfected and submitted to starvation.

3.2.4 Viability of H4 cells is compromised after transfection

During the transfection protocol, it was noticed that once transfected, there was a lot of death in H4 cells. For that reason, a MTT assay was performed.

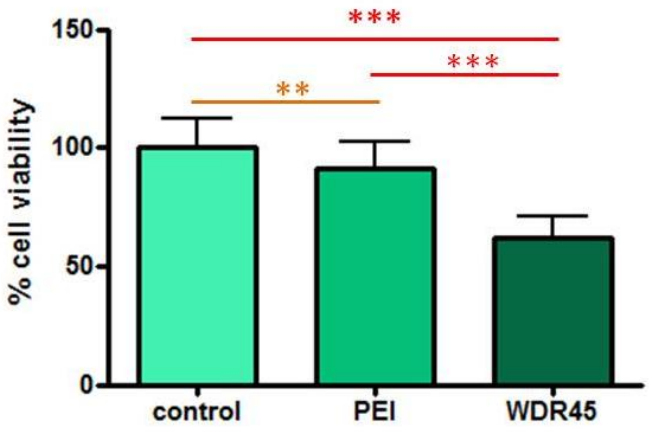

Figure 3.10 – Viability of H4 cells is compromised after transfection.

Analysis by Bonferroni's test – ANOVA (n=32) of cell viability in not transfected cells (control), cells treated only with the transfection reagent –PEI (PEI) and cells transfected with WDR45 (WDR45). The differences were considered statistically significant when P≤ 0.05 (*), very statistically significant when 0.001 ≤ P ≥ and 0.1 (**) and extremely statistically significant when P < 0.001. (***).The results are represented by their mean and its standard error.

By this MTT assay, it was possible to conclude that the plasmid WDR45, involved with PEI, was extremely toxic to H4 cells since it cuts the cell viability to half comparing with H4 cells non treated.

3.2.5 Localization of WDR45 and observation of induction of autophagy in H4 cells

Since, to our knowledge, the cellular localisation of WDR45 has not being investigated, with a hypothetical localization being in the endosomes (since Atg18 is located there), immunocytochemistry was performed in transfected H4 cells.

As there is no available antibody able to detect endogenous WDR45, the antibody used was the FLAG antibody since the cells were transfected with the plasmid of WDR4 followed by a FLAG epitope. Furthermore, to try to understand the connection between autophagy and WDR45, the antibody p62 was also used.

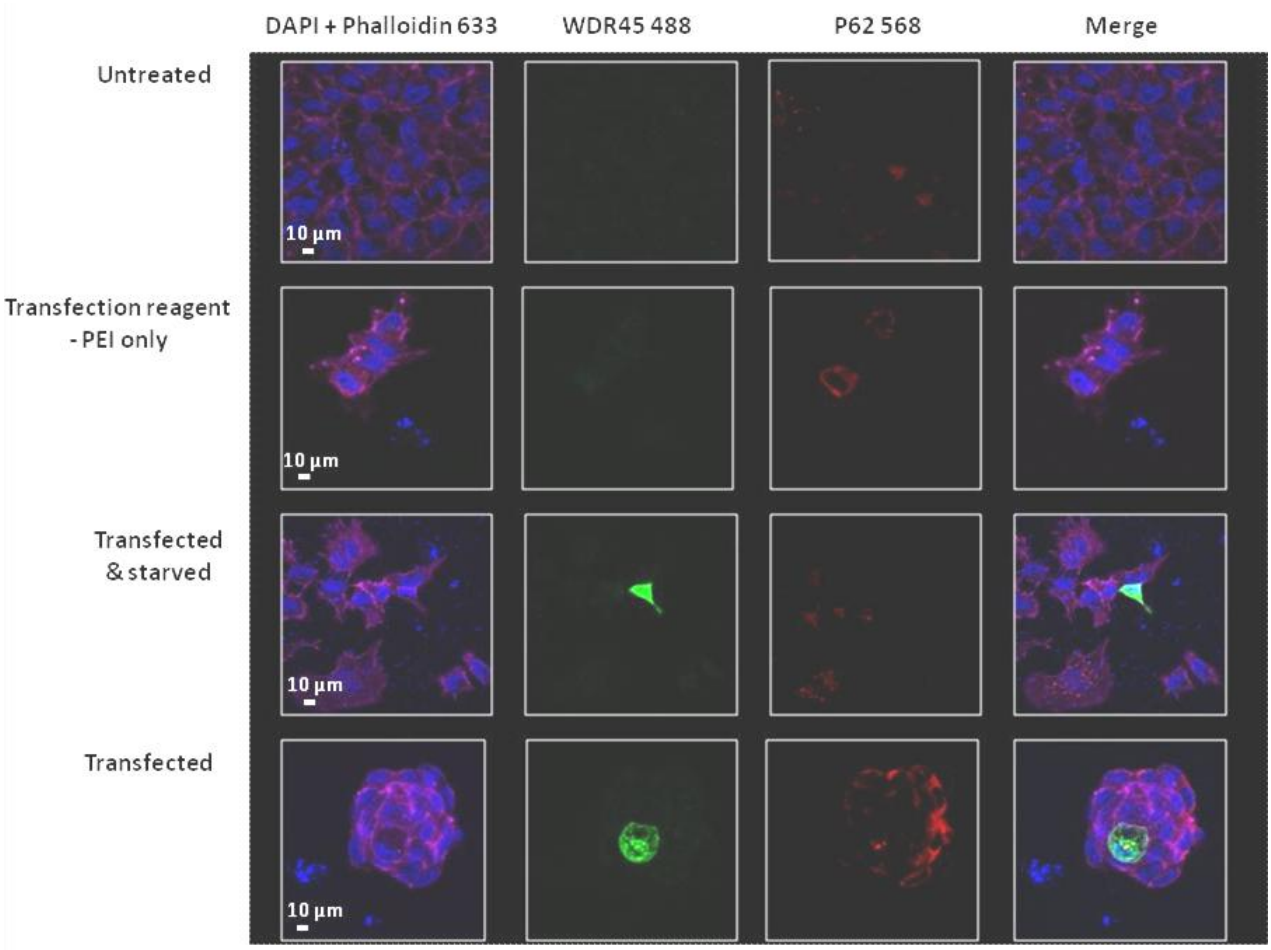

Figure 3.11 – Localization of WDR45 and observation of induction of autophagy in H4 cells.

H4 cells were immunostained against FLAG (green) to localize WDR45 transfected and with p62 (red) to observe the autophagy process. Cells were also counterlabeled with DAPI and phalloidin. Scale bar, 10 µm. All images were collected with 40x of magnification with the Zeiss LSM 710 confocal microscope and processed by the Zen 2009 software.

These results show that WDR45 seemed present in the nucleus and in the cytoplasm of cells. WDR45 and p62 seemed also co-localized in certain parts of the cytoplasm. In order to try to see in more detail a co-localization between these two proteins, a cell was examined at higher magnification as shown bellow.

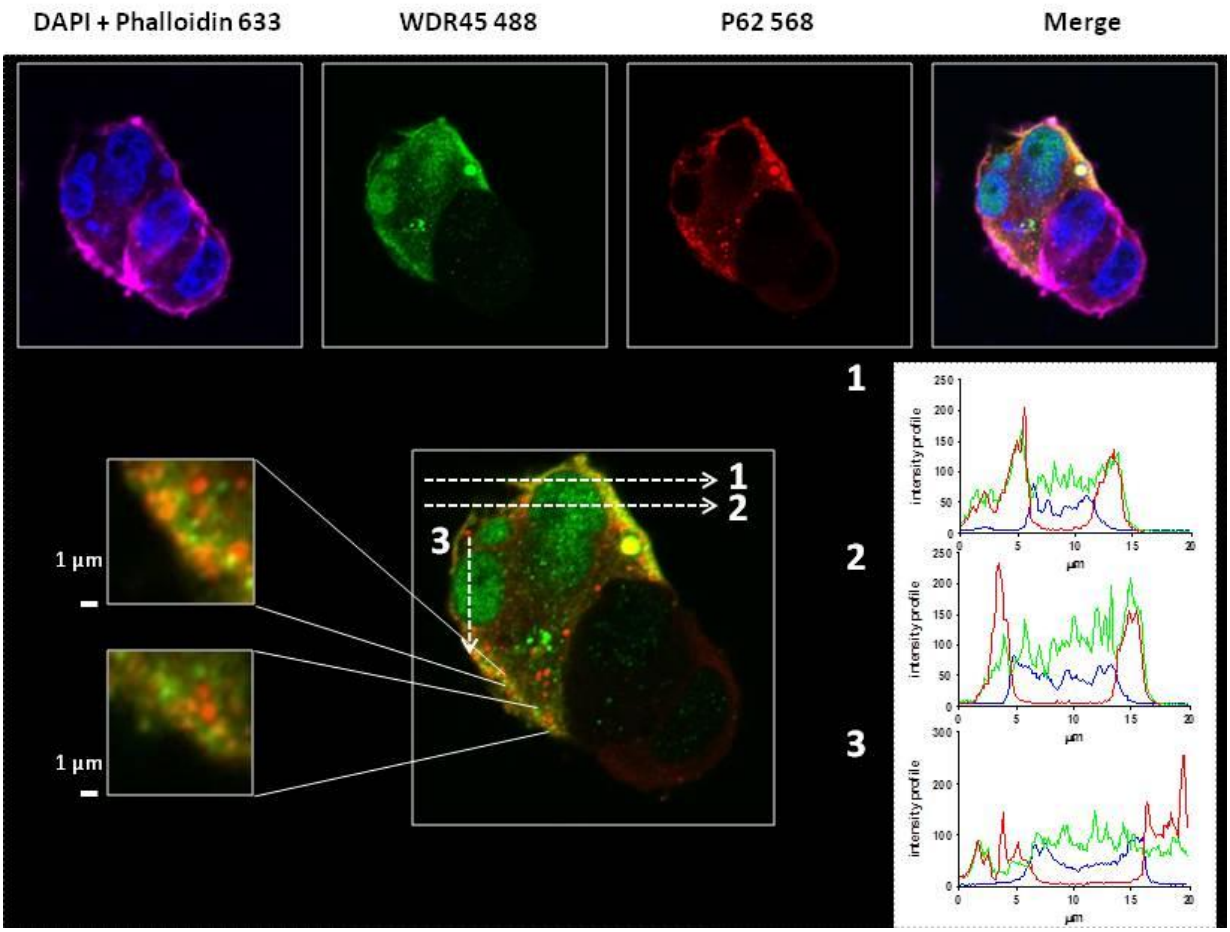

Figure 3.12 – Co-localization of WDR45 and p62 in H4 cells.

H4 cells were immunostained against FLAG (green) to localize WDR45 transfected and with p62 (red) to observe their co-localization. Cells were also counterlabeled with DAPI and phalloidin. Scale bar, 10 µm. All images were collected with 60x of magnification with the Zeiss LSM 710 confocal microscope and processed by the Zen 2009 software.

At a higher magnification, in several puncta a co-localization between WDR45 and p62 is observed.

3.2.6 Level of transfected cell is much lower than non-transfected cells level

Since the results of MTT assay showed that cell viability was reduced when the cells were transfected with WDR45, cell counts were carried out using immunocytochemistry images. The cells transfected and the non transfected were counted by a triplicate of random images obtained with the immunocytochemistry assay. A graphic was done with these data, as showed bellow:

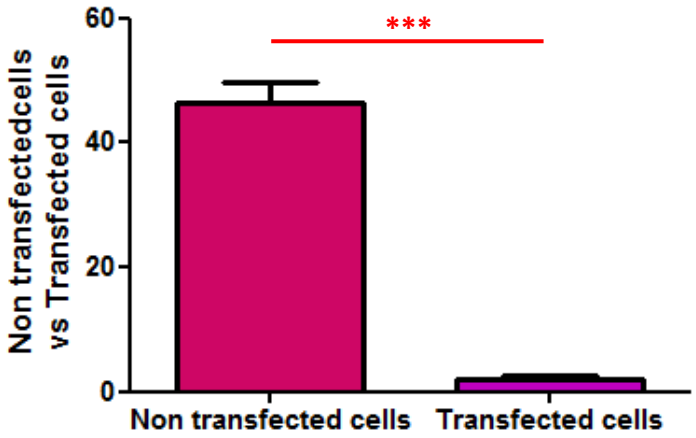

Figure 3.13- Cell count of transfected cells is much lower than non-transfected cells.

Analysis by unpaired t test – (n=3) of transfection's degree in H4 cells. The differences were considered statistically significant when $P \leq 0.05$ (*), very statistically significant when $0.001 \leq P \geq$ and 0.1 (**) and extremely statistically significant when $P < 0.001$. (***).The results are represented by their mean and standard error.

IV. DISCUSSION & CONCLUSION

Brain disorders, such as Parkinson's disease and dementia, are one of the major health problems in Europe [72].With an aging population, brain disorders cases will grow over the coming decades and represent an ever larger social and economical cost to society. It is estimated that in total 800 billion euros are used to treat brain diseases where 13.933 and 105.163 millions are used to treat Parkinson's disease and dementia respectively [72]. These data clearly suggest that research in these fields is really important and necessary in order to the social and economical charges to the society go down. As stated in the introduction, this thesis attempts to understand the mechanisms that are underlying dystonia 16 and BPAN in order to try to understand the different types of mechanisms that can lead to Parkinson's disease.

In the experiments investigating PRKRA mutations in which cause a complex dystonia - parkinsonism, WT and MT *PRKRA* plasmids were successfully transfected as it can be seen in figure 3.1. However, downstream readouts for this pathway did not present any significant difference. Based on these data, overexpressing PRKRA, in HT1080 cells, does not have a major impact on the levels of PKR total and phosphorylated (figure 3.2 and 3.3). The levels of eIF2α and eIF2α phosphorylated also did not present any significant alteration between all the conditions, adding the suggestion that in HT1080 cells, high levels of PRKRA do not induce changes in the PKR pathway.

These results can be due to several reasons. It can be explained by the fact that, since there is PRKRA endogenous in the cell that do not have the mutation; it can rescue the supposed phenotype of MT PRKRA plasmid inserted. By consequence the induction of autophagy will also not be seen. It also cannot be forgotten that the model used, that is HT1080 cells, maybe not be the best model to use in this experiments. Since it is a cell line that is derived from a fibrosarcoma, it has a lot of pathways already modified meaning that perhaps the autophagy pathway is one of them and that over expression of PRKRA will not be able to cause any alteration within the autophagy pathway already modified.

Also, visible alterations in autophagy process due to PRKRA maybe only accessed when the cells undergo a stress situation as for example starvation or heat shock. These stress conditions were not performed in these PRKRA experiments and that can also be one of the reasons of why autophagy levels were not altered between the different conditions.

In the case of WDR45 experiments, the cells were equally successfully transfected with the plasmid as it can be seen in figure 3.6.However, as shown by the MTT assay, the transfection method by PEI is extremely toxic to the cells.

After analysing all the graphics obtained from the steady and starvation conditions, it could be accessed that the conversion from LC3-I to LC3-II was not significant in both conditions. This fact could suggest that WDR45 is may be capable of substituting for LC3, being part of a parallel pathway for autophagy.

In the immunocytochemistry experiments, it was observed that the p62 staining was increased when the cells were transfected with WDR45 and starved, comparing with the cells only treated with PEI. This observation can suggest that p62 is trying to eliminate the excess of WDR45 but can also corroborate the MTT assay results. That is, since WDR45 coated with PEI is extremely toxic to the cells, p62 is increased because the cells are dying and not necessarily because there is an excess of WDR45.

The immunocytochemistry assays also showed that probably WDR45 would be localized inside the autophagic vesicle since it was co-localized with p62 (as shown in figure 3.11). p62 is known to be localized inside the vesicles [73]. That would be interesting since its other family members (wipi2 and wipi3) are known to be outside the autophagic vesicles [60].

Finally, by the images obtained from immunocytochemistry, it is clear that WDR45 can be localised to the nucleus. However, it cannot be judged from data here presented if this represents the normal function of WDR45 or if it is due to the over-expression of WDR45. In order to try to respond to this uncertainty, the sequence of the plasmid used was analysed in the MultiLoc program [74] in order to predict the protein sub cellular localization (see Anex III). However, the localization predicted

was not for nucleus but for the Golgi, an intriguing result that merits further investigation.

As mentioned above for PRKRA, is important to note that the cell lines used were cells that already have major alterations in cellular pathways, including in the autophagy, which could alter the response to the over-expression of WDR45.

All these data presented in this thesis shows that there is much to investigate in order to understand how the molecular pathways function and are connected. Even if a lot is already known about Parkinson's disease, a lot has to be found and understand in order to find a cure. It is urgent to keep studying this subject, no matter the angle, since it would be a disease that would affect more thousands and thousands of people every year.

4.1 Future work

Future experiments would be necessary to corroborate these data and try to better understand the mechanisms underlying PRKRA and WDR45.

For future PRKRA experiments, it will be necessary to try to find a cell line that does not possess PRKRA endogenous or try to completely knock out the PRKRA endogenous in the cell line that would be used. This precaution will prevent the rescue of MT PRKRA phenotype and will allow to really observed the effect of RKRA within the cell. If working with a cell line has its advantages (cost, easy to preserve and grow, for instance) it also has disadvantages: for example not replicating real conditions within the brain and presenting with a significantly altered growth rate and gene expression profile compared to primary cells. In order to mitigate these problems, these experiments could be done in a primary tissue culture (for example neurons). This will more closely model the conditions found in disease of human body and a lot of pathways.

For future experiments with WDR45, it will be better to try another method of transfection as for example electroporation technique since the MTT results showed that transfection of WDR45 coated with PEI is extremely toxic to the cells. It

will also allow passing the doubt if the toxicity is due to the excess of WDR45 or due to the transfection method.

It would also be necessary to have a good antibody which would be able to detect the endogenous levels of WDR45. With this antibody, it would be possible to produce a knockout cell line for WDR45 and induce the starvation to see what would happen to autophagy. It would also be possible to access the localization of the endogenous WDR45 and therefore better understand the role of WDR45 within the cell.

For confirming the fact that WDR45 may be capable of substituting the role of LC3, it would be necessary to perform a bafilomycin treatment. Bafilomycin is able to block degradation of lysosomes by changing their pH and by consequence unable LC3 to be recycled and the lysosomes to degrade the proteins inside [75]. In that case, if the levels of LC3-II were the same in the untreated condition and in the over-expression WDR45 condition, it would mean that the autophagy was induce rapidly since there was an increase in the recycling of LC3. If the levels of LC3-II were lower in the over-expression of WDR45 condition than the untreated condition, it would mean that there was a decrease in autophagy.

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APPENDICES

APPENDIX I- WDR45 plasmid

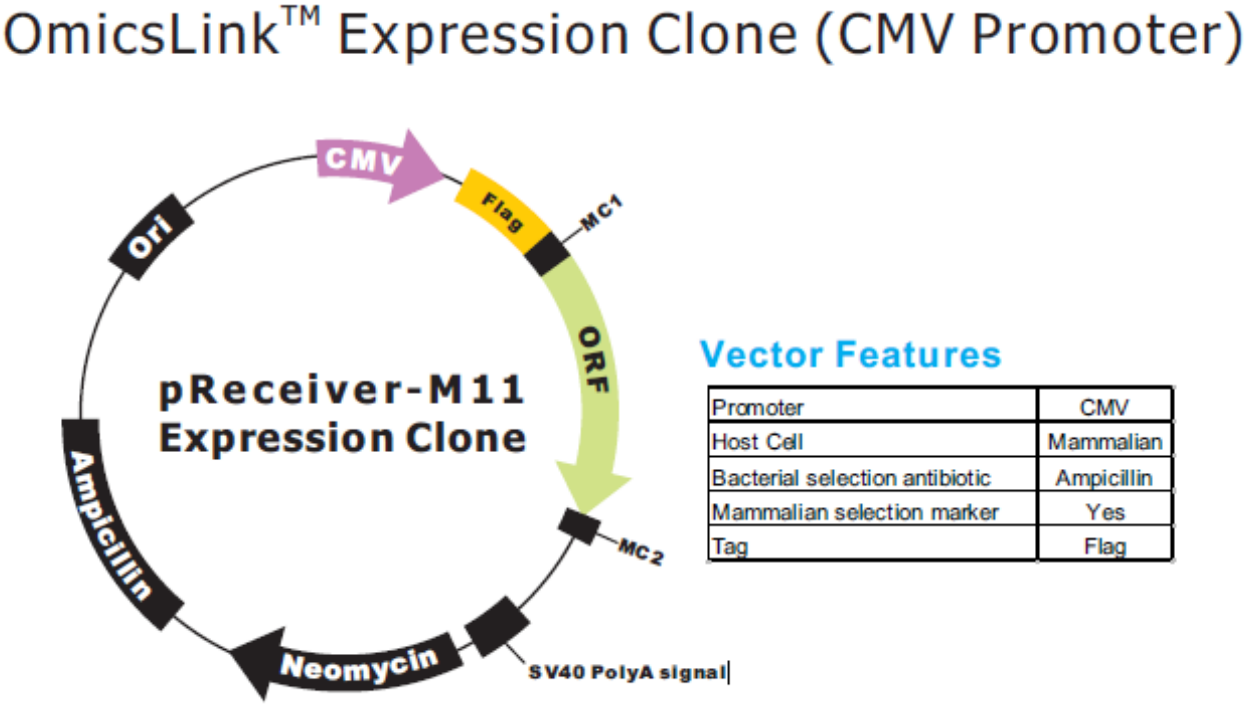


Figure A1 – WDR45 plasmid

Plasmid of WDR45 with the FLAG epitope and resistant to ampicillin and neomycin.

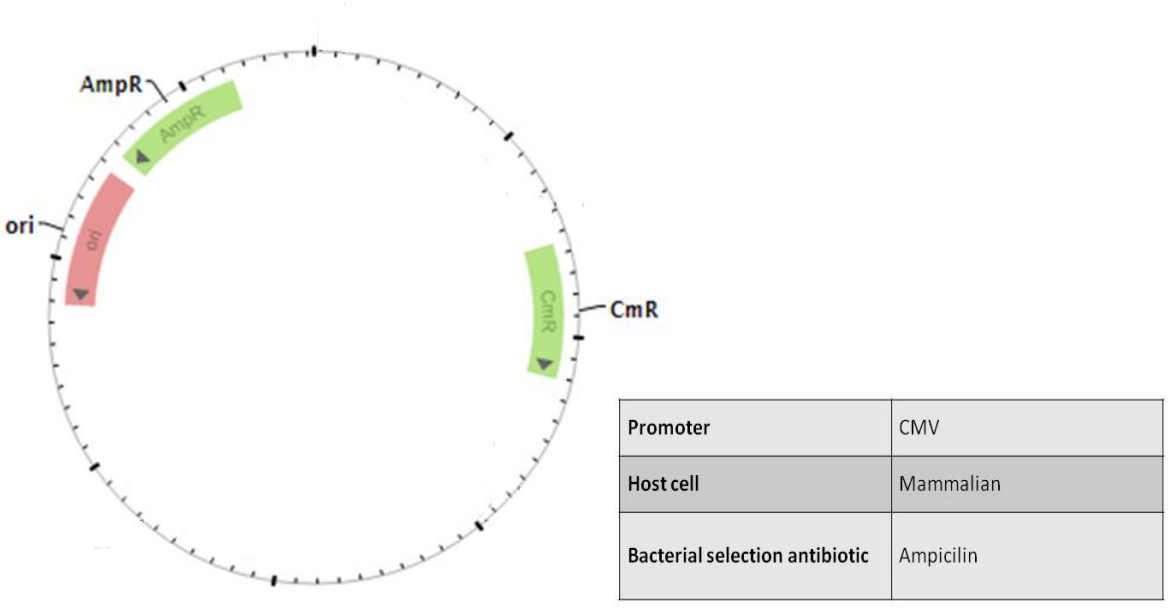


Figure A2 – PRKRA plasmid

Example of a plasmid of PRKRA used in the experiments, resistant to ampicilin.

APPENDIX II – Quality of DNA obtained

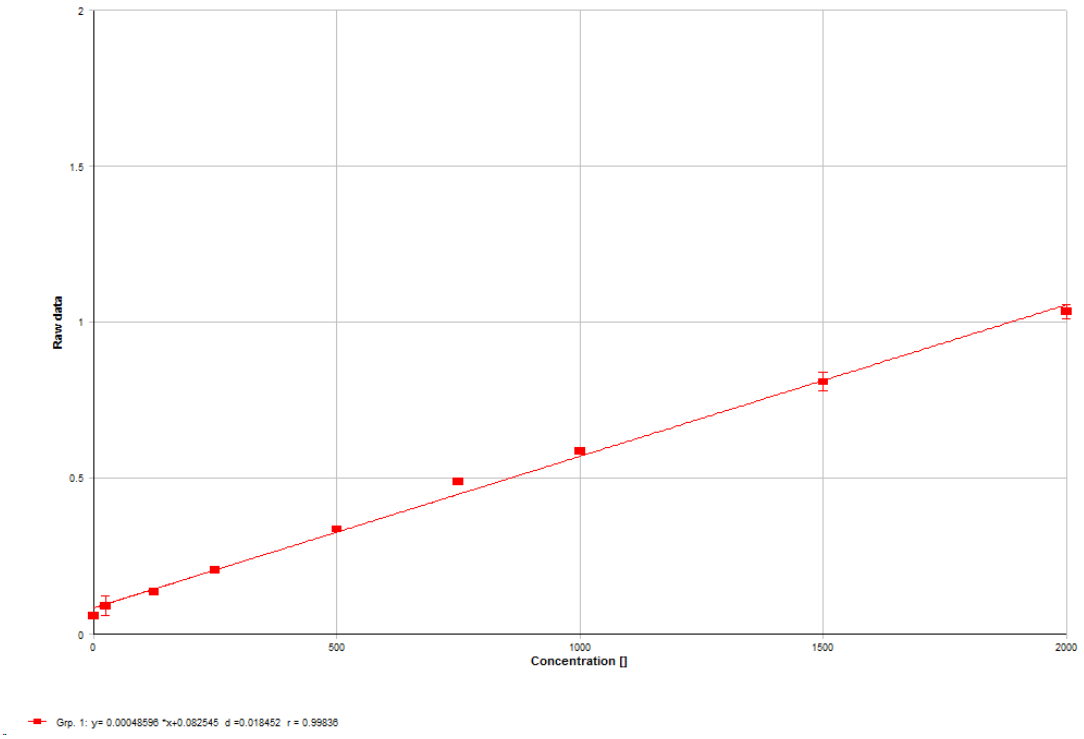


Figure A3 – Example of a standard curve for PRKRA's DNA.

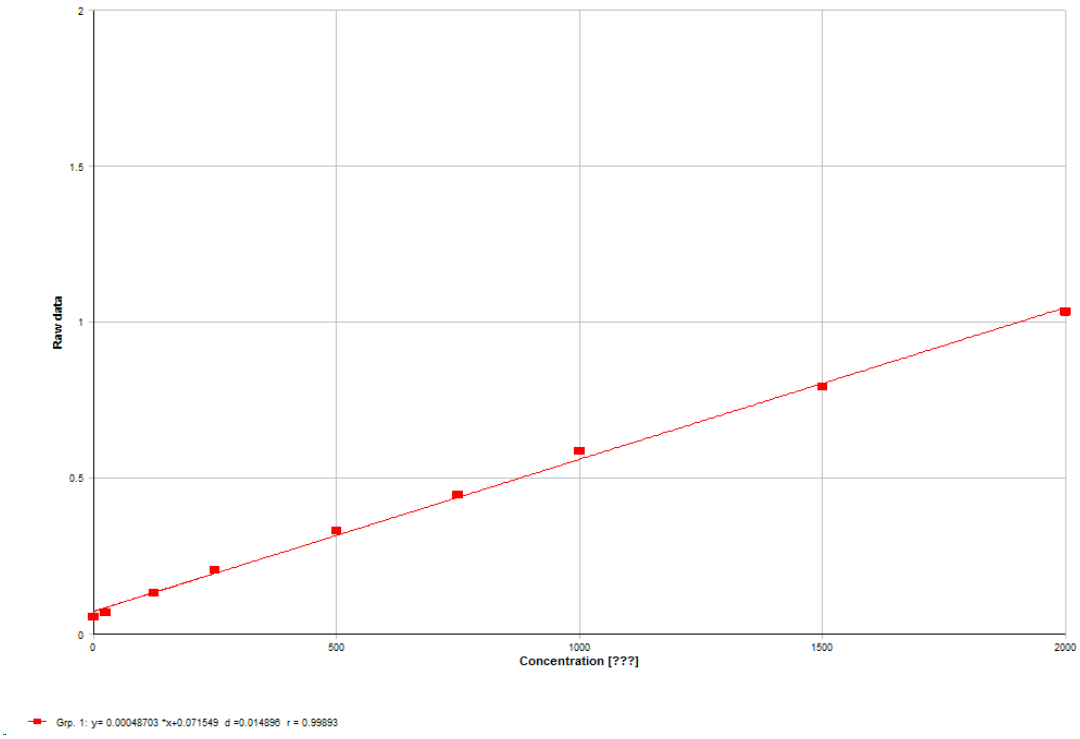
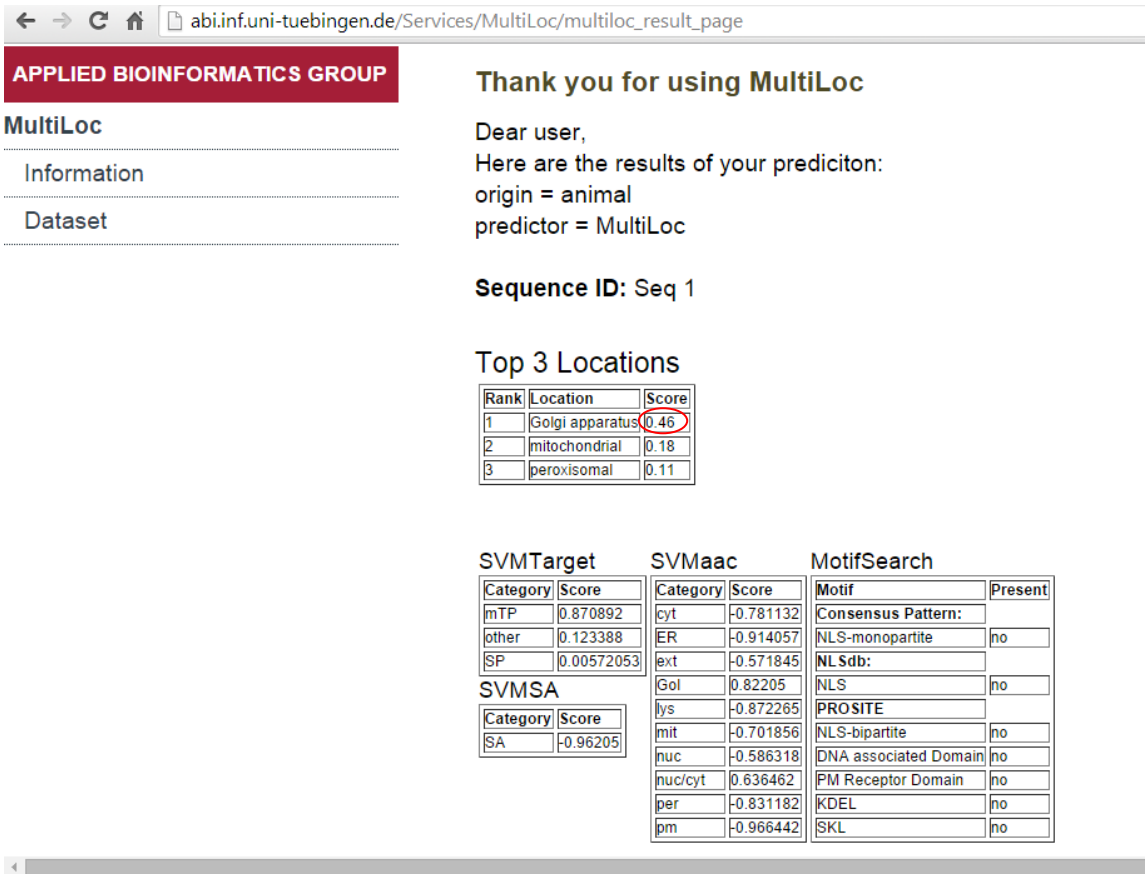


Figure A4 – Example of a standard curve for WDR45's DNA.

APPENDIX III – Prediction of WDR45's localisation



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MultiLoc

Information

Dataset

Thank you for using MultiLoc

Dear user,
Here are the results of your prediciton:
origin = animal
predictor = MultiLoc

Sequence ID: Seq 1

Top 3 Locations

Rank	Location	Score
1	Golgi apparatus	0.46
2	mitochondrial	0.18
3	peroxisomal	0.11

SVMTarget

Category	Score
mTP	0.870892
other	0.123388
SP	0.00572053

SVMSA

Category	Score
SA	-0.96205

SVMaac

Category	Score
cyt	-0.781132
ER	-0.914057
ext	-0.571845
Gol	0.82205
lys	-0.872265
mit	-0.701856
nuc	-0.586318
nuc/cyt	0.636462
per	-0.831182
pm	-0.966442

MotifSearch

Motif	Present
Consensus Pattern:	
NLS-monopartite	no
NLSdb:	
NLS	no
PROSITE	
NLS-bipartite	no
DNA associated Domain	no
PM Receptor Domain	no
KDEL	no
SKL	no

Figure A5 – Prediction of WDR45's localisation by MultiLoc program.