Investigation of the Protective Effect of Specific Inhibitors of α-Synuclein Aggregation

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Front page: Phase contrast image of OLN-AS7 cells with 90 % confluence. The color and contrast have been modified.

Preface

This report has been completed as documentation for the master thesis project by Maria Svanborg at the Section of Biotechnology (Department of Biotechnology, Chemistry and Environmental Engineering), Faculty of Engineering, Science and Medicine at Aalborg University from September 2010 to October 2011. The report titled "Investigation of the Protective Effect of Specific Inhibitors of α-Synuclein Aggregation" addresses both fellow students and supervisors.

The report is divided into chapters, sections and subsections. References are denoted using the Harvard method (author, published year). Undated references are abbreviated to Undat. References with identical author and published year are distinguished with capital letters, e.g. (author A, published year). Appendix B containing additional results can be found in the report after the references, while appendix A is enclosed on a data disc and contain the report in pdf format, raw data, Excel data sheets, laboratory protocols, product sheets, vector maps, list of chemicals, and additional plots.

I would like to thank the following people for advice and help with the project: my supervisor Associate Professor Allan Stensballe, PhD student Anders Dahl Knudsen, PhD student Mia Larsen, PhD Mette Sondrup, PhD student Anne Louise Revenfeld, laboratory technician Anne Rusborg Nygaard, laboratory technician Hanne Krone Nielsen, laboratory technician Charlotte Sten, Associate Professor Kåre Lehmann Nielsen at Aalborg University and also Postdoc Suzette Sørensen at Hematological Research Laboratory, Aalborg Hospital.

Aalborg, October 18th, 2011

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Maria Svanborg

Abstract

The neurodegenerative disease, Parkinson's disease, is characterized by a loss of dopaminergic neurons in the substantia nigra part of the mid-brain causing motor disturbances and in later stages dementia for the affected individuals. Currently, diagnosis of Parkinson's disease is based on clinical symptoms at a stage where the disease has already progressed substantially. A definite diagnosis can only be performed by conducting a post-mortem examination. α-synuclein (α-syn) aggregation is directly involved in the pathogenesis of Parkinson's disease and research has shown that the oligodendroglial protein p25α (TPPP) stimulates α-syn aggregation *in vitro*. The model rat oligodendroglial cell lines OLN-AS expressing both α-syn and tau40 and the control cell line OLN-t40 only expressing tau40 have been applied for investigating the cytotoxic effect of transient transfection with p25α.

The α-syn aggregation inhibitory effect of the compounds ASI1D, EGCG (polyphenol (-)-epigallocatechin gallate), and baicalein, respectively, has been investigated using a cell-based XTT viability assay on p25α transfected OLN-AS and OLN-t40 cell lines. No considerable differences in relative viability for both the p25α transfected cells treated with ASI1D and the EGCG, respectively, one hour prior to transfection were observed when compared to the untreated transfected cells, which indicates low transfection efficiency. However, 2 μM ASI1D showed a possible rescue of the cells, when compared to the untreated negative control. p25α transfected OLN-AS treated with baicalein one hour prior to transfection exhibited a relative viability of 45% ± 25 % indicating that this compound had a cytotoxic effect in connection with the transient transfection. It has not been possible to verify a successful transfection of OLN-AS with p25α using FuGENE® HD transfection reagent (Roche) in this thesis. The expression of p25α was investigated by Western blotting where only expression of α-syn and α-tubulin was clearly apparent. Screening of two million putative α-syn aggregation inhibitors by members of the innovation consortium CureND has given 56 CureND inhibitor compounds, which in this project have been applied for cytotoxicity tests using OLN-AS.

The cell line OLN-AS7 not expressing tau40 was received and used in further experiments. The doubling time for OLN-AS7 was determined to be 18.7 hours. To enable an MS-based mitochondrial sub-proteomic investigation (SILAC) of the protective effect of α-syn aggregation inhibitors, the development of an inducible expression system using the Complete Control Inducible Mammalian Expression System kit (Agilent Technologies) was initiated. p25α-pEGSH and p25α-GFP-pEGSH constructs were prepared applying restriction enzyme digestion and ligation, followed by culturing and cloning in *E. coli* with subsequent plasmid DNA purification. The constructs were verified by colony PCR and restriction analysis and subsequent sequencing of the construct inserts. The construct vectors were extracted using phenol and chloroform followed by precipitation using isopropanol and ethanol to enable subsequent transfection into OLN-AS7 cells for the establishment of an inducible expression system; an inducible expression of p25α in OLN-AS7 and a control cell line OLN-AS7 expressing p25α-GFP when induced. To facilitate selection of stable OLN-AS7 transformants containing p25α and p25α-GFP, respectively, both a hygromycin and geneticin cytotoxicity test was performed. The LD_{100} of hygromycin was 200 μg/mL after 11 days, while for geneticin it was 600 μg/mL after 15 days.

Resumé

Den neurodegenerative sygdom, Parkinsons sygdom, er karakteriseret ved et tab af de dopaminergiske neuroner i substantia nigra pars compacta i midthjernen, hvilket forårsager motoriske forstyrrelser og i senere stadier demens for de berørte individer. Diagnosticering af Parkinsons sygdom er i dag baseret på kliniske symptomer på et stadie, hvor sygdommen allerede er betydelig fremskreden. Kun ved en obduktion kan en endelig diagnose stilles. α-synuclein (α-syn) aggregering er direkte involveret i patogenesen for Parkinsons sygdom og forskning har vist, at det oligodendrogliale protein p25α (TPPP) stimulerer α-syn aggregering *in vitro*. De oligodendrogliale model-rottecellelinjer OLN-AS, udtrykkende hhv. både α-syn og tau40 og OLN-t40, udtrykkende tau40 er blevet anvendt i undersøgelsen af den cytotoxiske effekt af transient transfektion med p25α.

Den α-syn aggregeringsinhibitoriske effekt af hhv. ASI1D, EGCG (polyphenol (-)-epigallocatechin gallate) og baicalein er blevet undersøgt ved brug af et cellebaseret XTT viabilitetsassay på de p25α-transfekterede OLN-AS og OLN-t40 cellelinjer. Ingen væsentlige forskelle i relativ viabilitet kunne observeres for celler transfekteret med p25α og behandlet med hhv. ASI1D og EGCG en time før transfektion, når disse blev sammenlignet med transfekterede celler, der ikke blev behandlet med inhibitor. Dette indikerer at transfektionseffektiviteten var lav. Dog viste 2 μM ASI1D en mulig redning af cellerne, når denne blev sammenlignet med den negative kontrol med ubehandlede celler. OLN-AS transfekteret med p25α og behandlet med baicalein en time før transfektion udviste relativ viabilitet på 45 % ± 25 %, hvilket indikerede at dette stof havde en cytotoxisk effekt i forbindelse med den transiente transfektion. Det har i dette speciale ikke været muligt at verificere en succesfuld transfektion af OLN-AS med p25α ved brug af FuGENE® HD transfektionsreagens (Roche). Udtrykkelsen af p25α blev undersøgt vha. Western blotting, hvor kun udtrykkelsen af α-syn og α-tubulin kunne ses tydeligt. Screening af to millioner formodede α-syn aggregeringsinhibitorer af medlemmer af innovationskonsortiet CureND har givet 56 CureND inhibitorforbindelser, som i dette projekt er blevet anvendt til cytotoxicitetstests i OLN-AS.

Cellelinjen OLN-AS7, som ikke udtrykte tau40, blev modtaget og anvendt i yderligere eksperimenter. Fordoblingstiden for OLN-AS7 blev bestemt til at være 18,7 timer. For at muliggøre en MS-baseret mitokondriel subproteomisk undersøgelse (SILAC) af den beskyttende effekt af α-syn aggregeringsinhibitorer, blev udviklingen af et inducerbart ekspressionssystem ved brug af Complete Control Inducible Mammalian Expression System kit (Agilent Technologies) påbegyndt. Constructs p25α-pEGSH og p25α-GFP-pEGSH blev fremstillet ved anvendelse af fordøjelse med restriktionsenzymer og ligering, efterfulgt af dyrkning og kloning i *E. coli* og herefter plasmid DNA oprensning. Constructs blev verificeret vha. koloni-PCR og restriktionsanalyse, efterfulgt af sekventering af inserts i constructs. Construct-vektorerne blev ekstraheret ved brug af phenol og chloroform med efterfølgende udfældning ved brug af isopropanol og ethanol, for at muliggøre oprettelsen af et inducerbart ekspressionssystem med inducerbar udtrykkelse af p25α i OLN-AS7 og en kontrolcellelinje OLN-AS7 med inducerbar udtrykkelse af p25α-GFP. For at muliggøre selektion af OLN-AS7 stabiltransfekteret med hhv. p25α og p25α-GFP, blev en cytotoxicitetstest af både hygromycin og geneticin udført. LD_{100} for hygromycin var 200 μg/mL efter 11 dage, mens den for geneticin var 600 μg/mL efter 15 dage.

Contents

List of Abbreviations

AD:	transcriptional activation domain
α-syn:	α-synuclein
α-tub:	α-tubulin
ASI1D:	α-synuclein inhibitor 1D
CSF:	cerebrospinal fluid
DBD:	DNA-binding domain
DreamFect:	DreamFect™ transfection reagent
E/GRE:	modified ecdysone responsive element
EcR:	ecdysone receptor
EcRE:	ecdysone responsive element
EGCG:	polyphenol (-)-epigallocatechin gallate
FCS:	fetal calf serum
FuGENE:	FuGENE® HD transfection reagent
G418:	geneticin
GR:	glucocorticoid receptor
IES:	inducible expression system
LBs:	Lewy bodies
LDB:	dimerization and ligand-binding domain
LRRK2:	leucine-rich repeat kinase 2
MAP:	microtubule-associated protein
MAPT:	microtubule-associated protein tau
MCS:	multiple cloning site
MS:	Mass spectrometry
ORF:	open reading frame
PCR:	polymerase chain reaction
PD:	Parkinson's disease
PINK1:	PTEN-induced kinase 1
ponA:	ponasterone A
RE:	restriction enzyme
ROS:	reactive oxygen species
RXR:	retinoid x receptor
TPPP:	Tubulin Polymerization-Promoting Protein (p25α)
UCHL1:	ubiquitin C-terminal hydrolase-1
UPS:	ubiquitin proteasome system
WB:	Western blot

1. Introduction

1.1 Parkinson's Disease

Parkinson's disease (PD) is a disease characterized by degeneration of the dopaminergic neurons in a specific part of the mid-brain, substantia nigra pars compacta. This leads to several unfavorable physical symptoms, such as rigidity in the limbs, tremor in different parts of the body, bradykinesia, loss of balance and coordination control, and changes in posture. Several other severe symptoms occur in later stages of the disease. (M P Singh et al. 2006) The symptoms of PD occur when 70-80 % of the dopaminergic neurons are already lost (Srivastava et al. 2010). A European study from 1991 showed that the frequency of the disease increased with age, where it affected 0.6 % of people from 65-69 years of age, 1.0 % of people from 70-74 years of age, 2.7 % of people from 75-79 years of age, 3.6 % of people from 80-84 years of age and 3.5 % of people from 85-89 years of age (de Rijk et al. 1997). A prognosis by the United Nations Department of Economic & Social Affairs suggests that an increase of people above 60 years of age in the more developed countries will occur, causing a population increase from 245 million people in 2005 to 406 million in 2050 (United Nations 2007). This inevitable entails an increase in people with PD.
Parkinson's disease appears in two forms, an idiopathic and an inherited form, where more than 90 % of the PD patients have the idiopathic form, where the cause of the disease is unknown. Despite the fact that the term idiopathic is used for the majority of PD cases, unknown and known gene defects might have an influence together with environmental impacts. Several different factors have been linked to the development of PD but no single cause of the idiopathic form has been detected. (Greenamyre & Hastings 2004; M P Singh et al. 2006)

In the search of specific biomarkers in PD disease as a possible early diagnostic tool, both increased and decreased expression of several different proteins has been identified in the cerebrospinal fluid (CSF) or plasma of PD patients (Srivastava et al. 2010). In spite of this, these proteins cannot yet be classified as PD biomarkers because more extensive research on different populations and more stage-specific studies need to be performed. Many different factors may induce specific components in the proteomic profile of body fluids in PD patients, such as lifestyle, environmental exposure to chemicals, and ethnicity, which makes it difficult to find specific biomarkers of the disease. It is probable that the disease stage and time of collection of sample have an influence on the proteomic profile of body fluids which also has to be taken into account when searching for PD-specific biomarkers. In addition to that, possible biomarkers present at an early stage of the disease or prior to actual symptoms are yet to be discovered. Such biomarkers would aid in both early diagnosis and treatment, since no cure is currently available. (Srivastava et al. 2010)

Current diagnosis of PD is based on symptom occurrence and diminution in symptoms when treated with present PD medicine, e.g. Levodopa. To obtain a definite diagnosis, an autopsy is necessary. Treatment of PD is based on symptom alleviation, where the application of specific drugs is dependent on the stage of the disease. When no symptoms that complicate daily routines are apparent, the disease is treated to a less extent. Levodopa and dopamine agonists are currently used in treatment of motor-related symptoms, however, adverse effects have been observed in the form of motor problems caused by the drugs, which means that other drugs are taken into use to relief these adverse effects. Surgical treatment in the form of deep brain stimulation, high frequency brain stimulation, is also applied, but definite clinical symptoms and other criteria have to be apparent before this method is applied. In addition, several side effects have been observed by deep brain stimulation. (Jankovic & Aguilar 2008; Samii et al. 2004)

Human embryonic dopamine neurons have been transplanted into the brains of PD patients, where 85 % of the patients of 34-60 years of age, however only in this age group, had no reoccurring of the movement disorders, dyskinesia and dystonia, in the first year after transplantation. (Freed et al. 2001)

Studies have confirmed that environmental exposure to pesticides and other chemicals can lead to idiopathic PD and increased oxidative stress in the substantia nigra pars compacta may be involved in a cascade leading to degeneration of the dopaminergic neurons, and hereby loss of dopamine (Greenamyre & Hastings 2004; Peter Jenner 2003). In addition to that, the cytoplasmic inclusion bodies, Lewy bodies (LBs), and abnormal filament-containing neurites, Lewy neurites, are present in the substantia nigra and many other brain regions of PD patients, which seems to be a critical feature of the disease. It has been found that insoluble aggregates of α-synuclein (α-syn) are main constituents of LBs and Lewy neurites in idiopathic PD and dementia with LBs. (Baba et al. 1998; Krüger et al. 1998; Spillantini et al. 1998)

Human α-syn is a protein, which consists of 140 amino acids and is expressed in presynaptic nerve terminals and is involved in neural plasticity (George et al. 1995; M Goedert 1997). An investigation of knockout mice not expressing α-syn indicates that the protein has an inhibitory role as a negative regulator of dopamine transmission. The α-syn knockout mice were viable and fertile and did not show critical abnormalities. (Abeliovich et al. 2000) The primary structure of α-syn can be divided into three main parts, the N-terminal region, the central region, and the C-terminal region. The C-terminal region, also called the NAC region (non-Aβ component), differs in sequence and length dependent on species, whereas the N-terminal and central regions are conserved to a great extent. It has been discovered that the acidic C-terminal domain is linked to chaperone activity of α-syn (S. M. Park et al. 2002). The primary structure of α-syn is characterized by a repetitive sequence motif of six amino acids, KTKEGV, which seems to have an important role in the binding activity of α-syn. (Amer et al. 2006; George et al. 1995; P H Jensen et al. 1998)
Proteins with a natively unfolded structure are often involved in protein-protein interactions, and the natively unfolded structure of α-syn makes it likely to share this property (Weinreb et al. 1996). In addition, aggregation of α-syn seems to remove the chaperone activity of the protein, which also indicates that the natively unfolded structure of α-syn could be important for protein-protein binding (T. D. Kim et al. 2000). Studies have shown that in connection with PD, α-syn displays a conformational change into a partially folded state, which leads to the formation of non-fibrillar oligomeric aggregates (protofibrils), see Figure 1. These oligomeric aggregates, which mainly have anti-parallel β-sheet structure, are intermediates in the fibrillation process in PD. The formation of the α-syn aggregates is nucleation-dependent, which means that the growth of the fibrils is dependent on the presence of these oligomeric aggregates or nuclei. Once these nuclei are formed, the fibrils grow rapidly. (S.-J. Lee 2003; Volles et al. 2001; S. J. Wood et al. 1999)

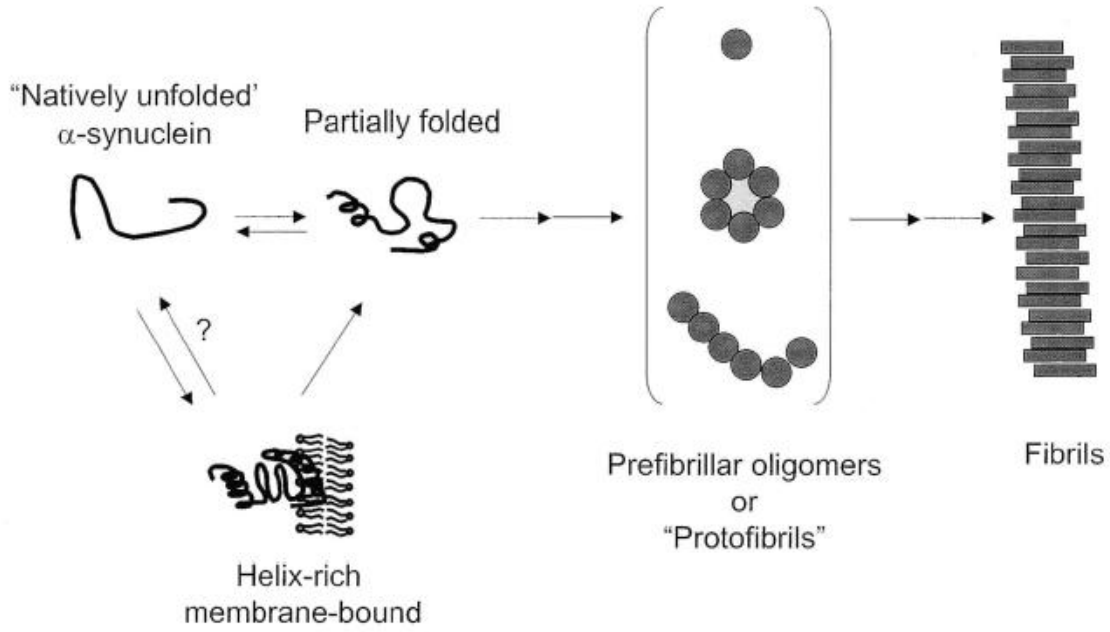

Figure 1: The formation of the natively unfolded α-syn into fibrils (S.-J. Lee 2003).

It is speculated whether it primarily is these oligomeric aggregates that are cytotoxic towards dopaminergic neurons as opposed to the actual fibrils. The monomeric α-syn does not display any toxic properties in studied cell cultures and animals. (S.-J. Lee 2003)
The amino acid substitutions Ala53Thr, Ala30Pro, and Glu46Lys in the gene encoding α-syn have been proven to induce an inherited form of PD (O. M. El-Agnaf & G B Irvine 2000; Zarranz et al. 2004). The mutations Ala53Thr and Ala30Pro in α-syn lead to an accelerated formation of oligomeric aggregates, compared to wild-type α-syn (Conway et al. 2000). New studies have revealed that phosphorylation of Ser129 in α-syn renders the cytotoxic effect of α-syn (Kragh et al. 2009).

The protein p25α (TPPP, Tubulin Polymerization-Promoting Protein) consists of 219 amino acids and is mainly expressed in the specific glial cell type oligodendrocytes. Glial cells are the major cell constituent of the nervous system. Studies indicate that the oligodendrocytes are involved in regulation of the microenvironment around neurons. p25α is involved in abnormal assembly of microtubules and a recent study has shown that co-expression of p25α and α-syn in the oligodendroglial cell line, OLN-93, leads to microtubule retraction followed by cellular degeneration and apoptosis (Kragh et al. 2009). p25α has been located in neuronal Lewy neurites, Lewy bodies, and glial cytoplasmic inclusions together with α-syn in synucleinopathies, including PD. It seems that p25α preferentially binds the aggregated form of α-syn, but it can also bind the monomeric form of α-syn. Furthermore, the binding of p25α to α-syn seems to be pro-aggregatory, leading to a nucleation-competent oligomer. (Baumann & Pham-Dinh 2001; Desai & Mitchison 1997; Hlavanda et al. 2002; Lindersson et al. 2005; D. E. Otzen et al. 2005)

The tau protein is primarily found in neurons and is a microtubule-associated protein (MAP) (Buée et al. 2000). Tau apparently acts as a ligand to α-syn, and α-syn is contributory in phosphorylation of tau (P H Jensen et al. 1999). An *in vitro* study showed that incubation of α-syn together with tau40 induced fibrillation of both proteins, where tau40 did not fibrillate in the absence of α-syn, which indicates that α-syn promotes fibrillation of tau40, and thus may be involved in the pathology in PD (Giasson et al. 2003)

Furthermore, the genes encoding DJ-1, PINK1, parkin, and ubiquitin C-terminal hydrolase-1 (UCHL1) have been linked to PD disease. The lack of expression of DJ1 or mutations in the gene encoding DJ1 causing a loss of function leads to neurodegeneration, and it is suspected that the protein is involved in oxidative stress response (Bonifati et al. 2003).
The protein PTEN-induced kinase 1 (PINK1) is located in mitochondria and mutations in the gene encoding the protein have been found in PD patients. In connection with cellular stress, PINK1 presumably has a role of phosphorylating mitochondrial proteins to prevent mitochondrial dysfunction, and mutations in PINK1 with functional effects may lead to mitochondrial dysfunction. (S Gandhi et al. 2006; Valente et al. 2004)
Parkin is a part of the ubiquitin proteasome system (UPS) and acts as an E3 ubiquitin-ligating enzyme. The UPS is responsible for non-lysosomal degradation of misfolded proteins or proteins with other abnormalities. Inactivation of parkin will lead to dysfunction in the mitochondria, causing the development of early onset autosomal recessive PD. (Greenamyre & Hastings 2004; Kitada et al. 1998; McNaught et al. 2001; Shimura et al. 2000)
Ubiquitin C-terminal hydrolase-1 is also a part of the UPS, where it disassembles polyubiquitin into monomeric ubiquitin. The mutation Ile93Met in the gene encoding this protein has been identified as a probable cause of PD, however, the research involving this mutation is contradictory. (Leroy et al. 1998; Y. Liu et al. 2002; McNaught et al. 2001)

An overview of known proteins, in which genetic mutations are contributing to the development of PD, can be seen in Table 1.

Table 1: An overview of characteristics of proteins, which cause the development of PD, when one or more mutations are present in the gene encoding the specific protein. AR = autosomal recessive. AD = autosomal dominant. Modified from Knudsen (2007). (M. Farrer et al. 2001; Sonia Gandhi & Nicholas W Wood 2005; Rachakonda et al. 2004; Schapira 2008)

Gene Product	Locus	Chromosome Location	Inheritance	Age of Onset	Lewy Bodies
α-synuclein	PARK1	4q21-q23	AD	40s	Yes
parkin	PARK2	6q25.2-27	AR	20s+	No*
unknown	PARK3	2p13	AD	60s	Yes
α-synuclein triplication	PARK4	4p14-p16.3	AD	40s	Yes
UCHL1	PARK5	4p14	AD	50s	-
PINK1	PARK6	1q35-36	AR	30s	-
DJ1	PARK7	1p36	AR	30s	-
Leucine-rich repeat kinase 2 (LRRK2)	PARK8	12p11.2-q13	AD	Late	No
ATPase type 13A2	PARK9	1p36	AR	Juvenile	-
Unknown	PARK10	1p32	-	Late	-
Unknown	PARK11	2q36-q37	-	-	-
NR4A2	-	2q22-23	AD	Late	-

* One patient with Lewy bodies has been reported (M. Farrer et al. 2001).

The above-mentioned proteins with genetic mutations are all suspected to be involved in mitochondrial activity and it has been discovered that the activity of the mitochondrial complex I is decreased in PD patients. The mitochondrial complex I is a part of the mitochondrial electron transfer chain, which is crucial for the production of ATP in the cell. (Sherer et al. 2002)
Drug users, which unintentionally consumed the drug MPP^+, showed symptoms of PD and this lead to further investigation of MPP^+. It was discovered that addition of the drug MPP^+ to rat hepatocytes (liver cells) lead to a loss of ATP in the cells followed by cell death. MPP^+ is presumably transferred to the mitochondria, where it inhibits NADH dehydrogenase in the electron transfer chain, prompting a decrease of ATP in the cell, leading to cell death. These findings indicated that MPP^+ induces the symptoms of PD and that mito-

chondria are involved in the pathogenesis of PD (Di Monte et al. 1986; E. Hasegawa et al. 1990; Langston et al. 1983).
Studies have suggested that reactive oxygen species (ROS) give rise to oxidative damage in PD and it is assumed that an increase in the production of these ROS is due to dysfunction of the mitochondria or dopamine metabolism. It is assumed that improper handling of dopamine vesicles causes increased levels of dopamine in the cytoplasm and thus formation of ROS, leading to oxidative stress. (Dauer & Przedborski 2003; Greenamyre & Hastings 2004; E. Hasegawa et al. 1990; Lotharius & Brundin 2002)

An overview of many of the different pathways in the pathology of PD which have been suggested can be seen in Figure 2. Mutations in the genes encoding α-syn, parkin (PARK2), DJ1, UCHL1, PINK1, and LRRK2 are all suspected to be important factors in PD, together with reactive oxygen species, accumulation of α-syn aggregates and Lewy body formation, fibrillation of the tau protein, dysfunction of the UPS, and mitochondrial dysfunction. (M. J. Farrer 2006)

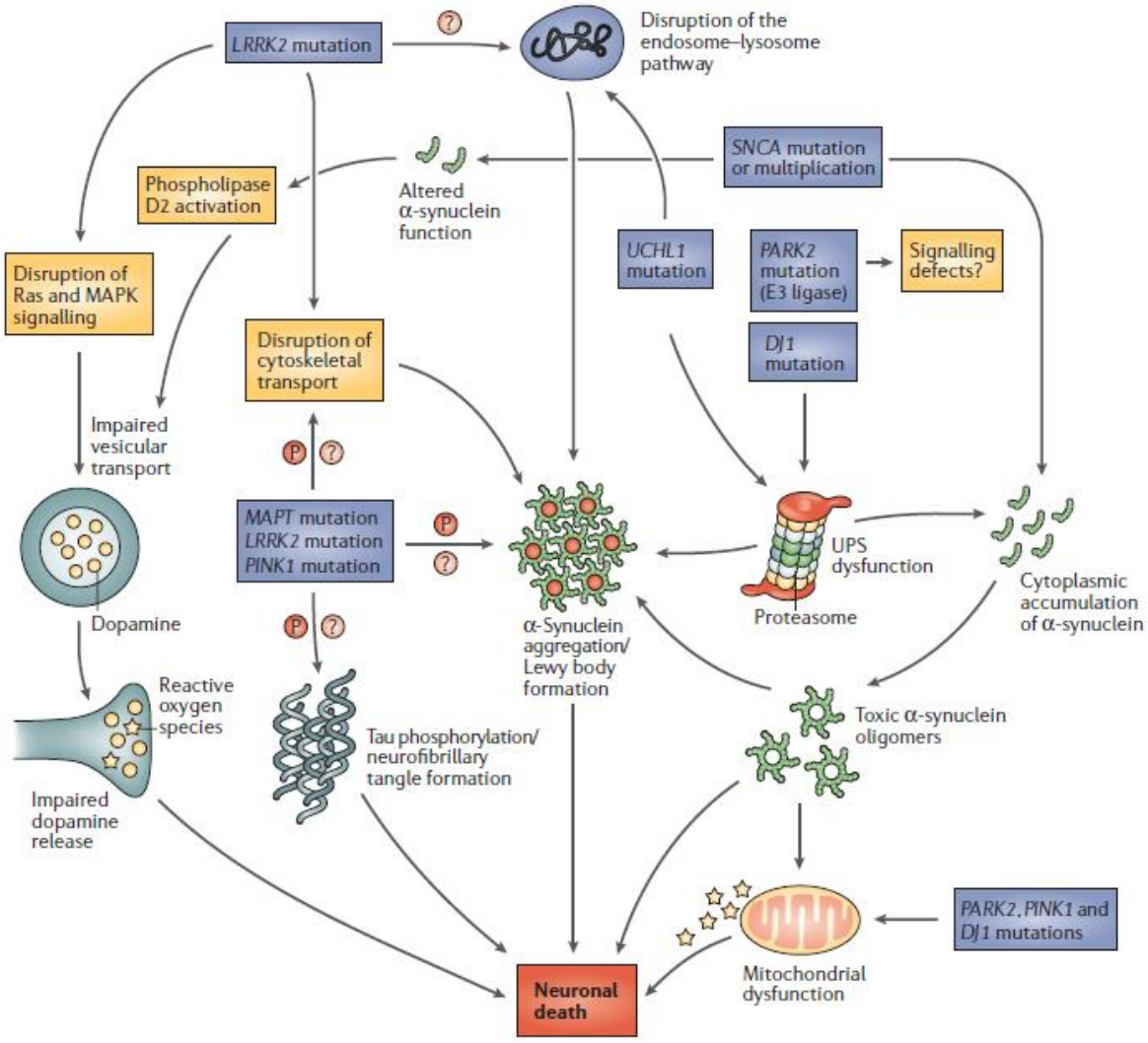

Figure 2: Suggested pathological pathways of neurodegeneration in Parkinson's disease. In this model, M. J. Farrer has suggested the linkage between the different causes of PD. Mutations in the genes encoding α-syn, parkin (PARK2), DJ1, UCHL1, PINK1, and LRRK2 are all suspected to be important factors in PD, together with the development of reactive oxygen species, accumulation of α-syn aggregate and Lewy body formation, fibrillation of the tau protein, dysfunction of the UPS, and mitochondrial dysfunction. Mutations in α-syn can lead to the formation of α-syn oligomers which display cytotoxic properties. It is assumed that the cell will react by degrading the monomeric α-syn or an increased formation of the fibrillar α-syn will be apparent. Degraded monomeric α-syn may be degraded via the UPS or the endosome-lysosome pathway. As described earlier, parkin and UCHL1 are involved in the UPS and mutations in the genes encoding these proteins may therefore be contributory in the further formation of α-syn oligomers. The microtubule-associated protein tau (MAPT) also seems to be involved in the pathogenesis with its interaction with α-syn. PINK1 is implicated as mutations in the gene encoding this protein may lead to mitochondrial dysfunction. The role of DJ1 and LRRK2 is not fully understood. An altered form of α-syn is likely to be involved in impairment of dopamine handling, leading to an increased formation of ROS, which causes degeneration of the dopaminergic neurons. (M. J. Farrer 2006)

As a strategy towards developing novel treatment strategies for PD, inhibitors of α-syn aggregation are one of the main focus areas. By inhibiting or blocking the α-syn aggregation, it may be possible to put the disease in arrest before it shows any symptoms or at least before symptoms worsen.

2. Strategy

This master thesis has two main objectives:

1) **To further develop and apply a cell-based assay enabling screening of known compounds (EGCG, baicalein and ASI1D) and 56 non-peptide inhibitor lead compounds for cytotoxicity and efficacy as α-synuclein aggregation inhibitors.**

2) **To further develop a model cell line, OLN-AS7, to contain inducible expression of p25α for the purpose of future mitochondrial quantitative sub-proteomic studies of the α-synuclein aggregation inhibitor effect in the cell model system.**

The already developed experimental setup, an XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) cell-based assay, acquired from PhD students Henrik Kjeldal and Anders Dahl Knudsen at AAU will be applied to test the effect of EGCG, baicalein, and ASI1D as α-syn aggregation inhibitors in the cell line OLN-AS transiently transfected with p25α. Furthermore, the expression of α-syn and p25α in the OLN-AS cell line will be investigated by Western blotting. The cytotoxic effect of 56 non-peptide inhibitor lead compounds, which inhibit α-syn aggregation *in vitro*, cf. the innovation consortium CureND (in collaboration with Wyeth (Pfizer)), will be examined using the abovementioned cell-based XTT assay for the purpose of selecting the leads suitable for further investigation. The selected lead inhibitor compounds will eventually be applied for testing the α-syn aggregation inhibition in cell culture.

The development of a novel model cell line stably expressing p25α, when induced, and α-syn will be initiated for the purpose of future examination of the protective effect of different α-syn aggregation inhibitors in the cell line by conducting mitochondrial sub-proteomic studies using SILAC labeling. This mitochondrial sub-proteomic investigation will be carried out to display the mitochondrial protein expression pattern in the model cell line, when introducing an α-syn aggregation inhibitor and when not, and also to possibly verify a linkage between mitochondrial dysfunction and α-syn aggregation.
Previous studies using transient transfection of OLN-AS with p25α demonstrate low transfection efficiency, thus few cells are targeted for rescue by an inhibitor compound (Knudsen, A. D., unpublished data). Consequently, it is difficult to use the XTT assay to measure a difference between transfected cells that are rescued and the transfected cells that are not rescued, because the majority of the cell population will be comprised of non-transfected cells. In addition, the cells are markedly stressed by the transient transfection.

The advantage of developing a model cell line with stable expression of both α-syn and p25α by induction is that the following experiments will be reproducible, all cells will be affected equally by an inhibitor and the cells will not be potentially stressed by a transient transfection of p25α prior to or after the addition of an inhibitor compound, which allows proteomic experiments.
Additionally, a control cell line stably expressing a p25α-GFP fusion protein when induced together with α-syn will be established. The expression and possible leakage expression can hereby be tested by fluorescence microscopy. The protein expression can also be tested using Western blotting or Northern blotting.

In connection with the establishment of the inducible expression system, the transfection efficiency of three different transfection methods will be evaluated by image analysis of both fluorescence microscopy and phase contrast images, where the most efficient method will be applied in the development of the novel cell lines. See Figure 3 for an outline of the aims of the project.

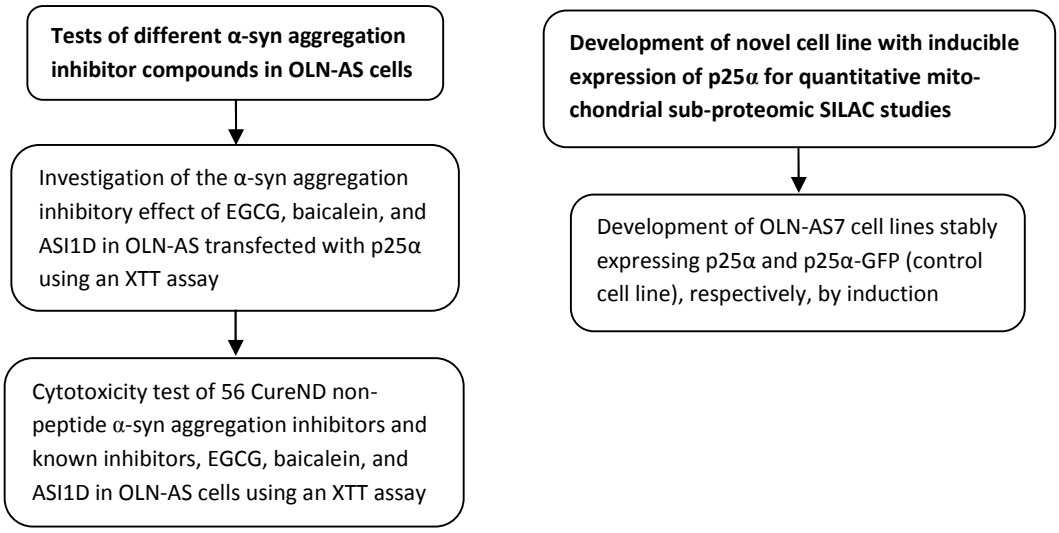

Figure 3: Outline of the aims of the project.

In the following, the applied model cell lines will be described together with the known α-syn aggregation inhibitors, which will be applied in this thesis. Subsequently, the experimental setup will be described in detail.

2.1 OLN Model Cell System

Cellular models are advantageous for investigating PD since they are easy to maintain and more accessible than blood, CSF, and brain of actual PD patients. Only a limited quantity of tissue from PD patients is available for examination and the brain tissue is very heterogeneously organized, making it difficult to fractionate the specific cells of interest. Tissue handling and preparation are also crucial steps for obtaining interpretable data, leaving cell models an advantageous choice in studying PD. However, model cell lines may not show the exact expression pattern as actual cells of the human brain, since the different cells may affect each other at different levels and at different stages of the disease. Conversely with cell models, it is possible to investigate one feature of the disease at a time, obtaining simpler data with less unknown factors involved. Additionally, proteins not normally present in the cell can be expressed. (Srivastava et al. 2010)

In the study of Parkinson's disease, different cell models have been applied. SH-SY5Y and M17-A53T are human neuroblastoma cell lines, which have been applied for testing the cytotoxicity of the α-syn aggregation inhibitor ASI1D. M17-A53T has been created by stable transformation of the BE(2)-M17cell line to overexpress the mutant α-syn Ala53Thr (Biedler et al. 1978; O. M. A. El-Agnaf et al. 2004). The human embryonic kidney cells, HEK-293, have been transfected to overexpress α-syn in order to investigate the effect of the α-syn aggregation inhibitor EGCG in a cell model system (Bieschke et al. 2010). Another cell line utilized in the study of Parkinson's disease, the PC12 cell line, originates from rat adrenal pheochromocytoma cells (tumor cells from a specific part of the adrenal glands), which is suitable for neurobiological studies (L. A. Greene & Tischler 1976), and has been applied by stably transfecting the cell line with mutant α-syn (Kostka et al. 2008). The model cell line OLN-93 was developed due to the lack of oligodendroglial cell line model systems and thus to enable studies of differentiation and proliferation in oligodendroglial cells. The OLN-93 cell line was established from primary rat brain glial cultures and is characterized by a doubling time of 16-18 hours. The cell line is stable when cultured over a period of approximately two years and displays a stable phenotype up to 40 passages. (C Richter-Landsberg & Heinrich 1996)

Subsequently, the OLN-tau40 (OLN-t40) cell line has been developed from the OLN-93 cell line and applied in the investigation of the formation of tau protein aggregates, the characteristic of tauopathies and Alzheimer's disease, in oligodendroglial cells. Tauopathies are neurodegenerative diseases, which are characterized

by the development of abnormal tau protein aggregates. For synucleinopathies, it is the protein α-synuclein which is present abnormally aggregated in the cell. Tauopathies and synucleinopathies may display similar pathology. Consequently, as PD is a synucleinopathy, research with the application of the OLN-t40 cell line may also be relevant for PD research. In order to establish the OLN-t40 cell line, the OLN-93 cell line was co-transfected with a pcDNA3 vector containing a neomycin resistance gene and cDNA encoding the human tau40 protein. (Galpern & Lang 2006; Goldbaum et al. 2003; V. M.-Y. Lee et al. 2004)
This was followed by the development of the OLN-AS cell line to enable an investigation of the role of α-syn aggregates in connection with oxidative stress in synucleinopathies, including PD. (Riedel et al. 2007) OLN-t40 was stably transfected with the gene encoding the human α-syn by lentiviral transduction, as it was not possible to create a stable OLN-93 cell line solely expressing α-syn (Riedel et al. 2009). Eventually, the OLN-AS7 cell line was developed by stable transformation of OLN-93 with the pcDNA3.1/Zeo(-) vector containing the gene encoding the human α-syn and did not express tau40 (P.H. Jensen, pers. commun.). The expression of both α-syn and p25α in an oligodendroglial cell is not normal in human, cf. section 1.1 Parkinson's Disease, but was originally developed to mimic the pathological pathway of the synucleinopathy, multiple system atrophy (Kragh et al. 2009). However, it is assessed that this model is also suitable in the examination of the role of α-syn in PD.

An experimental setup involving a transient transfection of OLN-AS with a plasmid vector containing the gene encoding p25α has been developed, where microtubule retraction is induced, leading to apoptosis and cell death 72 hours after transfection. The principle of the setup is to add an inhibitor one hour prior to transfection to potentially inhibit the toxic effects of α-syn/p25α induced cell death (Kragh et al. 2009; Knudsen, A. D., unpublished data). The experimental setup can be viewed in Figure 4.

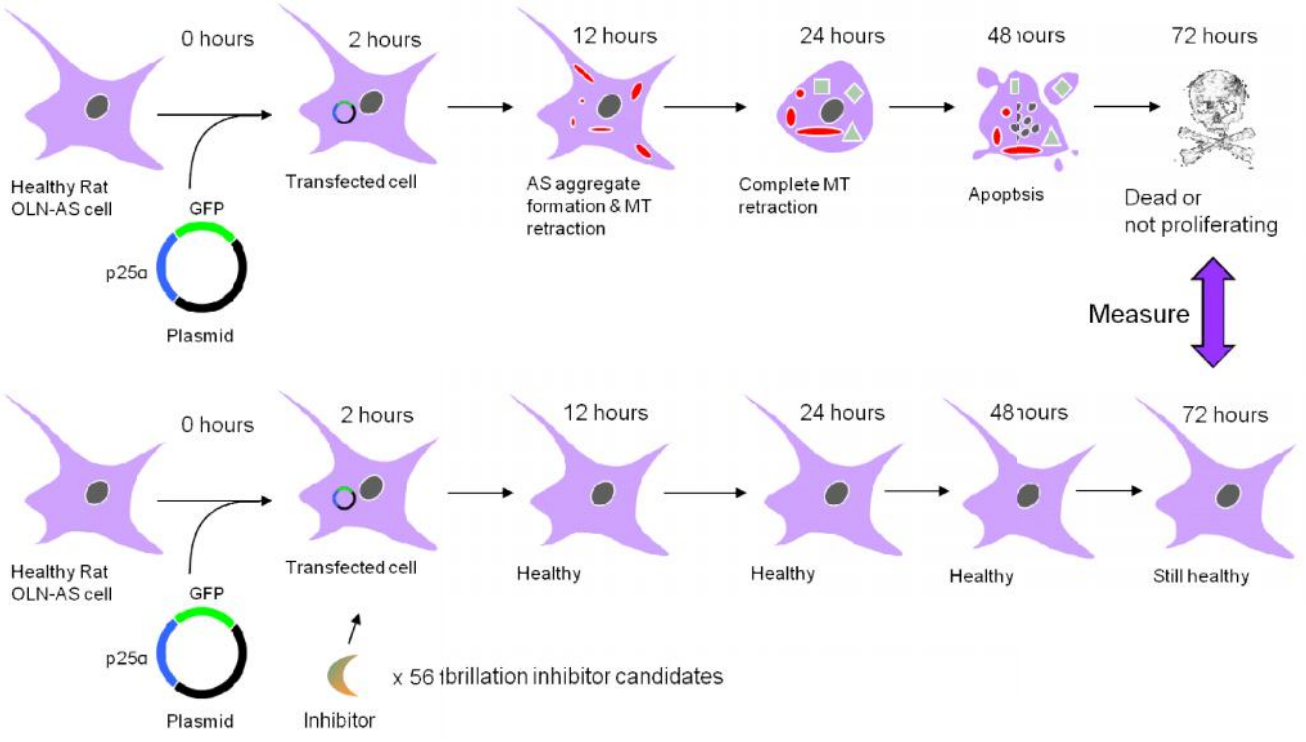

Figure 4: The experimental setup involving transient transfection of OLN-AS with p25α, in this example p25α-GFP. The upper panel shows the pathway of an OLN-AS cell expressing p25α. The cell is initially transfected with the plasmid vector. After 12 hours the α-syn aggregates have formed and microtubule retraction starts. After 24 hours, complete microtubule retraction has occurred and within 48 hours, apoptosis takes place. The cells will be dead or not proliferating after 72 hours. The lower panel suggests a pathway rescuing the OLN-AS cell by adding an α-syn aggregation inhibitor. The cell is transfected with the plasmid vector. After two hours, the inhibitor is added. The cells will be viable, still healthy and proliferating after 72 hours. Modified from Knudsen, A. D. (unpublished data) and Kragh et al. 2009.

2.2 α-Synuclein Aggregation Inhibitors

The described pathogenesis of PD involving α-syn aggregation, cf. chapter 1. Introduction, and the knowledge of α-syn oligomer formation facilitates an investigation of possible α-syn aggregation/oligomer inhibitors. The requirements of an α-syn aggregation inhibitor comprise that it needs to be non-cytotoxic, cell-permeable, and effective at low dosage (Knudsen, A.D., unpublished data).

Several possible inhibitor candidates have been developed in the form of synthetic peptides. Their binding ability towards the NAC region (residue 69-72) of α-syn has been tested and one of those was further tested in cell-based experiments. The ASI1 peptide (RGGAVVTGR-NH_2) binds to residue 68-72 of the NAC region of α-syn and is an inhibitor of α-syn aggregation *in vitro*. An altered version of ASI1, ASI1D (RGGAVVTGRRRRRR-NH_2), contains six arginine residues in the C-terminal, enabling the inhibitor to enter the cell membrane. The ASI1D inhibitor was tested on the cell lines M17-A53T and SHSY-5Y, where it showed no cytotoxicity in the concentration range 0.01-10 μM within 24 hours. Additionally, pretreatment of cell line OLN-AS with 10 μM ASI1D prior to transfection with p25α indicated a rescue of the cells. (O. M. A. El-Agnaf et al. 2004; Kragh et al. 2009)

The compound EGCG (polyphenol (-)-epigallocatechin gallate) is an antioxidant of green tea that have been demonstrated to inhibit α-syn aggregation *in vitro.* EGCG does not bind to a specific sequence of the target protein. Several studies have proven that EGCG inhibits α-syn aggregation *in vitro* and has a higher binding-affinity towards unfolded proteins, such as α-syn, compared to folded proteins. Furthermore, a cell-based study revealed that 20 μM EGCG inhibits the formation of α-syn aggregates in HEK-293 cells and convert these α-syn aggregates into smaller aggregates that are non-cytotoxic. (Bieschke et al. 2010; Ehrnhoefer et al. 2006; Ehrnhoefer et al. 2008; Masuda et al. 2006)

Baicalein is a component of the specific Chinese herbal medicine, *Scutellaria baicalensis*. It has been proven to have several positive benefits, as both anticarcinogenic and anti-HIV features have been reported. Baicalein reportedly inhibits α-syn aggregation and the binding of baicalein to α-syn seems to involve one or more Tyr residues in α-syn, and furthermore cause a structural change (Zhu et al. 2004). An *in vitro* study of several different α-syn aggregation inhibitor compounds, including baicalein, display an IC_{50} <10 μM (Masuda et al. 2006), which may be suitable for further cell-based research. A cell culture based study with PC12 cells stably expressing Glu46Lys α-syn by induction showed that treatment with 10 μM baicalein for six days reversed the toxicity of the α-syn aggregation (Kostka et al. 2008), which strongly indicated a potential in baicalein as a future clinical drug for treatment of PD. In addition to that, a recent study with OLN-AS cells pretreated with 100 μM baicalein followed by transfection with p25α reduced MT retraction and thereby indicated that cells were rescued (Kragh et al. 2009). However, a novel investigation shows that the addition of 25-100 μM baicalein to OLN-AS cells four hours after transfection with p25α does not save the cells (Knudsen, A. D., unpublished data).

Consequently, it is in interest to further investigate the α-syn aggregation inhibitory effect of EGCG, ASI1D and, baicalein in a cell model system. In the following sections, the different strategy methods will be described in detail.

2.3 Experimental Approach

2.3.1 Transfection of OLN Cells

To transiently express p25α and p25α-GFP in the rat OLN-AS cell line, an efficient transfection setup is to be established. The plasmid vectors p25α-pcDNA3.1/Zeo(-) and p25α-pAcGFP1-N1 acquired from PhD student Anders Dahl Knudsen will be applied for the transient expression of p25α and p25α-GFP. p25α was

inserted into pcDNA3.1/Zeo(-) using the restriction sites *Bam*HI and *Xba*I, while p25α was inserted into pAcGFP1-N1 using the restriction sites *Eco*RI and *Bam*HI. Further information concerning the plasmid vectors can be found in appendix A.6.
Different ratios of FuGENE HD® Transfection Reagent:plasmid DNA will be used to test which is the most effective in this system. These ratios will be evaluated by Western blotting with cell lysate, using antibodies against α-synuclein, p25α, and α-tubulin (loading control). The ratio with the highest expression of p25α will be further applied in the XTT assay experiments. Furthermore, a comparative transfection efficiency analysis of the three different transfection methods, FuGENE HD® Transfection Reagent (Roche), DreamFect™ Transfection Reagent (OZ Biosciences) and the Calcium Phosphate Transfection Method (Invitrogen) will be performed on the OLN-AS7 cell line to evaluate the most effective method for creating the novel cell line stably expressing p25α by induction. The ratio of FuGENE HD® Transfection Reagent:plasmid DNA from the prior experiment will be applied in this experiment, while one ratio of DreamFect™ Transfection Reagent:plasmid DNA will be applied, according to prior experiments with the OLN-93 cell line (Knudsen, A.D., unpublished data). See section 2.3.4 Examination of Transfection Efficiency for elaboration of the transfection efficiency experiment.

FuGENE HD® Transfection Reagent (Roche) (FuGENE) is a lipid-based transfection reagent containing 80 % ethanol which forms a complex with DNA after which this complex is transported into the cell. This transfection reagent can be applied with serum present in the media, but addition of antibiotics and fungicides in the transfection media is not recommended. (Roche 2007)
The DreamFect™ Transfection Reagent (OZ Biosciences) is a lipopolyamine-based transfection reagent, containing both a lipophilic and a polyamine part. It forms a positive charged complex with DNA and is transported into the cell by endocytosis, causes destabilization of the endosomal membrane and is released into the cytosol, where some of the DNA is transported to the nucleus. (OZ Biosciences, Undat)
The calcium phosphate transfection method is based on co-precipitation of DNA with calcium phosphate, where this complex adsorbs to the cell membrane. This is calcium-dependent. The cells are assumed to take up the DNA by endocytosis in the media at 37 °C. (Graham & van der Eb 1973)

2.3.2 XTT Cell Viability Assay

In the XTT cell viability assay, metabolic active cells react with the yellow tetrazolium salt, XTT (sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate), which is water soluble, and the orange formazan dye is formed. The mitochondrial dehydrogenase in the cells will cleave XTT and the activity of the cells can be measured by a shift in absorbance. Hereby, the viability of the cells can be quantified using an ELISA plate reader. The electron coupling reagent PMS (phenazine methosulfate) is added together with XTT to enhance the bioreduction of XTT. The absorbance maximum of formazan is between 440-490 nm. This assay is suitable for examining the cytotoxicity and the growth inhibitory effect of a compound. (Roche 2005; Roehm et al. 1991; Scudiero et al. 1988)

This assay will be utilized to examine the cytotoxicity of the 56 α-syn aggregation inhibitor compounds in the OLN-AS cell line and both the cytotoxicity and the effect of the known inhibitors, EGCG, baicalein, and ASI1D, on the viability of p25α transfected OLN-AS cells and untransfected cells. The experimental setup implies the addition of inhibitor compound to the OLN-AS cells one hour prior to transfection with p25α, followed by incubation for 72 hours before addition of XTT/PMS and subsequent measuring of the absorbance of the produced orange formazan. The absorbance measurement will be performed 72 hours post-transfection, as p25α transfected cells not rescued by addition of inhibitor should have undergone apoptosis followed by cell death after this period of time, cf. Figure 4. Furthermore, the difference between untransfected cells and transfected cells will be more apparent after 72 hours, compared to a shorter period of time,

as a higher absorbance due to higher cell viability in untransfected and inhibitor-rescued cells should be evident compared to non-rescued transfected cells. This should entail higher reproducibility and lower error margins of the experiment.

2.3.3 Inducible Expression System

The inducible expression system will be developed by applying the Complete Control Inducible Mammalian Expression System kit (Stratagene, Agilent Technologies). The concept of this inducible expression system (IES) kit is to transfect the OLN-AS7 cell line with a receptor expressing vector, pERV3, followed by transfection with the construct vector pEGSH containing the gene of interest. When the two vectors have been stably introduced into the cell line, the expression of the insert gene can be induced by adding ponasterone A (ponA). The system will be elaborated in the following sections.

The pERV3 vector contains a modified version of the ecdysone receptor (EcR), which is normally only found in insect cells, where it is formed as a heterodimeric transcription factor bound to the promoter. The heterodimer consists of the ecdysone receptor encoded by *ecr* and the ultraspiracle encoded by *usp*. In insect cells the receptor binds co-repressors, where transcription is activated in the presence of the hormone ecdysone or an analog to this hormone. Ecdysone binds to a specific domain of the EcR, which releases the co-repressors and co-activators enable transcription. The EcR consists of three domains, an N-terminal transcriptional activation domain (AD), a DNA-binding domain (DBD) and a dimerization and ligand-binding domain in the C-terminal (LBD). When the EcR is expressed in mammalian cells, it forms a heterodimer with the mammalian homolog of ultraspiracle, the retinoid X receptor (RXR), which is present in the pERV3 vector. The EcR in the IES has been modified so that it does not activate host genes or is involved in endogenous pathways of the host cells. The DNA-binding specificity of EcR DBD has been altered, mimicking that of the glucocorticoid receptor (GR) and the EcR AD has been replaced by the VP16 AD of the herpes simplex virus. The LBD domain is that of the wild-type *D. melanogaster* ecdysone receptor. The modified EcR is denoted VgEcR. In the mammalian cell, the VgEcR will bind to the RXR, forming a heterodimer, as mentioned. This complex will bind to five copies of a modified version of the wild-type ecdysone responsive element (EcRE) on the pEGSH vector, denoted E/GRE. See Figure 5. (Agilent 2008; Primrose & Twyman 2006)

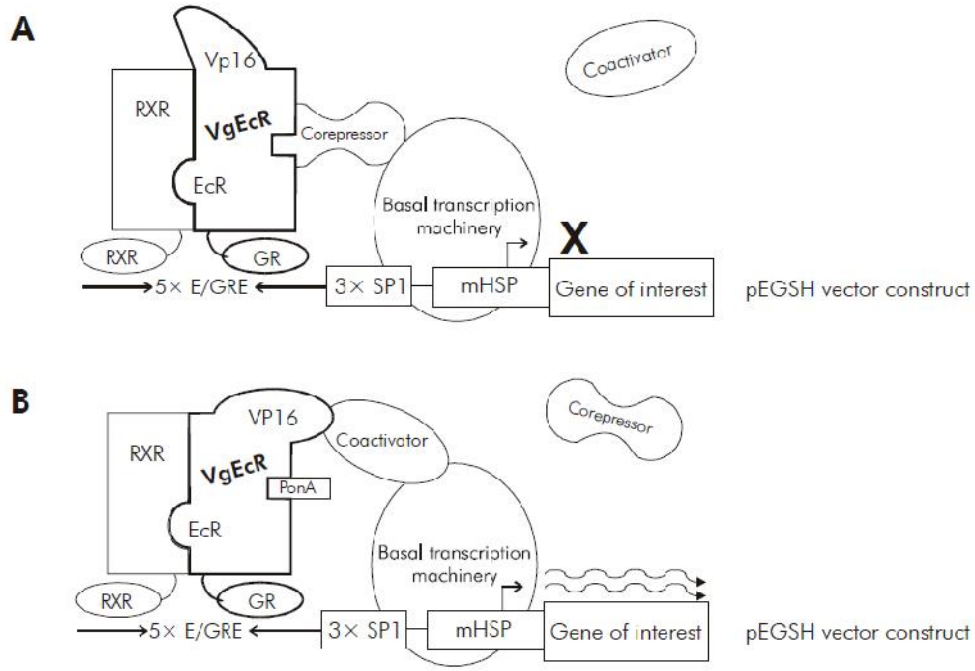

Figure 5: Transcriptional regulation in the inducible expression system. A. The modified ecdysone receptor, VgEcR, consisting of the VP16 AD domain, the EcR LBD domain and the GR DBD domain binds to the RXR. This complex recognizes five copies of the E/GRE sequence in the pEGSH vector. Co-repressors suppress transcription until ponasterone A is present. B. Ponasterone A binds to the VgEcR and co-repressors are released and co-activators bind to VgEcR enabling transcription. (Agilent 2008)

The vector map of the pERV3 vector can be viewed in Figure 6. Inserts containing the sequences encoding the VgEcR and RXR can be found in the pERV3 vector. These genes are transcribed from the CMV promoter. A ribosomal entry site is placed between the open reading frames (ORF) of VgEcR and RXR to enable internal translation of the RXR. The SV40 promoter allows transcription of a gene encoding neomycin phosphotransferase (*neo*) to select stable transformants in mammalian cells using geneticin (G418). If the vector is cloned in *E. coli*, the transformants can be selected using kanamycin, which the β-lactamase (*bla*) promoter facilitates. (Agilent 2008; Primrose & Twyman 2006)

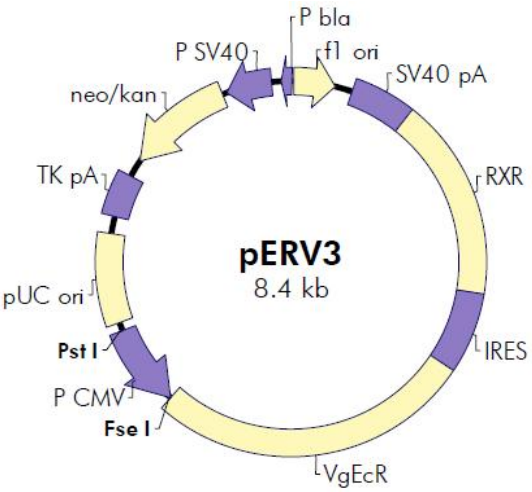

Figure 6: The pERV3 vector expressing the VgEcR and RXR proteins. Stable transformants in *E. coli* can be selected using kanamycin and in mammalian cells by using geneticin (G418). (Agilent 2008)

The pEGSH vector can be viewed in Figure 7. This vector contains a multiple cloning site where a gene of interest can be placed. Additional information about the restriction sites in the multiple cloning site (MCS) of

pEGSH can be found in the Complete Control Inducible Mammalian Expression System manual (Agilent 2008) in appendix A.4. The five copies of the E/GRE enable the induction of transcription by ponA, when the VgEcR-RXR complex binds to this sequence. Downstream of the E/GRE, a minimal promoter consisting of three SP1 sites is present. The pEGSH vector contains a HSV-thymidine kinase (TK) promoter for transcription of the hygromycin gene, which allows selection of stable transformants in mammalian cells. If the vector is cloned in *E. coli*, the transformants can be selected using ampicillin. The vector contains two primer binding sites and matching primers, which are supplied in the IES kit for e.g. PCR. (Agilent 2008)
The expressed VgEcR and RXR should not be involved in endogenous pathways, since they contain genetically modified domains; however, expression leakage could be controlled or examined by Western blotting or Northern blotting. (No et al. 1996)

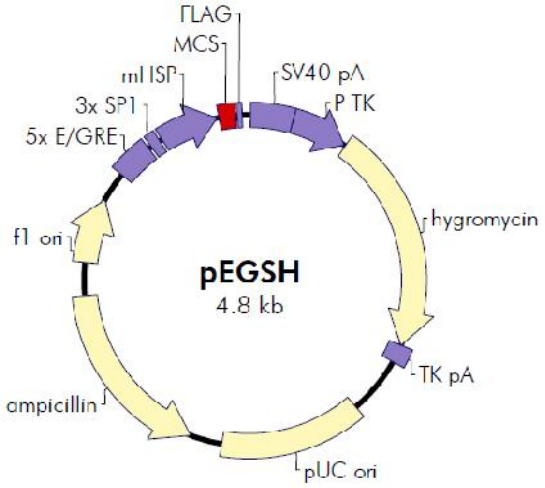

Figure 7: The pEGSH vector expressing the gene of interest. The gene of interest can be placed in the MCS. The hygromycin gene enables selection of stable transformants in mammalian cells, while the ampicillin gene enables selection of stable transformants in *E. coli*. The E/GRE allows the binding of the VgEcR-RXR complex, followed by expression of the gene of interest if ponA is present. (Agilent 2008)

Application of the Inducible Expression System

In the following, the different steps necessary for developing the inducible expression system (IES) are lined up.

1) Performance of a restriction site analysis to determine which restriction enzymes that are appropriate to obtain the p25α and the p25α-GFP inserts from p25α-pAcGFP1-N1 to enable insertion into the pEGSH vector. The p25α gene was inserted into the multiple cloning site of pAcGFP1-N1 using the restriction sites *Eco*RI and *Bam*HI. The vector map and sequence of pAcGFP1-N1 can be found in appendix A.6 on the enclosed data disc.
2) Restriction enzyme (RE) digestion of the p25α-pAcGFP1-N1vector to obtain the p25α and the p25α-GFP inserts, respectively, and RE digestion of the pEGSH vector to obtain compatible overhangs with the inserts.
3) Agarose gel electrophoresis of the RE digested p25α-pAcGFP1-N1 and pEGSH vectors.
4) Extraction and purification of the p25α and the p25α-GFP insert digests and the pEGSH construct digests from the agarose gel pieces.

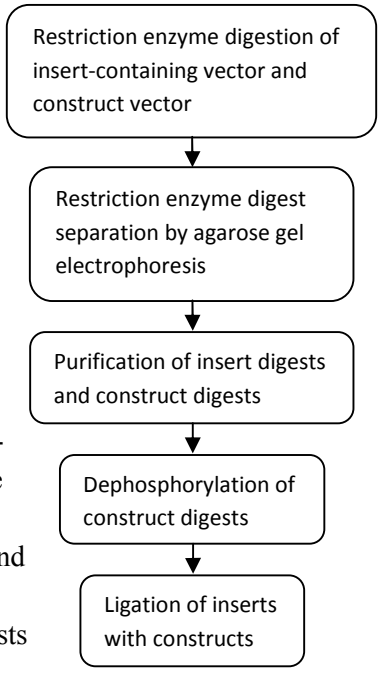

Figure 8: Flowchart of the first step in the IES. Preparation of the construct vectors.

5) Dephosphorylation of pEGSH construct digests to avoid self-ligation.
6) Ligation of p25α and the p25α-GFP insert digests with the pEGSH construct digests, respectively.
   See Figure 8.
7) Transformation of competent *E. coli* with two different ligation mixes containing either p25α-pEGSH or p25α-GFP-pEGSH, respectively, using heat shock transformation or electroporation.
8) Plating the transformed *E. coli* on ampicillin-containing LB agar plates.
9) Selecting ampicillin-resistant colonies for colony PCR. Primers from the IES kit will be applied for PCR.
10) Agarose gel electrophoresis of PCR products to verify that the selected colonies display PCR pro-ducts of the expected size.
11) Culturing selected colonies for cloning of the construct vectors p25α-pEGSH and p25α-GFP-pEGSH.
12) Plasmid DNA purification of the construct vectors.
13) Restriction analysis of the purified construct vectors to verify the expected sequence length of the digested constructs.
14) Sequencing of the construct vector inserts to verify the DNA sequence of the inserts.
    See Figure 9.
15) To avoid contamination of the constructs with endotoxins and other unwanted components, the construct vectors are extracted using phenol-chloroform and precipitated using isopropanol and ethanol.
16) Determination of an optimal transfection method for stable transfection of the OLN-AS7 cell line with the pERV3 and p25α-pEGSH/p25α-GFP-pEGSH vectors. The p25α-pAcGFP1-N1 vector will be applied in the testing of three different transfection methods and the expression of GFP will be examined using fluorescence microscopy, cf. section 2.3.4 Examination of Transfection Efficiency.

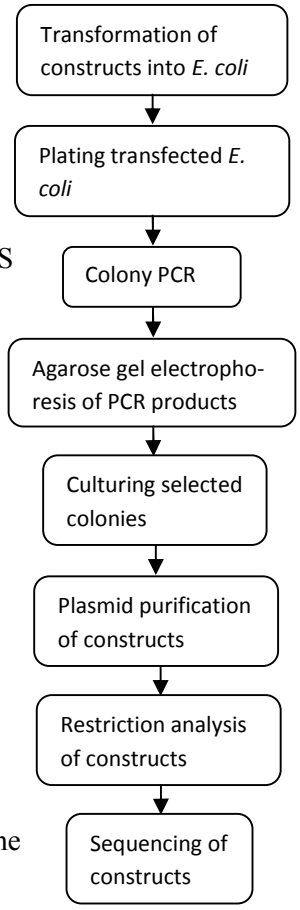

Figure 9: Flowchart of the second step in the IES. Cloning, purification, and verification of the constructs.

17) The lethal dosage of geneticin and hygromycin will examined to obtain the antibiotic concentration necessary to select stable OLN-AS7 transformants.
    See Figure 10.
18) Stable transformation of the OLN-AS7 cell line with the pERV3 vector and isolation of geneticin-resistant clones.
19) Examination of VgEcR and RXR expression in the pERV3 transfected OLN-AS7 cells by transfection with the pEGSH-Luc vector followed by induction of luciferase transcription by addition of ponasterone A (ponA). The OLN-AS7 cells containing both the pERV3 and the pEGSH-Luc vector will display expression of luciferase when induced with ponA, which can be monitored using a luciferase assay kit.
20) The OLN-AS7 cells stably expressing the pERV3 vector is then stably transformed with the p25α-pEGSH vector and p25α-GFP-pEGSH vector for the control cell line, respectively.
21) The OLN-AS7 cell line stably expressing both the pERV3 vector and the p25α-pEGSH, and the OLN-AS7 cell line stably expressing both

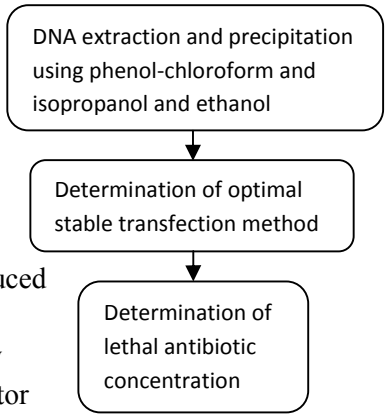

Figure 10: Flowchart showing the third step in the IES.

the pERV3 vector and the p25α-GFP-pEGSH vector are cultured with geneticin and hygromycin until stable transformants have been obtained.

22) Inducible expression of p25α and p25α-GFP, respectively, by addition of ponA is examined by performing Western blotting or Northern blotting on cell lysate of induced and uninduced OLN-AS7-p25α and OLN-AS7-p25α-GFP cells.
23) Inducible expression of p25α-GFP in the OLN-AS7-p25α-GFP cells will be studied by applying fluorescence microscopy to examine expression leakage of uninduced cells.
See Figure 11.

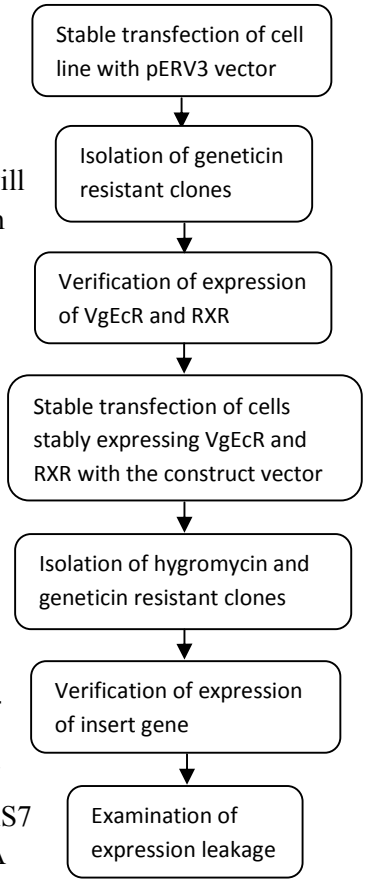

Figure 11: Flowchart of the fourth step in developing the IES.

(Agilent 2008)

2.3.4 Examination of Transfection Efficiency

The transfection efficiency by transiently transfecting OLN-AS7 with p25α-pAcGFP1-N1 plasmid vector using FuGENE® HD transfection reagent (Roche), DreamFect™ transfection reagent (OZ Biosciences), and the calcium phosphate transfection method (Invitrogen) will be investigated to determine the optimal transfection method and the appropriate ratio of transfection reagent:DNA for stable transformation of OLN-AS7 with the receptor expressing vector, pERV3, and the created constructs, p25α-pEGSH and p25α-GFP-pEGSH, respectively. The transfection method with the most advantageous transfection efficiency will be used for the development of an OLN-AS7 cell line stably expressing p25α when induced with ponasterone A. An OLN-AS7 control cell line stably expressing p25α-GFP when induced with ponasterone A will also be developed.
Cell suspension of transiently transfected OLN-AS7 cell will be examined by taking phase contrast images and fluorescence images using fluorescence microscopy. The images can be overlaid and a cell counting script received from and previously applied by Anders Dahl Knudsen (unpublished data) can be used in the program ImageJ to calculate the transfection efficiency of the different transfection methods.

3. Materials and Methods

Detailed protocols can be found in appendix A.4 Laboratory Protocols. The list of chemicals can be found in appendix A.8.

3.1 Cell Culturing

OLN-AS, OLN-t40, and OLN-AS7 were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose, UltraGlutamine (or L-glutamine) and sodium pyruvate (BioWhittaker®, Lonza or Gibco®, Invitrogen). Additionally, the medium contained 10 % (v/v) fetal calf serum (FCS) (PAA Laboratories), 50 µg/mL gentamicin (Invitrogen), 100 µg/mL streptomycin (Invitrogen), and 100 U/mL penicillin (Invitrogen). The different components were either sterile filtered and added to the DMEM or the final mixture was sterile filtered. For filtration, a 0.22 µM PEG membrane was used. The medium was stored for maximum one month at 4°C. The cells were grown in a humidified CO_2 heating incubator set to 5.0 % CO_2, 37 °C. The cell medium was changed every 3-5 days. The OLN-AS and OLN-t40 cell cultures contained 50 µg/mL geneticin (G418) to maintain selective pressure. The OLN-AS7 cell culture contained 100 µg/mL zeocin to maintain selective pressure.

3.1.1 Subculturing

All media from the flask was removed and discarded. The adherent cells were carefully washed with sterile room temperature 1xPBS twice (10xPBS was diluted with cell culture grade water and autoclaved). An appropriate volume of 1:1 mixture of 0.02 % EDTA and 0.25 % trypsin in 1xPBS was added to the flask with the adherent cells. The flask was tilted until the cells detached from the bottom. A volume of new medium was quickly added to the flask to stop the trypsin activity. The final volume of FCS in the added media was always above 2 % to assure trypsin inactivity. The cell clumps were dissolved by pipetting the cell medium using a stripette. A volume of cell suspension was mixed with 0.4 % trypan blue (Gibco, Invitrogen) 1:1 and inverted to assure even suspension. A volume of this was transferred to a hemocytometer and the viable cells were counted. The cells were subcultured at 80-90 % confluence. A volume of cell suspension containing a number of cells with minimum 1:40 confluence, e.g. 250,000 cells to a T75 flask (maximum 10 million cells) was added to a new flask with the appropriate volume of medium and selective antibiotic.

3.1.2 Freezing Procedure

A volume of cell culture was spun down at 4°C at 600 x g for 5 minutes. The supernatant was discarded. A volume of freezing media (media containing 30 % FCS and 5% DMSO) was added to the cell pellet. The cells were kept on ice at all times after adding the freezing media, since DMSO will stress and kill the cells if stored at temperatures above 5 °C. The mixture was aliquoted into cryo tubes and placed in a polystyrene box or in a mammalian cell freezing box at -80 °C for 24 hours. The freezing box assured that the cells were not frozen too fast, since that will kill the cells. Within a week, the cryo tubes were removed from the freezing box and placed in a -135 °C freezer for long-term storage.

3.1.3 Thawing Procedure

The cells were thawed by placing the cryo tubes in a polystyrene box with ice or for a couple of minutes at room temperature. The cell suspension was transferred to a centrifuge tube containing new media. The cell suspension was centrifuged for 5 minutes at 600 x g. The supernatant was carefully discarded and the cells were resuspended in new media. A volume of cell suspension was transferred to an eppendorf tube for cell counting. The remaining cell suspension was transferred to a new culture flask, selection antibiotic was added and the flask was placed in the incubator (37 °C, 5.0 % CO_2). The cells were counted according to proto-

col and the percentage of dead cells were counted (should not exceed 10-20 %). The media was replaced by new media after approximately 24 hours to bring down the concentration of DMSO.

3.1.4 Cell Doubling Rate Determination

The cell doubling rate was determined for the OLN-AS7 cell line. 100,000 cells were seeded in each well of two 6 well culture plates and in 4x T25 flasks (quadruple determination). Two wells in each plate were trypsinized every 24 hours and the cells were counted. The T25 flasks were trypsinized and counted on day 4. The cells were incubated for 0-4 days. The media contained 100 µg/mL zeocin. The well or T25 flask was washed twice with a volume of 1xPBS. The well or T25 flask was trypsinized in an appropriate volume of 0.01 % EDTA/0.125 % trypsin in 1xPBS. The trypsin in the well or T25 flask was further suspended in media and the cell suspension was transferred to eppendorf tubes. The cells were counted in a hemocytometer and the number of cells pr. mL in each well was calculated.
The cell doubling time was calculated by applying the equation:

$$t_2 = \frac{\ln(2)}{k}$$

where t_2 is the doubling time and k is a constant. The constant k can be acquired by performing linear regression of a plot displaying ln to the number of cells over time. This equation is derived from

$$N_t = N_0 \cdot e^{k \cdot t}$$

This describes the number of cells over time in an exponential growth phase. N_t is the number of cells as a function of time, N_0 is the number of cells at time 0, t is time and k is the above-mentioned constant. (Kielberg et al. 2001)

3.2 SDS-PAGE and Western Blotting

3.2.1 Transient Transfection of OLN-AS Cells and Cell Lysing with RIPA Buffer

FuGENE® HD transfection reagent (FuGENE) was applied in this experiment. A volume of cell suspension containing 500,000 OLN-AS cells was transferred to each well of a 6 well culture plate, except in the first experiment where a cell suspension volume containing 200,000 OLN-AS cells was applied. Different ratios of FuGENE transfection reagent:plasmid DNA was applied. In the first experiment, ratios of 3:1, 4:1, 5:1, 6:1 and 7:1 were applied. In the second and third experiment, ratios of 4:1, 5:1, 6:1 and 7:1 were applied. In the fourth experiment, a ratio of 5:1 was applied. In addition to that, eppendorf tubes were utilized instead of glass vials in the first experiment.
According to protocol, a volume of FuGENE was transferred to a volume of transfection media (DMEM with 4.5 g/L glucose, L-glutamine, and 10% FCS) in a glass vial. A volume of plasmid DNA was added to another glass vial containing a volume of transfection media. It was important to pipette both the FuGENE transfection reagent and the plasmid DNA in the middle of the transfection media, since the reagent or plasmid solution may stick to the surface of the vial. The plasmid/media mixture was transferred to the FuGENE/media mixture and pipetted up and down once, before leaving the glass vials for 15 minutes at room temperature. The transfection complex was then added to the wells containing cell suspension and the plate was incubated at 37°C, 5.0 % CO_2 for 24 hours.
The plate was placed on ice and the media was removed from the wells. The wells were carefully washed with 4 °C 1xPBS twice. A volume of 4 °C lysis buffer (RIPA) was added to each well and the cell lysate was collected after 5-10 minutes in eppendorf tubes on ice. A cell scraper was used to remove the cells from the

bottom of the plate. The samples were used for SDS-PAGE followed by Western Blotting. Until then, the samples were stored at -20 °C.

3.2.2 SDS-PAGE and Western Blotting of OLN-AS Cell Lysate

The glass plates were assembled and the 15 % resolving gels were prepared in a centrifuge tube containing MilliQ H_2O, 30 % acrylamide/bisacrylamide mix, 1.5 M Tris•Cl pH 8.8, a volume of 10% SDS and a volume of 10 % ammonium persulfate (APS) and TEMED. APS and TEMED were added just prior to casting of the gel. The solution was poured into the gap between the glass plates with a Pasteur pipette, while making space for the stacking gel. Water was poured over the resolving gel, and the gel was left to polymerize for 30 minutes. The overlay was poured off and the top of the gel was washed with milliQ water. The water was drained off the gel using a paper towel. The 5 % stacking gel was prepared in a centrifuge tube containing milliQ H_2O, 30 % acrylamide/bisacrylamide mix, 1.5 M Tris•Cl pH 6.8, a volume of 10% SDS, a volume of 10 % APS, and TEMED. See appendix A.4 for detailed information. A comb was placed between the gap of the glass plates and the stacking gel was poured onto the resolving gel. The stacking gel was left to polymerize for 30 minutes. The comb was removed and the gel was washed in milliQ water. If not used right away, the gel was wrapped in a wet paper towel and foil and saved at 4 °C. The gel was mounted in the electrophoresis apparatus and 1x running buffer (0.1 % SDS, 0.025 M Tris base, 0.192 M glycine in milliQ H_2O) was transferred to the vessel until the electrode was covered and the interior vessel was topped with running buffer. The cell lysate samples were spun down at 600x g for 5 minutes and the supernatant was removed. 2x SDS reducing sample buffer (0.2 M Tris, 2 % SDS, 0.2 % bromophenol blue, 20 % glycerol, 40 mM DTT), pH 6.8, was prepared. SDS reducing sample buffer was added to the sample and heated immediately for 3 minutes at 95 °C. The samples were spun down for 20 seconds before loading them into the gel. SM0671 marker (Fermentas) was applied. The experiments were carried out at 70 V for 10 min. followed by 150 V for 1 hr, except in the first experiment, where 70 V for 10 min, 130 V for 15 min., 70 V for 10 min. followed by 130 V for 1 hr was applied. When the electrophoresis had finished, the gel was washed in milliQ water for 10 minutes and used for Western Blotting.

For Western blotting, the primary antibodies anti-α-syn (Aarhus University, Department of Biochemistry) and anti-p25α-2 (Aarhus University, Department of Biochemistry) were applied with the dilutions 1:700 and 1:500, respectively, to detect the expression of the proteins α-syn, p25α and p25α-GFP the in OLN-AS cell line. The primary antibody anti-TPPP 166-180 (Sigma-Aldrich) was later applied with the dilution 1:700 to detect p25α in the OLN-AS cell line. The primary antibody anti-α-tubulin (Aarhus University, Department of Biochemistry) was used as a loading control to detect α-tubulin. A 1:3000 dilution of Alexa fluor 647 (Invitrogen) was applied as the secondary antibody for all experiments. The antibodies were in a binding buffer with 1 % skimmed milk powder.
The newly run 15% gel containing pre-stained protein ladder SM0671, two pieces of filter paper and two pads were equilibrated in transfer buffer (192 mM glycine, 25 mM Tris-base in milliQ water) for 20 minutes. The PVDF membrane was activated in MeOH for minimum10 minutes. The transfer sandwich was assembled (black side down: pad, paper, gel, membrane, paper, pad). Contact was assured by rolling first membrane, then paper with a 15 mL tube and air bubbles were removed. The tank was filled with cold transfer buffer, cooling element and magnet stir bar (max rpm) and transfer was carried out at 350 mA (~150V) for 3 hours at 4°C. The black side of the sandwich was placed towards the black side of the apparatus. The sandwich was disassembled, the protein side of the membrane was marked (corner was cut) and the membrane was blocked in a plastic sleeve for ~30 minutes on a rocking table. The gel was stained with coomassie brilliant blue (control). The membrane was cut in two halves. Blocking buffer (1 % (v/v) skim milk powder, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % (v/v) Tween 20 in milliQ water) was removed and primary

antibody was added and the membrane was stored overnight at 4°C on a rocking table followed by three times washing (washing buffer: 0.1 % (v/v) Triton X-100, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05 % (v/v) Tween 20 in milliQ water) (15, 30, 30 minutes). The plastic sleeve was covered with tin foil and secondary antibody was added and stored for 2 hours at 4°C on a rocking table followed by three times washing (15, 30, 30 minutes). The membranes were scanned in a Typhoon 8600 scanner (Molecular Dynamics) using the setting Cy5 670 BP30, red laser (633 nm), PMT 400-800.

3.3 XTT Cell Viability Assay

For the XTT assays, DMEM without phenol red was utilized.

3.3.1 Examination of Sensitivity of XTT Assay

The experiment was carried out using a 96 well culture plate. OLN-AS cells were applied. A volume of 1x PBS was added to all of the outer wells to avoid evaporation. An increasing number of cells was added to 10x 6 wells (not using the outer wells) of the 96 well culture plate. 2000 cells in the first 6 wells and increasing with 2000 cells pr. 6 wells, until 20,000 cells pr. 6 wells were obtained. The volume of cell suspension added to each well was calculated from counting the cells. A volume of media was transferred to each of the wells containing cells. The plate was incubated at 37°C, 5.0 % CO_2 for 24 hours. The media was carefully removed. If colored media was used, this was removed from the plates. The cells were washed two times with 1x PBS. A volume of colorless media containing 0.208 mg/mL XTT and 1.6 µg/mL PMS was transferred to a centrifuge tube and incubated at 37 °C until the solution was transparent (for approximately 10 minutes). A volume of this activated XTT solution was added to each well. 1x PBS was removed from the top 6 wells and the bottom 6 wells. An equal volume of colorless media without XTT solution was applied in each of the top 6 wells, while an equal volume of activated XTT solution was applied to each of the bottom 6 wells. The plate was gently swirled and incubated at 37°C, 5.0% CO_2 for 2 hours. The absorbance of the formed, orange formazan dye was measured at 450, 490 and 690 nm.

3.3.2 Examination of Cytotoxicity of 56 Inhibitor Compounds on OLN-AS Cells

The experiment was carried out with triplicate determination. Four replicates were performed, where PhD student Anders Dahl Knudsen performed the first two. EGCG was only included in the last replicate. ASI1D and baicalein were only included in the last two replicate experiments. The inhibitors were dissolved in DMSO for preparation of stocks.

All media from the culture flask was removed and the adherent cells were washed twice with sterile 1xPBS. The cells were trypsinized with an appropriate volume of 0.01 % EDTA/0.125% trypsin in 1xPBS and an appropriate volume of media, depending on the number of cells present, was added to the flask. The cells were counted according to protocol. A volume of 1xPBS was transferred to each of all the outer wells of four 96 well plates to avoid evaporation. For the two first replicates, the cell suspension was diluted and a volume of cell suspension containing 3000 cells was transferred to each well. For the two last replicates, the cell suspension was diluted and a volume of cell suspension containing 1500 cells was transferred to each well. The plates from the two last replicates were incubated at 37 °C and 5.0 % CO_2 for 24 hours, the plates from the two first replicates were not incubated for 24 hours.

A volume of media was added to an eppendorf and a volume of inhibitor stock leading to a 1000x dilution of inhibitor in the well was added to the eppendorf. This was carried out for all 56 inhibitor leads (10 mM stock), ASI1D (10 mM stock) and baicalein (100 mM stock). A 2000x dilution of EGCG (27 mM stock) was applied. This was mixed until the inhibitor was dissolved. A volume of the mixture was transferred to three wells for every inhibitor (triplicate determination) with the wells having a final concentration of 10 µM ASI1D, 100 µM baicalein, 13.5 µM EGCG, and 10 µM of each of the 56 inhibitor compounds, respectively. An equal volume of media was transferred to 6-12 wells pr. plate as a control. An equal volume of media

with a final 1000x dilution of DMSO in each well was added to 6-12 wells pr. plate as a control. The plates were placed in a humified incubator at 37 °C and 5.0 % CO_2 for 72 hours.
The media was carefully removed from the wells. The cells were washed by adding a volume of 1xPBS in each well and then gently swirled. All of the 1xPBS was carefully removed from every well. A volume of XTT (50 mg/mL stock), a volume of PMS (0.383 mg/mL stock), and a volume of colorless media was added to a Greiner tube with a final XTT concentration of 0.33 mg/mL and a final PMS concentration of 2.55 µg/mL. This was incubated at 37 °C until the solution was transparent. Some of the XTT/PMS/media solution was poured into a pipetting tub and a volume of this was transferred to each well using a multi channel pipette. The plates were incubated at 37 °C for 2 hours. The absorbance of the formed, orange formazan dye was measured at 450, 490 and 690 nm.

3.3.3 El-Agnaf Inhibitor Assay with Transfection Testing Viability of OLN-AS Cells

The experiment was carried out with six replicates of each sample. All media from the culture flask was removed and the adherent cells were washed twice with sterile 1xPBS. The cells were trypsinized with an appropriate volume of 0.01 % EDTA/0.125% trypsin in 1xPBS and an appropriate volume of media, depending on the number of cells present, was added to the flask. The cells were counted according to protocol. A volume of 1xPBS was transferred to each of all the outer wells of two 96 well culture plates to avoid evaporation. Only the 60 inner wells per plate were used for samples and controls. The cell suspension was diluted so that each well would have a final number of 4000 cells.
A volume of cell suspension was transferred to eppendorf tubes and a volume of the inhibitor stocks was transferred to each of the eppendorf tubes leading to a final concentration of 13.5 µM EGCG, 27 µM EGCG, 10 µM ASI1D, and 100 µM baicalein, respectively. Control samples were eppendorfs with cell suspension and an appropriate volume of DMSO, since the inhibitors were dissolved in DMSO. The eppendorfs were placed in a humified CO_2 incubator for 1 hr at 37 °C with 5.0 % CO_2. The tubes were inverted regularly to avoid cell adherence.
The controls containing FuGENE® HD transfection reagent (FuGENE), FuGENE+p25α-pcDNA3.1/Zeo(-) plasmid vector and transfection media (media without antibiotics) were prepared for the cell suspension samples: One glass vial containing transfection media and FuGENE reagent for two controls (FuGENE and FuGENE+DMSO); one glass vial containing transfection media and FuGENE reagent (for mixture with DNA) for six controls; one glass vial with transfection media and p25α-pcDNA3.1/Zeo(-) plasmid vector. The mixture in the vial containing plasmid was transferred to the vial containing FuGENE mixture for the six controls and was left to incubate for 15 minutes at room temperature. The final ratio of FuGENE:plasmid DNA was 5:1. The transfection media and the transfection media containing either FuGENE or FuGENE+p25α-pcDNA3.1/Zeo(-) was transferred to the eppendorfs with cell suspension and to those with cell suspension+inhibitor/DMSO. Equal volumes from the eppendorf tubes were transferred to the wells in the plates. The plates were incubated at 37 °C and 5.0% CO_2 for 72 hours.
The cell morphology was observed in the microscope. The colored media was carefully removed from the wells using a multi channel pipette and the cells were washed by adding a volume of 1xPBS in each well. The plates were hereafter gently swirled. The 1xPBS was completely removed. A volume of XTT stock (50 mg/mL) and PMS stock (0.383 mg/mL), respectively, was mixed with a volume of colorless media in a Greiner tube with the ratio 1:300 of each reagent, corresponding to the volume needed for the wells. This was incubated at 37 °C until the solution was transparent. Some of the XTT/PMS/media solution was poured into a pipetting tub and an appropriate volume was transferred to each well using a multi channel pipette. The plates were incubated at 37 °C for 2 hours and the absorbance was measured at 450, 490 and 690 nm.

3.3.4 ASI1D Inhibitor Assay with Transfection Testing Viability of OLN-AS and OLN-t40 Cells

The experimental setup was identical to the El-Agnaf experiment, cf. section 3.3.3 El-Agnaf Inhibitor Assay with Transfection Testing Viability of OLN-AS Cells, except ASI1D was the only inhibitor applied and the cell line OLN-t40 was also included in this experiment. 2 μM, 5 μM, 10 μM, and 15μM ASI1D was tested.

3.4 Inducible Mammalian Expression System

The Complete Control Inducible Mammalian Expression System kit (Agilent Technologies, Stratagene) was applied. The One Shot Top10 chemically competent and electro competent *E. coli* were used instead of the XL1-blue strain in the kit.

3.4.1 Cloning of the pEGSH Expression Vector

The vector, pEGSH, in which the p25α and p25α-GFP inserts, respectively, were to be placed was cloned and purified to obtain more material to enable further experiments. The pEGSH vector was transformed into One Shot Top10 chemically competent *E. coli* (Invitrogen) by heat shock transformation. The DNA was purified from the *E. coli* culture using the Plasmid DNA Purification protocol (Nucleospin® Plasmid, Macherey Nagel).

Preparation of LB Agar Plates and LB Medium

The LB agar plates were prepared by mixing sodium chloride (0.01 % w/v), tryptone (0.01 % w/v), yeast extract (0.005 % w/v), and agar (0.02 % w/v) in milliQ water. The pH was adjusted to 7.0 with 1 M sodium hydroxide and the LB agar was autoclaved. This was cooled to 55 °C and sterile filtered ampicillin was added to a final concentration of 100 μg/mL. The LB agar was poured into petri dishes.
The LB medium was prepared by mixing sodium chloride (0.01 % w/v), tryptone (0.01 % w/v), and yeast extract (0.005 % w/v) in milliQ water. The pH was adjusted to 7.0 with 1 M sodium hydroxide and the LB medium was autoclaved.

Heat Shock Transformation

A water bath was equilibrated to 42 °C. A vial of SOC medium was heated to room temperature. LB agar plates containing 100 μg/mL ampicillin was placed in a 37°C incubator to remove excess moisture. The vials containing plasmids were centrifuged briefly and placed on ice. Two vials of One Shot® Top10 chemically competent cells (Invitrogen) were thawed on ice. An amount of 10 pg pUC19 control plasmid was transferred to one vial competent cells and 25 ng pEGSH vector was transferred to the other vial with competent cells. The vials were tapped gently to mix the cells with the DNA. The cells were incubated on ice for 30 minutes. Subsequently, the vials were incubated for exactly 30 seconds in the 42 °C water bath without mixing or shaking. The vials were removed from the water bath and placed on ice. A volume of SOC medium was transferred to each vial and the cell suspension was gently transferred to a 12 mL sterile culture tube. The tubes were placed in a rack with tape and incubated in a rotary shaker incubator at 37 °C for 1 hour at 170 rpm. A specific volume of LB medium was mixed with four different volumes of cell suspension in four eppendorfs. This was carried out for each transformation mixture. The cell suspensions were spread using glass marbles on labeled LB agar plates with 100 μg/mL ampicillin. The plates were inverted and incubated at 37 °C overnight. Ten separate colonies from the pEGSH transformation were transferred to 12 mL sterile tubes with LB medium containing 100 μg/mL ampicillin. The tubes were incubated overnight at 37 °C at 200 rpm in a rotary shaker incubator. The cell suspensions were used for plasmid DNA purification.

Plasmid DNA Purification
The plasmid DNA purification was conducted according to the manual, Nucleospin® Plasmid (Macherey Nagel), high-copy plasmid. The washing step with Buffer AW preheated to 50 °C, followed by centrifugation for 1 minute at 11,000 x g was included.

Increasing the DNA Concentration
1 µL 20 g/L glycogen was added to 42 µL DNA sample (p25α-pEGSH and p25α-GFP-pEGSH constructs), obtained from the plasmid DNA purification. 4 µL 7.5 M NH_4Ac was added to the mixture and finally 100 µL 99.9 % EtOH was transferred to the mixture. This was spun down with maximum speed for 10 minutes at 14,000 rpm. The supernatant was carefully removed without touching the pellet. The pellet was washed with 500 µL 70 % EtOH. This was spun down with maximum speed for 10 minutes at 14,000 rpm. The supernatant was carefully removed without touching the pellet. The tubes were left at room temperature for 15 minutes. The pellet was redissolved in DNA water. A volume of the sample was used to measure the DNA concentration. To further dissolve the DNA, the samples were heated to 50 °C for 2 minutes.

3.4.2 Restriction Enzyme Digestion of pEGSH and pAcGFP1-N1 Vectors
The pEGSH construct vector and the p25α-pAcGFP1-N1 containing the p25α and the p25α-GFP insert were digested with restriction enzymes (RE) to enable ligation of the construct digest and the insert digest, respectively. Matching restriction sites between the pEGSH vector and the p25α-pAcGFP1-N1 vector was acquired, cf. appendix B.2 Restriction Site Analysis. FastDigest RE *Eco*RI, *Bam*HI, *Mun*I (*Mfe*I) and *Not*I (Fermentas) were applied. A volume of vector DNA corresponding to 1 µg/µL FastDigest buffer was applied and a volume of each RE corresponding to 0.5 µL/µg vector DNA was applied. Double digestion with two RE was carried out in all the experiments to generate compatible overhangs in the pEGSH vector and the p25α and p25α-GFP inserts, respectively. The overhang created by *Mun*I (*Mfe*I) is compatible to that of *Eco*RI. The different RE used to obtain the different vector construct digests and vector insert digests can be viewed in Table 2.

Table 2: Overview of the restriction enzymes applied for obtaining the different vector construct digests and vector insert digests.

For vector construct	Vector for digest	Restriction enzymes	Obtained insert/construct
p25α-pEGSH	pEGSH	*Mun*I and *Bam*HI	pEGSH for p25α
	p25α-pAcGFP1-N1	*Eco*RI and *Bam*HI	p25α
p25α-GFP-pEGSH	pEGSH	*Mun*I and *Not*I	pEGSH for p25α-GFP
	p25α-pAcGFP1-N1	*Eco*RI and *Not*I	p25α-GFP

In the following order, a volume of nuclease free water, a volume of 10x FastDigest buffer, a volume of plasmid vector (3 µg pEGSH or 6 µg p25α-pAcGFP1-N1) and a volume of each RE was transferred to an eppendorf tube. This was mixed gently by inverting the tube and spun down briefly. The mixture was incubated at 37 °C for 15 minutes in a heating block. The restriction enzymes were inactivated by heating at 80 °C for 10 minutes in a heating block. Except, the RE *Mun*I (*Mfe*I) could not be heat inactivated and star activity would occur after 16 hours of incubation. Although, the vector digests were separated by agarose gel electrophoresis immediately after digestion.
The RE digestions were carried out twice, the first as a pilot experiment. In the second experiment, the digestion of p25α-pAcGFP1-N1 with *Eco*RI and *Bam*HI was also carried out with a volume of each RE corresponding to 1 µL/µg vector DNA. Controls consisted of nuclease free water, 10x FastDigest buffer and vector plasmid (2 µg p25α-pAcGFP1-N1 or pEGSH).

3.4.3 Agarose Gel Separation of RE Digests and DNA Extraction from Gel

The Nucleospin® Extract II kit (Macherey Nagel) was applied when performing the DNA extraction from the agarose gels.

Agarose Gel Electrophoresis of RE Digests

Agarose gels with 1 % and 2.5 % agarose in 1x TAE buffer were applied. The 2.5 % agarose gel was applied for separating the p25α insert digest. The agarose mixed with 1x TAE buffer in a blue cap bottle was heated in a microwave oven until the agarose was dissolved. The flask was cooled for 10 minutes to 50-55 °C. A gel casting tray was prepared by sealing the ends with tape and the comb (25 µL pr. well) was placed in the tray. An appropriate volume of ethidium bromide was added to the cooled agarose gel solution, mixed and poured into the tray. This was left for 30 minutes to cool. The comb was removed and 1x TAE buffer was poured into the electrophoresis apparatus. For the gels for separating the pEGSH digests, extra ethidium bromide was added to the 1x TAE buffer in the chamber. The 1 kbp and 50 bp ladders (Fermentas) were loaded. A volume of 6x DNA loading buffer was mixed with a volume of each sample and controls from the RE digestion. These were loaded in each well and the gel was run at 80 V from – to + for approximately 1 hour. The DNA was visualized using a Bio Rad scanner.

DNA Extraction from Gel

The DNA extraction from the agarose gels was performed according to the Nucleospin® Extract II kit manual (Macherey Nagel). In the washing step, the column was washed two times with buffer NT3. After the drying step, the column was washed additionally with buffer NT3 followed by another drying step and the columns were incubated at 70 °C for 4 minutes to remove excess ethanol. Prior to elution, the elution buffer was preheated to 70 °C. The DNA concentration was measured using a NanoDrop spectrophotometer.

3.4.4 Dephosphorylation of RE Digests of pEGSH

The vector construct digests for p25α-pEGSH and p25α-GFP-pEGSH, respectively, were dephosphorylated using shrimp alkaline phosphatase (SAP) (Fermentas) to avoid self-ligation. Prior to the experiment, the volume of SAP required for 500 ng of each vector digest was calculated, when 1 µg linear 3 kbp DNA (approximately 1 pmol termini) is required for 1 u SAP. The reaction mixture was prepared in the following order. For each digest, a volume containing 0.5 µg RE digest of pEGSH, 2 µL 10x SAP buffer, 0.3 µL SAP and nuclease free water to a total volume of 20 µL was transferred to an eppendorf tube. This was thoroughly mixed by inverting, spun down briefly, and incubated at 37 °C for 30 minutes in a heating block (for 5' overhangs). The reaction was stopped by heating at 65 °C for 15 minutes.

3.4.5 Ligation of pEGSH Digests and p25α/ p25α-GFP Inserts

A molar vector:insert ratio of 1:3 was applied. The amount of insert DNA was calculated prior to the experiment, where 100 ng of each dephosphorylated construct vector digest was applied for each ligation, cf. section 3.4.4 Dephosphorylation of RE Digests of pEGSH. The insert digests were acquired according to section 3.4.3 Agarose Gel Separation of RE Digests and DNA Extraction from Gel.
The amount of insert was calculated using the following equation:

$$\text{ng insert} = \frac{(\text{ng vector} \cdot \text{bp size insert})}{(\text{bp size vector})} \cdot (\text{molar ratio of insert : vector})$$

(Promega 2005)

A volume containing 100 ng linearized dephosphorylated pEGSH vector digest, a volume containing either 41.7 ng p25α insert or 87.7 ng p25α-GFP insert, 2 µL 10x T4 DNA ligation buffer (Fermentas), 0.2 µL 5 u/µL T4 DNA ligase (Fermentas) and nuclease free water to a total of 20 µL was transferred to an eppendorf

tube. This was vortexed and spun briefly to collect the drops. The mixture was incubated at 22 °C (room temperature) for 10 minutes. The ligation mixture was stored at 4 °C until used for transformation. This was carried out for both vector construct digests and insert digests, i.e. the p25α insert + pEGSH for the p25α insert and the p25α-GFP insert + pEGSH for the p25α-GFP insert. Control ligation samples without insert DNA were also included.
A second ligation experiment was later conducted with incubation at 16 °C overnight in PCR tubes in a PCR machine (instead of 22 °C for 10 minutes in eppendorfs), followed by heat inactivation of the T4 DNA ligase at 65 °C for 10 minutes.

3.4.6 Cloning of p25α-pEGSH and p25α-GFP Constructs

The p25α-pEGSH and p25α-pEGSH vector constructs were transformed into One Shot® Top10 chemically competent *E. coli* (Invitrogen) by heat shock transformation and electroporation with One Shot® Top10 electro competent *E. coli* (Invitrogen).

Heat Shock Transformation

Heat shock transformation with the chemically competent *E. coli* was performed for all the ligation samples which were incubated at 22 °C for 10 minutes. In a second experiment, the ligation samples which were incubated at 16 °C overnight followed by heat inactivation were also used for heat shock transformation. A volume of 5 µL ligation mix was applied in both of these experiments. In a third experiment, only the ligation mix containing p25α-GFP-pEGSH construct (incubated at 16 °C overnight and heat inactivated) was applied, using 10 µL ligation mix for one vial of chemically competent cells.
A water bath was equilibrated to 42°C. A vial of SOC medium was heated to room temperature. LB agar plates containing 100 µg/mL ampicillin was placed in a 37°C incubator to remove excess moisture. The vials containing plasmids were centrifuged briefly and placed on ice. Five vials of One Shot® Top10 chemically competent cells (Invitrogen) were thawed on ice. An amount of 10 pg pUC19 control plasmid was transferred to one vial competent cells and a volume of each ligation mix was transferred to the four other vials with competent cells, the p25α-pEGSH ligation mix, the p25α-GFP-pEGSH ligation mix, and the two controls containing the pEGSH digests with ligase, but without inserts. The vials were tapped gently to mix the cells with the DNA. The cells were incubated on ice for 30 minutes. Subsequently, the vials were incubated for exactly 30 seconds in the 42 °C water bath without mixing or shaking. The vials were removed from the water bath and placed on ice. A volume of SOC medium was transferred to each vial and the cell suspension was gently transferred to a 12 mL sterile culture tube. The tubes were placed in a rack with tape and incubated in a rotary shaker incubator at 37 °C for 1 hour at 170 rpm. A specific volume of LB medium was mixed with four different volumes of each cell suspension in four eppendorfs. This was carried out for each transformation mixture. The cell suspensions were spread using glass marbles on labeled LB agar plates with 100 µg/mL ampicillin. The plates were inverted and incubated at 37 °C overnight. Colonies were selected for further analysis.

Electroporation

Electroporation of the electro competent *E. coli* was performed with the p25α-GFP-pEGSH construct (incubated at 16 °C overnight and heat inactivated), using 1 µL ligation mix for one vial of electro competent cells. The electroporator was set up for *E. coli* electroporation and a volume of ligation mix was transferred to the vial with the electro competent cells. The vials were tapped gently to mix the cells with the DNA. The cell suspension was transferred to the chilled electroporation cuvette on ice. The cells were electroporated and a volume of room temperature SOC medium was quickly added. This was gently mixed. The solution was transferred to a 12 mL sterile culture tube and placed in a rotary shaker incubator at 37 °C for 1 hour.

Two different volumes of the cell culture were spread on prewarmed LB plates containing 100 µg/mL ampicillin. The plates were incubated at 37 °C overnight. Colonies were selected for further analysis.

3.4.7 Colony PCR and Agarose Gel Electrophoresis

E. coli colonies transfected with p25α-pEGSH and p25α-GFP-pEGSH ligation mix from the heat shock transformation and electroporation were further verified by colony PCR. The PCR products were visualized by agarose gel electrophoresis.

Colony PCR

The primers applied for colony PCR were acquired from the inducible expression system kit. The primers were dissolved in DNA water (to 100 µM stocks). These stocks were further diluted into 10 µM aliquots. The pEGSH forward sequencing primer was 5'-CTCTGAATACTTTCAAAAGTTAC-3' and the T3 promoter reverse sequencing primer was 5'-AATTAACCCTCACTAAAGGG-3'. The PCR program was prepared using the formula $T_m = 4 \cdot (G + C) + 2 \cdot (A + T)$, where the letters A, C, G and T denotes the different DNA bases. The different program steps were as follows; 1. initialization: 94 °C for 1 minute, 2. denaturation: 94 °C for 30 seconds, 3. annealing: 52 °C for 30 seconds, 4. elongation: 72 °C for 2 minutes, 5. Final elongation: 72 °C for 10 minutes and 6. Pause: 4 °C for short term storage. Step 2-4 was repeated 30 times in total (30 cycles) with a total experiment duration of 2 hours, 2 minutes and 24 seconds.
Sixteen colonies from the LB agar plates (eight containing each construct, p25α-pEGSH and p25α-GFP-pEGSH) were chosen and transferred to a specific spot on a new LB agar plate containing 100 µg/mL ampicillin (four colonies pr. new plate). These plates were incubated overnight at 37 °C. Both 2 mM and 5 mM $MgCl_2$ were tested in the experiment, which means that four colonies of each construct was tested using 2 mM $MgCl_2$ and four colonies of each construct was tested using 5 mM $MgCl_2$. Each pipette tip used to pick out the sixteen colonies was placed in the PCR plate containing DNA water (for each of the sixteen tips). Two tubes contained only DNA water and no bacteria (negative control) for both $MgCl_2$ concentrations. Two tubes contained pure pEGSH vector (without insert, from stock containing 255 ng/µL) and DNA water (positive control) for both $MgCl_2$ concentrations. The tips in the PCR plate were each placed onto the pipette and the cells in DNA water were pipetted up and down. Subsequently, the PCR plate was placed on ice. PCR master mix was prepared, containing different volumes of 10x DreamTaq PCR buffer (Fermentas), a volume of 25 mM dNTP mix, a volume of 10 µM of each construct primer, a volume of 25 mM $MgCl_2$ (for 5 mM samples) and a volume of 5U/µL DreamTaq DNA polymerase (Fermentas). An example can be seen in Table 3. Taq polymerase was added just prior to starting the PCR cycle. A volume of PCR mix was transferred to each well in the PCR plate according to calculations. Both negative control, DNA water, and positive control, pure pEGSH plasmid construct, were applied in the PCR reaction. The length of the PCR fragments was examined using agarose gel electrophoresis.

Table 3: Preparation of PCR mixture for colonies with p25α-pEGSH construct. This was also prepared for the colonies containing the p25α-GFP-pEGSH construct (not shown).

PCR mixture for colonies with p25α-pEGSH construct				
	2 mM $MgCl_2$ Vol. pr. well	5 mM $MgCl_2$ Vol. pr. well	**2 mM $MgCl_2$ Vol. for 8* wells**	**5 mM $MgCl_2$ Vol. for 8* wells**
DNA water w. or w/o bacteria or ctrl plasmid	21 µL	18 µL	168 µL	144 µL
10xPCR buffer (20 mM $MgCl_2$)	2.5 µL	2.5 µL	20 µL	20 µL
dNTP (25 mM)	0.25 µL	0.25 µL	2 µL	2 µL
FW primer (10 µM)	0.5 µL	0.5 µL	4 µL	4 µL
RW primer (10 µM)	0.5 µL	0.5 µL	4 µL	4 µL
$MgCl_2$ (25 mM)	-	3 µL	-	24 µL
Taq polymerase (5 U/µL)	0.25 µL	0.25 µL	2 µL	2 µL
Total volume of PCR mix	**4 µL**	**7 µL**	32 µL	56 µL
Total volume	25 µL	25 µL	200 µL	200 µL

* Volumes pr. $MgCl_2$ concentration are in theory for **8 wells**, but approximately enough for 6 wells (4 samples + 2 ctrls).

Agarose Gel Electrophoresis of PCR Products

Agarose gels with 2.5 % agarose in 1x TAE buffer were applied. The agarose mixed with 1x TAE buffer in a blue cap bottle was heated in a microwave oven until the agarose was dissolved. The flask was cooled for 10 minutes to 50-55 °C. A gel casting tray was prepared by sealing the ends with tape and the comb (25 µL pr. well) was placed in the tray. An appropriate volume of ethidium bromide was added to the cooled agarose gel solution, mixed and poured into the tray. This was left for 30 minutes to cool. The comb was removed and 1x TAE buffer was poured into the electrophoresis apparatus. The 1 kbp and 50 bp ladders (Fermentas) were loaded. A volume of 6x DNA loading buffer was mixed with a volume of each PCR product, including controls. These were loaded in each well and the gel was run at 80 V from – to + for approximately 1 hour. The gel containing the p25α-GFP-pEGSH PCR products was run for 1 hour and 20 minutes. The DNA was visualized using a Bio Rad scanner.

The colonies containing the correct insert were cultured and grown for propagation, followed by plasmid DNA purification and subsequent restriction analysis.

3.4.8 Plasmid DNA Purification and Sequencing of Constructs

The colonies of colony PCR with the PCR product length of interest were further cultured. Two colonies of with each construct, A4 and C2 containing p25α-pEGSH and A1 and A2 containing p25α-GFP-pEGSH, were grown for subsequent plasmid DNA purification using the Nucleobond® Xtra Midi kit (Macherey Nagel) (midiprep). The plasmids from the midiprep were further examined by restriction analysis, sequenced and the DNA was extracted using phenol and chloroform. Subsequently, plasmid DNA purification was performed using the Nucleospin® Plasmid mini kit (Macherey Nagel) (miniprep) to repeat the purification procedure in a smaller scale. Restriction analysis was performed on the miniprep plasmids, followed by sequencing. After this restriction analysis and sequencing, a plasmid purification using the Nucleobond® Xtra maxi kit (maxiprep) to obtain a greater amount of the p25α-pEGSH and p25α-GFP-pEGSH plasmid constructs for further experiments. The midiprep and maxiprep manuals were identical, except for the proportion of volumes applied. The isopropanol precipitation step in the Macherey Nagel manual for midiprep and maxiprep was not carried out.

Midiprep Plasmid Purification

The LB agar plates containing the colonies used for colony PCR were further used for cloning, colony A4 and C2 containing p25α-pEGSH and colony A1 and A2 containing p25α-GFP-pEGSH. Some material from two of the construct containing colonies were picked out and placed in a 12 mL sterile culture tube with 3 mL sterile LB media containing 3 µL ampicillin. This starter culture was incubated for approximately 6 hours at 37°C, 240 rpm in a shaking incubator. 100 µL of the starter culture was transferred to 100 mL LB media containing 100 µL ampicillin and incubated for 17 hours at 37°C, 210 rpm in a shaking incubator. 95 mL of the cell culture was used for plasmid purification. The Nucleobond® Xtra Midi kit (Macherey Nagel) manual was followed. The cell lysate was centrifuged at 5,000 x g for 15 minutes for clarification.

Miniprep Plasmid Purification

The LB agar plates containing the colonies used for colony PCR were further used for cloning, colony A4 and C2 containing p25α-pEGSH and colony A1 and A2 containing p25α-GFP-pEGSH. Some material from the construct containing colonies were picked out and placed in a 12 mL sterile culture tube with 3 mL sterile LB media containing 3 µL ampicillin. This was incubated overnight at 37°C, 200 rpm in a shaking incubator. The Nucleospin® Plasmid mini kit (Macherey Nagel) manual was followed.

Maxiprep Plasmid Purification

The LB agar plates containing the colonies used for colony PCR, colony C2 containing p25α-pEGSH and colony A2 containing p25α-GFP-pEGSH, were further used for cloning after verification by restriction analysis, cf. section 3.4.9 Restriction Analysis. Some material from the colonies were picked out and placed in a 12 mL sterile culture tube with 3 mL sterile LB media containing 3 µL ampicillin. This starter culture was incubated for approximately 8 hours at 37°C, 220 rpm in a shaking incubator. 300 µL of the starter culture was transferred to 300 mL LB media containing 300 µL ampicillin and incubated overnight at 37°C, 210 rpm in a shaking incubator. The entire cell culture was used for plasmid purification. The Nucleobond® Xtra Maxi kit (Macherey Nagel) manual was followed. The cell lysate was centrifuged at 5,000 x g at 4 °C for 15 minutes for clarification.

Sequencing of Constructs

The construct elution samples of midiprep and miniprep were diluted using the respective elution buffer of each kit to a concentration of 50-100 ng/µL in 15 µL in eppendorfs with a lock-cap. The samples were sequenced by European Eurofins MWG Operon. The primers used for colony PCR were applied for sequencing, cf. section 3.4.7 Colony PCR and Agarose Gel Electrophoresis. Each construct sample was sequenced twice using one of each primer.

3.4.9 Restriction Analysis

The purified plasmid DNA was examined by restriction analysis to verify that the correct inserts, p25α and p25α-GFP, respectively, were present in the pEGSH vector. Two colonies of each construct which were verified by colony PCR were used for each restriction analysis digestion. The restriction enzymes were chosen by examining restriction sites in the p25α-pEGSH and p25α-GFP-pEGSH construct sequences using BioEdit. The restriction enzyme *Acu*I cleaves in the sequence of p25α and p25α-GFP, respectively. Cleavage with both *Hind*III and *Bgl*II occurs outside the insert of the constructs. See Table 11 for an overview of the theoretical restriction digest products. Two experiments were carried out where the restriction enzyme *Acu*I was applied in the first experiment and the restriction enzymes *Hind*III and *Bgl*II were utilized in the second experiment.

Restriction Enzyme Digestion

For the digestion with *Acu*I, a volume of nuclease free water, 2 µL 10x FastDigest buffer, 1 µL 0.2 mM S-adenosylmethionine (SAM) (Fermentas), a volume of construct vector (750 ng) and 1 µL FastDigest *Acu*I (Fermentas) were gently mixed in the order mentioned. The total volume in each sample was 20 µL. The mixture was briefly spun down and then incubated at 37 °C in a heating block for 30 minutes. The midiprep plasmid samples were incubated for 30 minutes, while the miniprep plasmid samples were incubated for 45 minutes. The restriction enzyme was inactivated by heating at 65 °C for 5 minutes. The digested vector samples and the controls were mixed with 6x DNA loading buffer. The negative controls contained each construct vector from all of the chosen colonies without restriction enzyme. The positive control contained restriction enzyme and the pEGSH vector without insert. In the restriction analysis with the midiprep samples, 510 ng control vector was applied and 462.5 ng was applied in the experiment with the miniprep samples. The digests were visualized using agarose gel electrophoresis.

For the digestion with *Hind*III and *Bgl*II, a volume of nuclease free water, 2 µL 10x Buffer R (Fermentas), a volume of construct vector (750 ng) and a 1:2 ratio volume of *Hind*III (0.5 µL) and *Bgl*II (1 µL) (Fermentas), respectively, were gently mixed in the order mentioned. The total volume in each sample was 20 µL. The mixture was briefly spun down and then incubated at 37 °C in a heating block for 1 hour. The midiprep plasmid samples were incubated for 1 hour, while the miniprep plasmid samples were incubated for 2 hours. The restriction enzyme was inactivated by heating at 65 °C for 5 minutes. The digested vector samples and the controls were mixed with 6x DNA loading buffer. The negative controls contained each construct vector from all of the chosen colonies without restriction enzyme. The positive control contained restriction enzymes and the pEGSH vector without insert. In the restriction analysis with the midiprep samples, 510 ng control vector was applied and 462.5 ng was applied in the experiment with the miniprep samples. The digests were visualized using agarose gel electrophoresis.

Table 4: Overview of the theoretical sequence length of vector constructs and digests in the restriction analysis using restriction enzymes *Acu*I and *Hind*III+*Bgl*II.

Theoretical Sequence Length of Vector Constructs and Digests (bp)		
p25α-pEGSH construct		5488
p25α-GFP-pEGSH construct		6206
pEGSH vector		4829
Digest with *Acu*I	**Fragment size**	**Fragment position**
p25α-pEGSH construct	2923	4985-2420
	1009	2420-3429
	1556	3429-4985
p25α-GFP-pEGSH construct	3094	5532-2420
	1009	2420-3429
	1556	3429-4985
	547	4985-5532
pEGSH vector	3820	3429-2420
	1009	2420-3429
Digest with *Hind*III+*Bgl*II	**Fragment size**	**Fragment position**
p25α-pEGSH construct	4142	13-4155
	1346	4155-13
p25α-GFP-pEGSH construct	4142	13-4155
	2064	4155-13
pEGSH vector	687	4155-13
	4142	13-4155

Agarose Gel Electrophoresis of RE Digest

Two agarose gels with 1 % agarose in 1x TAE buffer were applied. The agarose mixed with 1x TAE buffer in a blue cap bottle was heated in a microwave oven until the agarose was dissolved. The flask was cooled for 10 minutes to 50-55 °C. A gel casting tray was prepared by sealing the ends with tape, and the comb (25 µL pr. well) was placed in the tray. An appropriate volume of ethidium bromide was added to the cooled agarose gel solution, mixed and poured into the tray. This was left for 30 minutes to cool. The comb was removed and 1x TAE buffer was poured into the electrophoresis apparatus. The 1 kbp ladder (Fermentas) was loaded. A volume of 6x DNA loading buffer was mixed with a volume of each restriction analysis product, including controls. These were loaded in each well and the gel was run at 80 V from – to + for approximately 1 hour. The DNA was visualized using a Bio Rad scanner. The colonies containing the construct vectors which were verified by both colony PCR and restriction analysis were further cloned and purified using the maxiprep plasmid purification, cf. section 3.4.8 Plasmid DNA Purification and Sequencing of Constructs.

3.4.10 DNA Extraction and Precipitation

The p25α-pEGSH and p25α-GFP-pEGSH construct elution samples from the maxiprep were purified by extracting the DNA using phenol and chloroform. Subsequently, the vector constructs were precipitated using first ethanol and then isopropanol.

DNA Extraction using Phenol-Chloroform and Ethanol Precipitation

The DNA extraction with phenol-chloroform was first carried out for the midiprep samples containing the p25α-pEGSH construct (colony A4 and C2) (first protocol), then for one of the midiprep samples containing the p25α-GFP-pEGSH construct (colony A1) (second protocol) and finally for the maxiprep samples containing the p25α-pEGSH and p25α-GFP-pEGSH constructs (third protocol).

First Protocol

A 1/10 of ice-cold 3 M NaOAc (pH 5.2) was added to the DNA sample and 1x volume phenol:chloroform:isoamyl (25:24:1) was also added. This was mixed for 20-30 seconds on the vortexer. The mixture was spun down in the mini centrifuge at 13,000 rpm for 3 minutes. The water phase (top phase) was transferred to a new tube. Nothing of the lower phase was transferred. 1x volume of phenol:chloroform:isoamyl (25:24:1) was added and mixed for 20-30 seconds on the vortexer. This was spun down in the mini centrifuge at 13,000 rpm for 3 minutes. The water phase was transferred to a new tube. 2.5x volume 99.9 % ethanol was added to the water phase and was precipitated overnight at -20 °C. This was spun down in the mini centrifuge at 13,000 rpm for 30 minutes at 4 °C. All liquid was removed so that only pellet remained. The pellet was washed in ice cold 70 % ethanol. This was spun down at 13,000 rpm for 10 minutes at 4 °C. The ethanol was removed completely and the pellet was left to dry at room temperature for 5-10 minutes. The pellet was resuspended in a volume of sterile filtered TE buffer, pH 7.5 (10 mM Tris, 1 mM EDTA). The DNA concentration was measured.

Second Protocol

A 1/10 of ice-cold 3 M NaOAc (pH 5.2) was added to the DNA sample and 1x volume phenol:chloroform:isoamyl (25:24:1) was also added. This was mixed for 20-30 seconds on the vortexer. The mixture was spun down in the mini centrifuge at 13,000 rpm for 3 minutes. The water phase (top phase) was transferred to a new tube. Nothing of the lower phase was transferred. 1x volume of phenol:chloroform:isoamyl (25:24:1) was added and mixed for 20-30 seconds on the vortexer. This was spun down in the mini centrifuge at 13,000 rpm for 3 minutes. The water phase was transferred to a new tube. 1x volume chloroform was added and mixed for 20-30 seconds on the vortexer. This was spun down in the mini centrifuge at 13,000 rpm for 3 minutes. The water phase was transferred to a new tube. This was spun down in the mini centrifuge at 13,000 rpm for 3 minutes and the water phase was transferred to a new tube. 2.5x volume 99.9 % ethanol was added to the water phase and was precipitated for 15 minutes at -80 °C. This was spun down in the mini centrifuge at 13,000 rpm for 15 minutes at 4 °C. All liquid was removed so that only pellet remained. The pellet was washed in ice cold 70 % ethanol. This was spun down at 13,000 rpm for 3 minutes at 4 °C. The ethanol was removed completely and the pellet was left to dry at room temperature for 5-10 minutes. The pellet was resuspended in a volume of sterile filtered TE buffer, pH 7.5 (10 mM Tris, 1 mM EDTA). The DNA concentration was measured.

Third Protocol

A 1/10 of ice-cold 3 M NaOAc (pH 5.2) was added to the DNA sample and 1x volume phenol:chloroform:isoamyl (25:24:1) was also added. This was mixed for 20-30 seconds by hand. The mixture was spun down in the centrifuge at 12,000 x g for 3 minutes at 4 °C. The water phase (top phase) was transferred to a new tube. Nothing of the lower phase was transferred. 1x volume of phenol:chloroform:isoamyl (25:24:1) was added and mixed for 20-30 seconds by hand. This was spun down in the centrifuge at 12,000 x g for 3 minutes at 4 °C. The water phase was transferred to a new tube. 1x volume chloroform was added and mixed for 20-30 seconds by hand. This was spun down in the centrifuge at 12,000 x g for 3 minutes at 4 °C. The water phase was transferred to a new tube. 2.5x volume 99.9 % ethanol was added to the water phase and was precipitated for 15 minutes at -80 °C. This was spun down in the centrifuge at 12,000 x g for 15

minutes at 4 °C. All liquid was removed so that only pellet remained. The pellet was washed in ice cold 70 % ethanol. This was spun down at 12,000 x g for 3 minutes at 4 °C. The ethanol was removed completely and the pellet was left to dry at room temperature overnight. A volume of DNA water was added for resuspension. The tube was vortexed and additionally heated for 5-10 minutes in a 42 °C water bath, followed by 20 minutes in a 65 °C water bath. The DNA concentration was measured. A volume of DNA water was added to the vector construct samples. The DNA samples were applied for further DNA precipitation using ethanol and isopropanol.

DNA Precipitation using Isopropanol and Ethanol
A 8/18 volume of room temperature isopropanol was added to precipitate a 10/18 volume of vector DNA in DNA water. This was vortexed well and incubated for 2 minutes at room temperature. The mixture was centrifuged at 12,000 x g for 30 minutes at 4 °C. The supernatant was discarded. A volume of room temperature 70 % ethanol was added to the pellet. This was vortexed to dissolve the pellet, followed by centrifugation at 12,000 x g at room temperature for 5 minutes. The ethanol was removed completely using a pipette tip. This was left to dry at room temperature for 15 minutes. The DNA pellet was dissolved in sterile filtered DNA water by pipetting and vortexing. The DNA concentration was measured.

3.4.11 Determination of Lethal Geneticin and Hygromycin Concentration for OLN-AS7

One experiment using geneticin and two experiments using hygromycin were conducted. The experimental setup was to trypsinize and count the cells, when the cells were confluent, and change the media when it was appropriate. The confluent cells were seeded out again after trypsination. The cells were cultured in 6 well culture plates, starting with 60,000 cells in each well. A volume of 2 mL growth medium containing cell suspension and 100 µg/mL zeocin was transferred to each well. The media was removed after 24 hours and new media containing 100 µg/mL zeocin and a given concentration of either geneticin or hygromycin was transferred to the wells. Eight different concentrations of geneticin (Invitrogen) and hygromycin (Invitrogen), respectively, were tested, 0 µg/mL (control), 50 µg/mL, 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL and 1000 µg/mL. Four 6 well culture plates were utilized for each experiment and the experiments were carried out with triplicate determination, i.e. three wells for each concentration. The media was changed every 4 days in the second hygromycin experiment and every 2-4 days in the geneticin experiment.

3.5 Examination of Transfection Efficiency

The transfection efficiency by transiently transfecting OLN-AS7 with p25α-pAcGFP1-N1 plasmid vector using FuGENE® HD transfection reagent (Roche), DreamFect™ transfection reagent (OZ Biosciences) and the calcium phosphate transfection method (modified from Invitrogen) was investigated by fluorescence microscopy. A transfection agent:plasmid DNA ratio of 5:1 was applied for both FuGENE® HD transfection reagent and DreamFect™ Transfection Reagent (Knudsen, A. D., unpublished data). The calcium phosphate protocol was scaled down from 60 mm culture dishes to 6 well culture plates.
A Leica DMR microscope with a Leica DC 200 camera was applied. Since the GFP in pAcGFP1-N1 is derived from *Aequorea coerulescens* (excitation maximum: 475 nm, emission maximum: 505 nm), a FITC filter was used to view the fluorescent cells. Both phase contrast images and fluorescent images were acquired. For the phase contrast images, an exposure time of 1/41 sec was applied and a gain of zero. For the fluorescence images, an exposure time of 1.50 minutes was applied and a gain of 85-97 %.

3.5.1 Transient Transfection of OLN-AS7 Cells

DreamFect™ Transfection Reagent

A volume of 2 mL cell suspension containing 250,000 cells was transferred to each well of a 6 well culture plate and incubated overnight at 37 °C, 5.0 % CO_2 in a humified incubator. The media was removed from the wells, and the wells were washed one time with 1x PBS. A volume of 2 mL transfection media (DMEM 4.5 g/L glucose, L-glutamine and 0.5 % FCS) was added to the each well. 10 µL DreamFect™ transfection reagent was pipetted into a glass vial containing 100 µL serum free media (DMEM 4.5 g/L glucose, 4mM L-glutamine) in the middle of the liquid. 2 µL p25α-pAcGFP1-N1 plasmid was pipetted into another glass vial containing 100 µL serum free media in the middle of the liquid. The plasmid solution was transferred to the solution containing DreamFect reagent within 5 minutes. This was pipetted up and down once and the glass vials were incubated for 15 minutes at room temperature. The transfection complex was added to the wells containing adherent cells. The plate was incubated at 37°C, 5.0 % CO_2 for 24 hours. The media was removed from the wells and the cells were washed twice with 1x PBS. The cells were trypsinized using 200 µL 0.01 % EDTA/0.125% trypsin in 1xPBS and resuspended in 300 µL media. Subsequently to increase the density of the cell suspension, the cell suspension was spun down at 600 x g for 2 minutes. The cells were resuspended in 1x PBS. The cell suspension was transferred to an eppendorf and a drop of cell suspension transferred to a cover slide. The transfection efficiency was examined using both phase contrast and fluorescence microscopy.

FuGENE® HD Transfection Reagent

A volume of 2 mL cell suspension containing 250,000 cells was transferred to each well of a 6 well culture plate and incubated overnight at 37 °C, 5.0 % CO_2 in a humified incubator. The media was removed from the wells, and the wells were washed one time with 1x PBS. A volume of 2 mL transfection media (DMEM 4.5 g/L glucose, 4 mM L-glutamine and 10 % FCS) was added to the each well. 5 µL FuGENE® HD transfection reagent was pipetted into a glass vial containing 50 µL serum free media (DMEM 4.5 g/L glucose, 4mM L-glutamine) in the middle of the liquid. 1 µL p25α-pAcGFP1-N1 plasmid was pipetted into another glass vial containing 50 µL serum free media in the middle of the liquid. The plasmid solution was transferred to the solution containing FuGENE reagent. This was pipetted up and down once and the glass vials were incubated for 15 minutes at room temperature. The transfection complex was added to the wells containing adherent cells. The plate was incubated at 37°C, 5.0 % CO_2 for 24 hours. The media was removed from the wells and the cells were washed twice with 1x PBS. The cells were trypsinized using 200 µL 0.01 % EDTA/0.125% trypsin in 1xPBS and resuspended in 300 µL media. Subsequently to increase the density of the cell suspension, the cell suspension was spun down at 600 x g for 2 minutes and the cells were resuspended in 1x PBS. A drop of cell suspension transferred to a cover slide. The transfection efficiency was examined using both phase contrast and fluorescence microscopy.

Calcium Phosphate Transfection

A volume of 2 mL cell suspension containing 250,000 cells was transferred to each well of a 6 well culture plate and incubated overnight at 37 °C, 5.0 % CO_2 in a humified incubator. The media was changed 3-4 hours prior to transfection. The transfection mixture was prepared in two eppendorfs. One eppendorf labeled A contained 7.2 µL 2 M $CaCl_2$ (Sigma), 4 µL p25α-pAcGFP1-N1 (4 µg) and 48.8 µL sterile cell culture grade water. Another eppendorf labeled B contained 60 µL 2x Hepes buffered saline (HBS) (Fluka). Solution A was added dropwise (15 µL every 20-30 seconds) to solution B while bubbling air through after every drop using a pipette. This was continued until solution A was depleted and carried out for three wells. The solution was incubated for 30 minutes at room temperature. The solution was added dropwise to the well containing media and cells. This was incubated overnight at 37 °C, 5.0 % CO_2 in a humified incubator. The

media was removed from the wells and the cells were washed twice with 1x PBS. The cells were trypsinized using 200 µL 0.01 % EDTA/0.125% trypsin in 1xPBS and resuspended in 300 µL media. Subsequently to increase the density of the cell suspension, the cell suspension was spun down at 600 x g for 2 minutes and the cells were resuspended in 1x PBS. A drop of cell suspension transferred to a cover slide. The transfection efficiency was examined using both phase contrast and fluorescence microscopy.

4. Results

4.1 Cell Culturing

The OLN-AS7 cells were tested for mycoplasma using the MycoAlert® Mycoplasma Detection Assay (Lonza) to ensure a non-contaminated cell culture before proceeding with experiments. The OLN-AS7 cell line was not infected with mycoplasma, see appendix B.1 Mycoplasma Test of OLN-AS7 Cell Line for additional information.

4.1.1 OLN Cell Line Characteristics

The OLN cell lines applied in this master thesis were characterized as adherent cells, cf. Figure 12. Additional information about the cell lines can be found in Table 5.

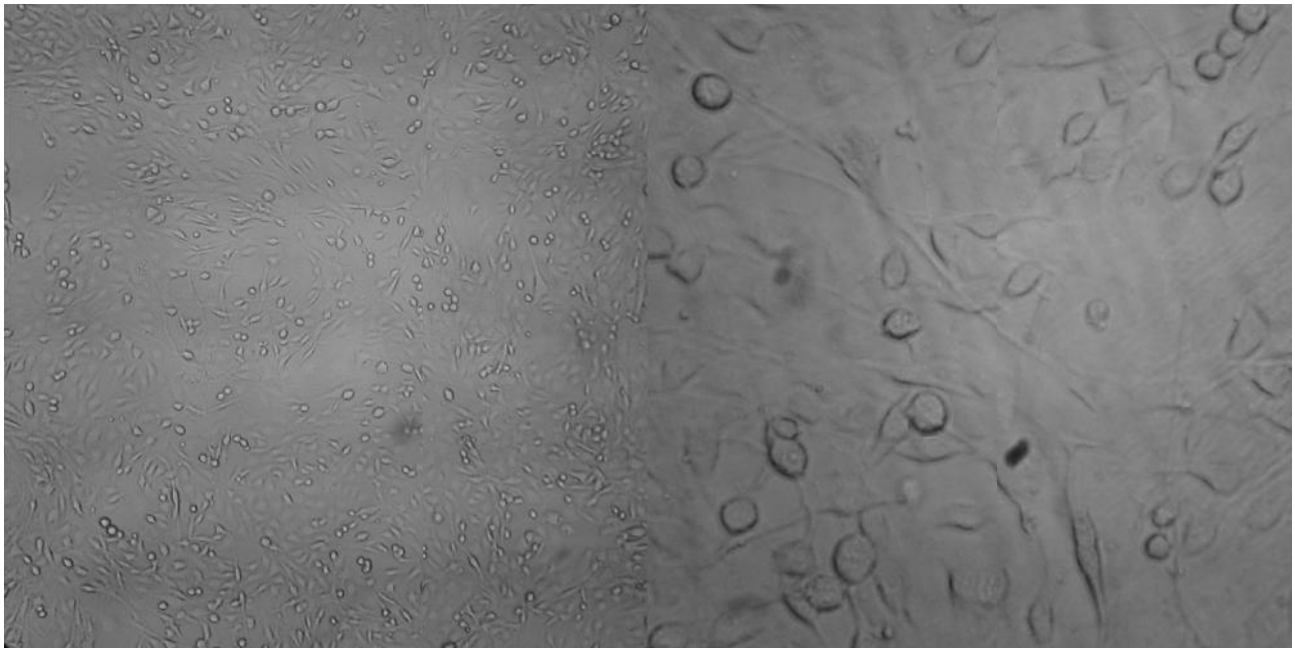

Figure 12: Phase contrast microscope images of approximately 90 % confluent OLN-AS7 cells with a magnification of 100x and 400x, respectively. The color has been altered to black/white.

Table 5: Cell line characteristics of the rat OLN cell lines. The vector maps of the pcDNA3.1/Zeo(-) can be found in appendix A.6. (Goldbaum et al. 2003; Kragh et al. 2009; P.H. Jensen, pers. commun.)

Cell Line	Origin	Inserted Gene	Insertion Method	Selection Antibiotic
OLN-AS	rat OLN-93	human α-synuclein human tau40	Retroviral transduction Plasmid vector, pcDNA3	N/A Geneticin
OLN-AS7	rat OLN-93	human α-synuclein	Plasmid vector, pcDNA3.1/Zeo(-)	Zeocin
OLN-t40	rat OLN-93	human tau40	Plasmid vector, pcDNA3	Geneticin

The OLN-AS and OLN-t40 cell line had a doubling time of approximately 24 hours (Knudsen, A. D., unpublished data). The doubling time for the OLN-AS7 cell line was determined to be 18.7 hours, see Excel file in appendix A.7 for calculations. The doubling time was determined from four data points, cf. Figure 13. The slope k = 0.0371 was applied for calculation of the doubling time. The doubling time experiment had been performed with triplicate determination.

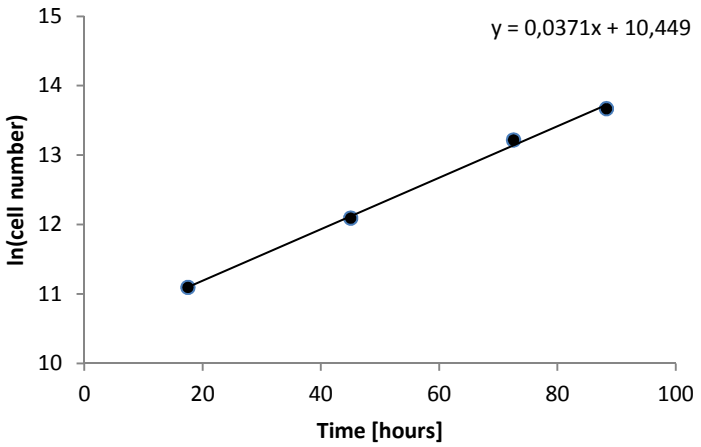

Figure 13: ln to the OLN-AS7 cell number over time. Four data points were applied for linear regression. The slope k = 0.0371 was applied to calculate the doubling time of OLN-AS7. The experiment was performed with triplicate determination.

4.2 Examination of p25α and p25α-GFP Expression in OLN-AS

The expression of α-syn, p25α, p25α-GFP, and α-tubulin in OLN-AS was examined by Western blotting. The transfection of the OLN-AS was performed using the FuGENE® HD transfection reagent (Roche) and different FuGENE reagent:plasmid ratios were tested to find the optimal ratio for further experiments. In the first experiment, see Figure 14, the FuGENE reagent was removed 4 and 24 hours, respectively, post-transfection to determine whether this had an effect on cell viability and 200,000 cells were applied per well 24 hours prior to collecting the cell lysate. In the remaining experiments, 500,000 cells per well were applied. The transfection and lysing of the cells applied on the Western blot in Figure 14 were prepared by Anders Dahl Knudsen (unpublished data).

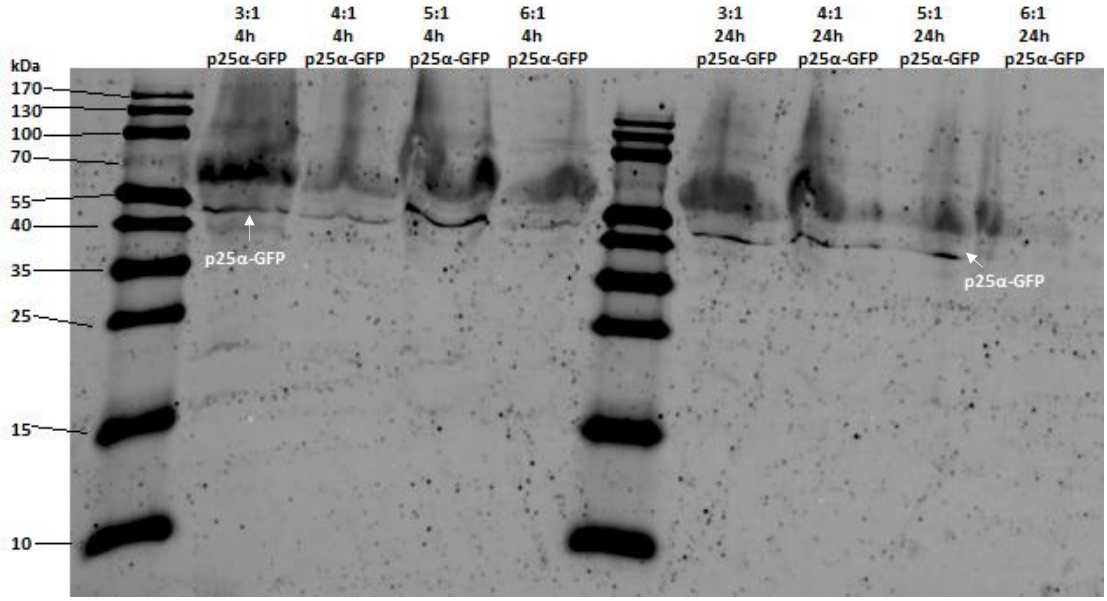

Figure 14: Verification of p25α-GFP expression in OLN-AS cells transiently transfected with p25α-pAcGFP1-N1 vector. The Western blot was prepared with primary antibody 1:500 Rbt anti-p25α-2 (Aarhus University, Department of Biochemistry). 1:3000 Alexa Fluor 647 was used as secondary antibody. From left to right, well 1 contained SM0671 protein ladder. Well 2, 3, 4 and 5 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α-GFP) ratios; 3:1, 4:1, 5:1 and 6:1, respectively, where the transformation media was removed after 4 hours. Well 6 contained SM0671 protein ladder. Well 7, 8, 9 and 10 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α-GFP) ratios; 3:1, 4:1, 5:1 and 6:1, respectively, where the transformation media was removed after 24 hours. p25α-GFP was identified at approximately 50 kDa.

In Figure 14, the expression of p25α-GFP in OLN-AS transiently transfected with p25α-pAcGFP1-N1 was examined. Well 1 and 6 contains prestained protein ladder, SM0671. Well 2-5 contains cell lysate of OLN-AS cells transfected with p25α-pAcGFP1-N1 using the FuGENE:plasmid ratios 3:1, 4:1, 5:1 and 6:1, respectively, where the transformation media was removed 4 hours post-transfection (4h). Well 7-10 contains cell lysate of OLN-AS cells transfected with p25α-pAcGFP1-N1 using the FuGENE:plasmid ratios 3:1, 4:1, 5:1 and 6:1, respectively, where the transformation media was removed 24 hours post-transfection (24h). The primary antibody anti-p25α-2 (Aarhus University, Department of Biochemistry) (1:500 dilution) targeting the human p25α protein and the secondary antibody Alexa fluor 647 (1:3000 dilution) were applied. A band at approximately 50 kDa can be viewed in well 2-5 and 7-9, which corresponds to the p25α-GFP fusion protein. The weak bands make it difficult to interpret which FuGENE:plasmid ratio that was the most effective and whether removing the FuGENE reagent 4 or 24 hours after transfection showed any difference.

In Figure 15, the expression of α-syn, α-tubulin, and p25α in OLN-AS was examined. OLN-AS displayed stable expression of α-syn and α-tubulin, however, for p25α expression the cell line was transiently transfected with the p25α-pcDNA3.1/Zeo(-) vector. Well 1 contained prestained protein ladder SM0671, while well 2-5 contained cell lysate of OLN-AS cells transfected with p25α-pcDNA3.1/Zeo(-) using the FuGENE:plasmid ratios 4:1, 5:1, 6:1 and 7:1, respectively. This was the case for all membranes in Figure 15. Figure 15A and B show the same membrane but with different PMT (photo multiplier tube) settings, 600 and 800 PMT, respectively. The primary antibodies anti-p25α-2 (1:500 dilution) and anti-α-tubulin (dilution 1:700) were applied in a blotting mixture in Figure 15A and B. The secondary antibody Alexa fluor 647 (1:3000 dilution) was applied. In Figure 15B, well 2, a weak band at approximately 30 kDa may be from p25α. The primary antibodies anti-α-syn (1:700 dilution) and anti-α-tubulin (1:700 dilution) were applied in a blotting mixture in Figure 15C. The secondary antibody Alexa fluor 647 (1:3000 dilution) was applied. In Figure 15A, B and C, the loading control α-tubulin was identified at approximately 55 kDa, and α-syn was identified at 14 kDa in C. The 15 % polyacrylamide gel applied for WB was stained using coomassie brilliant blue to examine the protein transfer. The ladder was successfully transferred; however, protein bands were still apparent in the gel.

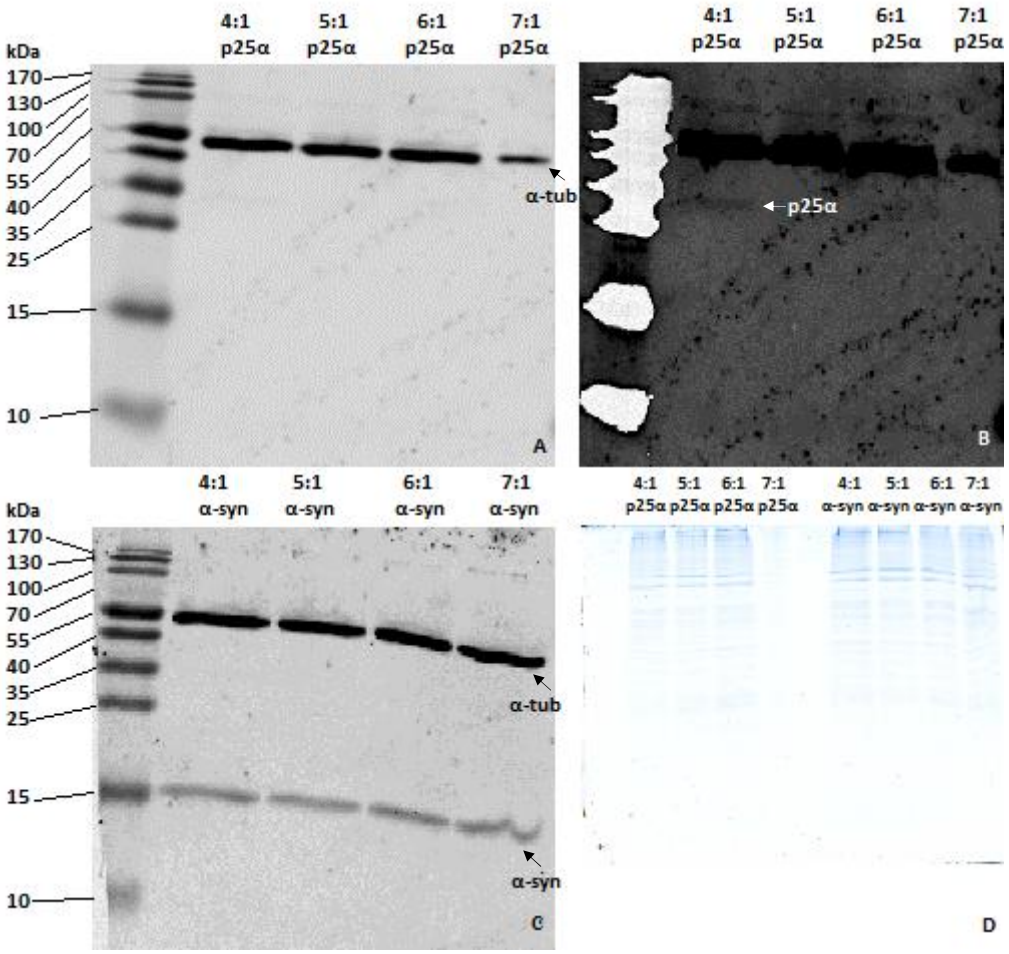

Figure 15: Verification of expression of α-syn, α-tubulin and p25α in OLN-AS cells transiently transfected with p25α-pcDNA3.1/Zeo(-) vector. A+B. Western blot with 1:500 Rbt anti-p25α-2 and 1:700 Rbt anti-α-tubulin. From left to right, well 1 contained SM0671 protein ladder, and well 2-5 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α) ratios; 4:1, 5:1, 6:1 and 7:1, respectively. α-tubulin was identified at approximately 55 kDa. A weak band at approximately 30 kDa was present, which corresponds to p25α (25 kDa). C. Western blot with 1:700 Rbt anti α-syn and 1:700 Rbt anti-α-tubulin. From left to right, well 1 contained SM0671 protein ladder and well 2-5 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α) ratios; 4:1, 5:1, 6:1 and 7:1, respectively. α-tubulin was identified at 55 kDa. α-syn was identified around 14 kDa. 1:3000 Alexa Fluor 647 was used as secondary antibody. The vector p25α-pcDNA3.1/Zeo(-) containing p25α was applied. D. The 15 % polyacrylamide gel applied for WB was stained using coomassie brilliant blue to examine the protein transfer. The ladder was successfully transferred; however, protein bands were still apparent in the gel.

In Figure 16, well 1 contained prestained protein ladder SM0671, while well 2-5 contained cell lysate of OLN-AS cells transfected with p25α-pcDNA3.1/Zeo(-) in glass vials using the FuGENE:plasmid ratios 4:1, 5:1, 6:1 and 7:1, respectively. This was the case for all membranes in Figure 16. In Figure 16F, the primary antibodies anti-α-syn (1:700 dilution) and anti-α-tubulin (1:700 dilution) were applied in a blotting mixture. The secondary antibody Alexa fluor 647 (1:3000 dilution) was applied. The loading control α-tubulin was identified at 55 kDa. α-syn was identified at 14 kDa. The band at approximately 30 kDa was believed to originate from α-tubulin, due to unspecific binding. In Figure 16G, the primary antibodies anti-p25α (1:500 dilution) and anti-α-tubulin (1:700 dilution) were applied in a blotting mixture. The secondary antibody Alexa fluor 647 (1:3000 dilution) was applied. α-tubulin was identified at 55 kDa. An unknown band was present at 30 kDa, possibly originating from α-tubulin, due to unspecific binding. The 15 % polyacrylamide gel applied for WB was stained using coomassie brilliant blue to examine the protein transfer. The ladder was successfully transferred; however, protein bands were still apparent in the gel.

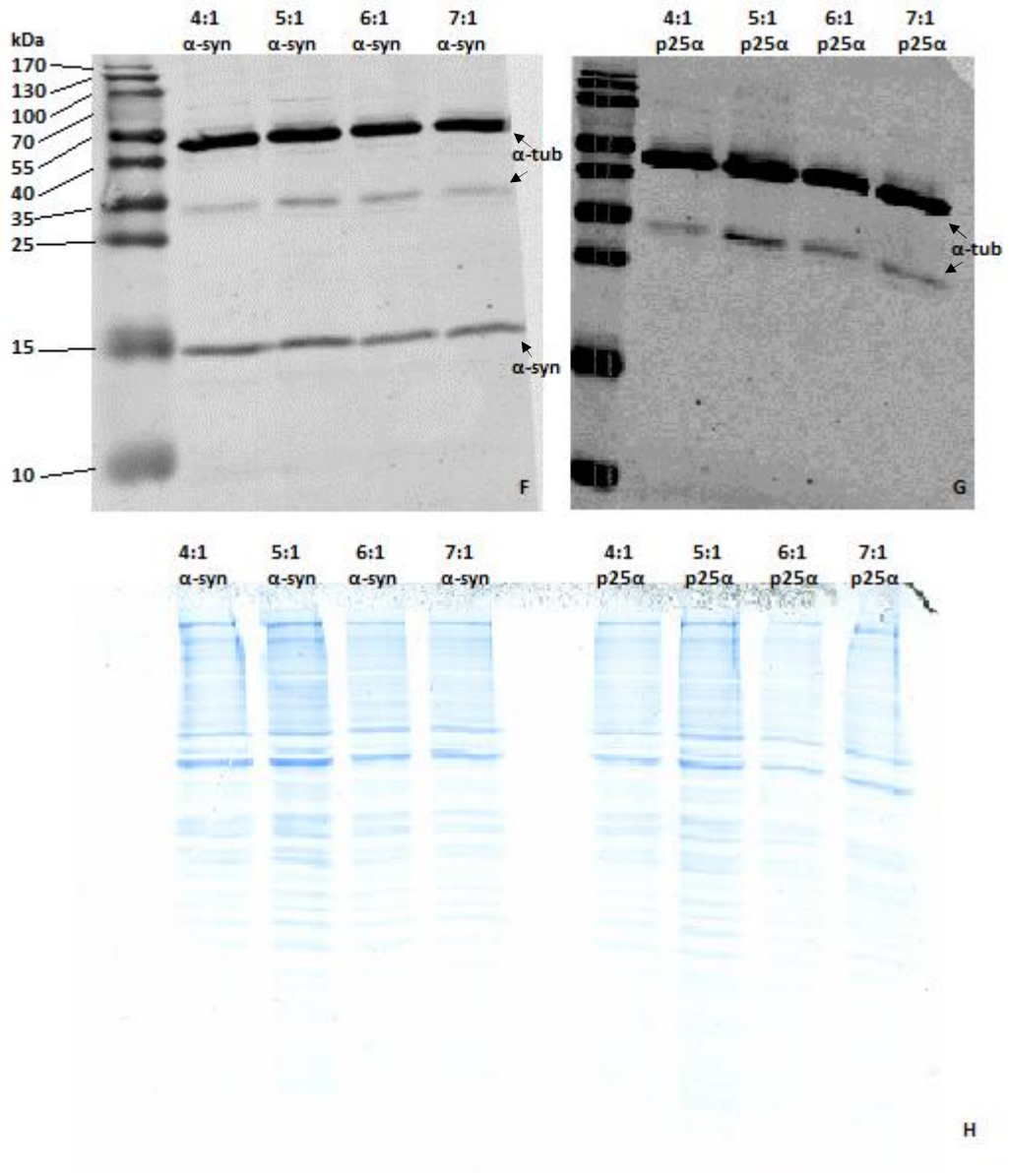

Figure 16: Verification of expression of α-syn, α-tubulin, and p25α in OLN-AS cells transiently transfected with p25α-pcDNA3.1/Zeo(-) vector. F. Western blot with 1:700 Rbt anti α-syn and 1:700 Rbt anti α-tubulin. From left to right, well 1 contained SM0671 protein ladder, and well 2-5 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α) ratios; 4:1, 5:1, 6:1 and 7:1, respectively. α-tubulin was identified at 55 kDa. α-syn was identified at 14 kDa. The band at approximately 30 kDa is believed to originate from α-tubulin. 1:3000 Alexa Fluor 647 was used as secondary antibody. G. Western blot with 1:500 Rbt anti-p25α and 1:700 Rbt anti-α-tubulin. From left to right, well 1 contained SM0671 protein ladder and well 2-5 contained cell lysate of OLN-AS cells transfected with different FuGENE reagent:plasmid (p25α) ratios; 4:1, 5:1, 6:1 and 7:1, respectively. α-tubulin was identified at approximately 55 kDa. The band at approximately 30 kDa was believed to originate from α-tubulin. 1:3000 Alexa Fluor 647 was used as secondary antibody. H. The 15 % polyacrylamide gel applied for WB was stained using coomassie brilliant blue to examine the protein transfer. The ladder was successfully transferred; however, protein bands were still apparent in the gel.

In Figure 17, well 1 contained prestained protein ladder SM0671. Well 2-4 contained cell lysate of OLN-AS cells transfected with p25α-pcDNA3.1/Zeo(-) in glass vials using the FuGENE:plasmid ratio 5:1. This was the case for both membranes in Figure 17. In Figure 17I, the primary antibody anti-α-tubulin (1:700 dilution) was applied. α-tubulin was identified at approximately 55 kDa. In Figure 17J, the primary antibody anti-TPPP (1:500 dilution) was applied. The secondary antibody Alexa fluor 647 (1:3000 dilution) was applied on both membranes. The expression of p25α could not be verified.

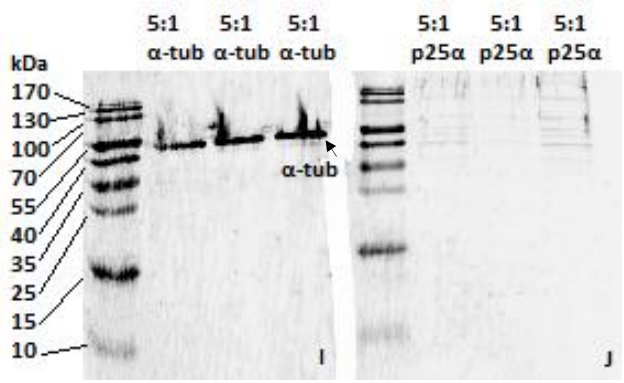

Figure 17: Verification of expression of α-tubulin and p25α in OLN-AS cells transiently transfected with p25α-pcDNA3.1/Zeo(-) vector. I. Western blot with 1:700 Rbt anti-α-tubulin. From left to right, well 1 contained SM0671 protein ladder and well 2-4 contained cell lysate of OLN-AS cells transfected with a FuGENE:p25α-pcDNA3.1/Zeo(-) ratio of 5:1. α-tubulin was identified at approximately 55 kDa. J. Western blot with 1:500 Rbt anti-TPPP (Sigma-Aldrich). From left to right, well 1 contained SM0671 protein ladder and well 2-4 contained cell lysate of OLN-AS cells transfected with a FuGENE:p25α-pcDNA3.1/Zeo(-) ratio of 5:1. 1:3000 Alexa Fluor 647 was used as secondary antibody.

4.3 XTT Cell Viability Assay

The absorbance was measured at 450, 490 and 690 nm, as the absorbance maximum of formazan is between 440-490 nm. The measurements values at 450 nm and 490 nm did not differ considerably, so only the 490 nm measurements were applied for further analysis. Light spreading from the cells was measured at 690 nm and this value was subtracted from the absorbance value measured at 490 nm. See section 2.3.2 XTT Cell Viability Assay. Raw data can be found in appendix A.3.

4.3.1 Examination of Sensitivity of XTT Assay

The sensitivity of the XTT assay was examined by seeding an increasing number of cells, followed by an absorbance measurement. The resolution of the assay was between 2000-4000 cells as the standard deviation overlapped for some of measurements, i.e. a difference in viability in different samples can be viewed when an increase or decrease of at least 2000-4000 cells was present. See Figure 18.

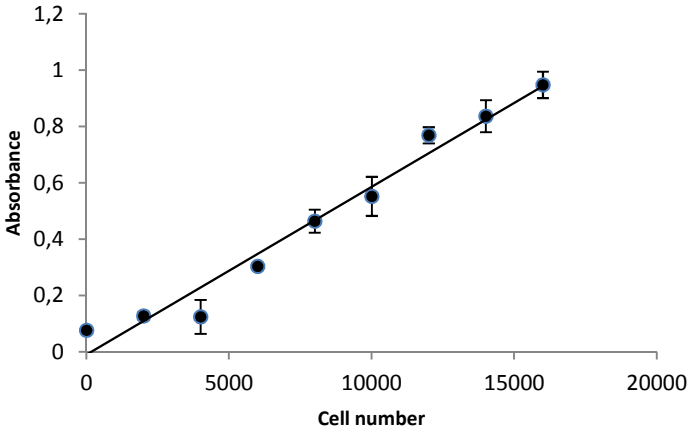

Figure 18: Sensitivity examination of the XTT assay. The absorbance of formazan for an increasing number of cells was measured.

4.3.2 Examination of Cytotoxicity of 56 Inhibitor Compounds

The experiment was repeated four times. The first two experiments were performed by PhD student Anders Dahl Knudsen and the handling of raw data and calculations were performed by both Anders Dahl Knudsen and the undersigned. The Excel data sheet with calculations can be found in appendix A.7. The viability data

were standardized according to the control samples containing colorless media, assigned “nul”, or colorless media and DMSO, assigned “DMSO”. The inhibitors were dissolved in DMSO, thus DMSO was used as a control. Since there was no considerable difference in the absorbance measurements of the control wells containing either DMSO or entirely media, these were applied as one single pool of control data for normalization. The inhibitor compounds ASI1D and baicalein were applied in the two last replicates. EGCG was applied in the last replicate. The 56 different CureND inhibitor compounds numbered from 1-59 were tested. Compound no. 14, 38, and 59 were not accessible for examination.

An overview of the relative viability plot of OLN-AS cells treated with 10 μM ASI1D, 100 μM baicalein, 13.5 μM EGCG or 10 μM CureND α-syn aggregation inhibitor compound can be viewed in Figure 19 and a more readable total plot can be found in appendix A.8. The CureND inhibitor leads have been sorted into three plots according to decreasing relative viability of the OLN-AS cells, when treated with these compounds, cf. Figure 20, Figure 21, and Figure 22. These three smaller plots enable a more detailed view of each tested compound. The 24 “non-toxic” compounds were selected with the requirement that the viability was minimum 100 % for at least two replicates and that no replicate was below 70 % viability, taking the coefficient of variation (CV or relative standard deviation) into account. The compounds resulting in cell viability of less than 75 % for at least two replicates and where one of the two other replicates did not exceed 100 % have been classified as “toxic”. The remaining compounds have been classified as “discrepant”. Detailed information on the relative viability of the different inhibitor compounds can be found in Table 6, Table 7 and Table 8. The relative viability was calculated by dividing the average absorbance values of the samples with the average control sample absorbance values. This was conducted for each separate 96 well culture plate to avoid possible plate-to-plate deviations. The average standard deviation values of the samples were divided by the average control sample values to calculate the coefficients of variation (relative standard deviations), which are displayed as error bars in Figure 20, Figure 21, and Figure 22. The measurements illustrated with circles have p-values below 0.05.

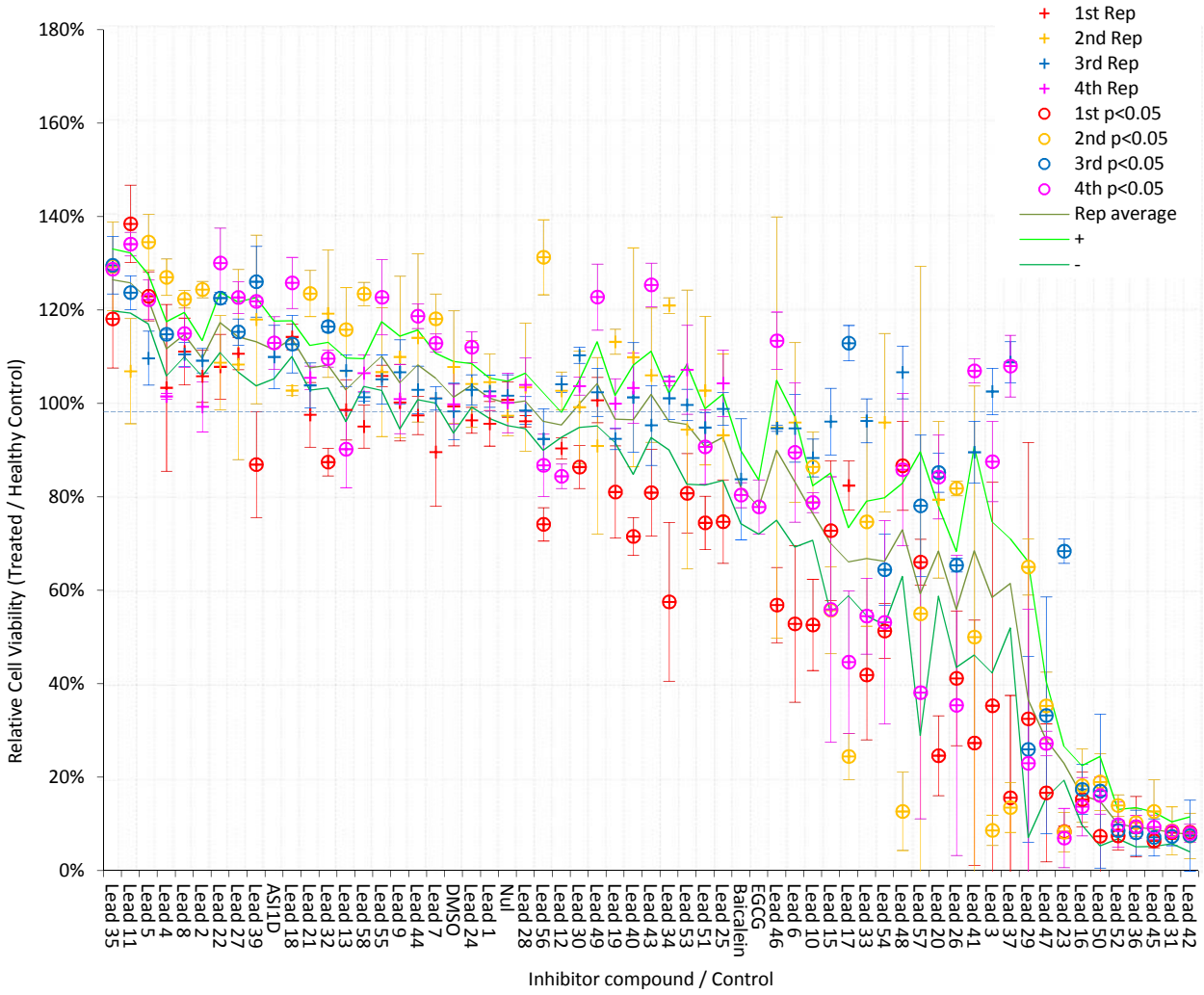

Figure 19: Comparison of four replicate experiments testing the relative viability of OLN-AS cells treated with 10 µM ASI1D, 13.5 µM EGCG, 100 µM baicalein or 10 µM CureND α-syn aggregation inhibitor compound. The relative viability is expressed in percentages. Overview of all tested inhibitor compounds. The crosses designate the four different replicate experiments, while the circles designate the samples with $p<0.05$. The middle green line represents the average mean of all four replicates, while the upper light (+) and lower dark (-) green lines represent the average relative viability +/- the average coefficient of variation (relative standard deviation).

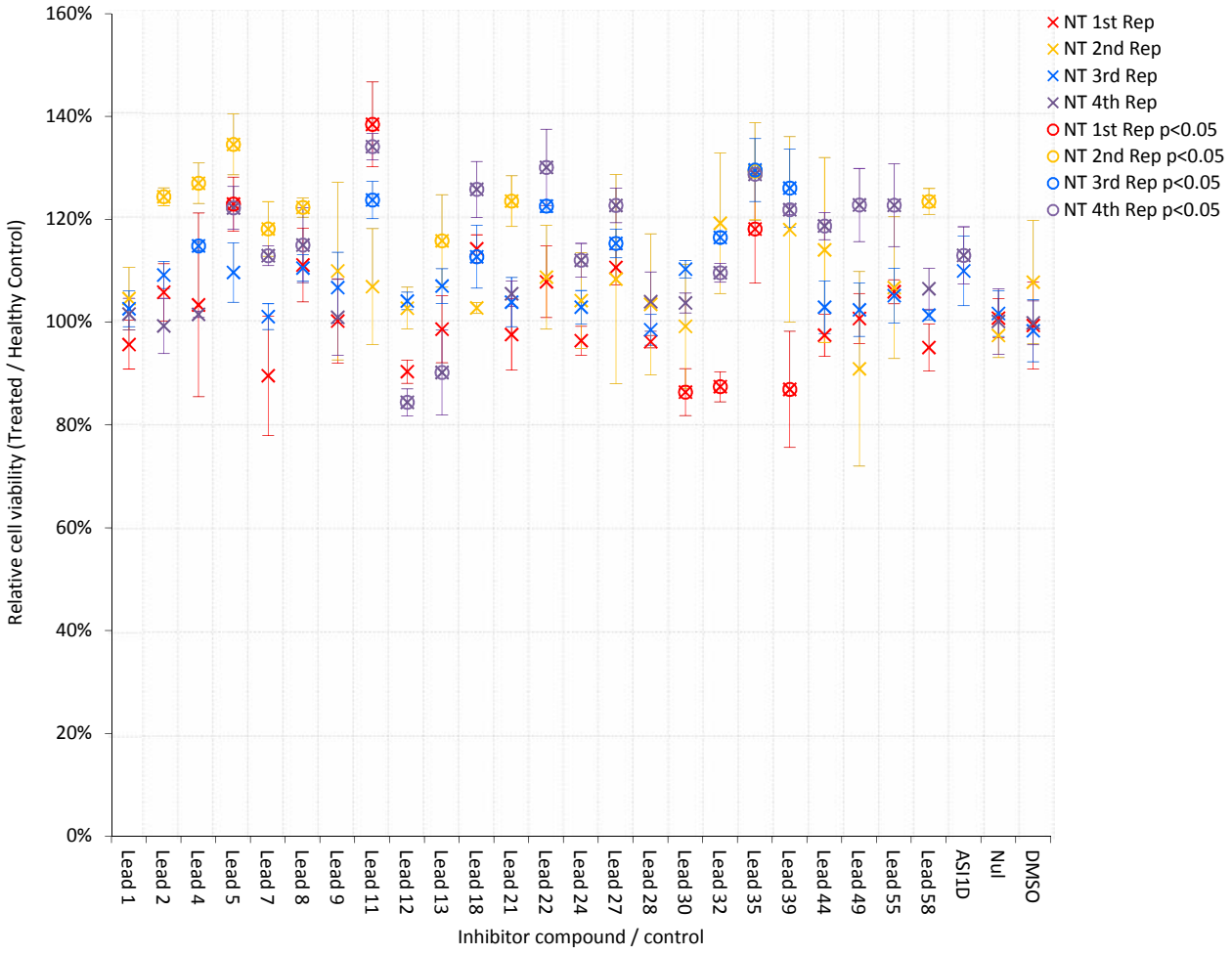

Figure 20: The relative viability of OLN-AS cells treated with 10 µM ASI1D or 10 µM CureND α-syn aggregation inhibitor compound. The relative viability is expressed in percentages. The NT designates that the compounds are non-toxic towards the OLN-AS cells. The 24 non-toxic compounds were selected with the requirement that the viability was minimum 100 % for at least two replicates and that no replicate was below 70 % viability, taking the coefficient of variation (CV or relative standard deviation) into account. The crosses designate the four different replicate experiments, while the circles designate the samples with $p<0.05$.

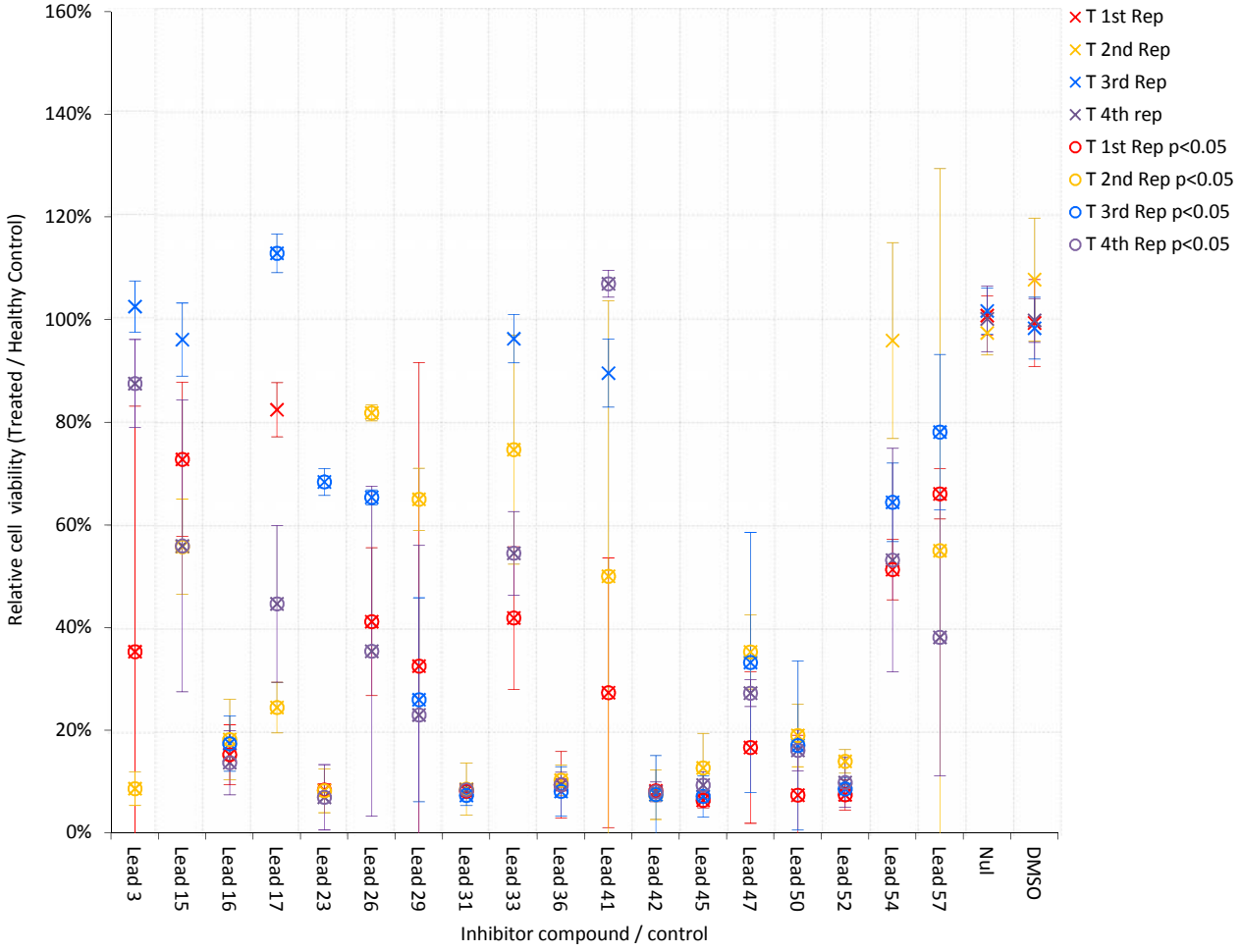

Figure 21: The relative viability of OLN-AS cells treated with 10 µM CureND α-syn aggregation inhibitor compound. The relative viability is expressed in percentages. The T designates that the compounds were toxic towards the OLN-AS cells. The compounds resulting in cell viability of less than 75 % for at least two replicates and where one of the two other replicates did not exceed 100 % were classified as "toxic". The crosses designate the four different replicate experiments, while the circles designate the samples with $p<0.05$.

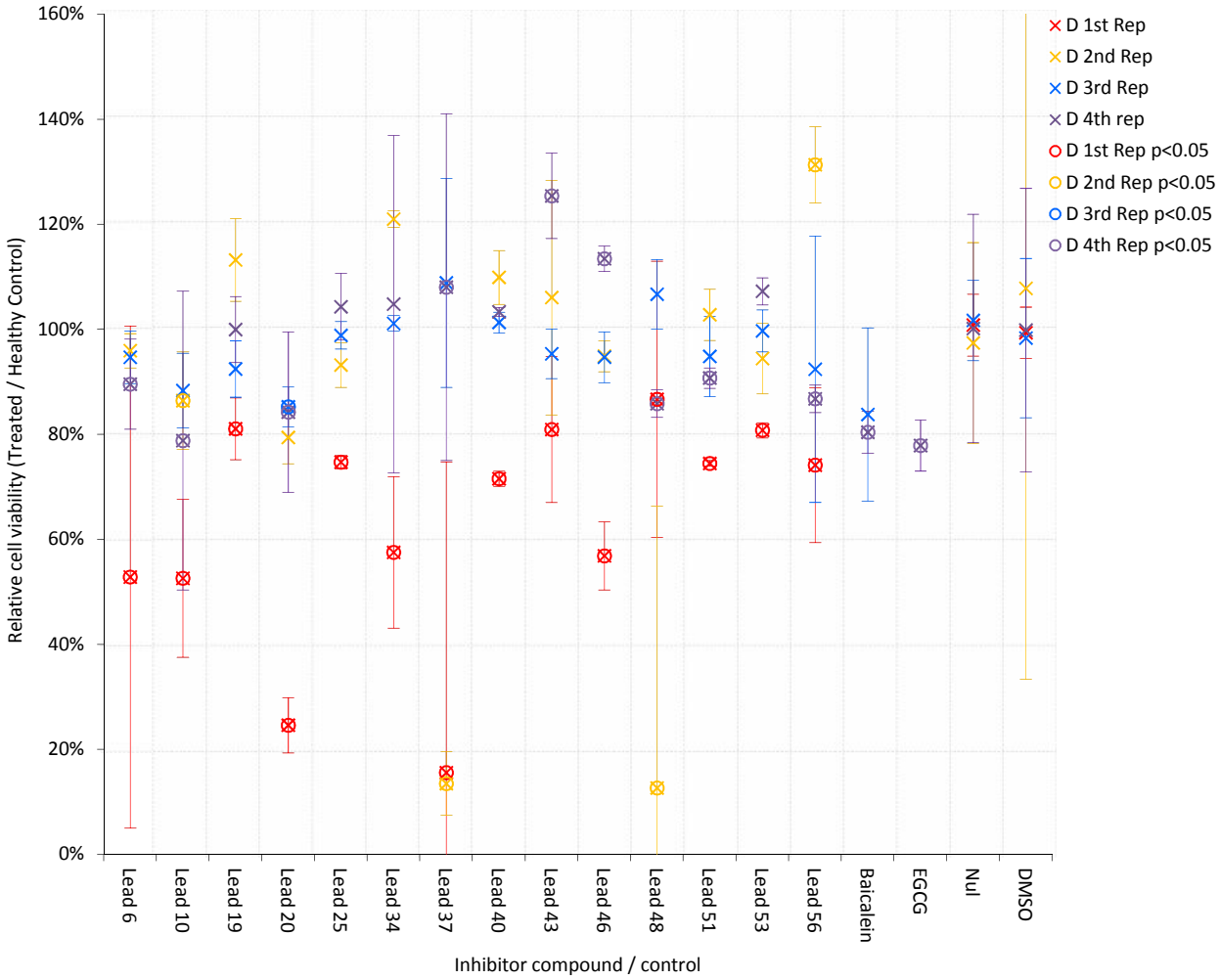

Figure 22: The relative viability of OLN-AS cells treated with 100 µM baicalein, 13.5 µM EGCG or 10 µM CureND α-syn aggregation inhibitor compound. The relative viability is expressed in percentages. The D designates that the compounds are discrepant. These compounds did not fulfill the requirements of being non-toxic or toxic towards the OLN-AS cells. The crosses designate the four different replicate experiments, while the circles designate the samples with $p<0.05$.

Table 6: The relative viability of OLN-AS cells treated with the non-toxic CureND α-syn aggregation inhibitor leads and ASI1D, respectively, in a concentration of 10 µM. The relative viability including the coefficient of variation and calculated p-value from each of the four replicate experiments are displayed. The p-value states the probability of the true value being equal to 100 % viability with a significance level of 0.05.

Non-toxic leads	1st replicate	p-value	2nd replicate	p-value	3rd replicate	p-value	4th replicate	p-value
Lead 1	96% ± 5%	0.47	105% ± 6%	0.28	103% ± 3%	0.66	102% ± 3%	0.70
Lead 2	106% ± 6%	0.34	124% ± 2%	3.84E-03	109% ± 3%	0.13	99% ± 5%	0.86
Lead 4	103% ± 18%	0.63	127% ± 4%	4.78E-06	115% ± 1%	0.02	102% ± 1%	0.70
Lead 5	123% ± 5%	4.78E-03	135% ± 6%	4.44E-07	110% ± 6%	0.12	122% ± 4%	2.89E-05
Lead 7	90% ± 12%	0.11	118% ± 5%	4.29E-04	101% ± 3%	0.85	113% ± 2%	4.13E-03
Lead 8	111% ± 7%	0.08	122% ± 2%	3.04E-05	111% ± 3%	0.09	115% ± 7%	2.43E-03
Lead 9	100% ± 8%	0.97	110% ± 17%	0.10	107% ± 7%	0.28	101% ± 7%	0.83
Lead 11	138% ± 8%	3.36E-06	107% ± 11%	0.16	124% ± 4%	8.79E-04	134% ± 3%	6.33E-08
Lead 12	90% ± 2%	0.12	103% ± 4%	0.50	104% ± 2%	0.49	84% ± 3%	8.94E-04
Lead 13	99% ± 6%	0.82	116% ± 9%	2.90E-03	107% ± 3%	0.24	90% ± 8%	0.03
Lead 18	114% ± 3%	0.07	103% ± 1%	0.77	113% ± 6%	4.97E-03	126% ± 5%	5.87E-05
Lead 21	98% ± 7%	0.76	124% ± 5%	0.03	104% ± 5%	0.32	106% ± 3%	0.27
Lead 22	108% ± 7%	0.32	109% ± 10%	0.38	123% ± 1%	1.14E-05	130% ± 7%	1.46E-05
Lead 24	96% ± 3%	0.64	104% ± 9%	0.67	103% ± 3%	0.44	112% ± 3%	0.02
Lead 27	111% ± 3%	0.17	108% ± 20%	0.44	115% ± 3%	6.99E-04	123% ± 3%	1.83E-04
Lead 28	96% ± 1%	0.36	103% ± 14%	0.73	99% ± 3%	0.69	104% ± 6%	0.43
Lead 30	86% ± 5%	4.57E-03	99% ± 12%	0.93	110% ± 2%	0.11	104% ± 2%	0.23
Lead 32	87% ± 3%	7.21E-03	119% ± 14%	0.08	116% ± 1%	0.01	110% ± 2%	5.69E-03
Lead 35	118% ± 11%	1.95E-03	129% ± 9%	0.01	130% ± 6%	2.40E-04	129% ± 1%	4.50E-08
Lead 39	87% ± 11%	0.01	118% ± 18%	0.12	126% ± 8%	8.85E-04	122% ± 1%	1.72E-06
Lead 44	97% ± 4%	0.62	114% ± 18%	0.08	103% ± 5%	0.44	119% ± 3%	8.48E-05
Lead 49	101% ± 5%	0.89	91% ± 19%	0.18	102% ± 5%	0.53	123% ± 7%	6.04E-05
Lead 55	106% ± 2%	0.26	107% ± 14%	0.27	105% ± 5%	0.19	123% ± 8%	9.24E-05
Lead 58	95% ± 5%	0.35	123% ± 3%	1.24E-04	101% ± 1%	0.70	107% ± 4%	0.08
ASI1D					110% ± 7%	0.08	113% ± 6%	5.47E-03

Table 7: The relative viability of OLN-AS cells treated with the toxic CureND α-syn aggregation inhibitor leads in a concentration of 10 μM. The relative viability including the coefficient of variation and calculated p-value from each of the four replicate experiments are displayed. The p-value states the probability of the true value being equal to 100 % viability with a significance level of 0.05.

Toxic leads	1st replicate			p-value	2nd replicate			p-value	3rd replicate			p-value	4th replicate			p-value
Lead 3	35%	±	48%	2.01E-09	9%	±	3%	6.82E-14	103%	±	5%	0.67	88%	±	9%	8.19E-03
Lead 15	73%	±	15%	2.04E-03	56%	±	9%	2.62E-04	96%	±	7%	0.52	56%	±	28%	3.58E-07
Lead 16	15%	±	6%	1.32E-10	18%	±	8%	1.92E-07	17%	±	5%	1.10E-13	14%	±	6%	2.48E-13
Lead 17	82%	±	5%	0.07	24%	±	5%	5.45E-07	113%	±	4%	3.09E-03	45%	±	15%	1.07E-09
Lead 23	8%	±	1%	2.94E-11	8%	±	4%	3.99E-08	68%	±	3%	1.68E-07	7%	±	6%	6.34E-14
Lead 26	41%	±	14%	1.01E-07	82%	±	2%	1.67E-02	65%	±	1%	4.59E-08	35%	±	32%	2.33E-10
Lead 29	32%	±	59%	4.04E-09	65%	±	6%	2.28E-03	26%	±	20%	1.25E-12	23%	±	33%	1.56E-13
Lead 31	8%	±	1%	1.30E-13	9%	±	5%	8.41E-07	7%	±	2%	5.34E-11	8%	±	1%	1.14E-15
Lead 33	42%	±	14%	2.93E-10	75%	±	22%	0.03	96%	±	5%	0.55	54%	±	8%	1.26E-10
Lead 36	9%	±	6%	1.61E-13	10%	±	3%	1.00E-06	8%	±	5%	6.08E-11	9%	±	2%	1.36E-15
Lead 41	27%	±	26%	4.62E-10	50%	±	54%	1.63E-03	90%	±	7%	0.11	107%	±	3%	0.04
Lead 42	8%	±	1%	1.30E-13	7%	±	5%	7.55E-07	7%	±	8%	5.50E-11	8%	±	2%	1.07E-15
Lead 45	6%	±	1%	3.07E-09	13%	±	7%	5.14E-10	7%	±	4%	1.22E-14	9%	±	3%	5.23E-13
Lead 47	17%	±	15%	1.06E-08	35%	±	7%	1.14E-08	33%	±	25%	1.40E-11	27%	±	3%	8.80E-12
Lead 50	7%	±	12%	3.46E-09	19%	±	6%	1.09E-09	17%	±	16%	9.06E-14	16%	±	4%	1.43E-12
Lead 52	7%	±	3%	3.45E-09	14%	±	2%	5.83E-10	9%	±	2%	1.56E-14	10%	±	5%	5.65E-13
Lead 54	51%	±	6%	1.81E-06	96%	±	19%	0.55	64%	±	8%	4.31E-08	53%	±	22%	7.85E-08
Lead 57	66%	±	5%	4.43E-05	55%	±	74%	5.48E-03	78%	±	15%	1.35E-04	38%	±	27%	1.50E-09

Table 8: The relative viability of OLN-AS cells treated with the discrepant CureND α-syn aggregation inhibitor leads in a concentration of 10 µM. It could not be stated whether the compounds were toxic or non-toxic. 100 µM baicalein and 13.5 µM EGCG were also tested. The relative viability including the coefficient of variation and calculated p-value from each of the four replicate experiments are displayed. The p-value states the probability of the true value being equal to 100 % viability with a significance level of 0.05.

Discrepant leads	1st replicate	p-value	2nd replicate	p-value	3rd replicate	p-value	4th replicate	p-value
Lead 6	53% ± 17%	1.02E-07	96% ± 17%	0.45	95% ± 7%	0.38	90% ± 15%	0.04
Lead 10	53% ± 10%	6.25E-08	86% ± 7%	4.49E-03	88% ± 4%	0.06	79% ± 2%	3.52E-05
Lead 19	81% ± 10%	0.02	113% ± 3%	0.18	92% ± 2%	0.05	100% ± 5%	0.99
Lead 20	25% ± 9%	1.18E-09	79% ± 17%	0.05	85% ± 4%	9.92E-04	84% ± 9%	5.74E-03
Lead 25	75% ± 9%	3.11E-03	93% ± 17%	0.51	99% ± 3%	0.75	104% ± 7%	0.41
Lead 34	58% ± 17%	7.98E-08	121% ± 2%	0.36	101% ± 5%	0.86	105% ± 1%	0.13
Lead 37	16% ± 22%	6.29E-13	13% ± 5%	1.41E-06	109% ± 4%	0.17	108% ± 7%	0.03
Lead 40	72% ± 4%	3.21E-06	110% ± 23%	0.41	101% ± 12%	0.85	103% ± 8%	0.35
Lead 43	81% ± 9%	5.13E-03	106% ± 14%	0.55	95% ± 9%	0.47	125% ± 5%	7.52E-07
Lead 46	57% ± 8%	6.54E-06	95% ± 45%	0.71	95% ± 1%	0.15	113% ± 6%	3.00E-03
Lead 48	87% ± 10%	0.03	13% ± 8%	5.20E-10	107% ± 6%	0.10	86% ± 16%	0.01
Lead 51	74% ± 6%	4.72E-04	103% ± 16%	0.66	95% ± 3%	0.17	91% ± 8%	0.03
Lead 53	81% ± 8%	4.47E-03	94% ± 30%	0.56	100% ± 3%	0.93	107% ± 10%	0.10
Lead 56	74% ± 4%	3.71E-04	131% ± 8%	7.98E-05	92% ± 6%	0.06	87% ± 7%	2.54E-03
Baicalein					84% ± 13%	0.10	80% ± 3%	7.27E-06
EGCG							78% ± 6%	2.17E-05

In general, some deviations between the four replicate experiments can be seen. Although, lead 16, 31, 36, 42, 45, 50, and 52 were clearly toxic in a concentration of 10 µM as the relative viability was below 20 % in all four replicates with these compounds and the p-value was maximum $1 \cdot 10^{(-6)}$ for these samples, cf. Figure 21 and Table 7.

The known inhibitor ASI1D (10 µM) showed properties as a non-toxic compound in the two replicates with a relative cell viability of 110 % ± 7 % and 113 % ± 6 % with a p-value of 0.005 in the last replicate. Baicalein (100 µM) was included in the two replicate experiments showing a relative cell viability of 84 % ± 13 % and 80 % ± 3 % with a p-value of $7.27 \cdot 10^{(-6)}$ in the last replicate, having a cytotoxicity towards the OLN-AS cells of 16 % and 20 %. EGCG (13.5 µM) was included in one experiment and showed a relative cell viability of 78 % ± 6 % with a p-value of $2.17 \cdot 10^{(-5)}$.

4.3.3 Inhibitory Effect of Known α-Syn Aggregation Inhibitors EGCG, Baicalein, and ASI1D

The viability data were standardized according to the control samples containing colorless media, assigned "untreated", or colorless media and DMSO, assigned "DMSO". The inhibitors were dissolved in DMSO, thus DMSO was used as a control. Since there was no considerable difference in the absorbance measurements of the control wells containing either DMSO or entirely media, these were applied as one single pool of control data for normalization. The remaining control samples contained media and FuGENE® HD transfection reagent ("fuGENE") or media, FuGENE® HD transfection reagent and DMSO (fuGENE+DMSO) or media, FuGENE® HD transfection reagent and p25α-pcDNA3.1/Zeo(-) plasmid ("fuGENE+p25α") or media, FuGENE® HD transfection reagent, DMSO and p25α-pcDNA3.1/Zeo(-) plasmid ("fuGENE+p25α+DMSO"). The samples assigned "pre. 27 µM EGCG", "pre. 13.5 µM EGCG", "pre. 10 µM ASI1D" and "pre. 100 µM baicalein" contained 27 µM EGCG, 13.5 µM EGCG, 10 µM ASI1D, and 100 µM baicalein together with media, FuGENE® HD transfection reagent and p25α-pcDNA3.1/Zeo(-) plasmid.

In Figure 23, the relative viability of OLN-AS cells treated with 10 µM ASI1D, 27 µM EGCG, 13.5 µM EGCG, and 100 µM baicalein one hour prior to transfection with p25α-pcDNA3.1/Zeo(-) can be viewed. No considerable difference in viability can be seen in the control samples. The control sample containing FuGENE+p25α was expected to lead to a decrease in cell viability with an expression of p25α, but this was not the case, which may be due to a poor transfection of the OLN-AS cells. When adding 100 µM baicalein to the cells, the cell viability decreased to approximately 45 %, which indicated that baicalein was cytotoxic when present together with FuGENE. The control cell line OLN-t40 was not applied in this experiment due to cell death caused by improper thawing.

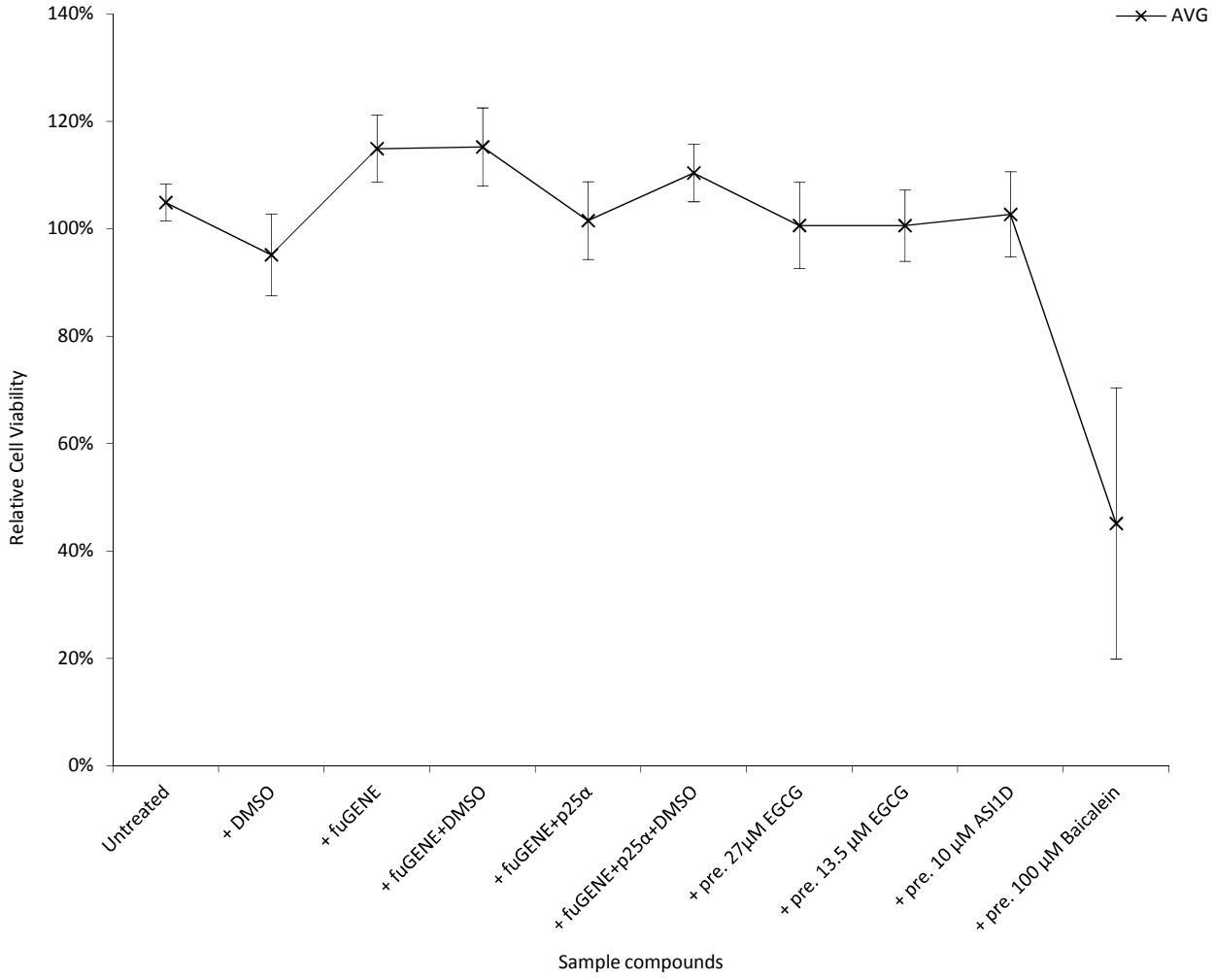

Figure 23: The relative cell viability of p25α-pcDNA3.1Zeo(-) transfected OLN-AS cells treated with 10 µM ASI1D, 27 µM EGCG, 13.5 µM EGCG, or 100 µM baicalein one hour prior to transfection. The plot shows the viability in percentages.

In Figure 24, the relative viability of OLN-AS and OLN-t40 cells treated with 15 µM, 10 µM, 5 µM, and 2 µM ASI1D one hour prior to transfection with p25α-pcDNA3.1/Zeo(-) can be viewed. The control sample containing FuGENE+DMSO showed a slight decrease in viability for the OLN-t40 control cell line. The OLN-t40 cell line should not be affected by the p25α transfection, since they do not express α-syn. ASI1D may have a rescuing influence on the p25α transfected OLN-AS cells, as the relative viability slightly increased with a decreasing concentration of ASI1D. Although, nothing conclusive can be stated about these data, since the relative standard deviations (coefficient of variation) of the inhibitor containing samples overlap. Also, the viability is in the 98-105 % range, except for the FuGENE+DMSO control sample with the

OLN-t40 cells, which may be caused by experimental errors. The OLN-t40 cells generally showed a higher viability compared to the OLN-AS cell line.

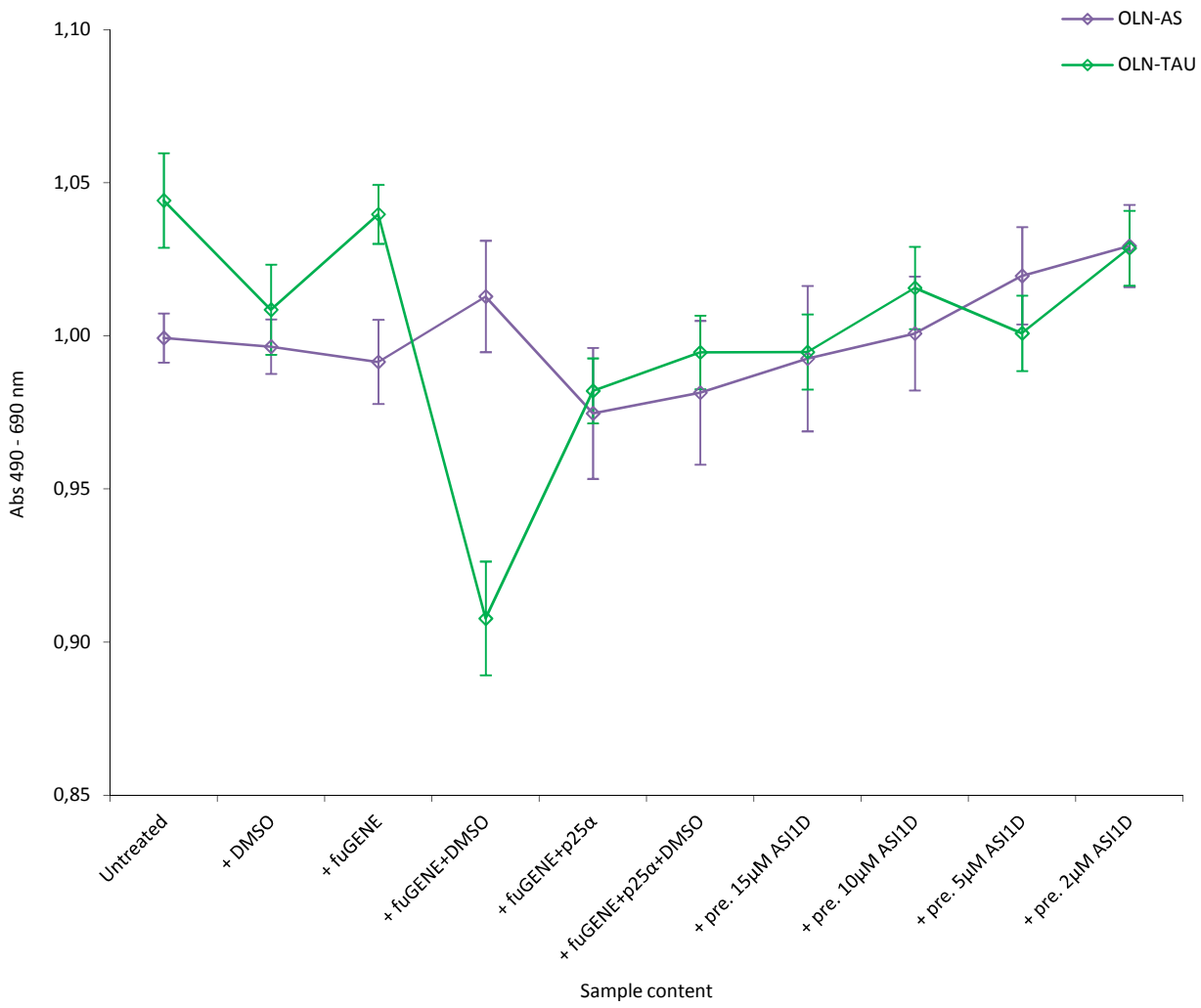

Figure 24: The absorbance measurements of p25α-pcDNA3.1Zeo(-) transfected OLN-AS and OLN-t40 cells treated with 15 µM, 10 µM, 5 µM, and 2 µM ASI1D one hour prior to transfection. The absorbance measurement at 690 nm was subtracted from the measurement at 490 nm. The data have been normalized.

4.4 Inducible Expression System

4.4.1 Cloning of the pEGSH Expression Vector

The pEGSH vector was cloned and purified using the Plasmid DNA Purification kit (Nucleospin® Plasmid, Macherey Nagel). The pEGSH vector concentration was measured, see Table 9.

Table 9: Concentration measurement using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

pEGSH sample	ng/µL	260/280	260/230
1	139.1	1.91	2.35
2	61.6	1.95	2.45
3	107.9	1.90	2.37
4	107.07	1.93	2.41
5	120.7	1.90	2.39
6	121.1	1.93	2.31
7	111.7	1.88	2.38
8	96.9	1.91	2.30
9	120.3	1.92	2.17
10	115.8	1.87	2.23

The plasmid DNA was additionally concentrated to enable further experiments and sample 1, 3, 4, 8 and 10 in Table 10 were pooled to one sample containing 10.98 µg DNA in 43 µL (0.255 µg/µL) and used in the subsequent experiments.

Table 10: Concentration measurement of the concentrated DNA samples using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

pEGSH sample	ng/µL	260/280	260/230
1	266.7	1.85	2.13
3	297.6	1.82	2.15
4	249.9	1.84	2.09
5	157.0	1.77	1.97
6	137.1	1.80	1.84
7	185.0	1.81	1.99
8	217.2	1.83	2.02
9	197.9	1.79	2.06
10	237.3	1.81	2.10

4.4.2 Restriction Enzyme Digestion of p25α-pAcGFP1-N1 Vector

Matching restriction sites between the pEGSH vector and the p25α-pAcGFP1-N1 vector were acquired, cf. appendix B.2 Restriction Site Analysis. FastDigest restriction enzymes *Eco*RI, *Bam*HI, *Mun*I (*Mfe*I) and *Not*I (Fermentas) were applied in the experiment. The length of the theoretical restriction enzyme digestion fragments can be viewed in Table 11.

Table 11: Length of calculated/theoretical restriction enzyme digestion fragments. The length of these fragments was calculated from the vector sequences in appendix B.2 Restriction Site Analysis.

Vector/Insert	Fragment Size (bp)
p25α-pAcGFP1-N1 digested with *Eco*RI and *Bam*HI	
-	4695
p25α insert	670
pEGSH digested with *Mun*I and *Bam*HI	
pEGSH for p25α	4818
-	11
p25α-pAcGFP1-N1 digested with *Eco*RI and *Not*I	
-	3961
p25α-GFP insert	1404
pEGSH digested with *Mun*I and *Not*I	
pEGSH for p25α-GFP	4802
-	27

4.4.3 Agarose Gel Separation of RE Digests and DNA Extraction

The restriction enzyme digests were separated on agarose gels to visualize the location of the different digests for later extraction. Figure 25 shows a 2.5 % agarose gel with restriction enzyme digests of p25α-pAcGFP1-N1 vector digested with *Eco*RI and *Bam*HI. Well 1 and 8 contained a 50 bp and 1 kbp ladder, respectively. Well 2 contained the p25α-pAcGFP1-N1 without enzymes (control). Well 3-5 contained a total of 6 µg p25α-pAcGFP1-N1 vector digested with 3 µL FastDigest *Eco*RI and 3 µL FastDigest *Bam*HI distributed into the three wells. Well 6-7 contained a total of 4 µg p25α-pAcGFP1-N1 vector digested with 4 µL FastDigest *Eco*RI and 4 µL FastDigest *Bam*HI distributed into the two wells. The p25α insert of 670 bp was apparent in well 3-7 just below 750 bp. The digest of 4695 bp can also be viewed below 8000 bp in well 3-7. Furthermore, a weak band which was apparent in well 2-7 just above 600 bp was a contaminant in the p25α-pAcGFP1-N1 vector sample. The undigested control vector can be viewed just below 8000 bp in well 2.

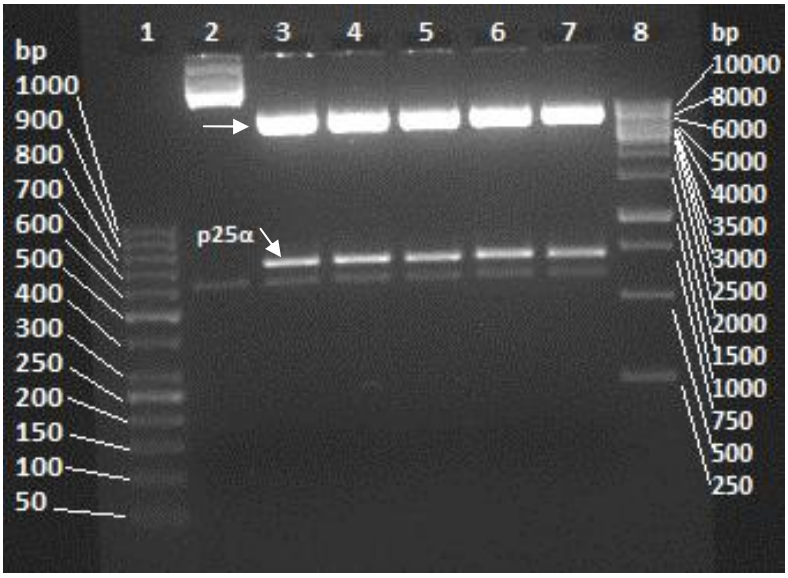

Figure 25: 2.5 % Agarose gel with restriction enzyme digests of p25α-pAcGFP1-N1 vector digested with *Eco*RI and *Bam*HI for obtaining the p25α insert. Well 1: 50 bp ladder. Well 2: 2 µg p25α-pAcGFP1-N1 vector without enzymes. Well 3-5: a total of 6 µg p25α-pAcGFP1-N1 vector digested with 3 µL FastDigest *Eco*RI and 3 µL FastDigest *Bam*HI. Well 6-7: a total of 4 µg p25α-pAcGFP1-N1 vector digested with 4 µL FastDigest *Eco*RI and 4 µL FastDigest *Bam*HI. Well 8: 1kbp ladder. The p25α insert of 670 bp was apparent in well 3-7 just below 750 bp. The digest of 4695 bp can also be viewed. Furthermore, a weak band which was apparent in well 2-7 just above 600 bp was a contaminant in the p25α-pAcGFP1-N1 vector sample. The undigested control vector of 5365 bp can be viewed just below 8000 bp in well 2.

Figure 26 shows a 1 % agarose gel with restriction enzyme digests of p25α-pAcGFP1-N1 vector digested with *Eco*RI and *Not*I. Well 1 and 6 contained a 1 kbp ladder. Well 2-4 contained a total of 6 µg p25α-pAcGFP1-N1 vector digested with 3 µL FastDigest *Eco*RI and 3 µL FastDigest *Not*I distributed into the three wells. Well 5 contained the p25α-pAcGFP1-N1 without enzymes (control). The p25α-GFP insert of 1404 bp was apparent in well 2-4 just below 1500 bp. The digest of 3961 bp can also be viewed in well 2-4 around 4000 bp. Furthermore, a weak band which was apparent in well 2-4 just above 500 bp is a contaminant in the p25α-pAcGFP1-N1 vector sample. The undigested control vector can be viewed just below 5000 bp in well 5.

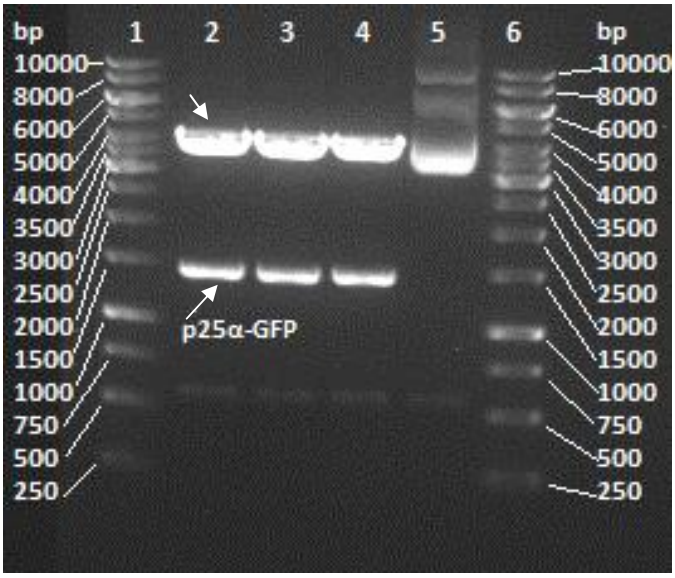

Figure 26: 1 % Agarose gel with restriction enzyme digests of p25α-pAcGFP1-N1 vector digested with *Eco*RI and *Not*I. Well 1: 1 kbp ladder. Well 2-4: a total of 6 µg p25α-pAcGFP1-N1 vector digested with 3 µL FastDigest *Eco*RI and 3 µL FastDigest *Not*I. Well 5: 2 µg p25α-pAcGFP1-N1 vector without enzymes. Well 6: 1 kbp ladder. The p25α-GFP insert of 1404 bp was apparent in well 2-4 just below 1500 bp. The digest of 3961 bp can also be viewed. Furthermore, a weak band which was apparent in well 2-4 just above 500 bp was a contaminant in the p25α-pAcGFP1-N1 vector sample. The undigested control vector of 5365 bp can be viewed just below 5000 bp in well 5.

Figure 27 shows a 1 % agarose gel with restriction enzyme digests of the pEGSH vector digested with *Mun*I and *Bam*HI for the p25α construct and with *Mun*I and *Not*I for the p25 α-GFP construct. Well 1 contained a 1 kbp ladder. Well 2-3 contained a total of 3 µg pEGSH vector digested with 1.5 µL FastDigest *Mun*I and 1.5 µL FastDigest *Bam*HI distributed into the two wells. Well 4 was blank. Well 5-6 contained a total of 3 µg pEGSH vector digested with 1.5 µL FastDigest *Mun*I and 1.5 µL FastDigest *Not*I distributed into the two wells. Well 7 contained 2µg pEGSH vector without enzymes (control). The pEGSH digests of 4818 bp and 4802 bp can be seen just below 6000 bp in well 2-3 and 5-6, respectively. The undigested control vector can be viewed around 3000 bp in well 7.

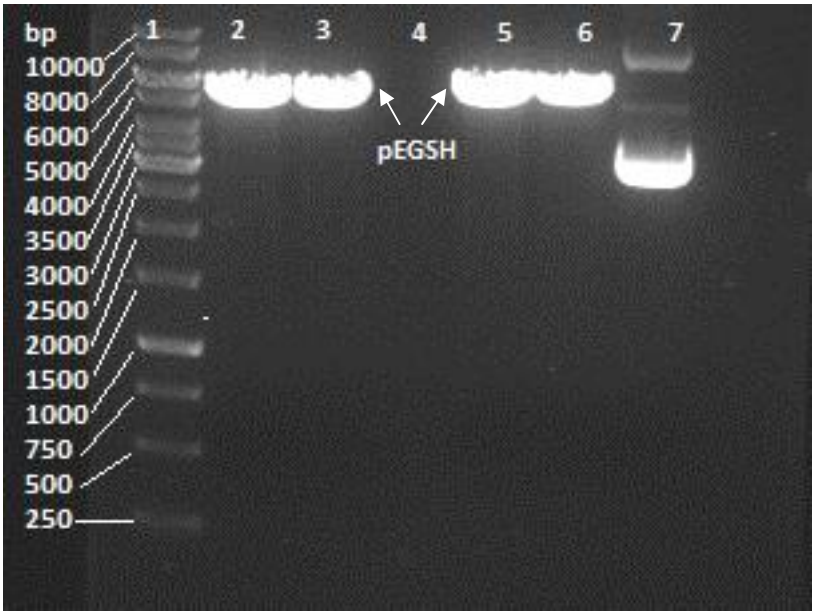

Figure 27: 1 % Agarose gel with restriction enzyme digests of pEGSH vector digested with *Mun*I and *Bam*HI for the p25α construct and with *Mun*I and *Not*I for the p25 α-GFP construct. Well 1: 1 kbp ladder. Well 2-3: a total of 3 µg pEGSH vector digested with 1.5 µL FastDigest *Mun*I and 1.5 µL FastDigest *Bam*HI. Well 4: Blank. Well 5-6: a total of 3 µg pEGSH vector digested with 1.5 µL FastDigest *Mun*I and 1.5 µL FastDigest *Not*I. Well 7: 2µg pEGSH vector without enzymes. The pEGSH digests of 4818 bp and 4802 bp can be seen just below 6000 bp. The undigested control vector of 4829 bp can be viewed around 3000 bp.

The p25α and p25α-GFP inserts together with the pEGSH digests for the two constructs were subsequently extracted from the gels for further analysis. The DNA concentration measurements of the extracted DNA can be seen in Table 12. The samples p25α insert BI, p25α-GFP insert, pEGSH for p25α and pEGSH for p25α-GFP were applied in the further experiments.

Table 12: DNA concentration measurement of the extracted restriction enzyme digest using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

DNA sample	ng/µL	260/280	260/230
p25α insert AI	7.1	1.63	7.13
p25α insert AII	5.0	1.63	0.05
p25α insert BI	4.9	1.77	3.21
p25α insert BII	5.3	1.55	1.35
p25α-GFP insert	18.1	1.86	2.28
pEGSH for p25α	33.0	1.89	2.91
pEGSH for p25α-GFP	31.9	1.85	1.06

4.4.4 Dephosphorylation of RE Digests of pEGSH

The calculations of the volumes of shrimp alkaline phosphatase necessary to dephosphorylate 500 ng vector can be found in appendix B.4 Calculations for Dephosphorylation and Ligation Experiment.
The pEGSH digests for the p25α and p25α-GFP insert, respectively, were dephosphorylated and the final product was 500 ng of each dephosphorylated pEGSH digest in 20 µL (25 ng/µL).

4.4.5 Ligation of pEGSH Digests and p25α/ p25α-GFP Inserts

Prior to the ligation of the pEGSH digests and the p25α and p25α-GFP inserts, respectively, the optimal amount of insert for the ligation was calculated, cf. appendix B.4 Calculations for Dephosphorylation and Ligation Experiments.

The ligation mix from the first ligation experiment was used for heat shock transformation of One Shot Top10 chemically competent cells (*E. coli*) where no colonies were formed after incubation overnight at 37 °C. Only the pUC19 control gave colonies.
After the second ligation experiment, the ligation mix was used for heat shock transformation of One Shot Top10 chemically competent cells (*E. coli*). Only colonies containing the p25α-pEGSH construct were present and not the p25α-GFP-pEGSH construct. The positive control, pUC19, gave colonies. The ligation mix containing the p25α-GFP-pEGSH construct was subsequently used for both heat shock transformation and electroporation of One Shot Top10 competent cells (*E. coli*), where only the electroporated *E. coli* cells gave colonies containing the p25α-GFP-pEGSH. The *E. coli* colonies containing the p25α-pEGSH and p25α-GFP-pEGSH construct, respectively, were further analyzed by colony PCR for verification.

4.4.6 Colony PCR and Agarose Gel Electrophoresis

The PCR products of colony PCR with colonies containing the p25α-pEGSH construct can be viewed in Figure 28. Well 1 and 14 contained a 50 bp and 1 kbp ladder, respectively. Well 2-5 contained four p25α-pEGSH construct colonies, where 2 mM $MgCl_2$ was applied in the PCR reaction. Well 6 and 12 contained negative controls with DNA water and 2 and 5 mM $MgCl_2$, respectively, applied in the PCR reaction. Well 7 and 13 contained positive controls with 2 and 5 mM $MgCl_2$, respectively, applied in the PCR reaction, DNA water and 1 µL pEGSH vector. Well 8-11 contained four p25α-pEGSH construct colonies, where 5 mM $MgCl_2$ was applied in the PCR reaction. In well 2-5 and 9, a clear band was apparent around 900 bp, which is consistent with the theoretical size of the p25α-pEGSH PCR product of 843 bp. The positive controls in well 7 and 13 displayed clear bands just below 200 bp, which is consistent with the theoretical size of the pEGSH PCR product of 184 bp. The negative controls did not show any bands.

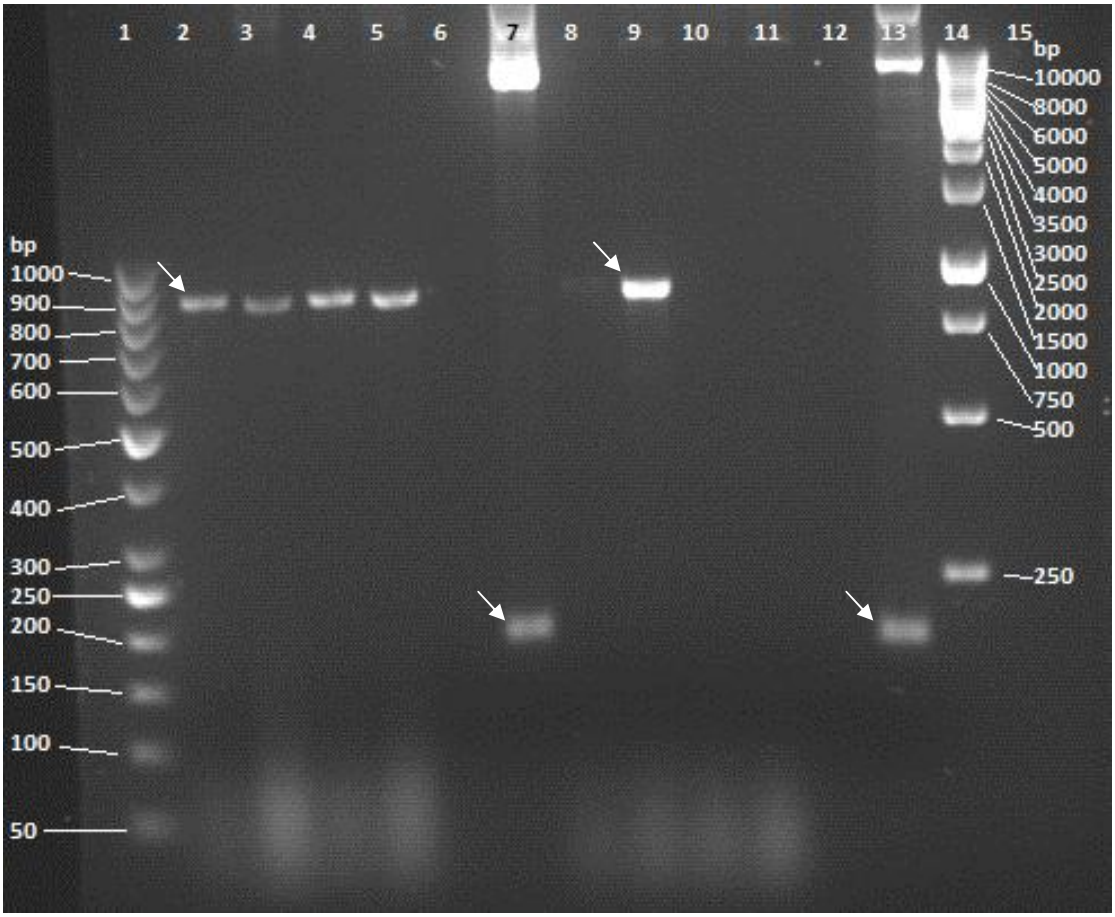

Figure 28: 2.5 % agarose gel displaying the PCR products of colony PCR with the colonies containing p25α-pEGSH. Well 1: 50 bp ladder. Well 2-5: Four colonies, A1-A4, respectively, transformed with the p25α-pEGSH ligation mix with 2 mM $MgCl_2$ applied in the PCR reaction. Well 6: Negative control with DNA water and 2 mM $MgCl_2$ applied in the PCR reaction. Well 7: Positive control with DNA water, 1 µL pEGSH vector and 2 mM $MgCl_2$ applied in the PCR reaction. Well 8-11: Four colonies, C1-C4, respectively, transformed with the p25α-pEGSH ligation mix with 5 mM $MgCl_2$ applied in the PCR reaction. Well 12: Negative control with DNA water and 5 mM $MgCl_2$ applied in the PCR reaction. Well 13: Positive control with DNA water, 1 µL pEGSH vector and 5 mM $MgCl_2$ applied in the PCR reaction. Well 14: 1 kbp ladder. In well 2-5 and 9, a clear band was apparent around 900 bp, which is consistent with the theoretical size of the p25α-pEGSH PCR product of 843 bp. The positive controls in well 7 and 13 displayed clear bands just below 200 bp, which is consistent with the theoretical size of the pEGSH PCR product of 184 bp. The negative controls did not show any bands.

In Figure 29, the PCR products of colony PCR with colonies containing the p25α-GFP-pEGSH construct can be viewed. Well 1 and 14 contained a 50 bp and 1 kbp ladder, respectively. Well 2-5 contained four p25α-GFP-pEGSH construct colonies, where 2 mM $MgCl_2$ was applied in the PCR reaction. Well 6 and 12 contained negative controls with DNA water and 2 and 5 mM $MgCl_2$, respectively, applied in the PCR reaction. Well 7 and 13 contained positive controls with 2 and 5 mM $MgCl_2$, respectively, applied in the PCR reaction, DNA water and 1 µL pEGSH vector. Well 8-11 contained four p25α-GFP-pEGSH construct colonies, where 5 mM $MgCl_2$ was applied in the PCR reaction. In well 2 and 3 a clear band was apparent just above 1500 bp, which is consistent with the theoretical size of the p25α-GFP-pEGSH PCR product of 1561 bp. The positive controls in well 7 and 13 and the colonies in well 4 and 11 displayed clear bands just below 200 bp, which is consistent with the theoretical size of the pEGSH PCR product of 184 bp. The negative controls did not show any bands.

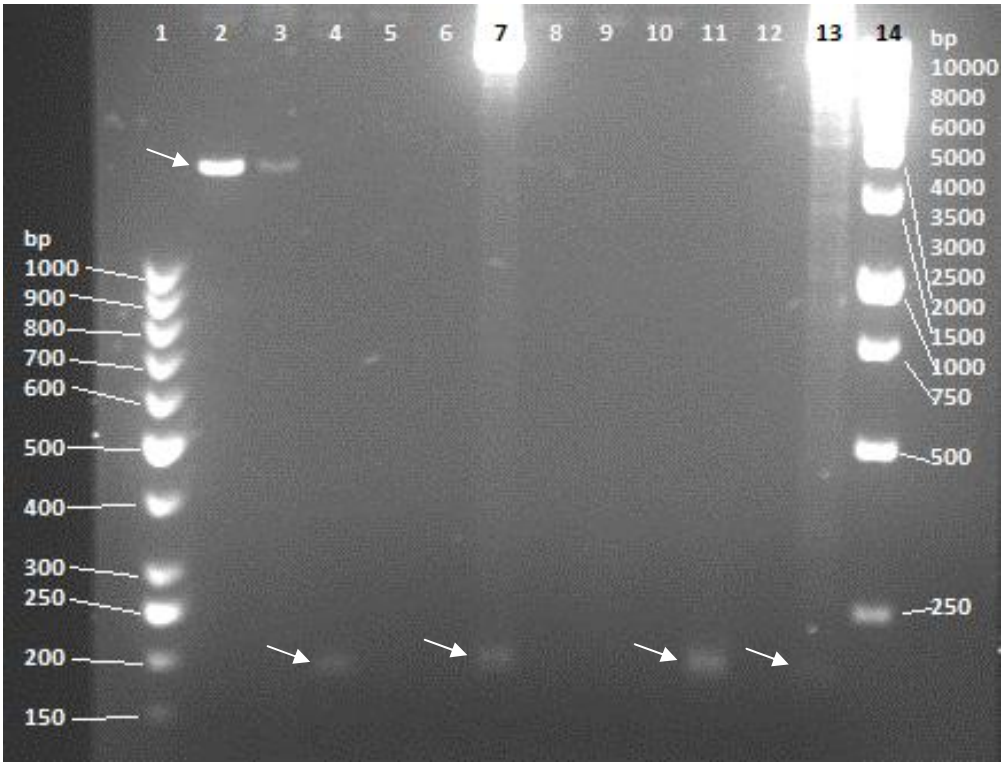

Figure 29: 2.5 % agarose gel displaying the PCR products of colony PCR with the colonies containing p25α-GFP-pEGSH. Well 1: 50 bp ladder. Well 2-5: Four colonies, A1-A4, respectively, transformed with the p25α-GFP-pEGSH ligation mix with 2 mM $MgCl_2$ applied in the PCR reaction. Well 6: Negative control with DNA water and 2 mM $MgCl_2$ applied in the PCR reaction. Well 7: Positive control with DNA water, 1 µL pEGSH vector and 2 mM $MgCl_2$ applied in the PCR reaction. Well 8-11: Four colonies, C1-C4, respectively, transformed with the p25α-GFP-pEGSH ligation mix with 5 mM $MgCl_2$ applied in the PCR reaction. Well 12: Negative control with DNA water and 5 mM $MgCl_2$ applied in the PCR reaction. Well 13: Positive control with DNA water, 1 µL pEGSH vector and 5 mM $MgCl_2$ applied in the PCR reaction. Well 14: 1 kbp ladder. In well 2 and 3 a clear band was apparent just above 1500 bp, which is consistent with the theoretical size of the p25α-GFP-pEGSH PCR product of 1561 bp. The positive controls in well 7 and 13 and the colonies in well 4 and 11 displayed clear bands just below 200 bp, which is consistent with the theoretical size of the pEGSH PCR product of 184 bp. The negative controls did not show any bands.

4.4.7 Plasmid DNA Purification and Sequencing of Constructs

Three plasmid DNA purifications were carried out to purify the construct vectors, p25α-pEGSH and p25α-GFP-pEGSH. First a midiprep purification was carried out. The colonies A4 and C2 containing the p25α-pEGSH construct and the colonies A1 and A2 containing the p25α-GFP-pEGSH construct were used. The DNA concentration of the midiprep samples was measured after elution, which can be viewed in Table 13.

Table 13: DNA concentration measurement of the midiprep purified constructs using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

Midiprep DNA	ng/µL	260/280	260/230	Total µg
p25α-pEGSH construct A4	107.0	1.96	2.23	1605
p25α-pEGSH construct C2	86.7	1.94	2.15	1300.5
p25α-GFP-pEGSH construct A1	134.9	1.93	2.17	2023.5
p25α-GFP-pEGSH construct A2	123.1	1.94	2.20	1846.5

The chemical content of the elution buffer in the Nucleobond® Xtra Midi kit (Macherey Nagel) was not provided and it was assumed that the elution buffer had the same content as the elution buffer of the Nucleospin® Plasmid Mini kit (Macherey Nagel), which was a solution with 5 mM Tris-HCl, pH 8.5. The construct samples of Table 13 were directly sent to sequencing and applied for restriction analysis. The sequencing results were poor and could not be used to verify the insert sequences, p25α and p25α-GFP, of the con-

structs. Consequently, a miniprep plasmid purification using the Nucleospin® Plasmid Mini kit (Macherey Nagel) was performed. The DNA concentration of the midiprep samples was measured after elution, which can be viewed in Table 14. These miniprep samples were applied for restriction analysis and sent to sequencing.

Table 14: DNA concentration measurement of the miniprep purified constructs using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

Miniprep DNA	ng/µL	260/280	260/230	Total µg
p25α-pEGSH construct A4	416.4	1.90	2.20	20.82
p25α-pEGSH construct C2	153.5	1.89	2.14	7.68
p25α-GFP-pEGSH construct A1	452.1	1.91	2.22	22.61
p25α-GFP-pEGSH construct A2	168.8	1.89	2.25	8.44

The sequencing results of the miniprep samples verified the insert sequences p25α and p25α -GFP of p25α-pEGSH and p25α-GFP-pEGSH and the inserts had no mutations. The sequencing raw data can be viewed in appendix A.2. Subsequently, a maxiprep plasmid purification was performed using the Nucleobond® Xtra Maxi kit (Macherey Nagel). The DNA concentration of the maxiprep samples was measured after elution, which can be viewed in Table 15.

Table 15: DNA concentration measurement of the maxiprep purified constructs using NanoDrop spectrophotometer. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

Maxiprep DNA	ng/µL	260/280	260/230	Total µg
p25α-pEGSH construct C2	87.0	1.80	2.18	1305
p25α-GFP-pEGSH construct A2	101.3	1.81	2.16	1519.5

4.4.8 Restriction Analysis

Restriction analyses of the midiprep and miniprep plasmid purification eluates with the p25α-pEGSH and p25α-GFP-pEGSH constructs were performed. The enzymatic digestion with *Acu*I and *Hind*III+*Bgl*II in the restriction analysis of the midiprep samples was not apparent from the agarose gels and the construct vectors could not be verified from this experiment. These data can be viewed in appendix B.5 Restriction Analysis of Constructs.

The 1 % agarose gels with the *Acu*I digests of the miniprep p25α-pEGSH and p25α-GFP-pEGSH constructs can be viewed in Figure 30. A 1 kbp ladder can be seen in well 1. Well 2 contained the p25α-pEGSH construct A4 with 1 µL *Acu*I and the appertaining control without enzyme in well 3. Well 4 contained the p25α-pEGSH construct C2 with 1 µL *Acu*I and the appertaining control without enzyme in well 5. Well 6 contained the p25α-GFP-pEGSH construct A1 with 1 µL *Acu*I and the appertaining control in well 7. Well 8 contained the p25α-GFP-pEGSH construct A2 with 1 µL *Acu*I and the appertaining control in well 9. Well 10 contained a positive control, pEGSH vector with 1 µL *Acu*I. Bands around 1000 bp and 4000 bp were apparent in well 10, which corresponded to the digests of the pEGSH vector of 1009 bp and 3820 bp, respectively. The negative controls showed bands around 4000 bp for p25α-pEGSH in well 3 and 5 and around 5000 bp for p25α-GFP-pEGSH in well 7 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively. Well 2 and 4 with the p25α-pEGSH construct from colony A4 and C2 digested with *Acu*I showed apparent bands around 3000 bp, 1500 bp and 1000 bp, which correspond to the theoretical sizes of the p25α-pEGSH digests of 2923 bp, 1556 bp, and 1009 bp. Well 6 and 8 with the p25α-GFP-pEGSH construct from colony A1 and A2 digested with *Acu*I showed apparent bands around 3000 bp, 1500 bp, 1000 bp and 500 bp, which corresponded to the theoretical sizes of the p25α-GFP-pEGSH digests of 3094 bp, 1556 bp, 1009 bp, and 547 bp.

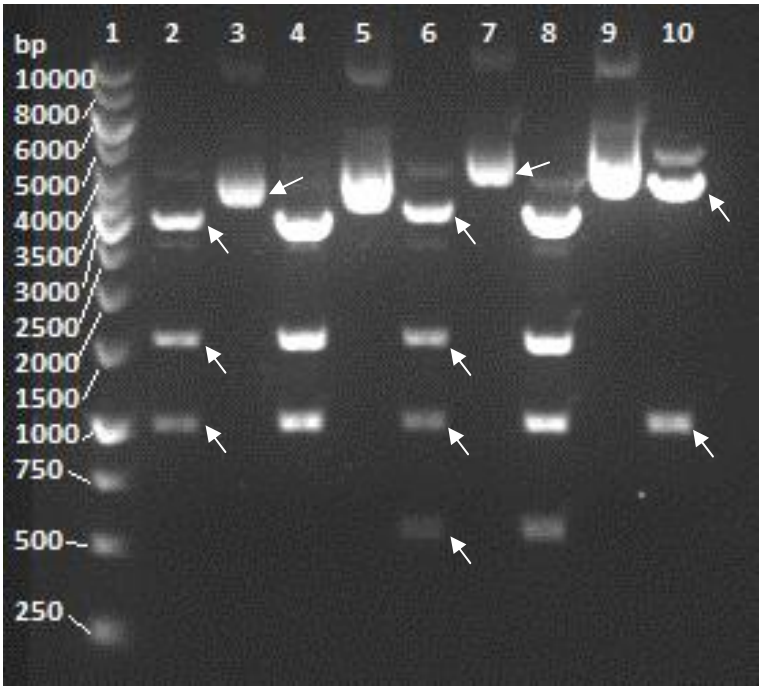

Figure 30: 1 % agarose gel displaying the *Acu*I digests of the p25α-pEGSH and p25α-GFP-pEGSH constructs from the miniprep plasmid purification. Approximately 462 ng of each construct vector was applied and 510 ng pEGSH control vector was applied. Well 1: 1kbp ladder. Well 2: p25α-pEGSH construct A4 with 1 µL *Acu*I. Well 3: p25α-pEGSH construct A4 without enzyme (ctrl). Well 4: p25α-pEGSH construct C2 with 1 µL *Acu*I. Well 5: p25α-pEGSH construct C2 without enzyme (ctrl). Well 6: p25α-GFP-pEGSH construct A1 with 1 µL *Acu*I. Well 7: p25α-GFP-pEGSH construct A1 without enzyme (ctrl). Well 8: p25α-GFP-pEGSH construct A2 with 1 µL *Acu*I. Well 9: p25α-GFP-pEGSH construct A2 without enzyme (ctrl). Well 10: pEGSH vector with 1 µL *Acu*I. In well 10, bands around 1000 bp and 4000 bp were apparent, which corresponded to the digests of the pEGSH vector of 1009 bp and 3820 bp, respectively. The negative controls showed bands around 4000 bp for p25α-pEGSH in well 3 and 5 and around 5000 bp for p25α-GFP-pEGSH in well 7 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively. Well 2 and 4 with the p25α-pEGSH construct from colony A4 and C2 digested with *Acu*I showed apparent bands around 3000 bp, 1500 bp and 1000 bp, which corresponded to the theoretical sizes of the p25α-pEGSH digests of 2923 bp, 1556 bp, and 1009 bp. Well 6 and 8 with the p25α-GFP-pEGSH construct from colony A1 and A2 digested with *Acu*I showed apparent bands around 3000 bp, 1500 bp, 1000 bp and 500 bp, which corresponded to the theoretical sizes of the p25α-GFP-pEGSH digests of 3094 bp, 1556 bp, 1009 bp, and 547 bp.

The 1 % agarose gels with the *Bgl*II and *Hind*III digests of the miniprep p25α-pEGSH and p25α-GFP-pEGSH constructs can be viewed in Figure 31. A 1 kbp ladder can be seen in well 1. Well 2 contained the p25α-pEGSH construct A4 with 1 µL *Bgl*II and 0.5 µL *Hind*III and the appertaining control without enzyme in well 3. Well 4 contained the p25α-pEGSH construct C2 with 1 µL *Bgl*II and 0.5 µL *Hind*III and the appertaining control in well 5. Well 6 contained the p25α-GFP-pEGSH construct A1 with 1 µL *Bgl*II and 0.5 µL *Hind*III and the appertaining control in well 7. Well 8 contained the p25α-GFP-pEGSH construct A2 with 1 µL *Bgl*II and 0.5 µL *Hind*III and the appertaining control in well 9.

Well 10 contained a positive control, pEGSH vector with 1 µL *Bgl*II and 0.5 µL *Hind*III. Bands around 5000 bp and just below 750 bp were apparent in well 10, which corresponded to the digests of the pEGSH vector of 4142 bp and 687 bp, respectively. The negative controls showed bands around 4000 bp for p25α-pEGSH in well 3 and 5 and around 5000 bp for p25α-GFP-pEGSH in well 7 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively. Well 2 and 4 with the p25α-pEGSH construct from colony A4 and C2 digested with *Bgl*II and *Hind*III showed apparent bands around 5000 bp and 1500 bp, which corresponded to the theoretical sizes of the p25α-pEGSH digests of 4142 bp and 1346 bp. Well 6 and 8 with the p25α-GFP-pEGSH construct from colony A1 and A2

digested with *Bgl*II+*Hind*III showed apparent bands around 5000 bp and 2000 bp, which corresponded to the theoretical sizes of the p25α-GFP-pEGSH digests of 4142 bp and 2064 bp.

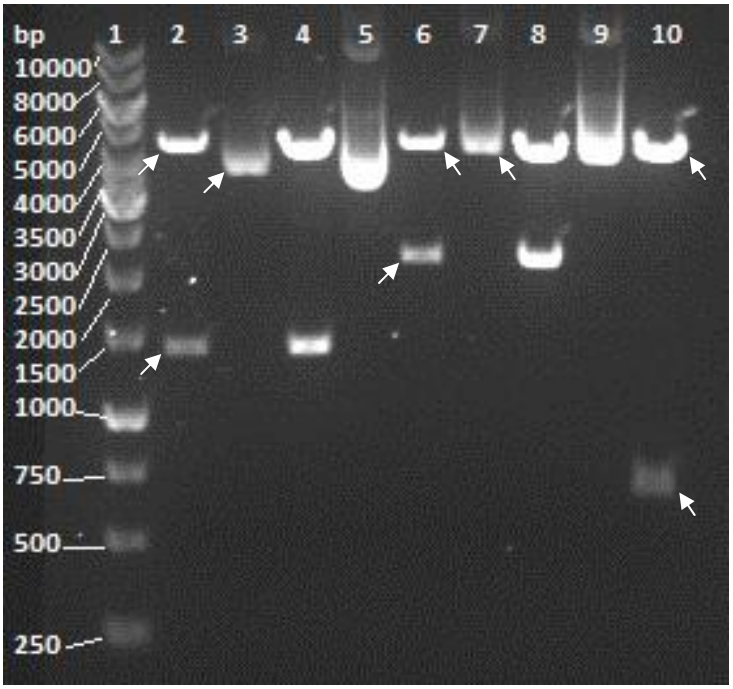

Figure 31: 1 % agarose gel displaying the *Bgl*II+*Hind*III digests of the p25α-pEGSH and p25α-GFP-pEGSH constructs from the miniprep plasmid purification. Approximately 462 ng of each construct vector was applied and 510 ng pEGSH control vector was applied. Well 1: 1kbp ladder. Well 2: p25α-pEGSH construct A4 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 3: p25α-pEGSH construct A4 without enzyme (ctrl). Well 4: p25α-pEGSH construct C2 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 5: p25α-pEGSH construct C2 without enzyme (ctrl). Well 6: p25α-GFP-pEGSH construct A1 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 7: p25α-GFP-pEGSH construct A1 without enzyme (ctrl). Well 8: p25α-GFP-pEGSH construct A2 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 9: p25α-GFP-pEGSH construct A2 without enzyme (ctrl). Well 10: pEGSH vector with 1 µL *Bgl*II and 0.5 µL *Hind*III. In well 10, bands around 5000 bp and just below 750 bp were apparent, which corresponded to the digest of the pEGSH vector of 4142 bp and 687 bp, respectively. The negative controls showed bands around 4000 bp for p25α-pEGSH in well 3 and 5 and around 5000 bp for p25α-GFP-pEGSH in well 7 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively. Well 2 and 4 with the p25α-pEGSH construct from colony A4 and C2 digested with *Bgl*II and *Hind*III showed apparent bands around 5000 bp and 1500 bp, which corresponded to the theoretical sizes of the p25α-pEGSH digests of 4142 bp and 1346 bp. Well 6 and 8 with the p25α-GFP-pEGSH construct from colony A1 and A2 digested with *Bgl*II+*Hind*III showed apparent bands around 5000 bp and 2000 bp, which corresponded to the theoretical sizes of the p25α-GFP-pEGSH digests of 4142 bp and 2064 bp.

4.4.9 DNA Extraction and Precipitation

DNA extraction using phenol and chloroform followed by precipitation using ethanol was performed for the midiprep plasmid purification eluates p25α-pEGSH A4 and C2 and p25α-GFP-pEGSH A1. The extraction and precipitation of the p25α-pEGSH A4 and p25α-pEGSH C2 midiprep constructs was carried out by following the first protocol, cf. 3.4.10 DNA Extraction and Precipitation. The DNA concentration of the samples considerably decreased, except for one sample, see Table 20, appendix B.6 DNA Extraction and Precipitation. Instead, the second protocol was applied for the p25α-GFP-pEGSH A1 midiprep construct, performing the ethanol precipitation by incubating the samples at -80 °C for 15 minutes, which gave a considerably higher DNA concentration, see Table 20, appendix B.6 DNA Extraction and Precipitation.
The third protocol was applied for the maxiprep plasmid purification eluates p25α-pEGSH C2 and p25α-GFP-pEGSH A2. Due to an oversaturated salt content in these samples, DNA water was added for dilution followed by isopropanol and ethanol precipitation to remove the excess salt, see Table 16. The final amount

of DNA obtained from the maxiprep after phenol-chloroform extraction and isopropanol and ethanol precipitation was 669 µg p25α-pEGSH construct and 664 µg p25α-GFP-pEGSH construct.

Table 16: DNA concentration of the maxiprep purified constructs, p25α-pEGSH and p25α-GFP-pEGSH, after phenol-chloroform extraction and ethanol precipitation, followed by dilution and isopropanol and ethanol precipitation. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

Maxiprep Phenol-Chloroform DNA	ng/µL	260/280	260/230	Total µg
p25α-pEGSH construct C2	474.0	1.86	2.08	900.6
p25α-GFP-pEGSH construct A2	427.1	1.85	2.09	640.7
After dilution				
p25α-pEGSH construct C2	85.5	1.84	2.13	855
p25α-GFP-pEGSH construct A2	90.1	1.89	2.20	901
After isopropanol and ethanol precipitation				
p25α-pEGSH construct C2	787.0	1.89	2.29	**669**
p25α-GFP-pEGSH construct A2	741.5	1.91	2.29	**664**

4.4.10 Determination of Lethal Geneticin and Hygromycin Concentration for OLN-AS7

To enable selection of OLN-AS7 stably transformed with the p25α-pEGSH construct, p25α-GFP-pEGSH construct and pERV3 vector, cytotoxicity tests using hygromycin and geneticin (G418), respectively, were performed. Two experiments using hygromycin were conducted. The Excel data sheet with calculations can be found in appendix A.7. Figure 32 shows the cytotoxicity of hygromycin after four days. The relative cell viability decreased to 75 % with 50 µg/mL, 39 % with 100 µg/mL, 28 % with 200 µg/mL, 6 % with 400 µg/mL, 2 % with 600 µg/mL, 1 % with 800 µg/mL and 0 % with 1000 µg/mL hygromycin, respectively.

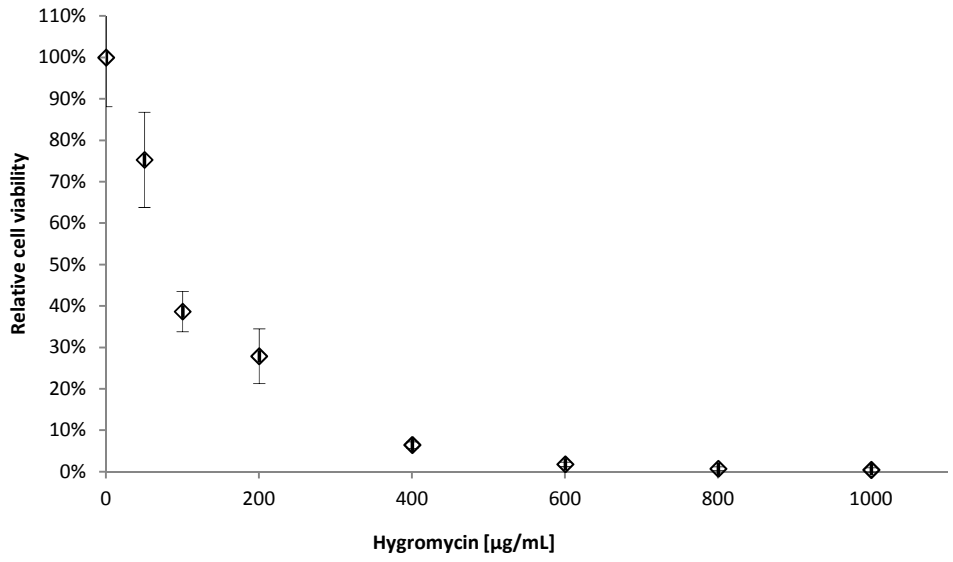

Figure 32: Hygromycin cytotoxicity towards OLN-AS7 cell line after 4 days. The relative cell viability was 75 % with 50 µg/mL, 39 % with 100 µg/mL, 28 % with 200 µg/mL, 6 % with 400 µg/mL, 2 % with 600 µg/mL, 1 % with 800 µg/mL and 0 % with 1000 µg/mL hygromycin, respectively.

A second experiment with hygromycin was conducted (data not shown). All of the cells were trypsinized and counted after 11 days. No adherent cells were apparent in the wells with 200, 400, 600, 800, and 1000 µg/mL hygromycin.

In the geneticin experiment, the cells with 0, 50, 100, and 200 µg/mL geneticin were trypsinized and counted continually. All of the cells were trypsinized and counted after 15 days. No adherent cells were apparent in the wells with 800 and 1000 µg/mL geneticin. Two of the wells with 600 µg/mL geneticin contained no cells, while one well contained 1250 cells.

4.5 Examination of Transfection Efficiency

Three different transfection methods were applied, the calcium phosphate transfection method, DreamFect™ transfection reagent, and FuGENE® HD transfection reagent. In Figure 33, a phase contrast and fluorescence image of OLN-AS7 cell transiently transfected with the p25α-pAcGFP1-N1 vector using the calcium phosphate transfection method can be viewed. Two cells out of 23 cells did show clear expression of the p25α-GFP fusion protein.

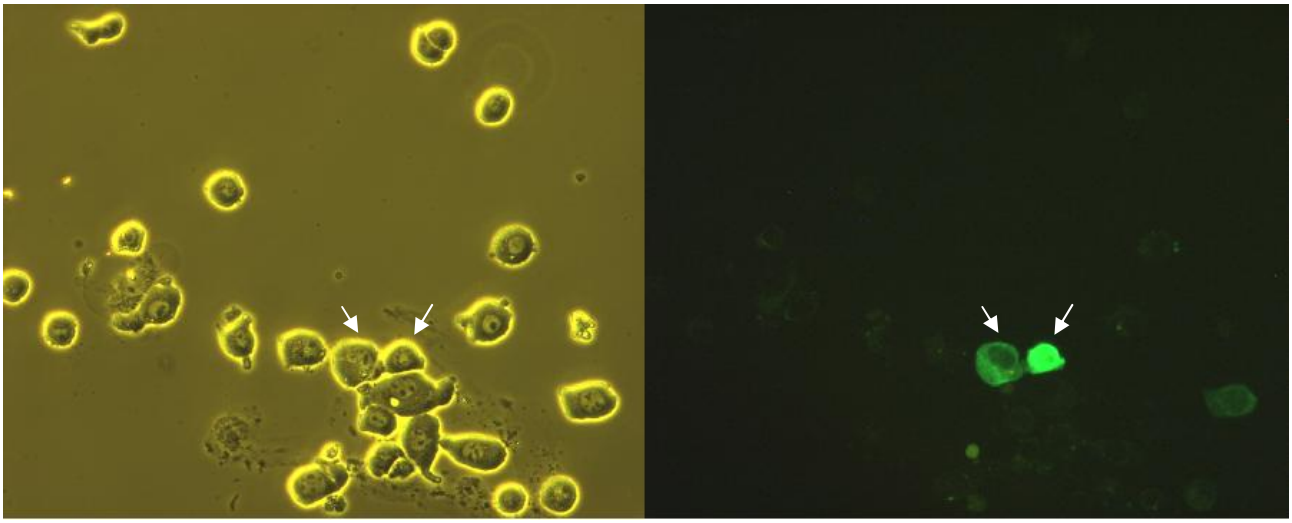

Figure 33: Phase contrast and fluorescence image of OLN-AS7 cells transiently transfected with p25α-pAcGFP1-N1 vector using the calcium phosphate transfection method. 400x magnification. Two cells displayed clear expression of GFP.

In Figure 34, a phase contrast and fluorescence image of OLN-AS7 cell transiently transfected with the p25α-pAcGFP1-N1 vector using the DreamFect transfection reagent in a 5:1 reagent to plasmid ratio can be seen. One out of six cells in the image showed clear expression of the p25α-GFP fusion protein.

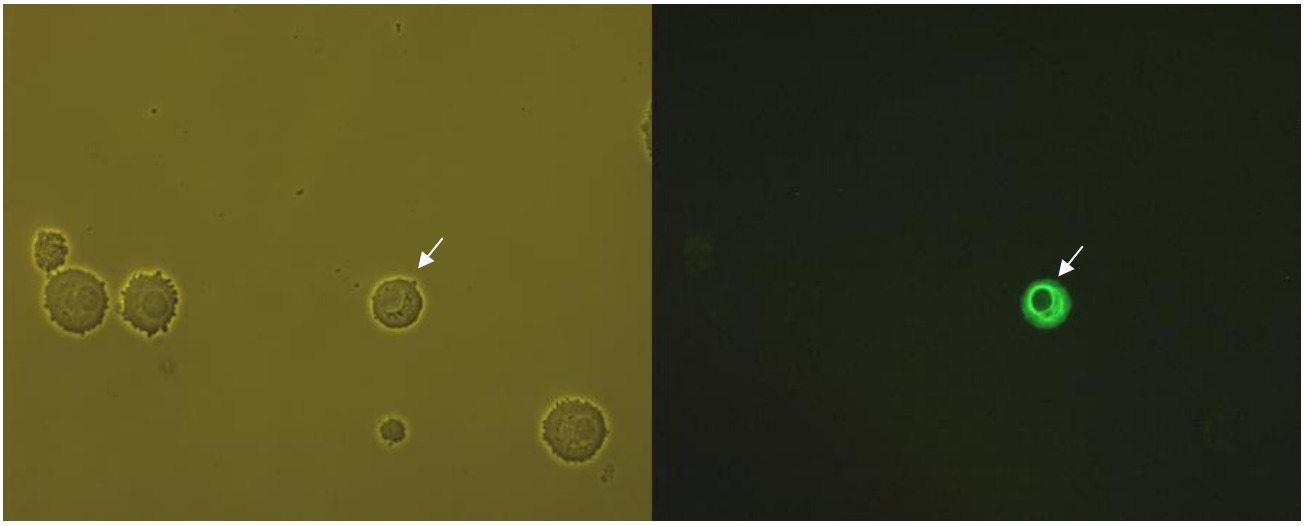

Figure 34: Phase contrast and fluorescence image of OLN-AS7 cells transiently transfected with p25α-pAcGFP1-N1 vector using DreamFect transfection reagent in a 5:1 reagent:plasmid ratio. 400x magnification. One out of six cells displayed clear expression of GFP.

In Figure 35, a phase contrast and fluorescence image of OLN-AS7 cell transiently transfected with the p25α-pAcGFP1-N1 vector using the FuGENE transfection reagent in a 5:1 reagent to plasmid ratio can be viewed. One out of 12 cells in the image showed clear expression of the p25α-GFP fusion protein.

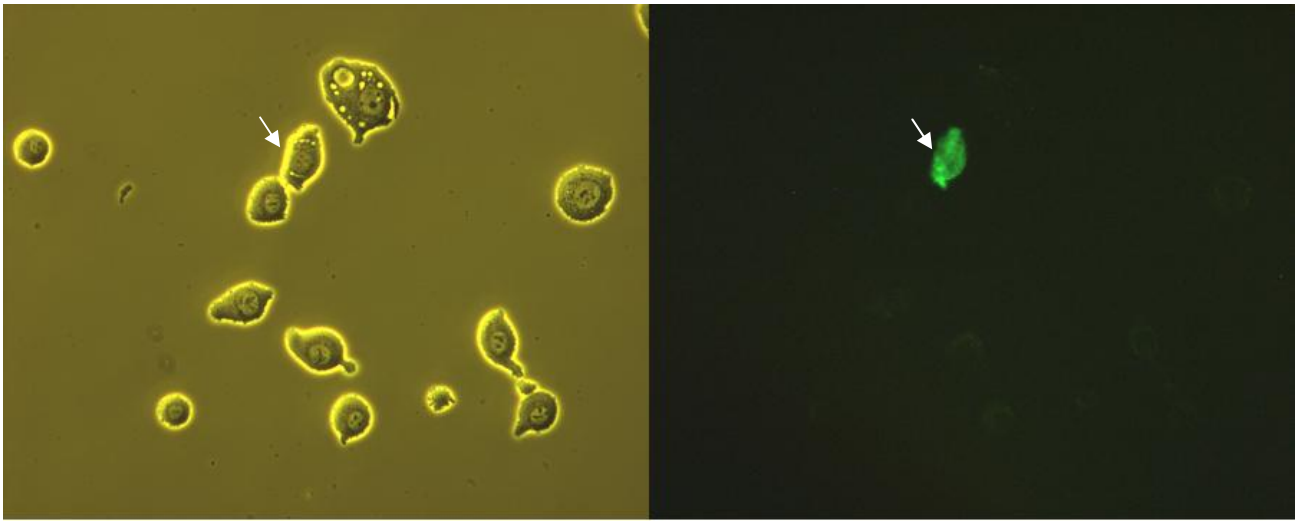

Figure 35: Phase contrast and fluorescence image of OLN-AS7 cells transiently transfected with p25α-pAcGFP1-N1 vector using FuGENE transfection reagent in a 5:1 reagent:plasmid ratio. 400x magnification. One out of 12 cells displayed clear expression of GFP.

Due to very low transfection efficiencies or no apparent transfection in the experiments, the remaining data is not shown. More experiments displaying higher cell densities must be performed to attain enough data to enable calculations of the transfection efficiency in the different experiments.

5. Discussion

Examination of p25α and p25α-GFP Expression in OLN-AS

The examination of the transient expression of p25α in OLN-AS could not be fully verified from the Western blots as presented in section 4.2 Examination of p25α and p25α-GFP Expression in OLN-AS. A weak band around 30 kDa was seen in Figure 15B, which may have been p25α, although the protein has previously been verified at 25 kDa and 23.7 kDa (Skjoerringe et al. 2006). In Figure 14, the expression of the p25α-GFP fusion protein was apparent and measurable in these cell lysate samples. Since the GFP of *Aequorea coerulescens* has a molecular weight around 27 kDa and the p25α molecular weight is around 25 kDa, the p25α-GFP fusion protein molecular weight would be ~52 kDa (Gurskaya et al. 2003). In Figure 14, a band around 50 kDa was visible in well 2-5 and 7-9 containing cell lysate, indicating that the transfection had been successful in that specific experiment applying the FuGENE:plasmid ratios of 3:1, 4:1, 5:1 and 6:1. The band in well 4 with the 5:1 ratio appeared to be more visible than the other bands, indicating that this transfection ratio was preferable over the other ratios, when applying the p25α-pAcGFP1-N1 vector. Fluorescence microscopy experiments testing different ratios of FuGENE:p25α-pAcGFP1-N1 vector in OLN-AS showed that the 5:1 ratio was preferable and that a transient transfection with p25α in OLN-AS using the FuGENE transfection reagent displayed an expression of p25α-GFP to an extent of 5-15 % of the cell population (Knudsen, A. D., unpublished data). Consequently, the 5:1 ratio was applied in the following experiments. The transfection complex of FuGENE and plasmid was removed from the cells 4 and 24 hours, respectively, after transfection to examine whether the FuGENE transfection reagent showed cytotoxic properties towards the cells. The cytotoxicity was examined using the XTT assay to display the growth of the cells or lack hereof. (Knudsen, A. D., unpublished data)

The absence of bands corresponding to p25α on the remaining Western blots may be due to a poor transient transfection of the OLN-AS cells, and accordingly an expression of p25α which could not be detected using Western blotting. The transfection method using FuGENE had been optimized using glass vials instead of plastic eppendorf tubes, as the lipid-based FuGENE transfection reagent could have adsorbed to the plastic surface, although the use of glass vials did not seem to make a difference. Also, Figure 17 displays a Western blot where another anti-TPPP (anti-p25α) antibody (Sigma-Aldrich) was applied to investigate whether a different antibody would yield a band displaying an expression of p25α, which did not seem to be the case. Since the experiment using anti-TPPP antibody was only performed once, it should be repeated to provide more replicates for comparison. Also, the Western blotting protocol should be optimized for further experiments, such as concentrating the anti-p25α primary antibody content of the binding buffer. In general more biological replicates should be performed.

Examination of Sensitivity of XTT Assay and Determination of Doubling Time

The XTT assay displayed a sensitivity of 2-4000 cells, i.e. a difference in cell viability can be seen when an increase or decrease of 2-4000 cells is apparent. However, errors in cell seeding can entail misinterpretation of the data. As the cell viability was measured 72 hours after transfection and/or treatment with inhibitor compound, the possible seeding error would be more apparent after 72 hours than it would be after 24 hours. However, the difference in untreated or rescued and treated cells would in theory also be more apparent after 72 hours compared to 24 hours, consequently, the advantages of measuring after 72 hours were preferred. After 72 hours, each well with 100 % viable cells should contain approximately 24,000 cells (with a doubling time of 24 hours).

From the data Excel file “OLN-AS7 Doubling Time” in appendix A.7, fewer cells were obtained by the first trypsination than those transferred to the wells when initiating the experiment. This was due to a loss of cells

which was apparent in general by trypsination, which also meant that the first data point from seeding could not be utilized for determination of the OLN-AS7 doubling time.

Examination of Cytotoxicity of α-Synuclein Aggregation Inhibitors

As one out of several future evaluations of the 56 CureND inhibitor compounds, the cytotoxicity was tested in a concentration of 10 μM. Optimally, a range of concentrations of each compound should have been tested, since some of the compounds which were toxic at 10 μM may have been effective and non-toxic at a lower dosage. However, due to limited availability of the test compounds and a confined time span to conduct the experiments, only one concentration was tested. The known α-syn aggregation inhibitor ASI1D was tested in a concentration of 10 μM, as previous studies have shown that the compound is non-toxic towards the M17-A53T and SHSY-5Y cell lines (O. M. A. El-Agnaf et al. 2004) and has displayed non-toxic α-syn aggregation inhibitory effect, when incubating the OLN-AS cell line with this inhibitor one hour prior to transient transfection with p25α (Kragh et al. 2009). The cytotoxicity experiment with ASI1D, cf. Figure 20 and Table 6, confirmed that ASI1D did not show cytotoxic properties towards the OLN-AS cells in a 10 μM concentration. Previous studies have shown that incubation with either 10 μM ASI1D or 100 μM baicalein after transient transfection of OLN-AS with p25α does not rescue the cells (Knudsen A.D., unpublished data).

The α-syn aggregation inhibitor EGCG caused 78 % ± 6 % cell viability of the OLN-AS cells when applying a concentration of 13.5 μM. As only one experiment was carried out, more replicates should be performed before anything conclusive can be stated. Other studies involving this compound indicated that 25 μM EGCG was not cytotoxic towards SH-SY5Y cells (Chung et al. 2007) and 20 μM EGCG showed an α-syn aggregation inhibitory effect in HEK-293 cells (Bieschke et al. 2010).

The 100 μM baicalein was tested to verify a preceding investigation, where treatment of OLN-AS with 100 μM baicalein one hour prior to transfection with p25α displayed an α-syn aggregation inhibitory effect by measuring microtubule retraction (Kragh et al. 2009). In this thesis, 100 μM baicalein displayed 84 % ± 13 % relative cell viability with a p-value of 0.10 and 80 % ± 3 % relative cell viability with a p-value of $7.27 \cdot 10^{(-6)}$ in the two replicate experiments, indicating that baicalein is somewhat cytotoxic. A recent study indicated that 100 μM baicalein displayed no general cytotoxic effect towards OLN-AS, although when applying this inhibitor one hour prior to transfection with p25α using FuGENE® HD transfection reagent (FuGENE), baicalein showed toxic properties towards the OLN-AS cells (Knudsen, A. D., unpublished data). The latter was also seen in Figure 23, where the relative viability of the OLN-AS cells decreased to approximately 45 % when the cells were incubated with 100 μM baicalein one hour prior to transfection with p25α, also indicating that baicalein is toxic when applied prior to the p25α transfection using FuGENE.

The classification of the CureND inhibitor compounds as non-toxic, toxic and discrepant was performed to obtain a clearer overview of the different compounds and is influenced by a subjective approach. No upper boundary was set when distinguishing between toxic and non-toxic compounds, however, a significant increase in cell viability is not necessarily favorable. Increased cell division may cause an adverse effect *in vivo* and possible future medication compounds should entail cell viability of 100 % or close to.

Some of the compounds stand out, e.g. lead 37, which entails cell viability around 100 % in two replicate experiments, while it displays cell viability far below 100 % in the two others. More experiments should be performed in those cases. The inhibitor treatment of the cells in suspension (replicate 3 and 4), not in the exponential growth phase, instead of treatment of adherent cells (replicate 1 and 2), in the exponential growth phase, may have an influence on the results. However, no general error specific for replicate 3 and 4 can be observed when comparing the data.

The untreated cells and the DMSO treated cells display an average relative standard deviation of ±5 % and ±8 %, respectively, in the four replicates, which mean that this deviation also should be taken into account when selecting the CureND inhibitor compounds for further experiments.

Inhibitory Effect of Known α-Syn Aggregation Inhibitors EGCG, Baicalein, and ASI1D

In Figure 23, p25α-transfected OLN-AS cells treated with 13.5 μM and 27 μM EGCG, 10 μM ASI1D, and 100 μM baicalein one hour prior to transfection can be viewed. The relative viability of DMSO-treated cells seems to slightly decrease compared to the untreated cells, however, not below 95 % viability. Also, the standard deviation of the DMSO-treated cells overlaps the measurement of the untreated cells, which means that this decrease may be due to a seeding error. The FuGENE- and FuGENE+DMSO-treated cells (negative controls) seem to show an increase in viability to approximately 115 %, whereas with the addition of p25α+FuGENE the cell viability decreases to 101 %, which is within the standard deviations of the controls. By adding p25α+FuGENE+DMSO to the cells, the viability increased to 110 %, although this measurement is within the standard deviations of the FuGENE and FuGENE+DMSO treated cells, which means that these measurements were difficult to distinguish and was consequently unreliable. The positive controls with both FuGENE+p25α did not show a decrease in relative cell viability considerably below the untreated control cells, which implies poor transfection efficiency. As this is the case for both positive controls, it is highly feasible that the transfection efficiency was undetectably low for all of the samples. The addition of 13.5 μM and 27 μM EGCG and 10 μM ASI1D one hour prior to transfection entailed a cell viability of 101-103 %, however, the coefficient of variation of the untreated cells was ± 3 %, so the cell viability should be estimated to be around 100 % for these inhibitor treated cells. This may indicate that the cells were rescued by EGCG and ASI1D, but it can also signify an unsuccessful transfection, possibly due to a disturbance in transfection caused by the inhibitor compound. This problem could be circumvented by applying a cell line stably expressing p25α. However, the positive controls also indicated poor transfection efficiency. A previous study, involving the transfection of OLN-AS with p25α-pAcGFP1-N1 and testing the transfection efficiency using fluorescence microscopy, implied a transfection efficiency below 10 % when applying the FuGENE® HD transfection reagent with different ratios of FuGENE:plasmid, where a 5:1 ratio gave the highest transfection efficiency with overnight incubation (Knudsen, A. D., unpublished data). Consequently, this 5:1 ratio was applied in this study; however, the cells were not incubated with the transfection complex overnight, but for 72 hours to be able to see a difference in absorbance between the transfected cells and the inhibitor-treated transfected cells. It has not been examined whether the 72 hours incubation with FuGENE had a negative effect on the cells. Additionally, the transfection of OLN-AS with p25α using FuGENE could not be verified by Western blotting, cf. section 4.2 Examination of p25α and p25α-GFP Expression in OLN-AS, also implying low transfection efficiency. The 100 μM baicalein applied one hour prior to transfection was clearly cytotoxic for the cells, as the relative viability decreased to 45 % ± 25 %. The cytotoxicity of 100 μM baicalein in OLN-AS, when applied both one hour prior to transfection with p25α-pcDNA3.1/Zeo(-) and four hours after transfection, have been reported in a recent study (Knudsen, A. D., unpublished data), implying that one hour pre-transfection treatment with baicalein does not rescue the cells, and that the four hour post-transfection treatment either has no rescuing effect or displays a cytotoxicity towards the OLN-AS cells. The former supports the findings in this study cf. Figure 23, if the low transfection efficiency is not taken into consideration. However, previous studies have implied that baicalein inhibits α-syn aggregation *in vivo* in 10 μM and 100 μM concentrations, respectively, without displaying cytotoxicity (Kostka et al. 2008; Kragh et al. 2009). Additional cell-based experiments involving this compound are necessary to verify any tendency.

In Figure 24, p25α-transfected OLN-AS and OLN-t40 cells treated with 2, 5, 10, and 15 μM ASI1D one hour prior to transfection can be viewed. The measurements have been normalized between the two cell lines. In general, the absorbance values were higher for the OLN-t40 cell line, which was probably due to a lower doubling time of the OLN-t40 cell line. The DMSO and FuGENE-treated cells appeared to show a level of viability to that of the untreated cells. The cells treated with FuGENE+DMSO showed a slight increase in cell viability, however, the standard deviations were in the range of the untreated, DMSO-treated, and FuGENE-treated controls. This implies that the FuGENE reagent alone was not toxic towards the cells. The p25α+FuGENE and p25α+FuGENE+DMSO positive controls displayed an absorbance slightly below that of the untreated cells; however, the standard deviations were in the range of the untreated cells, making it infeasible to distinguish the positive controls from the untreated negative control. As the positive control did not show a considerable decrease in cell viability, it also indicated low transfection efficiency in these samples. An inverse proportion between the ASI1D concentration and the cell viability was apparent from Figure 24, displaying increasing cell viability with decreasing ASI1D concentration, although this was not a considerable discovery, since the standard deviations of the ASI1D-treated cells overlapped. However, the standard deviation of the untreated cells did not overlap with the 2 μM ASI1D sample, signifying that this concentration may contribute to rescuing the cells. As mentioned above, 10 μM ASI1D displayed non-toxic α-syn aggregation inhibitory effect, when incubating the OLN-AS cell line with this inhibitor one hour prior to transient transfection with p25α (Kragh et al. 2009). Although the inhibitory effect of ASI1D could not be examined properly due to poor transfection efficiency, the compound displayed no cytotoxicity which was consistent with the discovery in Kragh et al., 2009.

When examining the OLN-t40 control cell line, the FuGENE+DMSO negative control showed a decrease in absorbance, indicating a cytotoxic effect of the FuGENE+DMSO mixture. However, this was not the case for the OLN-AS cell line. Also, none of the other controls showed a decrease to such an extent, indicating that this was an outlier. A distinct decrease in viability was seen for the transfected cells, FuGENE+p25α and FuGENE+p25α+DMSO, compared to the untreated OLN-t40 cells, cf. Figure 24, which was not consistent with the fact that OLN-t40 does not overexpress the human α-syn like OLN-AS. Accordingly, the transfection with p25α should in theory not cause a decrease in cell viability of OLN-t40. OLN-t40 may display a wild-type expression of α-syn prompting α-syn aggregation when p25α is present. A previous study showed that a transient transfection of OLN-t40 with p25α did not cause apoptosis and only had a minor effect towards the OLN-t40 cells, compared to the OLN-AS cells (Kragh et al. 2009), contradictory to the findings in this thesis. The presence of ASI1D seems to have a rescuing effect towards the transfected OLN-t40 cells in an inverse manner like for OLN-AS, except with 5 μM ASI1D. However, the standard deviations of the ASI1D-treated cells overlap. The OLN-t40 cells treated with 2 μM ASI1D displayed cell viability at the level of the untreated cells and above the standard deviations of the transfected positive controls, implying that this concentration rescued the transfected cells.

The low transfection efficiency of the control samples for both cell lines indicates general low transfection efficiency in all of the samples, making it difficult to substantiate the cell rescuing effect of the inhibitor compounds. Ideally, the transfection efficiency has to be improved to achieve a clear effect of the inhibitor compounds and the experiments should be repeated with narrower error margins to distinguish the different samples, if this experimental setup was to be continued. Furthermore, high reproducibility is important in this assay. The Western blots in the earlier experiments confirmed the low transfection efficiency, also implying that the transient transfection setup has too low reproducibility and that the establishment of an inducible expression system with stable expression of p25α would be preferred. Stressing the cells by the transient transfection could be avoided and consistent results would be obtainable.

OLN Model Cell System

The OLN-AS model cell line was applied to investigate α-syn aggregation, although the cell line expresses both the human α-syn and the human isoform of the tau40 protein, as the expression of tau40 could not be circumvented (Kragh et al. 2009). As the tau protein acts as a ligand to α-syn and the fibrillation of tau protein presumably is promoted by α-syn, cf. chapter 1. Introduction, more rapid neuronal degeneration may possibly be obtained from this, if the aggregation and oligomer formation occur to a greater extent in the presence of tau40. This may be a key feature of PD, making the expression of tau40 in OLN-AS a better PD model, than if tau40 was not expressed. However, from the XTT experiments it was unfeasible to interpret whether tau40 was influenced by the α-syn aggregation inhibitors tested or vice versa. Consequently, working with the OLN-AS7 cell line, solely overexpressing α-syn, produces data were only one variable, α-syn, is considered instead of two, α-syn and tau40. To circumvent that problem, the cell line OLN-t40 was applied as a negative control to monitor how the cell line would act in the absence of α-syn overexpression. However, the OLN-t40 cell line presumably displayed α-syn expression to some extent and a lower doubling time compared to OLN-AS. However, this finding was not significant and more biological replicates should be conducted. The OLN-AS7 cell line could have been applied as an extra control in the XTT assay experiments to examine whether this cell line behaved differently without tau40 expression, however, the OLN-AS7 cell line was applied later in the course of study, as the authorization to utilize the OLN-t40 and OLN-AS cell lines expired.

Oligodendroglial model cell lines have been applied as model cell lines for PD in this thesis, however, it may be assumed that some differences in expression pattern of other proteins than α-syn, p25α, and tau40 are present in oligodendroglial cells compared to dopaminergic neuronal cells. This disadvantage of applying a model cell line is difficult to evade, and results should in general be compared to other studies were different model systems have been applied to verify the findings.

Inducible Expression System

The digestion of p25α-pAcGFP1-N1 and pEGSH, purification, and ligation of the inserts and constructs was successfully carried out. This was followed by cloning in *E.coli* using ampicillin as selective antibiotic and colony PCR. The agarose gel electrophoresis of the colony PCR products displayed five colonies which presumably contained the p25α-pEGSH construct and two colonies, which presumably contained the p25α-GFP-construct, however, only two colonies with each construct were used for further examination. Midiprep plasmid DNA purification was performed on the cultured colonies. The final precipitation step with isopropanol and ethanol in the Nucleobond® Xtra Midi kit was skipped, causing poor sequencing results due to a high salt concentration in the elution buffer. No information on the elution buffer was informed in the kit, so it was wrongly assumed that the elution buffer was identical to that of the Nucleospin® Plasmid Mini kit (miniprep), which consisted of 5 mM Tris/HCl, pH 8.5. The restriction analysis of the midiprep samples did not give any of the expected bands on the agarose gel, except for the positive control, which was probably caused by loss of enzyme activity due to the high salt concentration. A miniprep plasmid purification was performed to verify that the unsuccessful restriction analysis and the poor sequencing results were caused by the elution buffer of the midiprep kit. The theoretical construct digestion was verified in the restriction analysis of the miniprep purified constructs, and the eluted constructs of midiprep were subsequently sequenced. The sequencing was successfully performed, verifying the insert sequences, p25α and p25α-GFP, of the constructs. Also, the DNA extraction and precipitation using phenol and chloroform was applied to remove proteins and lipids, and the isopropanol and ethanol precipitation to remove excess salt. The DNA content of the midiprep samples after phenol-chloroform extraction was lower than prior to the extraction, which may be due to high salt concentration, leaving the water phase containing DNA with a higher density than the phenol-phase. Accordingly, most of the DNA was probably lost in the lower water phase. The incubation at -80

°C may have contributed to precipitation of the DNA, yielding a higher DNA concentration, cf. Table 20. To obtain a larger amount of the p25α-pEGSH and p25α-GFP-pEGSH construct, maxiprep DNA purification was performed applying the Nucleobond® Xtra Maxi kit. The constructs were subsequently extracted by applying phenol-chloroform followed precipitation using isopropanol and ethanol. These processes should remove proteins, lipids, excess salts and also endotoxins originating from the *E. coli* applied for cloning to avoid contamination of the OLN-AS7 cells in further experiments (Sørensen, S., 2011).

The hygromycin and geneticin cytotoxicity experiments displayed a lethal concentration of 200 µg/mL and 600 µg/mL, respectively, towards the OLN-AS7 cells. It became apparent that the data points from the continual splitting of the wells with confluent cells could not be applied for further calculations, as a number of cells were lost from each trypsination. Also, reseeding the cells with 60,000 cells pr. well would display an altered experimental setup, as the cells would be in suspension and not adherent like on day 1 of the experiment. Thus, the cells would not be in the exponential growth phase. The cells in suspension (lag phase) may be more susceptible to the applied antibiotics and the data would not be comparable. Additionally, the lag phase period would also entail misinterpreted data if this was not taken into account when comparing the data. Optimally, the cells should be cultured for 10-14 days to determine the lethal antibiotic concentration, cf. the Complete Control Inducible Mammalian Expression System kit (Agilent Technologies); however, with the OLN-AS7 doubling time of 18.7 hours, the control with untreated cells could only be cultured for approximately 3-4 days before they displayed confluence. In the geneticin experiment, the 50, 100 and 200 µg/mL treated cells displayed decreasingly lower cell viability compared to the untreated cells, although they became confluent within the 14 days. This was also the case for the OLN-AS7 cells treated with 50 µg/mL hygromycin. Seeding the cells in a lower concentration from the beginning could be an option for improving the experimental setup, however, the cell concentration should not be too low, as it would stress the cells and they would not grow properly. Calculation uncertainties may also contribute to misinterpretation of data, since very few cells were counted in some of the wells, and the number counted was only an estimation of the actual number of cells in the entire well, as the counted number of cells was multiplied by a factor. The geneticin experiment should be performed with more replicates and the morphology of the cells should have been examined in detail with microscope images of the cells to support the findings. One well containing 600 µg/mL geneticin still contained very few viable cells, which may be due to poor resuspension after trypsination, however, it was estimated that this concentration can be applied for selection of stable transformants in further experiments.

The transfection efficiency experiments using FuGENE® HD transfection reagent (FuGENE), DreamFect™ transfection reagent (DreamFect), and the calcium phosphate precipitation method did not give clear results on which method was optimal for transfection of the OLN-AS7 cell line with the construct vectors. Very few cells displayed GFP expression. A too low cell density in the samples precluded collection of sufficient data to assess the transfection efficiency, even though increasing the cell density had been attempted. The project deadline did not allow further experiments, testing different ratios of transfection reagent:DNA and application of lower cell densities for examination. A recent study showed that the 5:1 ratio of FuGENE and DreamFect to DNA was optimal for transfection of OLN-AS with p25α-pAcGFP1-N1 (Knudsen, A. D., unpublished data), thus this ratio was applied in these experiments. Optimally, different ratios of FuGENE and DreamFect transfection reagent to DNA should be tested. Additional information from OZ Biosciences on the transfection efficiency using DreamFect Gold for OLN-93 cells showed that a transfection efficiency of 80 % could be obtained if a 3:1 transfection reagent to DNA ratio was applied, see appendix A.5. However, this new version of the DreamFect transfection reagent was not tested. When applying the calcium phosphate precipitation method, a visible white precipitate did not form as expected; however, the entire phos-

phate-calcium mixture was transferred to the cell-containing wells, which may have been cytotoxic for many of the cells (Jordan & Wurm 2004). In spite of this, some cells did show expression of GFP. The calcium phosphate transfection method was downsized from the Invitrogen protocol, making it possible to perform the experiment using 6 well culture plates instead of 60 mm or 100 mm culture dishes, which might have had a negative effect on the transfection efficiency. From a subjective view, the calcium phosphate precipitation method appeared to generate more transfected OLN-AS7 cells than the other methods.
The transfection method with the highest transfection efficiency using the p25α-pAcGFP1-N1 vector may not be optimal when transfecting OLN-AS7 with the actual construct vectors, p25α-pEGSH and p25α-GFP-pEGSH, and the pERV3 vector. The purity of the samples containing the construct vectors is highly important, since impurities may be cytotoxic and consequently preclude transient transfection. Studies have shown that the size of the plasmid vector has an influence on transient transfection efficiency, as very large constructs decrease transfection efficiency and a lower degree of expression per cell (Walker et al. 2004; Yin et al. 2005). The pERV3 vector, which the OLN-AS7 cell line initially has to be stably transfected with, has a size of 8.4 kbp, whereas the p25α-pAcGFP1-N1 vector has a size of 5365 bp. The pERV3 vector is 1.5 times as large as the p25α-pAcGFP1-N1 vector, which may cause lower transfection efficiency. The p25α-pEGSH and p25α-GFP-pEGSH construct vectors are 5488 bp and 6206 bp, respectively. The p25α-pAcGFP1-N1 vector was applied to test the transfection efficiency, since the construct vector p25α-GFP-pEGSH could not be tested in the OLN-AS7 cells without a stable expression of pERV3 in the cells, implying that the experimental conditions were only applied as a guideline for subsequent experiments with the pERV3 and the construct vectors. Additionally, the main purpose of transfection is to establish a stable expression of p25α and p25α-GFP, respectively, in OLN-AS7, which leaves the establishment of optimal transient transfection efficiency a subsidiary aim.

When the p25α-GFP-OLN-AS7 cell line has been established, expression leakage can be examined by fluorescence microscopy before and after induction of p25α-GFP expression. The expression of p25α in the p25α-OLN-AS7 cell line can be examined using the XTT assay before and after induction of p25α expression, where the cell viability can be examined after 72 hours incubation. The expression of p25α in the p25α-OLN-AS7 cell line can also be examined using Western blotting or Northern blotting. The use of endogenous inducible promoters may cause leaking; however, this problem has presumably been overcome by the development of recombinant inducible expression systems. The use of steroid hormones like ecdysone is advantageous, as it is degraded within few hours; it should not be involved in activation of endogenous signaling pathways, and reportedly displays a level of leakage close to zero (Primrose & Twyman 2006; No et al. 1996).

Future Perspectives
The objective of the cytotoxicity test of the 56 CureND α-syn aggregation inhibitors was to compare the results in this thesis with biophysical tests of the compounds, performed by Poul Henning Jensen's research group at the Department of Biochemistry at Aarhus University, and thus select the lead α-syn aggregation inhibitor compounds for further investigation using an OLN-AS7 cell model with inducible expression of p25α. In that connection, a range of concentrations of the CureND, ASI1D, baicalein, and EGCG inhibitors should be tested to examine the inhibition of α-syn aggregation in the model cell line, and furthermore quantify the up- and down-regulation of specific mitochondrial proteins in the presence and absence of the inhibitor compound using SILAC. It would then be feasible to investigate which proteins are expressed by searching protein databases.

The optimal transfection method for transfecting the OLN-AS7 cell line with the pERV3 vector, p25α-pEGSH, and p25α-GFP-pEGSH construct vector could be examined using fluorescence microscopy and

image analysis of p25α-GFP-transfected versus non-transfected cells. The next step in completing the development of the inducible expression system would be to create an OLN-AS7 cell line stably expressing pERV3, followed by stable transformation of this new cell line with p25α-pEGSH and p25α-GFP-pEGSH, respectively, creating two novel cell lines with inducible expression of p25α and p25α-GFP, respectively.

Parkinson's disease is characterized by the loss of dopaminergic neurons but the pathological pathway with formation of α-syn aggregates is displayed in other diseases such as dementia with Lewy bodies and multiple system atrophy, and a breakthrough in this area will influence the research of all synucleinopathies and possibly also tauopathies, as the tau fibrillar formation is presumably similar to that of α-syn (Galvin et al. 2001; Galpern & Lang 2006; V. M.-Y. Lee et al. 2004). The development of a novel oligodendroglial model cell line stably expressing both α-syn and p25α would be highly contributory in enabling the discovery of compounds that can possibly hinder the pathological pathway of α-syn aggregation.

6. Conclusion

The objective of developing two new OLN-AS7 cell lines with inducible expression of p25α and p25α-GFP, respectively, was partially achieved, as the construct vectors p25α-pEGSH and p25α-GFP-pEGSH were successfully developed and cloned for propagation. The constructs were verified by colony PCR and restriction analysis, followed by sequencing to confirm the insert sequences, p25α and p25α-GFP, respectively. The construct vectors were subsequently extracted and purified using phenol-chloroform extraction and then isopropanol and ethanol precipitation. Lethal concentrations of the selective antibiotics geneticin and hygromycin were achieved to enable selection of stable OLN-AS7 transformants containing the pERV3 vector and the p25α-pEGSH or p25α-GFP-pEGSH construct, respectively. Additionally, the OLN-AS7 cytotoxicity of 10 μM of each of the 56 CureND α-syn aggregation inhibitor compounds, 10 μM ASI1D, 100 μM baicalein, and 13.5 μM EGCG was examined applying an XTT assay, where ASI1D did not display cytotoxic properties, baicalein entailed 80 % and 84 % relative cell viability, and EGCG showed 78 % relative cell viability, however, only one experiment was performed for the latter. The α-syn aggregation inhibitory effect of 2, 5, 10, and 15 μM ASI1D, 100 μM baicalein, 13.5 and 27 μM EGCG in OLN-AS transiently transfected with p25α was investigated using the XTT assay, however, due to low transfection efficiency it was unfeasible to verify the inhibitory effect in the majority of the experiments. Though, 2 μM ASI1D seemed to display a rescuing effect of the p25α transfected OLN-AS cells, when treated one hour prior to transfection. When 100 μM baicalein was added one hour prior to transfection with p25α, the relative cell viability decreased to 45%, implying that baicalein was cytotoxic in connection with the transient transfection. The expression of α-syn, α-tubulin, and p25α-GFP was verified in the OLN-AS cell line, however, transient expression of p25α could not be performed successfully.

7. References

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A Contents of Data Disc

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A.3 XTT Raw Data

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A.5 Product Sheets

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B Additional Results

B.1 Mycoplasma Test of OLN-AS7 Cell Line

The manual for the mycoplasma detection kit, can be found in appendix A.5 Product Sheets. The mycoplasma test was carried out at the Department of Clinical Immunology at Aalborg Hospital.

The result of the mycoplasma test can be viewed in Table 17. MycoAlert® Reagent was added to the cleared media supernatant and reading A was performed after 5 minutes using a luminometer. MycoAlert® Substrate was added to the sample and reading B was performed after 10 minutes. The B/A ratio indicated whether the media supernatant contained mycoplasma.

As a guiding line, Aalborg Hospital uses the following ratios for indicating mycoplasma infection.
<1: negative. No infection.
1-2: Borderline. Retest after next passage.
>2: Positive. Mycoplasma infection.

The sample B/A ratio of 0.78 indicates that the OLN-AS7 media supernatant did not contain mycoplasma. The negative control did not show mycoplasma either, though the positive control, which was in the kit, did not show mycoplasma. This is probably due to degradation of enzymes in the positive control and the fact that the positive control expired August 2010. Table 18 shows earlier measurements using the same positive control as in this experiment, which supports the statement that the positive control had degraded over time and therefore was not reliable.

Table 17: The negative control contained DMEM media 1 g/L glucose containing penicillin/streptomycin and 10 % FCS. The positive control does not show a ratio >2 as expected, but this is probably due to degradation of enzymes in the positive control (the positive control expired August 2010). The following table shows previous readings using the positive control.

Sample	Reading A	Reading B	Ratio B/A
Negative control	76	64	0.84
OLN-AS7 media supernatant	80	62	0.78
Positive control	151	62	0.41

Table 18: The positive control showed lower B/A ratios over time. The enzymes in the positive control had degraded over time and it was not reliable as a control. Source: Anne (Laboratory technician, Clinical Immunology, Aalborg Hospital).

Positive control	Ratio B/A
May, 2009	~8
July, 2009	2.5
2010	1.52

B.2 Restriction Site Analysis

The nucleotide sequence of the original pAcGFP1-N1 without p25α is known in advance and shown below. Translation of this using ExPASy translation tool computed the amino acid sequence below, showing that it contains some mutations compared to the original sequence of Green fluorescent protein of *Aequorea coerulescens* (see below). This was carried out by the manufacturer to remove specific restriction sites. The sequence of pAcGFP1-N1 and the pEGSH vector are shown below.

p25α was inserted into pAcGFP1-N1 using restriction sites *Eco*RI and *Bam*HI. These are shown in colors pink and grey below**.** To retain the sequence of both p25α and GFP, restriction sites prior to p25α and after GFP are to be used. When digesting pAcGFP1-N1 with *Eco*RI and *Not*I (pink and blue-green colors) and subsequently digesting pEGSH with *Mun*I (blue color) and *Not*I the formed overhangs of digestion with *Eco*RI and *Mun*I are compatible (5' G'AATTC 3' and 5' C'AATTG 3', respectively).

To retain the sequence of p25α without GFP from pAcGFP1-N1, restriction sites *Eco*RI and *Bam*HI can be reused. Subsequently, pEGSH vector is digested with *Mun*I and *Bam*HI to enable ligation. The formed overhangs of digestion with *Eco*RI (5' G'AATTC 3') and *Mun*I (5' C'AATTG 3') are compatible.

It has been investigated whether the chosen restriction enzymes cleave the sequence of p25α and p25α-GFP and that is not the case. The overhangs prior to and right after the sequence of p25α on pAcGFP1-N1 are known, and none of the chosen restriction enzymes cleave in this area.

The restriction enzymes, *Bam*HI, *Not*I, *Eco*RI, and *Mun*I, are very specific and only cleave once which should not cause any problems in identifying the digestion products.
It has been examined whether the restriction enzymes can be used for double digest (buffer compatibility), which is possible.

Legend
Red: amino acid mutation
Green: GFP
Yellow: Original amino acid
Pink: Restriction site for *Eco*RI (5' G'AATTC 3')
Grey: Restriction site for *Bam*HI (5' G'GATCC 3')
Turquoise: Restriction site for *Xba*I (5' T'CTAGA 3')
Blue-green: Restriction site for *Not*I (5' GC'GGCCGC 3')
Blue: Restriction site for *Mun*I (5' C'AATTG 3')
Multiple cloning site is underlined.
Red bold letters indicate in which area p25a is located (but not exact area). This sequence is no longer present in the plasmid.
Blue bold letters indicate primer position
Purple bold is the p25a insert.

p25a TPPP_HUMAN Tubulin polymerization-promoting protein Homosapiens$
NCBI Reference Sequence: NP_008961.1
MADKAKPAKAANRTPPKSPGDPSKDRAAKRLSLESEGAGEGAAASPELSALEEAFRRFAV
HGDARATGREMHGKNWSKLCKDCQVIDGRNVTVTDVDIVFSKIKGKSCRTITFEQFQEAL
EELAKKRFKDKSSEEAVREVHRLIEGKAPIISGVTKAISSPTVSRLTDTTKFTGSHKERF
DPSGKGKGKAGRVDLVDESGYVSGYKHAGTYDQKVQGGK*

Nucleotide sequence of p25a TPPP (NCBI Reference Sequence:NM_007030.2):

ATGGCTGACAAGGCCAAGCCTGCCAAAGCTGCCAACAGGACGCCCCCCAAGTCCCCGGGG
GACCCCTCGAAGGACCGGGCAGCCAAGAGGCTGTCGCTGGAATCGGAGGGTGCTGGTGAG
GGGGCAGCCGCATCCCCTGAGCTCAGTGCCCTGGAGGAGGCCTTCCGGCGCTTTGCCGTG
CACGGGGACGCCAGGGCCACCGGGAGGGAGATGCACGGCAAGAACTGGTCGAAGCTGTGC
AAGGACTGCCAGGTGATCGACGGCAGGAACGTGACCGTCACTGACGTGGACATCGTCTTC
AGCAAGATCAAAGGGAAGTCTTGCCGGACCATCACCTTTGAGCAGTTCCAGGAGGCGCTG
GAGGAGCTCGCCAAGAAGCGATTCAAAGACAAGAGCAGCGAGGAGGCCGTTCGCGAGGTG
CACAGGCTCATCGAGGGCAAGGCGCCCATCATCTCAGGGGTGACGAAAGCCATCTCGTCG
CCCACAGTGTCGAGGCTCACGGACACCACCAAGTTCACGGGCTCCCACAAGGAGCGCTTC
GACCCCTCTGGCAAGGGCAAGGGCAAGGCTGGCCGCGTGGATCTGGTGGACGAGTCAGGC
TATGTGTCCGGCTACAAGCACGCAGGCACCTACGACCAGAAGGTGCAAGGGGGCAAGTAG

Q6YGZ0_9CNID Green fluorescent protein OS=Aequorea coerulescens PE=1SV=1$

GenBank: AAN41637.1 (original GFP sequence)

MSKGAELFTGVVPILIELNGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTL
VTTFSYGVQCFSRYPDHMKQHDFFKSAMPEGYIQERTIFFKDDGNYKSRAEVKFEGDTLV
NRIELTGTDFKEDGNILGNKMEYNYNAHNVYIMTDKAKNGIKVNFKIRHNIEDGSVQLAD
HYQQNTPIGDGPVLLPDNHYLSTQSTLSKDPNEKRDHMIYFEFVTAAAITHGMDELYK*

Translation of entire nucleotide sequence of pAcGFP1-N1 using ExPASy translation tool

5'3'Frame1

-LLIVINYGVISS-PIYGVPRYITYGKWPAWLTAQRPPPIDVNNDVCSHSNANRDFPLTS
MGGVFTVNCPLGSTSSVSYAKYAPY-RQ-R-MARLALCPVHDLMGLSYLAVHLRISHRYY
HGDAVLAVHQWAWIAV-LTGISKSPPH-RQWEFVLAPKSTGLSKMS-QLRPIDANGR-AC
TVGGLYKQSWFSEPSDPLALPDSDLELKLRILQSTVPRARDPPVMVSKGAELFTGIVPIL
IELNGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLSYGVQCFSRYP
DHMKQHDFFKSAMPEGYIQERTIFFEDDGNYKSRAEVKFEGDTLVNRIELTGTDFKEDGN
ILGNKMEYNYNAHNVYIMTDKAKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLL
PDNHYLSTQSALSKDPNEKRDHMIYFGFVTAAAITHGMDELYK-AAATLDHNQPYHICRG
FTCFKKPPTPPPEPET-NECNCCC-LVYCSL-WLQIKQ-HHKFHK-SIFFTAF-LWFVQT
HQCILRRKL-ALIFC-NSR-IFVKSAHFLTNRPKSAKSLINQKNRPR-G-VLFQFGTRVH
Y-RTWTPTSKGEKPSIRAMAHYVNHHPNQVFWGRGAVKH-IGTLKGAPDLELDGESRRTW
RERKGRKRKERALGRWQV-RSRCA-PPHPPRLMRRYRARQVALFGEMCAEPLFVYFSKYI
QICIRS-DNNPDKCFNNIEKGRVLRRKEPAVECVSVRVWKVPRLPSRQKYAKHASQLVSN
QVWKVPRLPSRQKYAKHASQLVSNHSPAPNSAHPAPNSAQFRPFSAPWLTNFFYLCRGRG
RLGL-AIPEVVRRLFWRPRLLQRSIKRQDEDRFA-LNKMDCTQVLRPLGWRGYSAMTGHN
RQSAALMPPCSGCQRRGARFFLSRPTCPVP-MNCKTRQRGYRGWPRRAFLAQLCSTLSLK
REGTGCYWAKCRGRISCHLTLLLPRKYPSWLMQCGGCIRLIRLPAHSTTKRNIASSEHVL
GWKPVLSIRMIWTKSIRGSRQPNCSPGSRRACPTARISS-PMAMPACRISWWKMAAFLDS
STVAGWVWRTAIRT-RWLPVILLKSLAANGLTASSCFTVSPLPIRSASPSIAFLTSSSER
DSGVRNDRPSDAQPAITRFRFHRRLL-KVGLRNRFPGRRLDDPPARGSHAGVLRPP-GEA
N-NTEGDNTGRNPRYDGNKKTE-NARCWVVCS-TRGSVPGLALCRYPTETPLGPIRPRFF
LFPTPPPKFG-RPRARSQRRGGRPCHSLRLLIYTLD-FKTSFLI-KDLGEDPF--SHDQN
PLT-VFVPLSVRPRRKDQRIFLRSFFSARNLLLANKKTTATSGGLFAGSRATNSFSEGNW
LQQSADTKYCPSSVAVVRPPLQELCSTAYIPRSANPVTSGCCQWR-VVSYRVGLKTIVTG
-GAAVGLNGGFVHTAQLGANDLHRTEIPTA-AMRKRHASRREKGGQVSGKRQGRNRRAHE
GASRGKRLVSL-SCRVSPPLT-ASIFVMLVRGAEPMEKRQQRGLFTVPGLLLAFCSHVLS
CVIP-FCG-PYYRHA

Nucleotide sequence of pAcGFP1-N1 containing p25α insert (5365 bp)

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATG-
GAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAAC-
GACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGG-
GACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACAT-
CAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT-
GACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACAT-
GACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTAC-
CATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACT-
CACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAAT-

CAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACG-
CAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTGGTTTAGT-
GAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTCAAGCTTCGAATTCCCAC-
CATGGCTGACAAGGCCAAGCCTGCCAAAGCTGCCAACAG-
GACGCCCCCCAAGTCCCCGGGGGACCCCTCGAAGGACCGGGCAGCCAA-
GAGGCTGTCGCTGGAATCGGAGGGTGCTGGTGAGGGGGCAGCCGCATCCCCTGAGCTCAGTGCCCTGGAGGA
GGCCTTCCGGCGCTTTGCCGTGCACGGGGACGCCAGGGCCACCGGGAGGGAGATGCACGGCAA-
GAACTGGTCGAAGCTGTGC
AAGGACTGCCAGGTGATCGACGGCAGGAACGTGACCGTCACTGACGTGGACATCGTCTTCAG-
CAAGATCAAAGGGAAGTCTTGCCGGACCATCACCTTTGAGCAGTTCCAGGAGGCGCTGGAG-
GAGCTCGCCAAGAAGCGATTCAAAGACAAGAGCAGCGAGGAGGCCGTTCGCGAGGTGCA-
CAGGCTCATCGAGGGCAAGGCGCCCATCATCTCAGGGGTGACGAAAGCCATCTCGTCG
CCCACAGTGTCGAGGCTCACGGACACCACCAAGTTCACGGGCTCCCACAAG-
GAGCGCTTCGACCCCTCTGGCAAGGGCAAGGGCAAGGCTGGCCGCGTGGATCTGGTGGAC-
GAGTCAGGCTATGTGTCCGGCTACAAGCACGCAGGCACCTACGACCA-
GAAGGTGCAAGGGGGCAAGCGGGATCCACCGGTCATGGTGAG-
CAAGGGCGCCGAGCTGTTCACCGGCATCGTGCCCATCCTGATCGAGCTGAATGGCGATGTGAATGGCCA-
GAGCTGAATGGCGATGTGAATGGCCACAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACC-
CAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCAC-
TACGGCAAGCTGACCCTGAAGTTCATCTGCAC-
CACCGGCAAGCTGCCTGTGCCCTGGCCCACCCTGGTGACCACCCTGAGC-
TACGGCGTGCAGTGCTTCTCACGCTACCCCGATCACATGAAGCAGCACGACTTCTTCAA-
GAGCGCCATGCCTGAGGGCTACATCCAGGAGCGCACCATCTTCTTCGAGGATGACGGCAACTA-
CAAGTCGCGCGCCGAGGTGAAGTTCGAGGGCGATACCCTGGTGAATCGCATC-
GAGCTGACCGGCACCGATTTCAAGGAGGATGGCAACATCCTGGGCAATAAGATGGAGTACAAC-
TACAACGCCCACAATGTGTACATCATGACCGACAAGGCCAAGAATGGCAT-
CAAGGTGAACTTCAAGATCCGCCACAACATCGAGGATGGCAGCGTGCAGCTGGCCGACCAC-
TACCAGCAGAATACCCCCATCGGCGATGGCCCTGTGCTGCTGCCCGATAACCAC-
TACCTGTCCACCCAGAGCGCCCTGTCCAAGGACCCCAACGAGAAGCGCGATCACATGATC-
TACTTCGGCTTCGTGACCGCCGCCGCCATCACCCACGGCATGGATGAGCTGTACAAGT-
GAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTA-
GAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAAT-
GAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAG-
CATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACT-
CATCAATGTATCTTAAGGCGTAAATTGTAAGCGTTAA-
TATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAA-
TAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGA-
TAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAA-
GAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCGATGGCCCAC-
TACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCAC-
TAAATCGGAACCCTAAAGGGAGCCCCCGATTTA-
GAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAG-
GAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCA-
CACCCGCCGCGCTTAATGCGCCGCTA-
CAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATA
TGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAA-
GAGTCCTGAGGCGGAAAGAACCAGCTGTGGAATGTGTGTCAGT-
TAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAAT-
TAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATG-
CATCTCAATTAGTCAGCAACCA-
TAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAAT
TTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTGAG-
GAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGATCGATCAAGAGACAGGATGAG-
GATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGA-
GAGGCTATTCGGCTATGACTGGGCACAACAGA-
CAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTC
CGGTGCCCTGAATGAACTGCAAGACGAGGCAGCGCGGCTATCGTGGCTGGCCAC-
GACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGG-
GACTGGCTGCTATTGGGCGAAGTGCCGGGGCAG-
GATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATG-
CAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAA-
CATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGAC-
GAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGAG-
CATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATAT-

CATGGTGGAAAATGGCCGCTTTTCTGGATTCATC-
GACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAA-
TATTGCTGAA-
GAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCT
ATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGC-
GACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTAT-
GAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGAT-
GATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCTAGGGGGAGGCTAACT-
GAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTATGACGGCAATAAAAAGACAGAA-
TAAAACGCACGGTGTTGGGTCGTTTGTTCA-
TAAACGCGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCCAA-
GACCCCATTGGGGCCAA-
TACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAGGCCCAGGGCTCGCAGCCAACGTCGGG
GCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATATACTTTAGATTGATT-
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CAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAG-
GATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAAC-
CACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGG-
TAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGT-
TAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTAC-
CAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGT-
TACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGC-
GAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGA-
GAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGG-
TATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTA-
GAGCTTCCAGGGGGAAACGCCTGGTATCTTTA-
TAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAA
AACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCA-
CATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCATGCAT

Nucleotide sequence of pEGSH vector (MCS from 4670-4820) (4829 bp)

http://www.ncbi.nlm.nih.gov/nuccore/AF104248.2
ATAATAAACCCAAGCTTGGCACTGGGATCTGCGAACGCAGCAAGACG-
TAGCCCAGCGCGTCGGCCCCGAGATGCGCCGCGTGCGGCTGCTGGAGATGGCGGACGCGATG-
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GATTGGCTCCAATTCTTGGAGTGGTGAATCCGTTAGC-
GAGGTGCCGCCCTGCTTCATCCCCGTGGCCCGTTGCTCGCGTTTGCTGGCGGTGTCCCCGGAA-
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CAAACCCCGCCCAGCGTCTTGTCATTGGCGAATTCGAACACGCAGATG-
CAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAA-
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TAGCTGCGCCGATGGTTTCTACAAA-
GATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGA-
CATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCA-
CAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATG-
GAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATG-
GATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAG-
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GACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTA-
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CAAGTGCATTGTTCTCTTGCTGAAAGCTCAGTACCCGGGTCGGAGTACTGCCCCGCCCCTAGC-
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GAAAGCTAAGCAAATAAACAAGCGCAGCTGAACAAGCTAAACAATCTGCAGTAAAGTGCAAGT-
TAAAGTGAATCAATTAAAAGTAACCAGCAACCAAGTAAATCAACTGCAACTACT-
GAAATCTGCCAAGAAGTAATTATTGAATACAAGAAGACAA**CTCTGAATACTTTCAAAAGT-**
TACCGAGAAAGAAGAACTCAGACACAGCAGAAGAGGCAATTGGTACCGGATCCGATATC-
GATGCGGCCGCTCGAGACTAGTGAGCTCGTCGACTCTAGACTCTTCTGGTTCTGGCGACTA-
TAAGGATGACGATGACAAGTAATAG**CCCTTTAGTGAGGGTTAATT**GCTAGC

Nucleotide sequence of pEGSH vector construct with p25α insert (5488 bp)

ATAATAAACCCAAGCTTGGCACTGGGATCTGCGAACGCAGCAAGACG-
TAGCCCAGCGCGTCGGCCCCGAGATGCGCCGCGTGCGGCTGCTGGAGATGGCGGACGCGATG-
GATATGTTCTGCCAAGGGTTGGTTTGCGCATTCACAGTTCTCCGCAAGAATT-

GATTGGCTCCAATTCTTGGAGTGGTGAATCCGTTAGC-
GAGGTGCCGCCCTGCTTCATCCCCGTGGCCCGTTGCTCGCGTTTGCTGGCGGTGTCCCCGGAA-
GAAATATATTTGCATGTCTTTAGTTCTATGATGACA-
CAAACCCCGCCCAGCGTCTTGTCATTGGCGAATTCGAACACGCAGATG-
CAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAA-
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TAGCTGCGCCGATGGTTTCTACAAA-
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CAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATG-
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GATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAG-
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GAGCTGATGCTTTGGGCCGAG-
GACTGCCCCGAAGTCCGGCACCTCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGA-
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GACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTA-
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CAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGA-
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CATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATC-
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CAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAA-
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TAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTA-
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TATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATT-
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CAAGTGCATTGTTCTCTTGCTGAAAGCTCGATGGA-
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TAAAGTGAATCAATTAAAAGTAACCAGCAACCAAGTAAATCAACTGCAACTACT-
GAAATCTGCCAAGAAGTAATTATTGAATACAAGAAGACAA**CTCTGAATACTTTCAAAAGT-**
TACCGAGAAAGAAGAACTCAGACACAGCAGAAGAG**CAATTG**CCACC**ATGGCTGA-**
CAAGGCCAAGCCTGCCAAAGCTGCCAACAG-
GACGCCCCCCAAGTCCCCGGGGGACCCCTCGAAGGACCGGGCAGCCAA-
GAGGCTGTCGCTGGAATCGGAGGGTGCTGGTGAGGGGGCAGCCGCATCCCCTGAGCTCAGTGCCCTGGAGGA
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GACGTGGACATCGTCTTCAGCAAGATCAAAGGGAAGTCTTGCCGGACCATCACCTTTGAG-
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GAGCGCTTCGACCCCTCTGGCAAGGGCAAGGGCAAGGCTGGCCGCGTGGATCTGGTGGAC-
GAGTCAGGCTATGTGTCCGGCTACAAGCACGCAGGCACCTACGACCA-
GAAGGTGCAAGGGGGCAAGCG**GGATCC**GATATCGAT**GCGGCCGC**TCGAGACTAGT-
GAGCTCGTCGACTCTAGACTCTTCTGGTTCTGGCGACTATAAGGATGACGATGACAAGTAA-
TAG**CCCTTTAGTGAGGGTTAATT**GCTAGC

Nucleotide sequence of pEGSH vector with p25α-GFP insert (6206 bp)

ATAATAAACCCAAGCTTGGCACTGGGATCTGCGAACGCAGCAAGACG-
TAGCCCAGCGCGTCGGCCCCGAGATGCGCCGCGTGCGGCTGCTGGAGATGGCGGACGCGATG-
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GATTGGCTCCAATTCTTGGAGTGGTGAATCCGTTAGC-
GAGGTGCCGCCCTGCTTCATCCCCGTGGCCCGTTGCTCGCGTTTGCTGGCGGTGTCCCCGGAA-
GAAATATATTTGCATGTCTTTAGTTCTATGATGACA-
CAAACCCCGCCCAGCGTCTTGTCATTGGCGAATTCGAACACGCAGATG-
CAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTCGAA-
CACCGAGCGACCCTGCAGCGACCCGCTTAACAGCGTCAACAGCGTGCCGCA-
GATCCCGGGGGGCAATGAGATATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGA-
GAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAA-
GAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAA-
TAGCTGCGCCGATGGTTTCTACAAA-
GATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGA-
CATTGGGGAATTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCA-
CAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATG-
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GACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCGGGCGTA-
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CAAGTGCATTGTTCTCTTGCTGAAAGCTCGATGGA-
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CAAGGCCAAGCCTGCCAAAGCTGCCAACAG-
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GAGGCTGTCGCTGGAATCGGAGGGTGCTGGTGAGGGGGCAGCCGCATCCCCTGAGCTCAGTGCCCTGGAGGA
GGCCTTCCGGCGCTTTGCCGTGCACGGGGACGCCAGGGCCACCGGGAGGGAGATGCACGGCAA-
GAACTGGTCGAAGCTGTGCAAGGACTGCCAGGTGATCGACGGCAGGAACGTGACCGTCACT-
GACGTGGACATCGTCTTCAGCAAGATCAAAGGGAAGTCTTGCCGGACCATCACCTTTGAG-
CAGTTCCAGGAGGCGCTGGAGGAGCTCGCCAAGAAGCGATTCAAAGACAAGAGCAGCGAG-
GAGGCCGTTCGCGAGGTGCACAGGCTCATCGAGGGCAAGGCGCCCATCATCTCAGGGGTGAC-
GAAAGCCATCTCGTCGCCCACAGTGTCGAGGCTCACGGACACCACCAAGTTCACGGGCTCCCACAAG-
CAAGGAGCGCTTCGACCCCTCTGGCAAGGGCAAGGGCAAGGCTGGCCGCGTGGATCTGGTGGAC-
GAGCGCTTCGACCCCTCTGGCAAGGGCAAGGGCAAGGCTGGCCGCGTGGATCTGGTGGAC-
GAGTCAGGCTATGTGTCCGGCTACAAGCACGCAGGCACCTACGACCA-
GAAGGTGCAAGGGGGCAAGCGGGATCCACCGGTCATGGTGAG-
CAAGGGCGCCGAGCTGTTCACCGGCATCGTGCCCATCCTGATCGAGCTGAATGGCGATGTGAATGGCCA-
GAGCTGAATGGCGATGTGAATGGCCACAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACC-
CAAGTTCAGCGTGAGCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCAC-
TACGGCAAGCTGACCCTGAAGTTCATCTGCAC-
CACCGGCAAGCTGCCTGTGCCCTGGCCCACCCTGGTGACCACCCTGAGC-
TACGGCGTGCAGTGCTTCTCACGCTACCCCGATCACATGAAGCAGCACGACTTCTTCAA-
GAGCGCCATGCCTGAGGGCTACATCCAGGAGCGCACCATCTTCTTCGAGGATGACGGCAACTA-
CAAGTCGCGCGCCGAGGTGAAGTTCGAGGGCGATACCCTGGTGAATCGCATC-
GAGCTGACCGGCACCGATTTCAAGGAGGATGGCAACATCCTGGGCAATAAGATGGAGTACAAC-
TACAACGCCCACAATGTGTACATCATGACCGACAAGGCCAAGAATGGCAT-
CAAGGTGAACTTCAAGATCCGCCACAACATCGAGGATGGCAGCGTGCAGCTGGCCGACCAC-
TACCAGCAGAATACCCCCATCGGCGATGGCCCTGTGCTGCTGCCCGATAACCAC-
TACCTGTCCACCCAGAGCGCCCTGTCCAAGGACCCCAACGAGAAGCGCGATCACATGATC-
TACTTCGGCTTCGTGACCGCCGCCGCCATCACCCACGGCATGGATGAGCTGTACAAGT-
GAGCGGCCGCTCGAGACTAGTGAGCTCGTCGACTCTAGACTCTTCTGGTTCTGGCGACTA-
TAAGGATGACGATGACAAGTAATAGCCCTTTAGTGAGGGTTAATTGCTAGC

B.3 Primer Calculations for Colony PCR

The primers used for colony PCR were included in the Complete Control Inducible Mammalian Expression System kit.

Preparation of Primer Stocks

pEGSH forward sequencing primer: 5´-CTCTGAATACTTTCAAAAGTTAC-3´
T3 promoter sequencing primer: 5´-AATTAACCCTCACTAAAGGG-3´
2.5 µg of each primer is provided in the kit.

Table 19: Calculation of volume of DNA water which should be added to the lyophilized primer to obtain a stock concentration of 100 µM. Calculated using http://www.basic.northwestern.edu/biotools/oligocalc.html (22-02-11 at 15:39)

Primer Stock Preparation				
Fw primer				
	M	6982 g/mol	C	$100*10^{-6}$ M
	m	$2.5*10^{-6}$ g	V	$3.5806*10^{-6}$ L = **3.58 µL**
	n	$3.5806*10^{-10}$ mol	n	$3.5806*10^{-10}$ mol
Rw primer				
	M	6094 g/mol	C	$100*10^{-6}$ M
	m	$2.5*10^{-6}$ g	V	$4.1024*10^{-6}$ L = **4.10 µL**
	n	$4.1024*10^{-10}$ mol	n	$4.1024*10^{-10}$ mol

Preparation of PCR Program

The formula for calculating the melting temperature T_m was acquired from PhD student Mia Larsen, Section of Biotechnology, AAU.

$$T_m = 4 \cdot (G + C) + 2 \cdot (A + T)$$

This equation can be used for primers with a length of max 25 bp.

Fw (23 bp): $T_m = 4 \cdot (7) + 2 \cdot (16) = 60°C$

Rw (20 bp): $T_m = 4 \cdot (8) + 2 \cdot (12) = 56°C$

Annealing temperature should be 3-5 °C below the melting temperature of the less stable primer.

B.4 Calculations for Dephosphorylation and Ligation Experiments

Calculation of volume of shrimp alkaline phosphatase (SAP) needed to dephosphorylate 500 ng vector

The molecular weight of the double stranded DNA was calculated using http://www.ambion.com/techlib/append/na_mw_tables.html (14-04-2011 at 11:10 AM). The only information given by the manufacturer of SAP is that 1 µg linear 3 kbp DNA (approximately 1 pmol termini) is required for 1 u SAP. The number of units SAP required for the dephosphorylation of the pEGSH digests is calculated in the following.

The approximate molecular weight of the pEGSH digest for p25α (4818 bp):

$$4818 \cdot 607.4 g/mol + 157.9 g/mol = 2.92661 \cdot 10^6 g/mol$$

The approximate molecular weight of the pEGSH digest for p25α-GFP (4802 bp):

$$4802 \cdot 607.4 g/mol + 157.9 g/mol = 2.91689 \cdot 10^6 g/mol$$

The approximate molecular weight of a 3000 bp plasmid:

$$3000 \cdot 607.4 g/mol + 157.9 g/mol = 1.82236 \cdot 10^6 g/mol$$

Number of moles in 1 μg 3000 bp linear plasmid: $\frac{(1\cdot 10^{(-6)})g}{1.82236\cdot 10^{6}\,g/mol} = 5.488\cdot 10^{(-13)}\,mol$

Number of moles in 500 ng 4818 bp linear pEGSH: $\frac{(0.5\cdot 10^{(-6)})g}{2.92661\cdot 10^{6}\,g/mol} = 1.70846\cdot 10^{(-13)}\,mol$

Number of moles in 500 ng 4802 bp linear pEGSH: $\frac{(0.5\cdot 10^{(-6)})g}{2.91689\cdot 10^{6}\,g/mol} = 1.71415\cdot 10^{(-13)}\,mol$

Molar ratio between 3000 bp plasmid and pEGSH digests:

pEGSH digest (4818 bp): $\frac{(1.70846\cdot 10^{(-13)})mol}{5.488\cdot 10^{(-13)}\,mol} = 0.3113$

pEGSH digest (4802 bp): $\frac{(1.71415\cdot 10^{(-13)})mol}{5.488\cdot 10^{(-13)}\,mol} = 0.3123$

1 μL (1 u) SAP necessary for 3000 bp plasmid.
0.3 μL SAP necessary for 4818 bp and 4802 bp linear pEGSH vectors.

Calculation of ng insert necessary to obtain a 1:3 molar vector:insert ratio for ligation
The following equation was obtained from the Promega ligation kit protocol (LigaFast™ Rapid DNA Ligation System), June, 2005.

$$\text{ng insert} = \frac{(\text{ng vector}\cdot\text{bp size insert})}{(\text{bp size vector})}\cdot(\text{molar ratio of insert : vector})$$

p25α insert:

$$\text{ng insert} = \frac{(100\text{ ng}\cdot 670\text{ bp})}{(4818\text{ bp})}\cdot\frac{3}{1} = 41.7\text{ ng}$$

p25α-GFP insert:

$$\text{ng insert} = \frac{(100\text{ ng}\cdot 1404\text{ bp})}{(4802\text{ bp})}\cdot\frac{3}{1} = 87.7\text{ ng}$$

B.5 Restriction Analysis of Constructs

The agarose gels applied for the restriction analysis of the midiprep plasmid purification eluates of the p25α-pEGSH and p25α-GFP-pEGSH constructs can be viewed in Figure 36 and Figure 37.

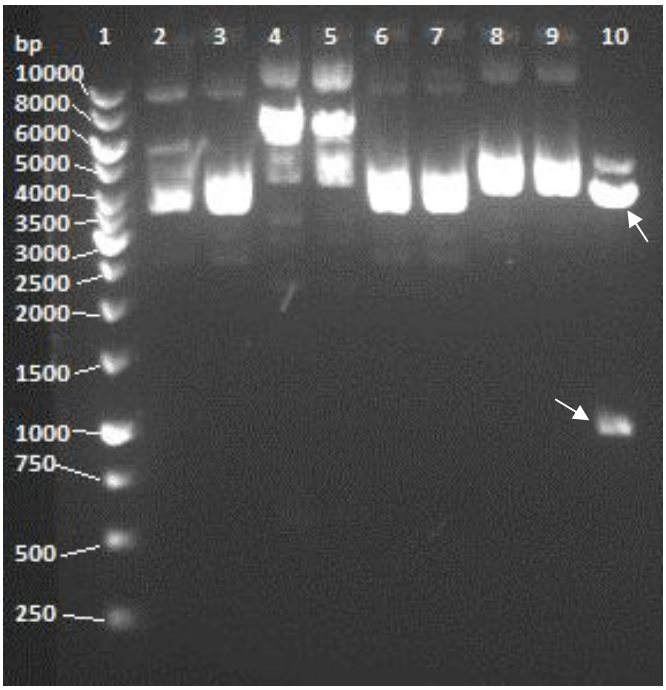

Figure 36: 1 % agarose gel displaying the *Acu*I digests of the p25α-pEGSH and p25α-GFP-pEGSH constructs from the midiprep plasmid purification. Approximately 750 ng of each construct vector was applied and 510 ng pEGSH control vector was applied. Well 1: 1 kbp ladder. Well 2: p25α-pEGSH construct A4 with 1 µL *Acu*I. Well 3: p25α-pEGSH construct C2 with 1 µL *Acu*I. Well 4: p25α-GFP-pEGSH construct A1 with 1 µL *Acu*I. Well 5: p25α-GFP-pEGSH construct A2 with 1 µL *Acu*I. Well 6: p25α-pEGSH construct A4 without enzyme (ctrl). Well 7: p25α-pEGSH construct C2 without enzyme (ctrl). Well 8: p25α-GFP-pEGSH construct A1 without enzyme (ctrl). Well 9: p25α-GFP-pEGSH construct A2 without enzyme (ctrl). Well 10: pEGSH vector with 1 µL *Acu*I. In well 10, bands around 1000 bp and 4000 bp were apparent, which correspond to the digest of the pEGSH vector of 1009 bp and 3820 bp, respectively. Well 2-5 containing construct vectors and *Acu*I did not show bands according to the theoretical digests. The negative controls showed bands around 5000 bp for p25α-pEGSH in well 6 and 7 and around 6000 bp for p25α-GFP-pEGSH in well 8 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively.

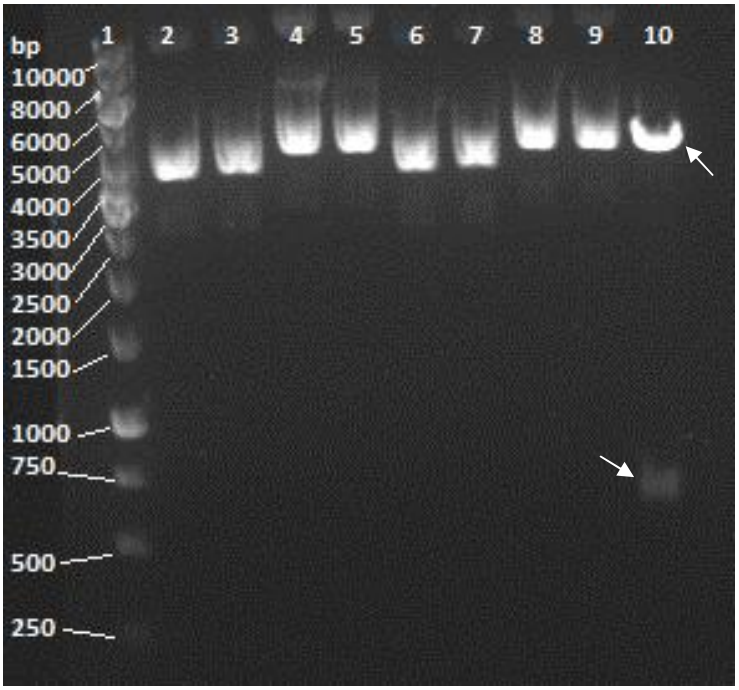

Figure 37: 1 % agarose gel displaying the *Hind*III and *Bgl*II digests of the p25α-pEGSH and p25α-GFP-pEGSH constructs from the midiprep plasmid purification. Approximately 750 ng of each construct vector was applied and 510 ng pEGSH control vector was applied. Well 1: 1 kbp ladder. Well 2: p25α-pEGSH construct A4 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 3: p25α-pEGSH construct C2 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 4: p25α-GFP-pEGSH construct A1 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 5: p25α-GFP-pEGSH construct A2 with 1 µL *Bgl*II and 0.5 µL *Hind*III. Well 6: p25α-pEGSH construct A4 without enzyme (ctrl). Well 7: p25α-pEGSH construct C2 without enzyme (ctrl). Well 8: p25α-GFP-pEGSH construct A1 without enzyme (ctrl). Well 9: p25α-GFP-pEGSH construct A2 without enzyme (ctrl). Well 10: pEGSH vector with 1 µL *Bgl*II and 0.5 µL *Hind*III. In well 10, bands around 5000 bp and just below 750 bp were apparent, which correspond to the digest of the pEGSH vector of 4142 bp and 687 bp, respectively. Well 2-5 containing construct vectors and *Bgl*II+*Hind*III did not show bands according to the theoretical digests. The negative controls showed bands around5000 bp for p25α-pEGSH in well 6 and 7 and around 6000 bp for p25α-GFP-pEGSH in well 8 and 9, respectively, which corresponded to the theoretical sizes of p25α-pEGSH and p25α-GFP-pEGSH of 5488 bp and 6206 bp, respectively.

B.6 DNA Extraction and Precipitation

Table 20 shows the DNA concentration of the p25α-pEGSH A4 and C2 and p25α-GFP-pEGSH A1 constructs, purified using the midiprep kit, Nucleobond® Xtra Midi kit (Macherey Nagel), and extracted by applying the first phenol-chloroform extraction protocol. The DNA concentrations of the p25α-pEGSH construct A4 and C2 samples had decreased compared to prior to the phenol-chloroform extraction, cf. Table 13. The second protocol was applied for the p25α-GFP-pEGSH A1 midiprep construct, performing the ethanol precipitation by incubating the samples at -80 °C for 15 minutes, which gave a considerably higher DNA concentration.

Table 20: DNA concentration measurement of the midiprep purified constructs after phenol-chloroform extraction. An Abs 260/280 value of ~1.8 indicated pure DNA and a value of ~2.0 indicated pure RNA. The expected value of Abs 260/230 was ~2.0-2.2.

Midiprep Phenol-chloroform DNA	ng/µL	260/280	260/230	Total µg
p25α-pEGSH construct A4				
1	65.8	1.91	2.21	2.63
2	38.6	1.90	2.28	1.54
3	9.4	2.14	2.19	0.38
4	38.8	1.90	2.11	1.55
5	17.0	1.89	2.40	0.68
6	927.8	1.87	2.30	37.1
7	16.6	1.94	2.08	0.66
p25α-pEGSH construct C2				
1	52.0	1.53	1.56	1.56
2	37.3	1.96	2.25	1.11
3	18.6	1.99	2.40	0.6
4	9.1	2.42	2.55	0.27
5	9.8	1.73	1.86	0.29
8	36.2	1.86	2.20	1.09
9	107.1	1.85	2.24	3.21
10	41.9	1.88	2.17	1.26
p25α-GFP-pEGSH construct A1				
1	640.4	1.83	2.22	14.7
2	511.7	1.90	2.26	11.8
3	479.1	1.90	2.32	11.0
4	459.4	1.93	2.41	10.6
5	508.1	1.88	2.30	11.7
6	513.5	1.90	2.31	11.8
7	480.9	1.89	2.27	11.1
8	499.0	1.88	2.27	11.5
9	534.5	1.88	2.27	12.3
10	586.1	1.87	2.27	13.5
11	1366.5	1.89	2.27	31.4
12	528.4	1.88	2.28	12.2
13	543.8	1.88	2.27	12.5
14	555.4	1.88	2.22	12.8
15	524.5	1.89	2.24	12.1
16	519.4	1.88	2.25	11.9