Chapter 1

Introduction
Introduction

Intellectual disability (ID) is present in 2-3 percent of the population and patients with ID show a significantly higher use of health care facilities compared to the general population\(^1\). The causes of intellectual disability are wide ranging and include genetic and environmental factors. ID can be caused by a myriad of factors including prenatal infections, prenatal uses of medicine and alcohol, brain malformation, monogenetic mutations in ID genes, chromosomal abnormalities and metabolic diseases. However, for 30-50% of diagnosed ID patients, the cause remains unknown\(^1-3\).

The Ubiquitin proteasome pathway

Before the Ubiquitin proteasome pathway (UPP) was identified, proteins were thought to solely undergo lysosomal degradation. Research in the late 1970's showed that reticulocytes lacking lysosomes had ATP dependent protein degradation, suggesting a second kind of degradation system in place\(^4-8\). Shortly thereafter, Ciechanover and colleagues described a heat stable polypeptide (APF-1), and Goldstein and colleagues described a ubiquitin peptide (first named UBIP; ubiquitous immunopoietic polypeptide) linked to proteins that were later degraded\(^9-12\). APF-1 and ubiquitin where later identified to be the same protein\(^6,13\).

The discovery of the UPP resulted in a shared Nobel prize for chemistry awarded to Aaron Ciechanover, Avram Hershko and Irwin Rose in 2004.

The Ubiquitin proteasome pathway functions in a two-step process. First the identification and tagging of proteins destined for degradation. Second, degradation of the protein by the 26S proteasome. Identification and tagging of proteins involves the addition of ubiquitin moieties which is a conserved 76-residue long peptide that can be linked to a protein by the formation of a bond between the C-terminal glycine of ubiquitin and a lysine on the substrate protein\(^5-9,14\). The ubiquitination of a protein is a three-step process initiated with the E1 ubiquitin-activating enzyme forming a thiol ester bond with the carboxyl group of glycine at amino acid 76 of ubiquitin (G76), activating the ubiquitin C-terminus; this process requires ATP\(^9,13\). In the second step a trans-esterification reaction transfers the activated ubiquitin to the E2 conjugating enzyme on a cysteine residue in the active site of the E2\(^8,15\). The E2 enzymes determine the
length and shape of the poly-ubiquitin chain and can thereby influence the fate of the ubiquitinated proteins. Finally the E2 enzyme ubiquitin complex transfers the activated ubiquitin to an E3 ligase-substrate complex (Figure 1).

**Figure 1**: Schematic overview of ubiquitination. An ubiquitin activating enzyme (E1) activates an ubiquitin. The activated ubiquitin is then transferred to the ubiquitin conjugating protein (E2). Subsequently the activated ubiquitin is transferred to an E3 ligase-subunit complex, either directly from the E2 to the substrate (RING ligase) or indirectly via the E3 ligase (HECT). After the substrate is sufficiently ubiquitinated the protein is degraded by the 26S proteasome. (Figure adapted from http://www.bikaken.or.jp)

There are three types of E3 protein ligases, classified on the presence of a HECT domain (Homologous to E6AP Carboxyl-Terminus), RING domain (Really Interesting New Gene) or Ring-in-between-Ring (RBR) domain. The RING ligases form multimeric complexes for its functioning, binding both the E2 and the target protein and catalyzing the direct transfer of the ubiquitin from the E2 to the target protein. The HECT ligases, on the other hand, accept the ubiquitin from the E2 conjugating enzymes and form an E3 thio-ester intermediate with the ubiquitin moiety prior to transferring the ubiquitin to the substrate. Ring between ring ligases share features of both HECT and RING E3 ligases. Although there are two RING domains present and an in between ring domain (IBR), this E3 ligase was found to contain a catalytic cysteine in the second RING that actually mediated ubiquitination in a HECT like manner.

The fate of the ubiquitinated protein depends on the particular lysine involved in chain formation and the length of the chain. There are seven lysines on the ubiquitin where a second ubiquitin can bind. When substrate proteins have only a single ubiquitin group, this mono-ubiquitination is a signal for...
endocytotic trafficking of plasma membrane proteins or initiation of DNA repair and may function as subsequent ubiquitin receptors in the endosome\textsuperscript{18,22}. Lysine 63 (K63)-linked ubiquitin chains can actually make novel ubiquitin chains for signaling in DNA repair and control of endocytosis\textsuperscript{19,20,23}. There is a form of linear ubiquitination that activates nuclear-factor kb (NF-kB), an important transcription factor involved in immune responses\textsuperscript{21}. Lysine 48 (K48) ubiquitination, however, is the most common modification and targets proteins for degradation\textsuperscript{22} (Figure 2). The high complexity of the ubiquitin chains and the possibility to build a ubiquitin chain on different lysine residues on the same protein can have many different consequences for different proteins\textsuperscript{23}.

![Figure 3: 3D representation of the 26S proteasome. On the left side the inner 20S core with the alpha en beta subunits. On the right side the two 19S regulatory subunits with the 20S subunit in the middle. (Figure adapted from Adams 200411)](image)

The second step in the UPP is the degradation of the proteins by the 26S proteasome into peptides varying in size between 3 and 23 amino acids. The ubiquitinated proteins are transported to the proteasome by shuttle proteins\textsuperscript{24,25}. The proteasome itself has two major units, the 20S core and two 19S regulatory domains on either end of the inner 20S cylinder, resembling two lids. The 19S unit recognizes the poly-ubiquitinated proteins and propels them towards the 20S core in an ATP-dependent mechanism\textsuperscript{26}. Degradation takes place in the inner rings of the 20S proteasome after removal of the ubiquitin peptides by
de-ubiquitinating enzymes (DUBs) and unfolding of the protein. The 20S part consists of two outer rings and two inner rings; the outer rings contain 7 alpha subunits and the inner ring 7 beta subunits. The catalytic activity results from the activation of 3 of the 7 beta subunits (1, 2 and 5). The inner core is very small, enabling the unfolded proteins to pass through\(^{27,28}\) (Figure 3).

**Figure 3:** 3D representation of the 26S proteasome. On the left side the inner 20S core with the alpha and beta subunits. On the right side the two 19S regulatory subunits with the 20S subunit in the middle. (Figure adapted from Adams 2004\(^{11}\))

In general the ubiquitin proteasome pathway (UPP) controls itself by specifically regulating the ubiquitination through the E3 ligases and the E2 ligases. General up-regulation of the whole UPP pathway is seen in muscle wasting as discussed later in this introduction and in cases of extreme tissue breakdown as is encountered during larval metamorphosis\(^{19}\).

There are a lot of specific regulation mechanisms in the UPP. For example some E3 ligases specialize in recognizing phosphorylated proteins\(^{30-34}\), destabilized residues of proteins or very specific sequences that are not always exposed\(^{35,36}\). Some E3 ligases bind only in trans when the target proteins need to bind first to ancillary proteins (like E6 mediated breakdown of p53 as described later). Another form of UPP regulation is seen in modification by ubiquitin-like proteins. These ubiquitin-like proteins conjugate to a protein in a ubiquitin like manner, locally changing the topography of a protein. Hereby these ubiquitin-like proteins can mask or open the binding sites of E3 ligases (review see Glickman et al 2002)\(^{37}\).
UPP in synaptic plasticity, learning and memory

That ubiquitination plays a role in neurodegenerative disorders like Alzheimer was already discovered in the late 1980s\textsuperscript{38-40}. However, it took almost a decade before the first description of the physiological role of the UPP was described in the nervous system. The first description of the involvement of the UPP was of degradation of a subunit of cAMP-dependent Protein Kinase A (PKA) in Long Term Facilitation (LTF) in Aplysia\textsuperscript{41-43}. Over the years the exact mechanism how the UPP controls synaptic plasticity remains unclear. One theory proposed by Dong et al (2008) suggests a potential mechanism starting with the protein Activating Transcription Factor 4 (ATF4) a known cAMP Response Element-Binding protein (CREB) inhibitor, which was shown to be degraded upon chemically induced Long Term Potentiation (LTP). This in turn promotes CREB activity and results in the expression of Brain Derived Neurotrophic Factor (BDNF) involved in the expression of LTP\textsuperscript{44}. Further evidence proving that UPP-mediated protein degradation is necessary for learning was shown by Banerjee et al (2009). They showed that the MOV10 (Moloney Leukemia Virus 10) helicase which is part of the RNA Induced Silencing Complex (RISC), is rapidly degraded when NMDA receptors are activated. Due to this degradation, a set of mRNA's selectively enters the polyribosome compartment (e.g. CaMK2A; alpha-Calcium/Calmodulin-dependent protein Kinase 2 and Limk1; Lysophospholipase 1). This takes place in dendrites, making MOV10 a potential pivotal control element in regulating local protein synthesis in dendrites\textsuperscript{45}. Additionally Ehlers et al (2003) showed that the Post Synaptic Density (PSD) is altered upon synaptic activity, which is accompanied by altered protein turnover. This was proven to take place through proteasome mediated degradation, thus showing a direct link between protein turnover and the organization of synapses\textsuperscript{46}. It was later shown that ubiquitination and degradation of PSD-associated proteins occurs during several learning paradigms\textsuperscript{47}.

The UPP system is also involved in Long-Term Depression (LTD)\textsuperscript{48}. LTD can be NMDA-dependent or mGluR-dependent. For NMDA-dependent LTD, Post-Synaptic Density protein 95 (PSD-95) is of major importance at the postsynaptic density where it tethers AMPA- and NMDA- glutamate receptors to signaling proteins and to the cytoskeleton. PD95 is recognized by the E3 ligase Mdm2. When the NMDA receptor is activated by an LTD-inducing stimulus, PSD-95 is degraded. Preventing ubiquitination or inhibiting the proteasome prevents
NMDA-receptor induced AMPA internalization, suggesting that the UPP is involved in regulating AMPA receptor surface expression upon LTD induction\(^49\). Whether mGluR-dependent LTD also depends on the UPP is not clear: some studies claim to see reduction when inhibiting the proteasome while others claim to see an enhancement \(^49,50\).

Finally, the proteasome itself is effectively recruited to the synapse in response to synaptic activity where it can remodel the synapses\(^51\). This could be mediated through NAC1, a proteasome-associated protein, because NAC1 knock out (KO) mice do not show activity dependent translocation. Alternately this could be through translocation of CaMK2A to the PSD\(^52\), since a Camk2a mutant, which cannot translocate to the PSD, results in less proteasome targeting in the PSD\(^53\). The increased activation of the proteasome upon NMDA receptor activation is thought to be dependent on CaMK2A that directly phosphorylates RPT6, a subunit of the before mentioned 19S complex of the 26S proteasome\(^53\).

Infusion with the proteasome inhibitor lactacystin of CA1 hippocampal region in rats causes retrograde amnesia of inhibitory avoidance learning\(^54\). This may be ascribed to increased ubiquitination and 26S proteasome proteolytic activity in hippocampus 4 hours after training\(^54\). Proteasome inhibitor administration also increased cell surface expression of the GluR1 subunit of the AMPA receptor, and augmented fear memory as measured by freezing after tone shock conditioning\(^55\). Fear extinction learning is also affected by proteasomal inhibition\(^56\). Moreover, infusion of proteasome inhibitor right after memory retrieval in CA1 region disrupts anisomysin-induced memory loss, suggesting that UPP underlies the destabilization process after fear memory retrieval\(^47\). Finally, blocking the entire proteasome increases synapse number and strength\(^57\). All these results indicate that proteasome activity is needed for long-term memory consolidation and the maintenance of late LTP (needed to retain memory) the UPP inhibits the induction of L-LTP, but enhanced the maintenance, by preventing degradation of transcription repressors and by stabilizing translation suppressors involved in the late stage of translation in both dendrites and the cell body\(^44,58\).

Further involvement of the UPP in learning and memory is demonstrated by the mouse mutant lacking a specific DUB (UCH-L3 ubiquitin C-terminal hydrolase L3), which has deficits in working memory. However hippocampal LTP is not affected in these mice\(^69\), indicating that de-ubiquitination is essential for working memory but this particular DUB not for LTP.
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To conclude, it is clear that ubiquitination and protein degradation is important for synaptic plasticity to occur and for learning and memory. The effect of proteasome inhibition is extensive, encompassing processes like LTD, LTP L-LTP, but the precise mechanism remain largely unknown. Besides its role in synaptic plasticity, the UPP has also been associated with postsynaptic density and stability, synapse formation, ubiquitination of AMPA and NMDA as reviewed by Mabb et al 2010.60

Dysfunction of the Ubiquitin proteasome pathway in disease

The UPP described above is well regulated. Therefore a disruption in this process can severely affect an individual. Two types of disruptions in the system are possible, the first being a gain of function mutation, resulting in accelerated degradation of proteins. The second is a loss of function mutation where the proteins accumulate and are not degraded. Given the essential role of the UPP system in the cell, it is likely that mutations that severely affect UPP function might not be compatible with life and therefore will never present as a disease.

Malignancies have been associated with deregulation in the ubiquitin proteasome pathway. It can be easily hypothesized that if growth factors (polypeptides that stimulate cell proliferation) are not properly degraded or tumor suppressor proteins are too heavily degraded that this could be the start of a malignancy.61 This is shown for human papilloma virus (HPV) known to cause cervical cancer. HPV expresses early and late proteins in its life cycle designated by either E1,2, 4-7 and Li-2. The E3 ligase linked to Angelman syndrome (AS) was first identified as a protein associated with the E6 oncoprotein (E6-AP) of the HPV virus.62,63 E6-AP promotes degradation of the p53 tumor suppressor in the presence of HPV, promoting tumor growth.63 Also the tumor suppressor protein p27 undergoes increased degradation in breast-, colorectal- and prostate tumors. The level of p27 determines the prognosis of these cancers, with low levels of this tumor suppressor having a bad prognosis.64 A third example is Von Hippel-Lindau (VHL) disease which is characterized by a wide range of malignancies caused by germ line mutations in the VHL gene. These tumors show a high vascularization due to high expression of Hypoxia inducible factor 1-α (HIF1-α) and vascular endothelial growth factor (VEGF). Much like the role E6AP plays in HPV induced cervical cancer, dysfunction of the E3 ligase VHL and promotes the
growth and the vascularization of the tumors\textsuperscript{65,66}. Many more malignancies have been associated with the UPP but that is beyond the scope of this thesis.

Besides malignancies, mutations in UPP genes can cause several other disorders like Liddle’s syndrome, a form of hereditary hypertension caused by deregulation of the endothelial Na\textsuperscript{+} channel (eNaC). The eNaC channel is normally short lived and targeted for degradation by NEDD4 (Neural precursor cell Expressed Developmentally Down-regulated protein 4) an E\textsubscript{3} HECT ubiquitin ligase. The mutations that cause Liddle’s syndrome disrupt the E\textsubscript{3} recognition of the eNaC thereby stabilizing the Na\textsuperscript{+} channel\textsuperscript{67}. Another disruption of the UPP is seen in severe muscle wasting as a result of long immobilization, denervation and severe metabolic stress, which activates the ubiquitin proteasome pathway resulting in muscle degradation. Research into proteasome inhibitors might be of great benefit to reduce muscle wasting\textsuperscript{68,69}.

Neurodegenerative disorders have been associated with the UPP as well. In Alzheimer disease (AD), misregulation of the UPP probably results from accumulation of tau aggregates. These tau aggregates in AD patients are immuno-reactive to ubiquitin antibodies. In relation to this, there is decreased hydrolyzing activity of the proteasome\textsuperscript{70-72}. Parkinson disease (PD) and Lewy body dementia, both known for Lewy body inclusions containing \textalpha-Synuclein (which is mutated in very rare forms of familiar PD), are ubiquitin-positive like the tau aggregates in AD\textsuperscript{73}. Indeed \textalpha-Synuclein protein has been shown to be targeted by the UPP\textsuperscript{74}. Additionally Parkin, a well-known gene causing a familiar form of PD is an E\textsubscript{3} ligase. Surprisingly however this form of PD develops without the presentation of the Lewy body inclusions\textsuperscript{75}. Mutations in Parkin result in loss of ubiquitation function\textsuperscript{76}. Other rare mutations causing familiar PD are located in \textit{UCH} isozyme \textit{UCH-L1}, a de-ubiquitinating enzyme, again demonstrating the importance of the UPP regulation in Parkinson\textsuperscript{77}. Huntington disease (HD) shows nuclear inclusions also reactive to ubiquitin much like the tau aggregates in AD and the Lewy body inclusions in PD. Like Huntington, both spinocerebral ataxia and spinobulbar muscular atrophy (Kennedy’s disease) are caused by expansions of polyglutamine tracts by different proteins: Huntingtin in HD; ataxins in spinocerebral ataxia and the androgen receptor in Kennedy’s disease\textsuperscript{78-80}. All of these proteins have been shown to be ubiquitinated, although it remains unclear if all these inclusions seen in these diseases are toxic or just a secondary reaction of the body in response to proteins that cannot be eliminated\textsuperscript{81-83}. Finally Amyloid lateral sclerosis (ALS) shows ubiquitin-
immunoreactive intra-neural inclusions like the AD, PD and HD inclusions but in a different area. These inclusions correspond with the progression of the disease, displaying clearly that ubiquitination and the lack of degradation by the proteasome of major importance.

Neurodevelopmental disorders of the central nervous system associated with the UPP are rare and the focus of this thesis. Autism spectrum disorders show a number of candidate genes in the ubiquitin proteasome pathway like PARK, UBE3A, RFWD2 and FBXO40. Kaufman syndrome was first described in 1971, subsequently many descriptions of patients with the same features followed. Kaufman oculocerebrofacial syndrome (KOS) is characterized by reduced growth, hypotonia, facial dismorphia, microcephaly, ocular anomalies and intellectual disability. In 2013 the syndrome was linked to loss of function of UBE3B protein. Very little is known about the precise function of this particular E3 ligase. Additional developmental disorders caused by the UPP are discussed in more detail below.

**UBE2A/HR6A**

The prevalence of mental retardation is significantly higher in males where 16% of male mental retardation is X-Linked Intellectual Disability. This is thought to be due to the sensitivity of gene defects on the X chromosome because of the lack of an alternate allele in males. One example is HR6A/UBE2A, located on the X chromosome, encoding an E2 ubiquitin conjugating enzyme. Males with a deletion or mutation in this gene suffer from a X-Linked Intellectual Disability disorder known as UBE2A deficiency syndrome. UBE2A deficiency syndrome is characterized by psychomotor retardation, severely impaired speech, synophrys, urogenital-, skin abnormalities and mild to severe intellectual disability.

**HR6A** (Human homologue to RAD6A) is a homologue of the *Saccharomyces cerevisiae* RAD6 gene sharing about 70% sequence homology. It is also highly homologous (95%) to HR6B (Human homologue to RAD6B). The *Drosophila* homologue Dhr6 closely resembles HR6A and HR6B proteins (85-87%). Based on the degree of divergence among the different RAD6 homologues, the duplication event of this gene must have occurred 200 x 10^6 years ago in the Jurassic era.

The first evidence of the importance of this gene comes from yeast studies. RAD6 yeast mutants are very sensitive to DNA damage and lack the ability to repair DNA damaged sites. They do not undergo meiosis or sporulation and
grow poorly\textsuperscript{102-104}. These activities are all regulated by the E2 activity of the RAD6 protein. When mutating Cys-88 to Val, the RAD6 protein no longer has E2 activity, causing a defect in DNA repair and sporulation\textsuperscript{105}. Adding either HR6A or HR6B rescues the DNA repair defect seen in yeast\textsuperscript{102}. RAD6 in yeast can poly-ubiquitinate histones H2A and H2B in an E3 independent manner. This is mediated by the highly acidic 23-residue carboxyl tail domain which is required for sporulation but not for DNA repair. Human HR6 does not contain this acidic tail and therefore by adding human HR6 to mutant RAD6 yeast, it can rescue the DNA repair deficit but only a small portion of the sporulation ability\textsuperscript{106,107}. Rad6 protein binds to one of 6 RING finger E3 ligases: Ubr1, Ubr2, Ubr3, Rad18, Rad5 and Bre1\textsuperscript{108}. Ubr1 and 2 are involved in the N-end rule degradation of proteins (proteolytic degradation based on N-terminal residues of short lived proteins) \textsuperscript{109}. Ubr3 regulates sensory pathways via APE1 protein (Apurinic/apyrimidinic Endonuclease 1)\textsuperscript{110}. Rad18 ubiquitinates PCNA (Proliferating Cell Nuclear Antigen) and is responsible for error prone replication of trans lesion synthesis. Rad5 is responsible for DNA repair processes \textsuperscript{111} and Bre1 is involved in histone mono-ubiquitination\textsuperscript{108,112}. In 2013, a seventh E3 ligase was identified that binds to UBE2A being Parkin. Parkin has been implied in the ubiquitination of mitochondrial proteins and mitophagy. Mitophagy deficits were also observed in Drosophila dRad6 mutants and in cells derived from patients with UBE2A deficiency syndrome. Notably, The Drosophila dRad6 mutants showed impaired neuronal vesicle trafficking, providing the first causal link between UBE2A and neuronal function. To sum up, Rad6 is involved in many cellular functions. The interaction of E2 and E3 ligases determines what proteins get ubiquitinated, in the case of Rad6 the functions of the E3 ligases it interacts with explain the deficits seen in the Rad6 mutants.

The involvement of the RAD6 protein in yeast sporulation sparked extensive studies of the human HR6A/B proteins for their involvement in gametogenesis. This resulted in the development of a HR6B mutant mouse; the first mutant in the UPP\textsuperscript{113}. This mutant showed male infertility. This was followed by the development of the HR6A mutant mouse displaying female infertility in homozygous state\textsuperscript{114}. This can be explained by the presence of the HR6A gene located on the X chromosome and the involvement in oogenesis. In spermatogenesis the X chromosome is silenced while in oocytes one active X chromosome is present. HR6B was shown to be important for X chromosome inactivation maintenance in spermatocytes\textsuperscript{115}.
The intellectual disability as seen in UBE2A deficiency syndrome suggests an important role for HR6A in brain function. However, the \textit{Hr6a} mouse mutant has only been studied in relation to reproduction. The role of this protein in brain function is part of this thesis.

\textbf{UBE3A}

In 1965 Dr. Harry Angelman (Figure 4A) described three children with similar features naming them Happy puppet children, after an oil painting (a boy with a puppet by the renaissance artist Giovanni Francesco Caroto) he saw on a vacation in Italy\textsuperscript{115} (Figure 4B). The initial diagnosis was purely clinical. Over the years many more patients were described to have the Angelman (puppet like) syndrome\textsuperscript{116-122}. Changing the name to Angelman syndrome was first suggested in 1982 by Charles Williams and Jaime L. Frias to avoid offending the family members\textsuperscript{117}. Angelman syndrome is characterized by severe developmental delay, absence of speech, epilepsy, movement and balance deficits and inappropriate laughter\textsuperscript{123}. The first clue to the underlying genetic defect came in 1987 when two articles described deletions on chromosome 15 that were not associated with Prader-Willi syndrome\textsuperscript{124,125}. Prader-Willi syndrome is characterized by failure to thrive in infancy and in a later stage by excessive eating and weight gain and developmental delay. Subsequent investigations showed that chromosome 15 is subjected to parent-of-origin imprinting, paternal deletions leading to Prader-Willi syndrome and maternal deletions causing Angelman syndrome\textsuperscript{126,127}. These syndromes were described as sister syndromes\textsuperscript{128}.

\textbf{Figure 4: A.} Dr. Harry Angelman, \textbf{B.} A boy with a puppet by Giovanni Francesco Caroto
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Angelman syndrome has a birth incidence rate of 1:25,000\(^{129}\) and results from maternal *de novo* gene deletions (70%); a point mutation in the *UBE3A* gene (10%); imprinting defects (5%); UPD (UniParental Disomy) (5%). In 10% of the cases no mutation is found in the *UBE3A* gene\(^{130}\). The chromosomal region associated with Angelman syndrome is highly imprinted, where some genes are only expressed from the paternal allele and some only from the maternal allele as reviewed by Horsthemke et al 2008\(^{127}\) (Figure 5). A single large transcript that includes *SNRPN* and snoRNA's is expressed from the paternal allele. This large transcript is an antisense transcript (*UBE3A-ATS*) of the *UBE3A* gene effectively silencing *UBE3A*\(^{131,132}\). Recent data shows that the *UBE3A* paternal allele is silenced in all mature neurons in all brain areas but remains bi-allelically expressed in astrocytes, mature myelinating oligodendrocytes and immature neurons in the postnatal stem cell niches although at very low levels\(^{133-135}\). This differential silencing is not due to different imprinting in different cell types but due to alternative splicing of the *UBE3A-ATS* in different tissues. This is illustrated by the SNURF/SNRPN gene which shows an ubiquitous pattern of expression unlike the distal part of the *UBE3A-ATS* which is only brain specific\(^{135}\).

*UBE3A*, also known as E6-associated protein (E6-AP) is an E3 ubiquitin ligase\(^37\). The targets of *UBE3A* could be misregulated by lack of ubiquitination and be causative for AS. Over the years many putative targets have been described (table 1). However, although many targets have been identified it becomes clear that a distinction should be made between ubiquitinated targets and proteins that are deregulated in AS mice, but are not directly ubiquitinated by *UBE3A*, as shown recently with the ARC protein\(^{136}\). An additional distinction is necessary between ubiquitinated targets that are degraded and that are not\(^{137}\). The complicated nature of interactions and the lack of replication of published findings is a major drawback in the progression in the field of research on Angelman syndrome. Confirmation of targets in *in-vivo* assays are necessary and even more important, investigation of these targets in the *Ube3a* maternal deficient mouse is essential.

In 1997 the first mouse model for Angelman was developed based on the uniparental disomy (UPD) seen in the Angelman patients\(^{131,132}\). This UPD mouse model displayed growth retardation, mild ataxia, hyperactivity, EEG abnormalities and gross obesity. This first UPD mouse model was soon followed in 1998 by a mouse model with a *Ube3a* deletion on the maternal chromosome, showing motor dysfunction, spatial learning deficit, inducible seizures and long-term potentiation (LTP) deficit\(^{138}\). To gain more insight into expression
patterns of the maternal versus the paternal *Ube3a*, Miura et al (2001) developed a LacZ reporter mouse\(^{139}\). All these mouse models have been an invaluable tool for research on Angelman syndrome.

Electrophysiological studies of *Ube3a* mice indicated that the LTP deficits result from mechanisms downstream of calcium influx. This was confirmed by the finding of a change in phosphorylation state of CaMK2A in the AS mice. CaMK2A showed diminished activity while the total CaMK2A levels were not different. The phosphorylation levels on the T286 site (activating phosphorylation site) and the TT305/6 site (the inhibitory phosphorylation site) were increased and the phosphatase activity of PP1 was reduced, resulting in increased inhibition of CaMK2A in AS mice\(^{140}\). A subsequent study showed that by introducing a mutation which prevents phosphorylation on the TT305/6 site of CaMK2A (CaMK2A-T305V/T306A) the watermaze-, fear conditioning-, rotarod- and bodyweight phenotype, CaMK2A activity and LTP deficits seen in AS mice could be rescued\(^{141}\).

**Figure 5:** Ideogram of chromosome 15, displaying imprinted genes. Maternally expressed genes displayed in red, paternally expressed genes in blue. The location of two breakpoints depicted by BPI-3, displaying type I and type II deletion. (Figure adapted from Cox 2015)
### Putative targets of UBE3A and association with E6

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<td>hTERT</td>
<td>+</td>
<td>Catalytic rate limiting unit of telomerase</td>
<td>Indirect by ubiquitination of NFX-91</td>
</tr>
<tr>
<td>MAPK6</td>
<td></td>
<td>Mitogen-activated protein</td>
<td>Binding</td>
</tr>
<tr>
<td>MCM7</td>
<td></td>
<td>DNA replication initiation</td>
<td>Ubiquitination</td>
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<tr>
<td>NEURL4</td>
<td></td>
<td>Neurolized E3 protein ligase</td>
<td>Binding</td>
</tr>
<tr>
<td>P27</td>
<td></td>
<td>Kinase inhibitor</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>P53</td>
<td>+</td>
<td>Tumor suppressor</td>
<td>Degradation</td>
</tr>
<tr>
<td>Pbl/ECT2</td>
<td></td>
<td>Neuronal outgrowth in post mitotic cells</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>PML</td>
<td></td>
<td>Regulation growth inhibition, senescence ect</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>PRDX1</td>
<td></td>
<td>Antioxidant enzyme</td>
<td>Ubiquitination/ Degradation</td>
</tr>
</tbody>
</table>
### Table 1: Putative targets of UBE3A, their association with E6 oncoprotein, main function of the protein and the nature of the interaction.

<table>
<thead>
<tr>
<th>Name</th>
<th>E6?</th>
<th>Function Description</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTPN3</td>
<td>+</td>
<td>Regulating tyrosine phosphorylation of growth factor</td>
<td>Degradation</td>
</tr>
<tr>
<td>Rhoa</td>
<td>-</td>
<td>Small GTPase involved in prostate cancer</td>
<td>Indirect regulation</td>
</tr>
<tr>
<td>Ring 1B</td>
<td>-</td>
<td>Ubiquitin ligase that modifies nucleosome histone H2A</td>
<td>Ubiquitination/Degradation</td>
</tr>
<tr>
<td>RPN 10</td>
<td>-</td>
<td>Proteasome shuttling agent</td>
<td>Ubiquitination/Degradation</td>
</tr>
<tr>
<td>Rps10b</td>
<td>-</td>
<td>Ribosomal subunit</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>Sacsin</td>
<td>-</td>
<td>Unknown function in the nervous system</td>
<td>Binding</td>
</tr>
<tr>
<td>SCR1B</td>
<td>+</td>
<td>Tumor suppressor</td>
<td>Ubiquitination/Degradation</td>
</tr>
<tr>
<td>SHR</td>
<td>-</td>
<td>Steroid hormone receptor</td>
<td>Coactivator</td>
</tr>
<tr>
<td>SOD1</td>
<td>-</td>
<td>Protein is linked to familial amyotrophic lateral sclerosis</td>
<td>Ubiquitination</td>
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<tr>
<td>TH1</td>
<td>-</td>
<td>Unclear/assembly functional negative elongation factor</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>TSC</td>
<td>-</td>
<td>Tumor suppressor</td>
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<td>UchL5</td>
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<td>Proteasome related protein</td>
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<td>Var1t</td>
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<td>Tumor suppressor</td>
<td>Ubiquitination</td>
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</tbody>
</table>

**Scope of this thesis**

The general goal of this thesis is to understand the role of UPP in two different neurodevelopmental disorders, in particular in the role of UBE3A causing Angelman syndrome. In **chapter 2** we characterize a potential mouse model for UBE2A deficiency syndrome. In **chapter 3** we make use of established and novel AS mouse models to elucidate the origin of the motor deficits seen in Angelman syndrome. In **chapter 4** we investigate PML, a potential target of UBE3A ligase, and the role of PML in learning and memory, together with its contribution to the phenotypes seen in AS mice. Finally in **chapter 5** we investigate to what extend the phenotypes of AS mice can be rescued by reactivating the *Ube3a* gene in a temporal manner.
References


Introduction

Chapter 1

Chapter 1


Introduction


175. Ramamoorthy. E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. Nucl Rec Sig 4, (2007).


