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 where 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 there after, 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[14,16]. Finally the E2 enzyme ubiquitin complex than transfers the activated ubiquitin to an E3 ligase-substrate complex[9,17] (Figure 1).
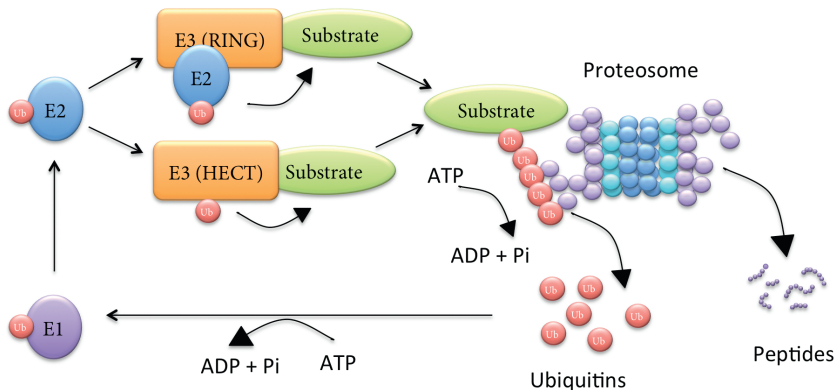

Figure 1: Schematic overview of ubiquitination. An ubiquitin activating enzyme (E1) activates an ubiquitin. The activated ubiquitin is than transferred to the ubiquitin conjugating protein (E2). Subsequently the activated ubiquitin is transferred to an E3 ligase-subunit complex, either directly from 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[15,18]; 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[16,19,20]. 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 cysine in the second RING that actually mediated ubiquiitnation in a HECT like manner[21].

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[18,22]. Lysine 63 (K63)-linked ubiquitin chains can actually make novel ubiquitin chains for signaling in DNA repair and control of endocytosis[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[21]. Lysine 48 (K48) ubiquitination, however, is the most common modification and targets proteins for degradation[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[23].
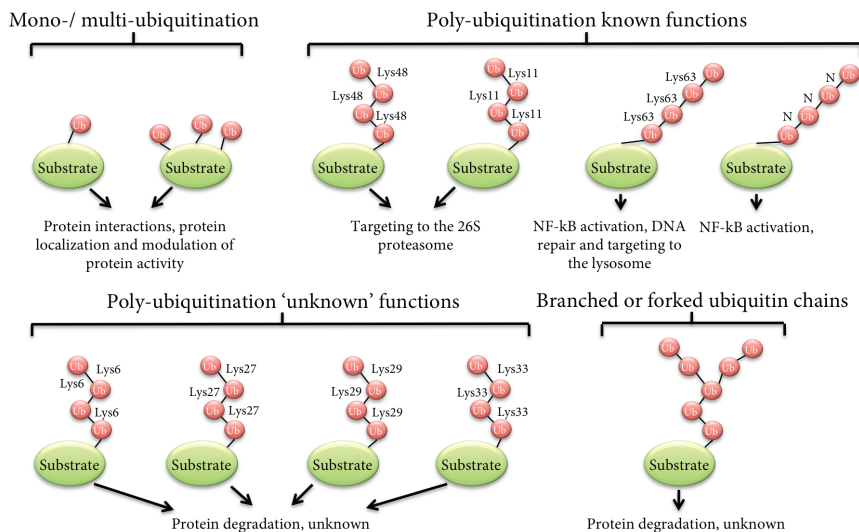

Figure 3: 3D representation of the 26S proteasome. On the left side the inner 20S core with the alpha en beta subunits. On the right side the two 19S regulatory subunits with the 20S subunit in the middle. (Figure adapted from Adams 2004[11])

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[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 a ATP-dependent mechanism[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[15]. 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).
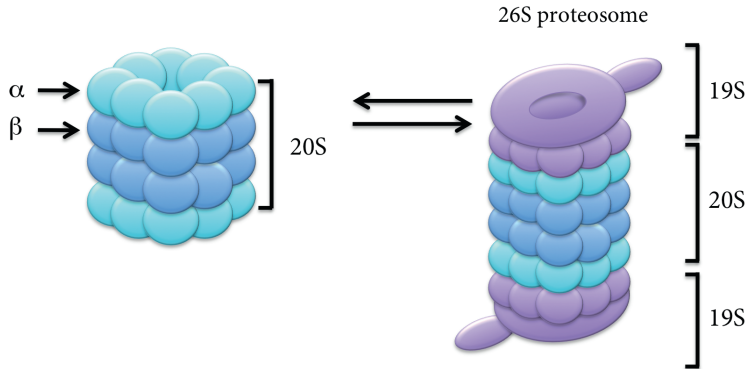

Figure 3: 3D representation of the 26S proteasome. On the left side the inner 20S core with the alpha en beta subunits. On the right side the two 19S regulatory subunits with the 20S subunit in the middle. (Figure adapted from Adams 200411)

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[29].

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 all 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[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[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[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 polysome 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[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[46]. It was later shown that ubiquitination and degradation of PSD-associated proteins occurs during several learning paradigms [47].

The UPP system is also involved in Long-Term Depression (LTD)[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[59], indicating that de-ubiquitination is essential for working memory but this particular DUB not for LTP.

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 L1-2. 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[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+ 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 E3 HECT ubiquitin ligase. The mutations that cause Liddle's syndrome disrupt the E3 recognition of the eNaC thereby stabilizing the Na+ channel[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[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[70-72]. Parkinson disease (PD) and Lewy body dementia, both known for Lewy body inclusions containing α-synuclein (which is mutated in very rare forms of familiar PD), are ubiquitin-positive like the tau aggregates in AD[73]. Indeed α-Synuclein protein has been shown to be targeted by the UPP[74]. Additionally *Parkin*, a well-known gene causing a familiar form of PD is an E3 ligase. Surprisingly however this form of PD develops without the presentation of the Lewy body inclusions[75]. Mutations in Parkin result in loss of ubiquitation function[76]. Other rare mutations causing familiar PD are located in *UCH* isozyme *UCH-L1*, a de-ubiquitinating enzyme, again demonstrating the importance of the UPP regulation in Parkinson[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 spinocereberal ataxia and spinobulbar muscular atrophy (Kennedey's disease) are caused by expansions of polyglutamine tracts by different proteins: Huntingtin in HD; ataxins in spinocerebral ataxia and the androgen receptor in Kennedys disease[78-80]. All of these proteins have been shown to be ubiquitinated, although it remains unclear if al these inclusions seen in these diseases are toxic or just a secondary reaction of the body in response to proteins that cannot be eliminated[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[84-86]. These inclusions correspond with the progression of the disease, displaying clearly that ubiquitination and the lack of degradation by the proteasome of mayor importance[87].

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*[88,89]. Kaufman syndrome was first described in 1971, subsequently many descriptions of patients with the same features followed[90-93]. Kaufman oculocerebrofacial syndrome (KOS) is characterized by reduced growth, hypotonia, facial dismorphia, microcephaly, ocular anomalies and intellectual disability[90-95]. In 2013 the syndrome was linked to los of function of UBE3B protein[95]. Very little is known about the precise function of this particular E3 ligase[92,94]. 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[96,97]. One example is *HR6A/UBE2A*, located on the X chromosome, encoding an E2 ubiquitin conjugating enzyme[14]. 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[98-101].

HR6A (Human homologue to RAD6A) is a homologue of the *Saccharomyces cervisiae RAD6* gene sharing about 70% sequence homology. It is also highly homologous (95%) to *HR6B* (Human homologue to RAD6B)[102]. 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[103].

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[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[105]. Adding either *HR6A* or *HR6B* rescues the DNA repair defect seen in yeast[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[106,107]. Rad6 protein binds to one of 6 RING finger E3 ligases: Ubr1, Ubr2, Ubr3, Rad18, Rad5 and Bre1[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) [109]. Ubr3 regulates sensory pathways via APE1 protein (Apurinic/apyrimidinic Endonuclease 1)[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 [111] and Bre1 is involved in histone mono-ubiquitination[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 form 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[113]. This mutant showed male infertility. This was followed by the development of the HR6A mutant mouse displaying female infertility in homozygous state[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[113].

The intellectual disability as seen in UBE2A deficiency syndrome suggests an important role for HR6A in brain function, However, the *Hr6a* mouse mutant has only been studied in relation to reproduction. The role of this protein in brain function is part of this thesis.

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[115] (Figure 4B). The initial diagnosis was purely clinical. Over the years many more patients were described to have the Angelman (puppet like) syndrome[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[117]. Angelman syndrome is characterized by severe developmental delay, absence of speech, epilepsy, movement and balance deficits and inappropriate laughter[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 [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[126,127]. These syndromes where described as sister syndromes[128].
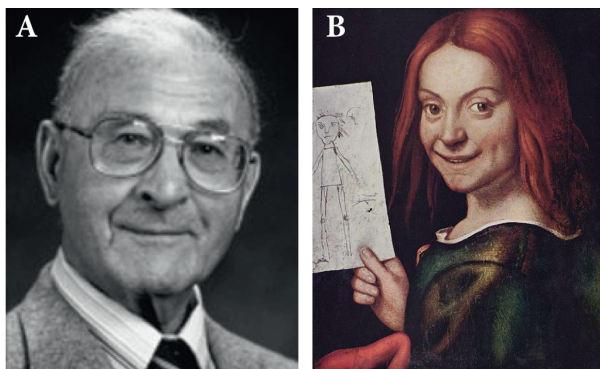

Figure 4: A. Dr. Harry Angelman, **B.** A boy with a puppet by Giovanni Francesco Caroto

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[132].

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 mayor 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.
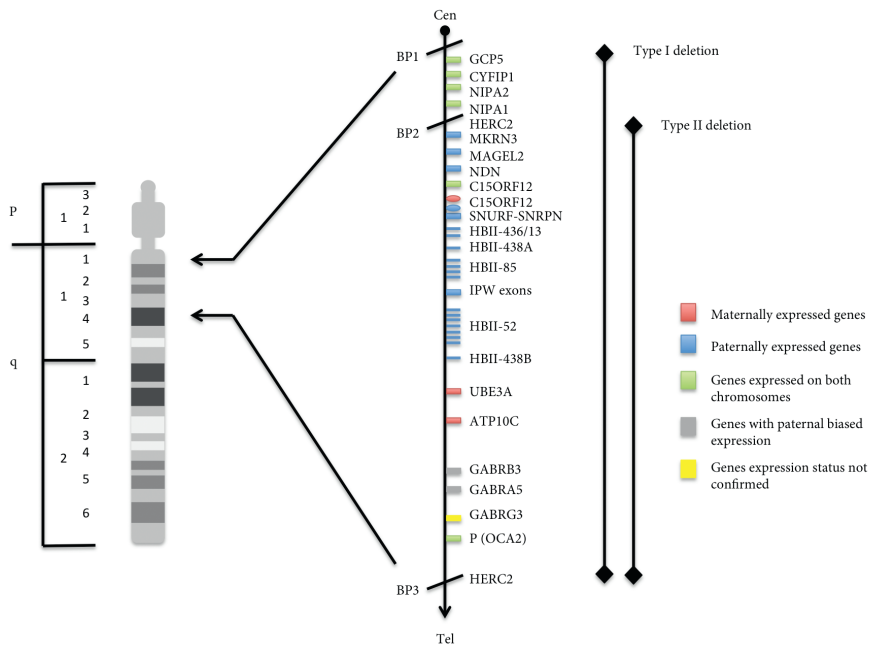

Figure 5: Ideogram of chromosome 15, displaying imprinted genes. Maternally expressed genes displayed in red, paternally expressed genes in blue. The location of two breakpoints depicted by BP1-3, displaying type I and type II deletion. (Figure adapted from Cox 2015)

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].

Putative targets of UBE3A and association with E6

Name	E6?	Function Protein	Interaction
AIBI	-	Steroid co-activator oncogene[142]	Ubiquitination
Annexin A1	-	Phospholipid binding ect[143]	Degradation
ARC	-	Internalization of AMPA[144]	Degradation
Rho-GEF pbl	-	Regulates cytokines[145]	Regulation
ASPM	-	Centrosomal related protien[146]	Binding
A synuclein	-	Unknown/ interacts with phospholipids[147]	Degradation
Bak	+	Pro-apoptotic protein[148]	Degradation
Be6	+	Transcriptional activation[149]	Binding
BLK	-	Regulates cellular processes like cytoskeleton organization[150]	Ubiquitination
BMAL	+	Core clock transcription factor[151]	Ubiquitination
C/EBPα	-	Transcription factor involved adipocyte differentiation[152]	Ubiquitination
CG8209	-	Proteasome related protein[137]	Ubiquitination
Disc	+	Large tumor suppressor[153]	Degradation
DLG4/PSD95	+	Tumor suppression[154]	Degradation
E6AP	-	Protein degradation[155]	Ubiquitination
Ephexin 5	-	Positive regulator of excitatory synapse development[156]	Ubiquitination
ESR1	-	Activating expression of ARC[136]	Inhibition
Grim-19	+	Tumor suppressor[157]	Ubiquitination/ Degradation
hADA3	+	Transcriptional co-activator[158]	Inhibition
HERC2	-	HECT E3 ligase[159]	Binding
HCV	-	Viral pathogenesis and hepatocarcinogenesis[160]	Ubiquitination/ Degradation
hDLG	+	Large tumor suppressor[161]	Binding
HHR23A	-	Cell cycle progression and DNA repair[162]	Ubiquitination
HIF1AN	-	Hypoxia induced factor[163]	Binding
Hsp70	-	Molecular chapperones[164]	Binding
hTERT	+	Catalytic rate limiting unit of telomerase[165]	Indirect by ubiquitination of NFX-91
MAPK6	-	Mitogen-activated protein[163]	Binding
MCM7	-	DNA replication initiation[166]	Ubiquitination
NEURL4	-	Neurolized E3 protein ligase[163]	Binding
P27	-	Kinase inhibitor[167]	Ubiquitination
P53	+	Tumor suppressor[168]	Degradation
Pbl/ECT2	-	Neuronal outgrowth in post mitotic cells[145]	Ubiquitination
PML	-	Regulation growth inhibition, senescence ect[169]	Ubiquitination
PRDX1		Antioxidant enzyme[170]	Ubiquitination/ Degradation

Putative targets of UBE3A and association with E6 *Continued*

Name	E6?	Function Protein	Interaction
PTPN3	+	Regulating tyrosine phosphorylation of growth factor[171]	Degradation
Rhoa	-	Small GTPase involved in prostate cancer[172]	Indirect regulation
Ring 1B	-	Ubiquitin ligase that modifies nucleosome histone H2A[173]	Ubiquitination/ Degradation
RPN 10	-	Proteasome shuttling agent[137]	Ubiquitination/ Degradation
Rps10b	-	Ribosomal subunit[137]	Ubiquitination
Sacsin	-	Unknown function in the nervous system[144]	Binding
SCR1B	+	Tumor suppressor[174]	Ubiquitination/ Degradation
SHR	-	Steroid hormone receptor[175]	Coactivator
SOD1	-	Protein is linked to familial amyotrophic lateral sclerosis[176]	Ubiquitination
TH1	-	Unclear/assembly functional negative elongation factor[177]	Ubiquitination
TSC	-	Tumor suppressor[178]	Ubiquitination
UchL5	-	Proteasome related protein[137]	Ubiquitination
Vartul	+	Tumor suppressor[179]	Ubiquitination

Table 1: Putative targets of UBE3A, their association with E6 oncoprotein, main function of the protein and the nature of the interaction.

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

1. Roeleveld, N., Zielhuis, G. A. & Gabreëls, F. The prevalence of mental retardation: a critical review of recent literature. *Dev. Med. Child Neuro.* **39,** 125–132 (1997).

2. Chiang, P.-H. *et al.* Healthcare utilization and expenditure analysis between individuals with intellectual disabilities and the general population in Taiwan: A population-based nationwide child and adolescent study. *Res. Dev. Dis.* **34,** 2485–2492 (2013).

3. Leonard, H. & Wen, X. The epidemiology of mental retardation: challenges and opportunities in the new millennium. *Ment. Retard. Dev. Disabil. Res Rev* **8,** 117–134 (2002).

4. Etlinger, J. D. & Goldberg, A. L. A soluble ATP-dependent proteolytic system responsible for the degradation of abnormal proteins in reticulocytes. *Proc. Natl. Acad. Sci. U.S.A.* **74,** 54–58 (1977).

5. Ciechanover, A., Elias, S., Heller, H., Ferber, S. & Hershko, A. Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *J. Biol. Chem.* **255,** 7525–7528 (1980).

6. Wilkinson, K. D., Urban, M. K. & Haas, A. L. Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *J. Biol. Chem.* **255,** 7529–7532 (1980).

7. Varshavsky, A. J., Bakayev, V. V. & Georgiev, G. P. Heterogeneity of chromatin subunits in vitro and location of histone H1. *Nucl. Acids Res.* **3,** 477–492 (1976).

8. Kornitzer, D. & Ciechanover, A. Modes of regulation of ubiquitin-mediated protein degradation. *J. Cell. Phys.* **182,** 1–11 (2000).

9. Pickart, C. M. Mechanisms Underlying Ubiquitination. *Annu. Rev. Biochem.* **70,** 503–533 (2001).

10. Ciehanover, A., Hod, Y. & Hershko, A. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem. Biophys. Res Comm.* **81,** 1100–1105 (1978).

11. Goldstein, G. *et al.* Isolation of a polypeptide that has lymphocyte-differentiating properties and is probably represented universally in living cells. *Proc. Natl. Acad. Sci. U.S.A.* **72,** 11-5(1975).

12. Schlesinger, D. H., Goldstein, G. & Niall, H. D. Complete amino acid sequence of ubiquitin, an adenylate cyclase stimulating polypeptide probably universal in living cells. *Biochem,* **14,** 2214–2218 (1975).

13. Haas, A. L., Warms, J. V., Hershko, A. & Rose, I. A. Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. *J. Biol. Chem.* **257,** 2543-2548 (1982).

14. Ye, Y. & Rape, M. Building ubiquitin chains: E2 enzymes at work. *Nat. Rev. Mol. Cell Biol.* **10,** 755–764 (2009).

15. Hershko, A. & Ciechanover, A. The ubiquitin system. *Annu. Rev. Biochem.* **67,** 425–479 (1998).

16. Scheffner, M., Nuber, U. & Huibregtse, J. M. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* **373,** 81–83 (1995).

17. Koegl, M. *et al.* A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96,** 635–644 (1999).

18. Haglund, K. Distinct monoubiquitin signals in receptor endocytosis. *Trends Biochem. Sci.* **28,** 598–604 (2003).

19. Haglund, K. & Dikic, I. Ubiquitylation and cell signaling. *EMBO J.* **24,** 3353–3359 (2005).

20. Hofmann, R. M. & Pickart, C. M. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96,** 645–653 (1999).

21. Tokunaga, F. *et al.* Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nature* **11,** 123–132 (2009).

22. Ikeda, F., Crosetto, N. & Dikic, I. What Determines the Specificity and Outcomes of Ubiquitin Signaling? *Cell* (2010).

23. Komander, D. Biochem. Soc. Trans (2009) 37, 937-953 - David Komander - The emerging complexity of protein ubiquitination. *Biochem. Soc. Trans.* **37,** 937-953 (2009).

24. Buchberger, A. From UBA to UBX: new words in the ubiquitin vocabulary. *Trends Cell Biol.* **12,** 216–221 (2002).

25. Kang, Y. *et al.* UBL/UBA ubiquitin receptor proteins bind a common tetraubiquitin chain. *J. Mol. Biol.* **356,** 1027–1035 (2006).

26. Gallastegui, N. & Groll, M. The 26S proteasome: assembly and function of a destructive machine. *Trends Biochem. Sci.* **35,** 634–642 (2010).

27. Cheng, Y. Toward an atomic model of the 26S proteasome. *Curr. Opin. Struct. Biol.* **19,** 203-208(2009).

28. Marques, A. J., Palanimurugan, R., Matias, A. C., Ramos, P. C. & Dohmen, R. J. Catalytic mechanism and assembly of the proteasome. *Chem. Rev.* **109,** 1509–1536 (2009).

29. Muller, S. & Schwartz, L. M. Ubiquitin in homeostasis, development and disease. *Bioessays* **17,** 677–684 (1995).

30. Sheaff, R. J., Groudine, M., Gordon, M., Roberts, J. M. & Clurman, B. E. Cyclin E-CDK2 is a regulator of p27Kip1. *Genes Dev.* **11,** 1464–1478 (1997).

31. Diehl, J. A., Zindy, F. & Sherr, C. J. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev.* **11,** 957–972 (1997).

32. Lanker, S., Valdivieso, M. H. & Wittenberg, C. Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science* **271,** 1597–1601 (1996).

33. Tyers, M., Tokiwa, G., Nash, R. & Futcher, B. The Cln3-Cdc28 kinase complex of S. cerevisiae is regulated by proteolysis and phosphorylation. *EMBO J.* **11,** 1773–1784 (1992).

34. Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M. & Roberts, J. M. Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev.* **10,** 1979–1990 (1996).

35. Breitschopf, K., Bengal, E., Ziv, T., Admon, A. & Ciechanover, A. A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. *EMBO J.* **17,** 5964–5973 (1998).

36. Johnson, P. R., Swanson, R., Rakhilina, L. & Hochstrasser, M. Degradation signal masking by heterodimerization of MATalpha2 and MATa1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell* **94,** 217–227 (1998).

37. Glickman, M. H. & Ciechanover, A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **82,** 373–428 (2002).

38. Upadhya, S. C. & Hegde, A. N. Role of the ubiquitin proteasome system in Alzheimer's disease. *BMC Biochem.* **8,** S12 (2007).

39. Perry, G., Rizzuto, N., Autilio-Gambetti, L. & Gambetti, P. Paired helical filaments from Alzheimer disease patients contain cytoskeletal components. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 3916–3920 (1985).

40. Lennox, G., Lowe, J., Morrell, K., Landon, M. & Mayer, R. J. Ubiquitin is a component of neurofibrillary tangles in a variety of neurodegenerative diseases. *Neurosci. Lett.* **94,** 211–217 (1988).

41. Hegde, A. N., Goldberg, A. L. & Schwartz, J. H. Regulatory subunits of cAMP-dependent protein kinases are degraded after conjugation to ubiquitin: a molecular mechanism underlying long-term synaptic plasticity. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 7436–7440 (1993).

42. Hegde, A. N. *et al.* Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in Aplysia. *Cell* **89,** 115–126 (1997).

43. Chain, D. G. *et al.* Mechanisms for generating the autonomous cAMP-dependent protein kinase required for long-term facilitation in Aplysia. *Neuron* **22,** 147–156 (1999).

44. Dong, C., Upadhya, S. C., Ding, L., Smith, T. K. & Hegde, A. N. Proteasome inhibition enhances the induction and impairs the maintenance of late-phase long-term potentiation. *Learn. Mem.* **15,** 335–347 (2008).

45. Banerjee, S., Neveu, P. & Kosik, K. S. A coordinated local translational control point at the synapse involving relief from silencing and MOV10 degradation. *Neuron* **64,** 871–884 (2009).

46. Ehlers, M. D. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat. Neurosci.* **6,** 231–242 (2003).

47. Lee, S. H. *et al.* Synaptic Protein Degradation Underlies Destabilization of Retrieved Fear Memory. *Science* **319,** 1253-1256 (2008).

48. Fioravante, D., Liu, R.-Y. & Byrne, J. H. The ubiquitin-proteasome system is necessary for long-term synaptic depression in Aplysia. *J. Neurosci.* **28,** 10245–10256 (2008).

49. Colledge, M. *et al.* Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron* **40,** 595–607 (2003).

50. Citri, A., Soler-Llavina, G., Bhattacharyya, S. & Malenka, R. C. N-methyl-d-aspartate receptor- and metabotropic glutamate receptor-dependent long-term depression are differentially regulated by the ubiquitin-proteasome system. *Eur. J. Neurosci.* **30,** 1443–1450 (2009).

51. Bingol, B. & Schuman, E. M. Activity-dependent dynamics and sequestration of proteasomes in dendritic spines. *Nature* **441,** 1144–1148 (2006).

52. Shen, H. *et al.* NAC1 Regulates the Recruitment of the Proteasome Complex into Dendritic Spines. *J. Neurosci.* **27,** 8903-8913(2007).

53. Djakovic, S. N., Schwarz, L. A., Barylko, B., DeMartino, G. N. & Patrick, G. N. Regulation of the proteasome by neuronal activity and calcium/calmodulin-dependent protein kinase II. *J. Biol. Chem.* **284,** 26655–26665 (2009).

54. Lopez-Salon, M. *et al.* The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. *Eur. J. Neurosci.* **14,** 1820–1826 (2001).

55. Yeh, S.-H., Mao, S.-C., Lin, H.-C. & Gean, P.-W. Synaptic Expression of Glutamate Receptor after Encoding of Fear Memory in the Rat Amygdala. *Mol. Pharmacol.* **69,** 299-308 (2006).

56. Mao, S.-C., Lin, H.-C. & Gean, P.-W. Augmentation of Fear Extinction by D-Cycloserine is Blocked by Proteasome Inhibitors. *Neuropsychopharm.* **33,** 3085–3095 (2008).

57. Zhao, Y., Hegde, A. N. & Martin, K. C. The ubiquitin proteasome system functions as an inhibitory constraint on synaptic strengthening. *Curr. Biol.* **13,** 887–898 (2003).

58. Merlo, E. & Romano, A. Long-term memory consolidation depends on proteasome activity in the crab Chasmagnathus. *Neuroscience* **147,** 46-52(2007).

59. Wood, M. A., Kaplan, M. P., Brensinger, C. M., Guo, W. & Abel, T. Ubiquitin C-terminal hydrolase L3 (Uchl3) is involved in working memory. *Hippocampus* **15,** 610–621 (2005).

60. Mabb, A. M. & Ehlers, M. D. Ubiquitination in postsynaptic function and plasticity. *Annu. Rev. Cell Dev. Biol.* **26,** 179–210 (2010).

61. Devoy, A., Soane, T., Welchman, R. & Mayer, R. J. The ubiquitin-proteasome system and cancer. *Essays Biochem.* **41,** 187–203 (2005).

62. Seedorf, K., Krämmer, G., Dürst, M., Suhai, S. & Röwekamp, W. G. Human papillomavirus type 16 DNA sequence. *Virology* **145,** 181–185 (1985).

63. Huibregtse, J. M., Scheffner, M. & Howley, P. M. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J.* **10,** 4129–4135 (1991).

64. Loda, M. *et al.* Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* **3,** 231–234 (1997).

65. Kondo, K. The von Hippel–Lindau Tumor Suppressor Gene. *Exp. Cell Res.* **264,** 117–125 (2001).

66. Kaelin, W. G. *et al.* Ubiquitination of hypoxia-inducible factor requires direct binding to the -domain of the von Hippel–Lindau protein. *Nat. Cell Biol.* **2,** 423–427 (2000).

67. Rotin, D. Regulation of the epithelial sodium channel (ENaC) by accessory proteins. *Curr. Opin. Nephrol. Hypertens.* **9,** 529–534 (2000).

68. Huang, L. & Chen, C. H. Proteasome regulators: activators and inhibitors. *Curr. Med. Chem.* **16,** 931 (2009).

69. Lecker, S. H., Solomon, V., Mitch, W. E. & Goldberg, A. L. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J. Nutr.* **129,** 227S–237S (1999).

70. de Vrij, F. M. S., Fischer, D. F., van Leeuwen, F. W. & Hol, E. M. Protein quality control in Alzheimer's disease by the ubiquitin proteasome system. *Progr. Neurobiol.* **74,** 249–270 (2004).

71. Lehman, N. L. The ubiquitin proteasome system in neuropathology. *Acta. Neuropathol.* **118,** 329–347 (2009).

72. Dennissen, F., Kholod, N. & van Leeuwen, F. W. The ubiquitin proteasome system in neurodegenerative diseases: Culprit, accomplice or victim? *Progr. Neurobiol.* **96,** 190-207 (2012).

73. Krüger, R., Müller, T. & Riess, O. Involvement of alpha-synuclein in Parkinson's disease and other neurodegenerative disorders. *J. Neural. Transm.* **107,** 31–40 (2000).

74. Bennett, M. C. *et al.* Degradation of α-Synuclein by Proteasome. *J. Biol. Chem.* **274,** 33855-33858 (1999).

75. Tanaka, K. *et al.* Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25,** 302–305 (2000).

76. Zhang, Y. *et al.* Parkin functions as an E2-dependent ubiquitin– protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 13354-13359 (2000).

77. Leroy, E. *et al.* The ubiquitin pathway in Parkinson's disease, *Nature* **395,** 451–452 (1998).

78. Ross, C. A. *et al.* Polyglutamine pathogenesis. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **354,** 1005–1011 (1999).

79. Lieberman, A. P. & Fischbeck, K. H. Triplet repeat expansion in neuromuscular disease. *Muscle Nerve* **23,** 843–850 (2000).

80. Cummings, C. J. *et al.* Mutation of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency while accelerating polyglutamine-induced pathology in SCA1 mice. *Neuron* **24,** 879–892 (1999).

81. Sherman, M. Y. & Goldberg, A. L. Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron* **29,** 15–32 (2001).

82. Alves-Rodrigues, A., Gregori, L. & Figueiredo-Pereira, M. E. Ubiquitin, cellular inclusions and their role in neurodegeneration. *Trends in Neurosciences* **21,** 516–520 (1998).

83. Kalchman, M. A. *et al.* Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *J. Biol. Chem.* **271,** 19385–19394 (1996).

84. Leigh, P. N. *et al.* Ubiquitin-immunoreactive intraneuronal inclusions in Amyotrophic Lateral Sclerosis. *Brain* **114,** 775-788 (1991).

85. Neumann, M. *et al.* Ubiquitinated TDP-43 in Frontotemporal Lobar Degeneration and Amyotrophic Lateral Sclerosis. *Science* **314,** 130–133 (2006).

86. Okamoto, K., Hirai, S., Yamazaki, T., Sun, X. & Nakazato, Y. New ubiquitin-positive intraneuronal inclusions in the extra-motor cortices in patients with amyotrophic lateral sclerosis. *Neurosci. Lett.* **129,** 233-236 (1991).

87. Stieber, A., Gonatas, J. O. & Gonatas, N. K. Aggregation of ubiquitin and a mutant ALS-linked SOD1 protein correlate with disease progression and fragmentation of the Golgi apparatus. *J. Neurol. Sci.* **173,** 53–62 (2000).

88. Glessner, J. T., Wang, K., Cai, G., Korvatska, O. & Kim, C. E. Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* **459,** 569-573 (2009).

89. Smith, S. E. P. *et al.* Increased Gene Dosage of Ube3a Results in Autism Traits and Decreased Glutamate Synaptic Transmission in Mice. *Sci. Trans. Med.* **3,** 103ra97–103ra97 (2011).

90. Jurenka, S. B. & Evans, J. Kaufman oculocerebrofacial syndrome: case report. *Am. J. Med. Genet.* **3,** 15–19 (1979).

91. Briscioli, V., Manoukian, S., Selicorni, A., Livini, E. & Lalatta, F. Kaufman oculocerebrofacial syndrome in a girl of 15 years. *Am. J. Med. Genet.* **58,** 21–23 (1995).

92. Pedurupillay, C. R. J. *et al.* Kaufman oculocerebrofacial syndrome in sisters with novel compound heterozygous mutation in UBE3B. *Am. J. Hum. Genet.* **167A,** 657–663 (2015).

93. Figuera, L. E., García-Cruz, D., Ramírez-Dueñas, M. L., Rivera-Robles, V. & Cantù, J. M. Kaufman oculocerebrofacial syndrome: report of two new cases and further delineation. *Clin. Genet.* **44,** 98–101 (1993).

94. Basel-Vanagaite, L. *et al.* Expanding the clinical and mutational spectrum of Kaufman oculocerebrofacial syndrome with biallelic UBE3B mutations. *Hum. Genet.* **133,** 939–949 (2014).

95. Flex, E. *et al.* Loss of function of the E3 ubiquitin-protein ligase UBE3B causes Kaufman oculocerebrofacial syndrome. *J. Med. Genet.* **50,** 493–499 (2013).

96. Ropers, H. H. & Hamel, C. J. X-Linked Memtal Retardatiom. *Nature Rev. Genet.* **6,** 46–57 (2005).

97. Stevenson, R. E. & Schwartz, C. E. X-linked intellectual disability: Unique vulnerability of the male genome. *Dev. Disabil. Res. Revs.* **15,** 361–368 (2009).

98. Honda, S. *et al.* Novel deletion at Xq24 including the UBE2A gene in a patient with X-linked mental retardation. *J. Hum. Genet.* **55,** 244–247 (2010).

99. Nascimento, R. M. P., Otto, P. A., de Brouwer, A. P. M. & Vianna-Morgante, A. M. UBE2A, Which Encodes a Ubiquitin-Conjugating Enzyme, Is Mutated in a Novel X-Linked Mental Retardation Syndrome. *Am. J. Hum. Genet.* **79,** 549–555 (2006).

100. de Leeuw, N. *et al.* UBE2A deficiency syndrome: Mild to severe intellectual disability accompanied by seizures, absent speech, urogenital, and skin anomalies in male patients. *Am. J. Hum. Genet.* **152A,** 3084–3090 (2010).

101. Budny, B. *et al.* Novel missense mutations in the ubiquitination-related gene UBE2A cause a recognizable X-linked mental retardation syndrome. *Clin. Genet.* **77,** 541–551 (2010).

102. Koken, M. H. *et al.* Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 8865–8869 (1991).

103. Koken, M. *et al.* Dhr6, a Drosophila homolog of the yeast DNA-repair gene RAD6. *Proc. Natl. Acad. Sci. U.S.A* **88,** 3832–3836 (1991).

104. Sullivan, M. L. & Vierstra, R. D. A ubiquitin carrier protein from wheat germ is structurally and functionally similar to the yeast DNA repair enzyme encoded by RAD6. *Proc. Natl. Acad. Sci. U.S.A* **86,** 9861–9865 (1989).

105. Sung, P., Prakash, S. & Prakash, L. Mutation of cysteine-88 in the Saccharomyces cerevisiae RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. *Proc. Natl. Acad. Sci. U.S.A.* **87,** 2695–2699 (1990).

106. Sung, P., Prakash, S. & Prakash, L. The RAD6 protein of Saccharomyces cerevisiae polyubiquitinates histones, and its acidic domain mediates this activity. *Genes Dev.* **2,** 1476–1485 (1988).

107. Robzyk, K., Recht, J. & Osley, M. A. Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287,** 501–504 (2000).

108. Hwang, W. W. *et al.* A conserved RING finger protein required for histone H2B monoubiquitination and cell size control. *Mol. Cell* **11,** 261–266 (2003).

109. Bartel, B., Wünning, I. & Varshavsky, A. The recognition component of the N-end rule pathway. *EMBO J.* **9,** 3179–3189 (1990).

110. Tasaki, T. *et al.* Biochemical and Genetic Studies of UBR3, a Ubiquitin Ligase with a Function in Olfactory and Other Sensory Systems. *J. Biol. Chem.* **282,** 18510–18520 (2007).

111. Durando, M., Tateishi, S. & Vaziri, C. A non-catalytic role of DNA polymerase in recruiting Rad18 and promoting PCNA monoubiquitination at stalled replication forks. *Nucleic Acids Res.* **41,** 3079–3093 (2013).

112. Hoege, C., Pfander, B., Moldovan, G.-L., Pyrowolakis, G. & Jentsch, S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419,** 135–141 (2002).

113. Mulugeta Achame, E. *et al.* The ubiquitin-conjugating enzyme HR6B is required for maintenance of X chromosome silencing in mouse spermatocytes and spermatids. *BMC Genomics* **11,** 367 (2010).

114. Roest, H. P. *et al.* The Ubiquitin-Conjugating DNA Repair Enzyme HR6A Is a Maternal Factor Essential for Early Embryonic Development in Mice. *Mol. Cell. Biol.* **24,** 5485–5495 (2004).

115. Angelman, H. 'Puppet' Children A Report on Three Cases. *Dev. Med. Child Neurol.* **7,** 681–688 (2008).

116. Willems, P. J., Dijkstra, I., Brouwer, O. F., Smit, G. P. A. & Reynolds, J. F. Recurrence risk in the Angelman ('happy puppet') syndrome. *Am. J. Med. Genet.* **27,** 773–780 (1987).

117. Williams, C. A., Frias, J. L. & Opitz, J. M. The Angelman ('Happy Puppet') syndrome. *Am. J. Med. Genet.* **11,** 453–460 (1982).

118. Bower, B. D. & Jeavons, P. M. The' happy puppet' syndrome. *Arch. Dis. Child.* **42,** 298-302 (1967).

119. Kuroki, Y., Matsui, I., Yamamoto, Y. & Ieshima, A. The 'happy puppet' syndrome in two siblings. *Hum. Genet.* **56,** 227–229 (1980).

120. Dooley, J. M. The Puppet-like Syndrome of Angelman. *Arch. Pediatr. Adolesc. Med* **135,** 621 (1981).

121. Fisher, J. A., Burn, J., Alexander, F. W. & Gardner-Medwin, D. Angelman (happy puppet) syndrome in a girl and her brother. *J. Med. Genet.* **24,** 294-298 (1987).

122. Dorries, A., Spohr, H. L. & Kunze, J. Angelman ('happy puppet') syndrome--seven new cases documented by cerebral computed tomography: review of the literature. *Eur. J. Pediatr.* **148,** 270–273 (1988).

123. Williams, C. A. *et al.* Angelman syndrome 2005: Updated consensus for diagnostic criteria. *Am. J. Hum. Genet.* **140A,** 413–418 (2006).

124. Kaplan, L. C. *et al.* Clinical heterogeneity associated with deletions in the long arm of chromosome 15: Report of 3 new cases and their possible genetic significance. *Am. J. Med. Genet.* **28,** 45–53 (1987).

125. Magenis, R. E. *et al.* Is angelman syndrome an alternate result of del(15)(qllql3)? *Am. J. Med. Genet.* **28,** 829–838 (1987).

126. Angulo, M. A., Butler, M. G. & Cataletto, M. E. Prader-Willi syndrome: a review of clinical, genetic, and endocrine findings. *J. Endocrinol. Invest.* **38,** 1249–1263 (2015).

127. Horsthemke, B. & Wagstaff, J. Mechanisms of imprinting of the Prader-Willi/Angelman region. *Am. J. Med. Genet.* **146A,** 2041–2052 (2008).

128. Cassidy, S. B., Dykens, E. & Williams, C. A. Prader-Willi and Angelman syndromes: sister imprinted disorders. *Am. J. Med. Genet.* **97,** 136–146 (2000).

129. Mertz, L. G. B. *et al.* Angelman syndrome in Denmark. Birth incidence, genetic findings, and age at diagnosis. *Am. J. Hum. Genet.* **161,** 2197–2203 (2013).

130. Lalande, M. & Calciano, M. A. Molecular epigenetics of Angelman syndrome. *Cell. Mol. Life Sci.* **64,** 947–960 (2007).

131. Runte, M. *et al.* The IC-SNURF-SNRPN transcript serves as a host for multiple small nucleolar RNA species and as an antisense RNA for UBE3A. *Hum. Mol. Genet.* **10,** 2687–2700 (2001).

132. Landers, M. Regulation of the large (1000 kb) imprinted murine Ube3a antisense transcript by alternative exons upstream of Snurf/Snrpn. *Nucleic Acids Res.* **32,** 3480–3492 (2004).

133. Dindot, S. V., Antalffy, B. A., Bhattacharjee, M. B. & Beaudet, A. L. The Angelman syndrome ubiquitin ligase localizes to the synapse and nucleus, and maternal deficiency results in abnormal dendritic spine morphology. *Hum. Mol. Genet.* **17,** 111–118 (2007).

134. Gustin, R. M. *et al.* Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome. *Neurobiol. Dis.* **39,** 283–291 (2010).

135. Judson, M. C., Sosa-Pagan, J. O., Del Cid, W. A., Han, J. E. & Philpot, B. D. Allelic specificity of Ube3a expression in the mouse brain during postnatal development. *J. Comp. Neurol.* **522,** 1874-1896 (2013).

136. Kuhnle, S., Mothes, B., Matentzoglu, K. & Scheffner, M. Role of the ubiquitin ligase E6AP/UBE3A in controlling levels of the synaptic protein Arc. *Proc. Natl. Acad. Sci.* **110,** 8888–8893 (2013).

137. Lee, S. Y. *et al.* Ube3a, the E3 ubiquitin ligase causing Angelman syndrome and linked to autism, regulates protein homeostasis through the proteasomal shuttle Rpn10. *Cell. Mol. Life Sci.* **71,** 2747-2758 (2013).

138. Jiang, Y. H. *et al.* Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 1–13 (1998).

139. Miura, K. *et al.* Neurobehavioral and Electroencephalographic Abnormalities in Ube3aMaternal-Deficient Mice. *Neurobiol. Dis.* **9,** 149–159 (2002).

140. Weeber, E. J. *et al.* Derangements of hippocampal calcium/calmodulin-dependent protein kinase II in a mouse model for Angelman mental retardation syndrome. *J. Neurosci.* **23,** 2634–2644 (2003).

141. van Woerden, G. M. *et al.* Rescue of neurological deficits in a mouse model for Angelman syndrome by reduction of αCaMKII inhibitory phosphorylation. *Nat. Neurosci.* **10,** 280–282 (2007).

142. Mani, A. E6AP Mediates Regulated Proteasomal Degradation of the Nuclear Receptor Coactivator Amplified in Breast Cancer 1 in Immortalized Cells. *Cancer Res.* **66,** 8680–8686 (2006).

143. Shimoji, T. *et al.* Identification of annexin A1 as a novel substrate for E6AP-mediated ubiquitylation. *J. Cell. Biochem.* **106,** 1123–1135 (2009).

144. Greer, P. L. *et al.* The Angelman Syndrome Protein Ube3A Regulates Synapse Development by Ubiquitinating Arc. *Cell* **140,** 704–716 (2010).

145. Reiter, L. T. Expression of the Rho-GEF Pbl/ECT2 is regulated by the UBE3A E3 ubiquitin ligase. *Hum. Mol. Genet.* **15,** 2825–2835 (2006).

146. Singhmar, P. & Kumar, A. Angelman syndrome protein UBE3A interacts with primary microcephaly protein ASPM, localizes to centrosomes and regulates chromosome segregation. *PLoS ONE* **6,** e20397 (2011).

147. Mulherkar, S. A., Sharma, J. & Jana, N. R. The ubiquitin ligase E6-AP promotes degradation of α-synuclein. *J. Neurochem.* **110,** 1955–1964 (2009).

148. Underbrink, M. P., Howie, H. L., Bedard, K. M., Koop, J. I. & Galloway, D. A. E6 proteins from multiple human betapapillomavirus types degrade Bak and protect keratinocytes from apoptosis after UVB irradiation. *J. Virol.* **82,** 10408–10417 (2008).

149. Ned, R., Allen, S. & Pol, S. V. Transformation by bovine papillomavirus type 1 E6 is independent of transcriptional activation by E6. *J. Virol.* **71,** 4866–4870 (1997).

150. Oda, H., Kumar, S. & Howley, P. M. Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc. Natl. Acad. Sci.* **96,** 9557–9562 (1999).

151. Gossan, N. C. *et al.* The E3 ubiquitin ligase UBE3A is an integral component of the molecular circadian clock through regulating the BMAL1 transcription factor. *Nucleic Acids Res.* **42,** 5765–5775 (2014).

152. Pal, P. *et al.* E3 ubiquitin ligase E6AP negatively regulates adipogenesis by downregulating proadipogenic factor C/EBPalpha. *PLoS ONE* **8,** e65330 (2013).

153. Gardiol, D. *et al.* Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene* **18,** 5487–5496 (1999).

154. Handa, K. *et al.* E6AP-Dependent Degradation of DLG4/PSD95 by High-Risk Human Papillomavirus Type 18 E6 Protein. *J. Virol.* **81,** 1379–1389 (2007).

155. Nuber, U., Schwarz, S. E. & Scheffner, M. The ubiquitin-protein ligase E6-associated protein (E6-AP) serves as its own substrate - Nuber - 2001 - European Journal of Biochemistry - Wiley Online Library. *Eur. J. Biochem.* **254,** 643–649 (1998).

156. Margolis, S. S. *et al.* EphB-Mediated Degradation of the RhoA GEF Ephexin5 Relieves a Developmental Brake on Excitatory Synapse Formation. *Cell* **143,** 442–455 (2010).

157. Zhou, Y. *et al.* GRIM-19 disrupts E6/E6AP complex to rescue p53 and induce apoptosis in cervical cancers. *PLoS ONE* **6,** e22065 (2011).

158. Hu, Y. *et al.* HPV16 E6-induced and E6AP-dependent inhibition of the transcriptional coactivator hADA3 in human cervical carcinoma cells. *Cancer Invest.* **27,** 298–306 (2009).

159. Kühnle, S. *et al.* Physical and functional interaction of the HECT ubiquitin-protein ligases E6AP and HERC2. *J. Biol. Chem.* **286,** 19410–19416 (2011).

160. Shirakura, M. *et al.* E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. *J. Virol.* **81,** 1174–1185 (2007).

161. Kiyono, T. *et al.* Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. *Proc. Natl. Acad. Sci.* **94,** 11612–11616 (1997).

162. Kumar, S., Talis, A. L. & Howley, P. M. Identification of HHR23A as a Substrate for E6-associated Protein-mediated Ubiquitination. *J. Biol. Chem.* **274,** 18785-18792(1999).

163. Martínez-Noël, G. *et al.* Identification and proteomic analysis of distinct UBE3A/E6AP protein complexes. *Mol. Cell. Biol.* **32,** 3095–3106 (2012).

164. Mishra, A., Godavarthi, S. K., Maheshwari, M., Goswami, A. & Jana, N. R. The ubiquitin ligase E6-AP is induced and recruited to aggresomes in response to proteasome inhibition and may be involved in the ubiquitination of Hsp70-bound misfolded proteins. *J. Biol. Chem.* **284,** 10537–10545 (2009).

165. Gewin, L. Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. *Genes Dev.* **18,** 2269–2282 (2004).

166. Kühne, C. & Banks, L. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J. Biol. Chem.* **273,** 34302–34309 (1998).

167. Mishra, A., Godavarthi, S. K. & Jana, N. R. UBE3A/E6-AP regulates cell proliferation by promoting proteasomal degradation of p27. *Neurobiol. Dis.* **36,** 26–34 (2009).

168. Scheffner, M. Ubiquitin, E6-AP, and their role in p53 inactivation. *Pharmacol. Ther.* **78,** 129–139 (1998).

169. Louria-Hayon, I. *et al.* E6AP promotes the degradation of the PML tumor suppressor. *Cell Death Differ.* **16,** 1156–1166 (2009).

170. Nasu, J. *et al.* E6AP ubiquitin ligase mediates ubiquitin-dependent degradation of peroxiredoxin 1. *J. Cell. Biochem.* **111,** 676–685 (2010).

171. Jing, M., Bohl, J., Brimer, N., Kinter, M. & Vande Pol, S. B. Degradation of Tyrosine Phosphatase PTPN3 (PTPH1) by Association with Oncogenic Human Papillomavirus E6 Proteins. *J. Virol.* **81,** 2231–2239 (2007).

172. Khan, O. Y. *et al.* Multifunction steroid receptor coactivator, E6-associated protein, is involved in development of the prostate gland. *Mol. Endocrinol.* **20,** 544–559 (2006).

173. Zaaroor-Regev, D. *et al.* Regulation of the polycomb protein Ring1B by self-ubiquitination or by E6-AP may have implications to the pathogenesis of Angelman syndrome. *Proc. Natl. Acad. Sci.* **107,** 6788–6793 (2010).

174. Matsumoto, Y. *et al.* Involvement of a cellular ubiquitin-protein ligase E6AP in the ubiquitin-mediated degradation of extensive substrates of high-risk human papillomavirus E6. *J. Med. Virol.* **78,** 501–507 (2006).

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

176. Mishra, A. *et al.* E6-AP association promotes SOD1 aggresomes degradation and suppresses toxicity. *Neurobiol. Aging* **34,** 1310.e11–23 (2013).

177. Yang, Y. *et al.* Ubiquitin-dependent proteolysis of trihydrophobin 1 (TH1) by the human papilloma virus E6-associated protein (E6-AP). *J. Cell. Biochem.* **101,** 167–180 (2007).

178. Zheng, L. *et al.* E3 ubiquitin ligase E6AP-mediated TSC2 turnover in the presence and absence of HPV16 E6. *Genes Cells* **13,** 285–294 (2008).

179. Nakagawa, S. & Huibregtse, J. M. Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol. Cell. Biol.* **20,** 8244–8253 (2000).

180. Berndsen, CE. & Wolberger, C. New insights into ubiquitin E3 ligase mechanism. *Nat. Struct Biol.* **21,** 310-307 (2014)