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COMPLEX LIPIDS

# Palmitoylation and depalmitoylation defects

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**Abstract** Palmitoylation describes the enzymatic attachment of a 16-carbon atom fatty acid to a target protein. Such lipidation events occur in all eukaryotes and can be of reversible (S-palmitoylation) or irreversible (N-palmitoylation) nature. In particular S-palmitoylation is dynamically regulated by two opposing types of enzymes which add (palmitoyl acyltransferases - PAT) or remove (acyl protein thioesterases) palmitate from proteins. Protein palmitoylation is an important process that dynamically regulates the assembly and compartmentalization of many neuronal proteins at specific subcellular sites. Enzymes that regulate protein palmitoylation are critical for several biological processes. To date, eight palmitoylation related genes have been reported to be associated with human disease. This review intends to give an overview on the pathological changes which are associated with defects in the palmitoylation/depalmitoylation process.

## Protein palmitoylation and depalmitoylation

The reversible enzymatic modification of proteins with a free fatty acid is generally described as S-acylation and occurs in all eukaryotes. It is different from other cellular lipidation events such as farnesylation and isoprenylation in which the lipid moiety is irreversibly attached to proteins via an amide or thioether bond. The most common modification is S-palmitoylation which describes the reversible attachment of a palmitic acid onto a cysteine residue via thioester linkage (Resh 2006; Conibear and Davis 2010; Salaun et al 2010). A diverse group of proteins undergo palmitoylation including signaling proteins, ion channels, scaffold proteins, proteins involved in vesicle trafficking and viral proteins (Mitchell Vasudevan et al.et al 2006; Charollais and Van Der Goot 2009; Fukata and Fukata 2010; Veit et al 2013). Up to date there is no consensus motive established which dictates S-palmitoylation. The attached palmitate serves as a hydrophobic anchor for proteins that lack transmembrane domains. The hydrophobicity of a single fatty acid alone is typically not sufficient to stably anchor a protein to a lipid bilayer. Therefore a second signal is normally required for stable membrane binding. Normally, this second signal is either a cluster of positively charged amino acids (lysines and arginines) or an attached palmitate (El-Husseini et al 2000; Fukata and Fukata 2010). Palmitoylation of a protein is typically associated with its transport to specific intracellular compartments. Soluble proteins are normally palmitoylated at the Golgi from where they traffic to the plasma membrane (Rocks et al 2010). The reversible nature of palmitoylation allows proteins to dynamically shuttle between intracellular compartments or to relocalize in physiological contexts. The small GTPases HRAS and NRAS shuttle between Golgi and plasma membrane due to a palmitoylation–depalmitoylation cycle (Rocks et al 2006). The dynamic nature of protein palmitoylation/depalmitoylation cycles resembles hereby the principle of regulating protein functions by a kinase and phosphatase mediated addition and removal of phosphate groups in cellular signaling events. Besides membrane interaction, palmitoylation also regulates protein stability, protein sorting, and the localization to specific membrane sub-

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domains (Linder and Deschenes 2007; Greaves et al 2009; Fukata and Fukata 2010). The segregation of plasma membrane proteins into lipid rafts is important for regulating many cell signaling events and often requires palmitoylation (Brown 2006; Levental et al 2010). Protein palmitoylation is important for the assembly and compartmentalization of many neuronal proteins at specific subcellular domains (Prescott et al 2009; Fukata and Fukata 2010) and plays a critical role in synaptic plasticity (El-Husseini and Bredt 2002; Huang and El-Husseini 2005). It is also important for neuronal developmental processes, such as neurite outgrowth, axon pathfinding, filopodia formation, and spine development (Kutzleb et al 1998; Kato et al 2000; Laux et al 2000; Gauthier-Campbell et al 2004; Arstikaitis et al 2008). It has been reported for numerous neuronal proteins, including signaling proteins (HRAS, NRAS and rhoB), synaptic scaffolding proteins (PSD95, GRIP1 and AKAP18), transmembrane proteins (G protein-coupled receptors), neuronal cell adhesion molecules (NCAMs), glutamate receptors (GluRs), synaptic vesicle proteins (SNAP25), and cysteine string proteins (CSP and synaptotagmin I) (El-Husseini and Bredt 2002; Linder and Deschenes 2007). In particular the postsynaptic targeting of PSD-95, a molecule involved in excitatory synapse development and plasticity, is regulated by palmitoylation (El-Husseini et al 2000; Kim and Sheng 2004). Glutamate receptor activation markedly accelerates depalmitoylation of PSD95 and causes endocytosis of the associated AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) glutamate receptor (AMPAR) which leads to a down regulation of the respective signaling pathway (El-Husseini et al 2002). Palmitoylation can also be influenced by alternative splicing. This is well described for Cdc42, a small Rho GTPase that is involved in neuronal morphogenesis. Cdc41 is normally prenylated but brain specific alternative splicing results in a variant that becomes palmitoylated instead. Both variants are expressed in developing neurons but only the palmitoylated isoform induces the extension of dendritic filopodia which then develop into dendritic spines (Kang et al 2008).

Instead of forming a thioester bond acylation may also occur at an N-terminal cysteine or glycine via an amide bond (N-palmitoylation). N-palmitoylation is primarily found in secreted proteins (Nadolski and Linder 2007) and is like prenylation and myristoylation not reversible and therefore not dynamic.

## Palmitoyl acyltransferases (PATs) and thioesterases

The lipidation of proteins was first observed 30 years ago (Schmidt and Schlesinger 1979; Schlesinger et al 1980) but only recently the relevant catalytic enzymes were identified. The first palmitoyl acyltransferase was identified in *S. cerevisiae* by forward genetic screens. This approach identified *Erf2 –Erf4* (Bartels et al 1999; Lobo et al 2002 and *Akr1* (Roth et al 2002) as palmitoyl acyltransferases (PATs) for the yeast proteins RAS2 and Yck2 (casein kinase 2). Erf2 and Akr1 share a 51-amino acid domain known as DHHC cysteine-rich domain (CRD) which is a variant of the $C_2H_2$ zinc finger motif (Putilina et al 1999). Erf2 and Akr1 strictly required an intact DHHC motif for activity and subsequent work showed that palmitoyl transferases are general members of the DHHC protein family (although commonly referred to as "DHHC PATs" the official gene name is "ZDHHC", e.g., *ZDHHC8*). The core DHHC motif is highly conserved and essential for catalytic function (Mitchell et al 2006). Seven DHHC genes have been identified in yeast and 23 DHHC proteins are predicted from mammalian genomes (human and mouse) (Fukata, Fukata et al 2004; Ohno et al 2006). DHHC palmitoyl transferases are membrane proteins with four to six predicted transmembrane domains (TMD) (Politis et al 2005; Conibear and Davis 2010) and expression studies showed that the majority of the mammalian PATs are localized at the ER and Golgi (Ohno et al 2006) with some exceptions like DHHC5, which is localized to the plasma membrane. Erf2 is present in the endoplasmic reticulum (ER), whereas Akr1 is localized at the Golgi. A precise consensus sequence for palmitoylation has not been identified yet and it seems that some DHHC proteins can palmitoylate rather a broad range of substrates whereas others are more selective. Also Erf2 and Akr1 showed a certain substrate specificity as Yck2 palmitoylation is unaffected by mutation in *Erf2* and Ras2 palmitoylation is not affected by *Akr1* mutants. However, the lack of a consensus sequence currently does not allow to predict whether a protein is a target for palmitoylation (Fig. 1).

In contrast to S-palmitoylation is N-palmitoylation mediated by set of multispanning transmembrane O-acyltransferase (MBOAT). Many members of the MBOAT family are lysophospholipid acyltransferases (Shindou and Shimizu 2009) and typically catalyze the addition of a fatty acid to the hydroxyl group of membrane lipids (Hofmann 2000).

In contrast to the great number of DHHC PATs only four acyl protein thioesterases (commonly referred to as thioesterases) have been identified to catalyze depalmitoylation. This is APT1 (LYPLA1) and APT2 (LYPLA2), and the protein palmitoyl thioesterases 1 (PPT1) and 2 (PPT2) (Zeidman et al 2009; Tomatis et al 2010). APT1 was first purified from the cytosol of rat hepatocytes based on its ability to remove palmitate from [$^3$H]-palmitoyl–G$\alpha_i$ (Duncan and Gilman 1998). APT1 is expressed in a wide range of mouse tissues and has been shown to depalmitoylate a list of proteins including H-Ras (Duncan and Gilman 1998) and endothelial NOS (Yeh et al 1999). APT2 is a homologue of APT1 and shows 64 % amino acid sequence identity. It has been shown to depalmitoylate GAP-43 and also H-Ras (Tomatis et al 2010; Rusch et al 2011). Also thioesterases seem not to have a defined substrate recognition sequence and the list of APT1 substrates contains proteins that are

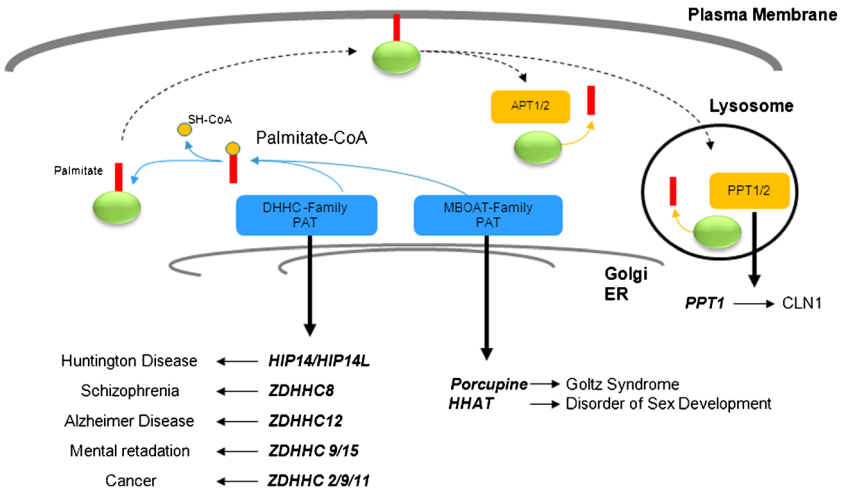


**Fig. 1** Schematic representation of the interplay between palmitoyltransferases (*PAT, blue*) and acyl protein thioesterases (*orange*) and associated pathologies

structurally diverse and containing different lipid modifications (Zeidman et al 2009). The third identified thioesterase is PPT1, which is a lysosomal enzyme and indiscriminately cleaves fatty acids from cysteine residues in the process of protein degradation (Zeidman et al 2009). As PPT1 is located in the lysosomes it is probably not involved in the dynamic depalmitoylation of cytoplasmic proteins (Hellsten et al 1996; Verkruyse and Hofmann 1996). However, PPT1 interacts with the F(1)-complex of the mitochondrial ATP synthase and the levels of F(1)-subunits alpha and beta on the plasma membrane were increased in neurons of PPT1 deficient mice (Lyly et al 2008). PPT2 is a homologue to PPT1 and shares about 20 % identity. Also PPT2 is a lysosomal protein but seems to have a distinct substrate specificity as it did not remove palmitate groups from proteins that are substrates for PPT1 (Soyombo and Hofmann 1997).

Enzymes that regulate protein palmitoylation are critical for several biological processes. To date, nine palmitoylation related genes have been reported to be associated with human disease (Liu et al 2002; Mansouri et al 2005; Yanai et al 2006; Raymond et al 2007; Mizumaru et al 2009; Singaraja et al 2011; Callier et al (2014).

## Huntington's disease

Huntington's disease (HD) is a neurodegenerative disease that presents with cognitive, motor, and psychiatric signs and symptoms (Roos 2010; Sturrock and Leavitt 2010). The prevalence of HD in Caucasian European populations is 5–7 per 100,000 individuals (Warby et al 2011). HD is caused by mutations in the HTT (huntingtin or HD) gene located on chromosome 4. The exact function of HTT is not clear. It is essential for development and the absence of HTT is lethal in mice (Nasir et al 1995). The protein has no sequence homology to other proteins and is highly expressed in neurons and testes of humans and rodents (Cattaneo et al 2005). CAG repeats which exceed the number of 36 lead to the production of an HTT protein with an abnormally long N-terminal polyglutamine tail which is unstable. Individuals with 36 to 40 CAG repeats may or may not develop HD whereas individuals with more than 40 repeats will develop the disorder. Sixty and more CAG repeats lead to a severe form of juvenile HD.

Huntingtin has been found to interact with a number of other proteins including HIP14 (ZDHHC17) (Kalchman et al 1996; Singaraja et al 2002). HIP14 and its homologue HIP14L are human orthologues of the yeast proteins Akr1 and Akr2. HIP14L is an atypical ZDHHC protein as it has a DQHC instead of the typical DHHC motive (Mitchell et al 2006; Greaves and Chamberlain 2011). The role of HIP14 in HD gained focus when it was shown that the HIP14-HTT interaction correlated inversely with the number of CAG repeats. This suggests an aberrant palmitoylation to be involved in the pathogenesis of HD (Singaraja et al 2002). HIP14 is expressed in brain and in particular in medium spiny neuron (MSN) cells, special inhibitory neurons which are primarily present in corpus striatum of the basal ganglia. MSN cells play a key role in initiating and controlling movements of the body, limbs, and eyes and are the earliest cell population affected in HD. Further work showed that HTT is primarily palmitoylated at Cys214 and the mutation of this site was associated with increased inclusion formation in COS cells and neuronal cultures and increased NMDA-induced toxicity in cultured rat cortical neurons (Yanai et al 2006). The silencing of HIP14 in cortical neuron cultures of YAC128 mice, a mouse model which is transgenic for the human huntingtin protein, resulted in increased inclusion formation (Slow et al 2003; Yanai et al 2006). Palmitoylation of HIP14 is reduced in brains of YAC128 mice, suggesting a reduced HIP14 activity in the presence of mutant HTT. The overexpression of HIP14 reduced inclusion formation in cultured neurons (Yanai et al 2006). HIP14 itself is autopalmitoylated (Huang et al 2004) — a conserved feature that is correlated with PAT activity (Fukata, Fukata et al 2004; Huang et al 2004).

Autopalmitoylation is reduced in HIP14 isolated from YAC128 brains. HIP14 appears to be predominantly a neuronal PAT and its confirmed substrates include SNAP25, GAD65, HTT, STREX-BK potassium channel, and GluR1/2 AMPA receptor subunits (Fukata, Fukata et al 2004; Huang et al 2004; Huang et al 2009; Greaves et al 2010; Tian et al 2010; Singaraja et al 2011). HIP14 may also be a PAT for PSD-95 as the knockdown of HIP14 reduced palmitoylation of PSD-95 (Huang et al 2004, 2009). In return it appears that HTT modulates the palmitoylation and activity of HIP14 itself. Palmitoylation of HIP14 was reduced in heterozygous $Hdh^{+/-}$ mice. $Hip14^{-/-}$ mice revealed a reduced brain weight at 1 month of age (Singaraja et al 2011).

### Schizophrenia

For three SNPs in the palmitoyl acyltransferases *ZDHHC8* a significant association with schizophrenia was found in a US and South African population (Liu et al 2002). The association with schizophrenia was further confirmed for one of these SNPs (rs175174) in an American and South African cohort. An association between *ZDHHC8* and schizophrenia was also found in the Han Chinese population (Chen et al 2004b). Also the association of the 22q11 microdeletion region, which bears the *ZDHHC8* gene and schizophrenia was reported. The identified SNP was functional and influenced alternative splicing of *ZDHHC8* which led to the retention of intron-4 and the introduction of a premature termination codon. Female *ZDHHC8*-deficient mice showed abnormalities in fear-related measures of spontaneous activity which were absent in male mice. This was believed to be related to an influence of ZDHHC8 on glutamatergic signal transmission, as female *ZDHHC8*-deficient mice also appeared to be less sensitive to an NMDAR blocker (Mukai et al 2004). A follow-up study demonstrated that *ZDHHC8*-deficient mice have a decreased density of dendritic spines (Mukai et al 2008). Furthermore it was shown that a polymorphism in *ZDHHC8* is associated with nystagmus (abnormalities in smooth eye movements) which is common in schizophrenia (Shin et al 2010). However, several other studies failed to identify an association of *ZDHHC8* with schizophrenia in other populations (Glaser et al 2005, 2006; Otani et al 2005; Saito et al 2005; Demily et al 2007; Xu et al 2010).

### Alzheimer's disease

A number of studies have explored the role of palmitoylation in the pathogenesis of Alzheimer's disease (AD). Alzheimer's disease is a neurodegenerative type of dementia in which the death of brain cells causes memory loss and cognitive decline. The disease starts typically mild and gets progressively worse. A typical pathogenic step in AD is the generation of neurotoxic beta-amyloid (Aβ) from amyloid precursor protein (APP) by the sequential cleavage of two proteases (β- and γ-secretase). No genetic link has yet been found between PATs and AD but there is evidence that the β- and γ-secretase enzymes are palmitoylated. Whether this palmitoylation is altered in AD is currently unclear. ZDHHC12 has been linked to APP trafficking by retaining APP in the Golgi and to prevent its further trafficking to the trans Golgi network and PM in neuroblastoma cells (Mizumaru et al 2009). However, this is probably not a direct effect of APP palmitoylation by ZDHHC12 as APP does not contain cytosolic cysteines.

Interestingly the major APP cleaving enzyme BACE1 is palmitoylated at four sites: three within the C-terminal cytosolic tail (Benjannet et al 2001) and one on its TMD which seems to be critical for targeting BACE1 to lipid rafts. Five PATs (ZDHHC3, 4, 7, 15, and 20) have been identified by co-expression studies to potentially enhance the palmitoylation of BACE1 (Vetrivel et al 2009).

### Goltz syndrome

Human focal dermal hypoplasia or Goltz syndrome is an X-linked dominant form of ectodermal dysplasia. It is transmitted as an X-linked dominant trait and is lethal in utero for male fetuses. It is a multisystem disorder, primarily characterized by skin manifestations as atrophic and hypoplastic areas (Wang et al 2007). Clinical signs constitute areas of cutaneous atrophy and periorificial papillomas around the mouth, genitalia, and/or anus. Osseous defects include scoliosis, hypoplastic clavicles and ribs, and a deformed thorax. Dental anomalies are typical and may include malpositioned teeth, extra teeth, and enamel defects. Eyes are affected by coloboma of the iris, microphthalmia, and/or strabismus. Goltz syndrome is caused by mutations in the *Porcupine* (*Porcn*) gene. Porcn is a multipass membrane protein and a member of the MBOAT family. Porcupine (Porcn) has been implicated in fatty acid modifications of Wnt proteins (Willert et al 2003; Takada et al 2006; Galli et al 2007) which are dual acylated with palmitate and palmitoleate. This modification is required for Wnt protein secretion and signaling (Takada et al 2006; Doubravska et al 2011) and dysregulation of the Wnt signaling pathways is associated with oncogenesis (Polakis 2007). The deletion of *Porcn* in mice is embryonically lethal due to the failure to secrete functional Wnt proteins (Biechele et al 2011) but symptoms of Goltz syndrome can be recapitulated in mice with a conditional disruption of *Porcn* (Barrott et al 2011).

### Other diseases related to PAT mutations

The post-translational attachment of cholesterol and palmitate to the Hedgehog (Hh) family of secreted proteins is critical for multimerization and long range signaling potency (Chen et al 2004a). Hh proteins act as morphogens to control embryonic

patterning and development in a variety of organ systems. A very recent study reported the case of an autosomal recessive syndromic 46,XY disorder of sex development (DSD) with testicular dysgenesis and chondrodysplasia which was associated with a homozygous missense mutation in the hedgehog acyl-transferase (HHAT) gene (Callier et al 2014). The mutation (G287V) was found in a conserved membrane bound O-acyltransferase (MBOAT) domain and in vitro studies showed that the mutations disrupted the ability of HHAT to palmitoylate Hh proteins such as DHH and SHH.

A translocation between chromosome X and a region 15,2442 bp upstream of the ZDHHC15 gene which resulted in the absence of ZDHHC15 transcripts was reported in the case of a 29-year old woman with severe non-syndromic mental retardation (Mansouri et al 2005). Mutations in another X-chomosomal PAT (*ZDHHC9*) were found in four of 250 families with X-linked mental retardation. Two missense mutations were located in the DHHC-CR domain and one was located at a highly conserved residue (Mitchell et al 2006; Raymond et al 2007). However, the effect of these mutations on enzyme activity was not yet validated in functional assays.

Mutations in three PATs (ZDHHC2, 9, 11) have been implicated in various forms of human cancer (Oyama et al 2000; Mansilla et al 2007; Yamamoto et al 2007; Zhang et al 2008) and one study reported HIP14 to be an oncogene in vitro and in vivo in mice (Ducker et al 2004). Mutations in HIP14L (ZDHHC13) and ZDHHC21 have been shown to result in dermatological and related phenotypes in inbred mice (Mill et al 2009; Saleem et al 2010).

## Ceroid lipofuscinosis type 1 (*CLN1*)

Besides defects in the palmitoylation reaction is also the opposite step — the removal of the S-linked palmitate — associated with pathological conditions. Mutations in the PPT1 gene result in infantile neuronal ceroid lipofuscinosis type 1 (CLN1 or Batten disease) (Vesa et al 1995). CLN1 is a rare disease (prevalence one out of 100,000 births) and belongs to the family of neuronal ceroid lipofuscinosis (NCL) a genetically distinct group of neurodegenerative diseases featured by epilepsy, progressive blindness and premature death. Collectively, they represent the most common group of hereditary encephalopathies in childhood, with an incidence of up to 1/12,500 (Haltia 2006). At the ultrastructural level the disease is associated with the lysosomal accumulation of lipofuscin — a granular autofluorescent lipopigment. CLN1 is caused by homozygous or compound heterozygous mutations in the *PPT1* gene on chromosome 1p32. To date 64 PPT mutations are annotated in the NCL mutation database (http://www.ucl.ac.uk/ncl/mutation.shtml). Many of these mutations are private mutations although a c.451 C>T (R151X) exchange seems to be the most prevalent mutation in non-related carriers. A c.364 A>T (R122W) transversion seems to cluster in Finnish patients. Both mutations represent about 20 % of the total CLN1 cases. CLN1 has an early-onset and symptoms appear typically within the first 6–12 months of life with previously normal development (Mitchison et al 1998). The disease is initially characterized by the delay of developmental progress, microcephaly, and the loss of motor function leading to hypotonia. Vision loss becomes apparent from the 12th month and progresses to blindness until 2 years of age. At that time the child usually starts to loose previously acquired skills (speech and movements) and most affected children die by the age of seven (Santavuori 2011). The autopsy shows a shrunken brain and diffuse cortical and cerebellar atrophy. Electron microscopy of brain and other tissues like blood lymphocytes demonstrates accumulation of granular osmiophilic dense (GROD) bodies. Palmitoylated peptide intermediates accumulate in the lysosomes along with sphingolipid activator proteins (saposin) A and D (Tyynela et al 1993). After the crystal structure of PPT1 was identified, a correlation was observed between the severity of the infantile CLN1 phenotype and the effect of the various mutations on the catalytic site (Bellizzi et al 2000). The pathogenesis of the disease may be due to activation of an apoptosis pathway. PPT1 deficient mice demonstrate abnormal ER morphology and an accumulation of palmitoylated GAP-43 in the ER. This might lead to activation of unfolded protein response in the ER and subsequently activation of caspase-3 and apoptosis (Zhang et al 2006). The same group reported that caspase-9 is activated following increased production of reactive oxygen species and disruption of calcium homeostasis. PPT1 may therefore help to protect against apoptosis. Neuroblastoma cells which overexpress PPT1 showed reduced cell death, reduced activation of caspase-3, and increased phosphorylation of the anti-apoptotic protein Akt when treated with the apoptosis-inducing agent $C_2$ ceramide (Cho and Dawson 2000). On the other hand, inhibition of PPT1 either via PPT1 antisense RNA or a PPT1 inhibitor resulted in enhanced apoptosis (Cho et al 2000).

Experimental PPT2 deficiency in mice causes an unusual form of neuronal ceroid lipofuscinosis with striking visceral manifestations but no PPT2 deficiency has been described in humans yet (Gupta et al 2003).

Currently there is no treatment for CNL1. However, a recently concluded pilot study investigated the combination of phosphocysteamine and N-acetylcysteine as a therapy in nine patients with CNL1 (Levin et al 2014). The results showed a remarkable clearance of GRODs in blood cells, a reduced irritability of the patients and a slowdown of the disease course. In particular the reduced irritability in response to the treatment might affect present clinical practice. Besides these promising results seizures may be controlled or reduced with use of anti-epileptic drugs and physical, speech, and occupational therapies may help affected patients retain their cognitive and motor functions as long as possible.

In summary six PATs (HIP14, HIP14L, ZDHHC8, ZDHHC9, ZDHHC12, and ZDHHC15) which catalyze S-palmitoylation have been implicated in the neuropsychiatric diseases like Alzheimer's and Huntington's disease, schizophrenia, and mental retardation. Mutations in Porcupine and HHAT which both belong to the MBOAT family cause human focal dermal hypoplasia (Goltz syndrome) and testicular dysgenesis and chondrodysplasia, respectively. On the opposite side are defects in the thioesterase PPT1 associated with infantile NCL (CLN1). This highlights the importance of protein palmitoylation in particular for neurons as a disturbance of this process results in severe, mostly neuronal pathologies.

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