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Review

Proteostasis plays an important role in demyelinating Charcot Marie Tooth disease

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ABSTRACT

Type 1 Charcot-Marie-Tooth disease (CMT1) is the most common demyelinating peripheral neuropathy. Patients suffer from progressive muscle weakness and sensory problems. The underlying disease mechanisms of CMT1 are still unclear and no therapy is currently available, hence patients completely rely on supportive care. Balancing protein levels is a complex multistep process fundamental to maintain cells in their healthy state and a disrupted proteostasis is a hallmark of several neurodegenerative diseases. When protein misfolding occurs, protein quality control systems are activated such as chaperones, the lysosomal-autophagy system and proteasomal degradation to ensure proper degradation. However, in pathological circumstances, these mechanisms are overloaded and thereby become inefficient to clear the load of misfolded proteins. Recent evidence strongly indicates that a disbalance in proteostasis plays an important role in several forms of CMT1.

In this review, we present an overview of the protein quality control systems, their role in CMT1, and potential treatment strategies to restore proteostasis.

1. Introduction

Despite major efforts in the field of neuroscience, the translation of new therapies towards clinical applications has lagged behind. At least partially, this is due to the fact that for many neurological diseases the exact pathological mechanism(s) are not yet fully understood. Interestingly, several well-known neurodegenerative diseases have one particular pathophysiological mechanism in common: a **disrupted proteostasis** [1,2]. As the accumulation of dysfunctional proteins is cytotoxic for cells, protein homeostasis is important to preserve cellular health in general [3]. To safeguard protein integrity, an equilibrium between protein synthesis, folding and degradation has to be maintained [4,5]. To do so, cells possess several protein quality control systems that prevent and clear dysfunctional proteins and hence maintain cells in a healthy state.

Charcot-Marie-Tooth disease type 1 (CMT1) is the most common inherited peripheral demyelinating neuropathy [6]. As both motor and sensory peripheral nerves are affected, patients typically suffer from

Abbreviations: Akt, protein kinase B; ATF6, Activating transcription factor 6; BiP, Immunoglobulin heavy chain binding protein; CHOP, CCAAT-enhancer binding protein homologues protein; CMA, Chaperone-mediated autophagy; CMT1, Charcot-Marie-Tooth disease type 1; CMT2, Charcot-Marie-Tooth disease type 2; Cnx, Calnexin; Crt, Calreticulin; DSS, Déjerine Sottas syndrome; DMSO, Dimethylsulfoxide; eIF2α, Eukaryotic initiation factor 2α; EGR2, Early Growth Response Protein 2; ER, Endoplasmic reticulum; ERAD, Endoplasmic reticulum- associated degradation; GADD34, Growth arrest DNA damage-inducible protein; GJB1, Gap Junction Beta 1; GRP78, 78kD glucose-regulated protein; HSF1, Heat shock factor 1; HSC70, Heat shock cognate protein 70; HSP, Heat shock protein; IRE1α, Inositol requiring enzyme 1α; LAMP2a, Lysosomal associated membrane protein 2a; LITAF, Lipopolysaccharide induced tumor necrosis factor α; LRP1, Low density lipoprotein receptor-regulated protein 1; MBP, Myelin basic protein; MMP-9, Matrix metalloproteinase 9; MPZ, Myelin Protein Zero; MVC, Motor Condution Velocity; PERK, Protein kinase RNA-like ER kinase; PI3K, phosphoinositol 3 kinase; PMP22, Peripheral Myelin Protein 22; PNS, Peripheral nervous system; Rer1, Retention in ER sorting receptor 1; SIMPLE, small integral membrane protein of the lysosome/late endosome; Tr, Trembler; Trj, Trembler J; UPR, Unfolded protein response; USP14, Proteasomal deubiquitinating enzyme; XBP1, X-Box binding protein 1.

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distally progressing muscle weakness and sensory reduction. To date, no effective treatment is available and patients completely rely on supportive care such as orthopaedic devices and physical therapy [7]. This highlights the importance to thoroughly understand the underlying disease pathology. In CMT1 genetic alterations are seen in several genes expressed by **Schwann cells**, the myelin-producing cells of the peripheral nervous system [8]. Schwann cells ensure proper signal propagation along the axon, by wrapping their lipid-rich membrane around axons. During myelination, these cells produce a massive bulk of myelin membrane proteins, lipids and cholesterol, which requires intensive use of the secretory pathway [9]. This could make Schwann cells more susceptible to dysfunctions in the cellular mechanism of proteostasis [9].

Recent evidence strongly indicates that cellular proteostasis is dysfunctional in demyelinating disorders, including several forms of CMT1 [9,10]. In this review, we give an overview of the general proteostasis and specific adaptations of the peripheral myelinating cells to deal with stressful circumstances. Thereafter, we focus on recent findings in relation to dysfunctional proteostasis in CMT1 and provide an overview on rebalancing proteostasis as a potential therapy for CMT1.

2. Charcot Marie Tooth disease type 1: A demyelinating pathology

Charcot-Marie-tooth disease (CMT) is the most common peripheral neuropathy affecting 1 in 2500 people worldwide [6]. Patients typically suffer from progressive muscle weakness, muscle atrophy, *pes cavus*, decreased reflexes and sensory problems [6]. In addition, CMT is heterogenous in age of onset, and in general disease symptoms are discernible in the second decade of life. However, less obvious symptoms such as general clumsiness and frequent falls have usually been reported earlier in life [11]. Until now, over 100 genes have been linked to CMT and the exact disease mechanisms of the different CMT forms are unknown [12,13]. Hence, there are no effective treatments available and patients completely rely on supportive care such as orthopaedic devices and physical therapy.

CMT is classified into two main groups, CMT1 and CMT2 of which CMT1 is the most frequent form and represents 80% of all CMT cases [14]. CMT1 is primarily demyelinating while CMT2 mainly comprises an axonal pathology, nevertheless in CMT1 demyelination eventually leads to secondary axonal damage [15,16]. Hence, diagnosis and discrimination between both types cannot be performed based on specific clinical features. Therefore, diagnosis occurs via electrophysiological examination based on the motor conduction velocity (MCV). In healthy patients the MCV is above 45 m/s. In CMT1 patients, the MCV values are below 38 m/s, whereas in CMT2 patients, MCV values above 38 m/s are measured [15]. Classification in different groups and their subtypes can be further based on the genetic cause underlying the disease [6].

As CMT1 is characterized by peripheral nerve demyelination, Schwann cells are specifically affected in this disease. In this review, we focus on the disturbed proteostasis in the most common CMT1 forms (see overview in Table 1).

Table 1
Overview of the most common CMT1 neuropathies, involved genes and relevant rodent models.

CMT1-form	Involved Gene	Most relevant rodent models	Sources
CMT1A	PMP22 duplication of 1 allele	C22, C3, CMT1A-rat, C61, My41, TgN248	[178–183]
CMT1B	MPZ mutation	MPZ^{R98C}, MPZ^{S63Del}, MPZS63C, $MPZ^{Ile106L}$, MPZ^{null}, MPZ^{D61N}, MPZ^{T124M}	[69,135,184,185]
CMT1C	LITAF mutation	$LITAF^{W116G}$, $LITAF^{P135T}$	[186,187]
CMT1D	EGR2 mutation	EGR2null, EGR2lo/lo	[178]
CMT1E	PMP22 mutation	$PMP22^{L16P}$ (Trj *L16P*), $PMP22^{G150D}$ *(Tr G150D)*	[188,189]
CMT1X	GJB1 mutation	$Cx32^{R142W}$, $Cx23^{null}$, $Cx32^{T55I}$, $Cx32^{R75W}$	[149,158,159,190]

PMP22: peripheral myelin protein 22; MPZ: myelin protein zero; LITAF: lipopolysaccharide-induced tumor necrosis factor α; EGR2: early growth response protein 2; GJB1: gap junction beta1 gene.

3. Cellular mechanisms to maintain proteostasis in healthy cells

Both secreted and transmembrane proteins enter the endoplasmic reticulum (ER) as unfolded proteins and exit as either folded proteins to be transferred to their organelles or as misfolded proteins targeted for degradation [17,18]. To ensure proper protein folding and prevent aggregation of proteins in the ER lumen, numerous chaperones are involved in every aspect of the ER quality control.

3.1. Endoplasmic reticulum–associated protein degradation and the unfolded protein response

When proteins enter the ER, the chaperones Calnexin (Cnx) and Calreticulin (Crt) ensure proper (re)folding by shuttling the proteins back and forth in the so-called “calreticulin/calnexin cycle” [19–23] (Fig. 1). If proper folding fails, **endoplasmic reticulum–associated protein degradation (ERAD)** signalling is activated [19,21,24,25]. Hence, misfolded proteins are *retro*-translocated towards the cytosol, polyubiquitinated and degraded by large oligomeric structures: the proteasomes [24,26]. When aggregation-prone or mutated proteins accumulate in the ER, ER stress is induced and the unfolded protein response (UPR) is activated to restore ER homeostasis [27,28]. **Activation of the UPR** depends on three ER stress sensor proteins, activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK), and inositol requiring enzyme 1α (IRE1α, Fig. 1).

The most abundant and well-known chaperone is immunoglobulin heavy chain binding protein (BiP), also known as the glucose-regulated protein 78 kDa (GRP78). BiP is involved in the translocation of nascent proteins, the facilitation of protein folding and targeting misfolded proteins to the ERAD machinery (Fig. 1) [29,30].

Under normal physiological conditions, ATF6, PERK and IRE1α are individually associated with BiP. However, upon ER stress, BiP dissociates from the UPR signal sensors to bind misfolded proteins, thereby activating the ATF6, IREα and PERK pathways [28,31]. Upon ER stress, **ATF6** is translocated to the Golgi apparatus where it is cleaved and its cytosolic part is transported into the nucleus. This induces ERAD-related protein transcription and consequently boosts the capacity of the ERAD system [32–34] (Fig. 1).

When activated, **PERK** oligomerizes and autophosphorylates. This leads to phosphorylation of the eukaryotic initiation factor (eIF2α) [28,35], reducing general protein translation and a reduction in ER stress [36]. However, when eIF2α is inactivated, proteins including ATF4 are preferentially translated. ATF4 has two important downstream factors: C/EBP homologous protein (CHOP) and growth arrest DNA damage- inducible protein (GADD34). CHOP is involved in apoptosis and terminal UPR signalling, while GADD34 is responsible for dephosphorylating eIF2α and, activating autophagy [37–41]. The third and most studied pathway is the **IRE1α** pathway. Following ER stress, IRE1α is activated, inducing its RNAse activity which in turn leads to the alternative splicing of X-Box binding protein 1 (XBP1) into its active variant. XBP1 is involved in blocking general protein translation and inducing ERAD-related gene expression [42]. Interestingly, XBP1 is also transcriptionally upregulated by activated ATF6, highlighting an important yet complex interplay between the different UPR pathways [43,44].

The UPR is a cytoprotective mechanism that enables cells to adapt to conditions that perturb protein folding in the ER. However, during

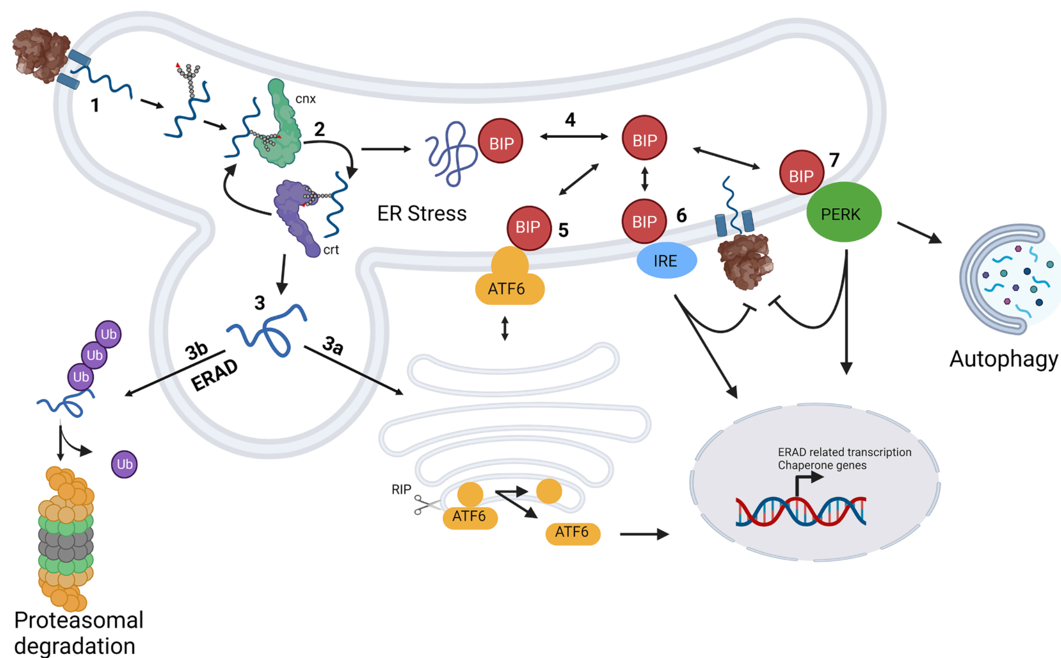

Fig. 1. Endoplasmic reticulum (Re)folding and the UPR. Nascent proteins enter the endoplasmic reticulum **(1)**, and after trimming by glucosidases I and II, proteins are recognized by two chaperones that assist folding: Cnx, Crt. Here, a back-and forth cycle is proceeded **(2)**. Next, correctly folded proteins are transported towards the Golgi apparatus for further processing **(3a)**. In contrast, misfolded proteins are exported towards the cytosol, where degradation is exceeded via the proteasomal system, also called ERAD **(3b)**. Overwhelming of the ERAD system causes ER stress and thereby activation of the UPR to restore ER homeostasis. To date, three crucial UPR sensors are described: ATF6, IRE1α and PERK. In basal conditions, the three UPR sensors are associated with BiP. Upon ER stress, BiP will be translocated towards misfolded proteins **(4)**. Hereby, the three individual pathways are activated. Activated ATF6 is translocated towards the Golgi apparatus, cleaved and its cytoplasmic domain travels towards the nucleus where it will induce ERAD-related transcription **(5)**. PERK activation leads to a reduction in general protein translation and induction of ERAD-related transcription **(7)** and activates autophagy. IRE1α is activated leading to ERAD-related gene transcription **(6)**. ATF6: Activating transcription factor 6, Bip: Binding protein, Cnx: Calnexin, Crt: Calreticulin, ER: endoplasmic reticulum, ERAD: endoplasmic reticulum associated degradation, PERK: protein kinase RNA-like ER kinase, UPR: unfolded protein response. Created with BioRender.com.

severe and prolonged ER stress, the UPR can turn into a cytotoxic mechanism inducing terminal UPR and apoptosis [31].

3.2. Autophagy-lysosomal pathway

The autophagy-lysosomal system is crucial for degradation of lipids, damaged organelles, and is a major pathway for the degradation of aggregated proteins. Hence, there has been intense interest in understanding its role in neurodegenerative disease.

The lysosomal autophagy system encompasses different pathways that are classified based on their cargo delivery to the lysosomes [45], including macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. After entering the lysosomes, the targeted cargo is degraded via hydrolytic enzymes such as proteases, phospholipases, phosphatases, nucleases, lipases and glycosidases [46–49].

Macroautophagy is the most studied lysosomal autophagy system and has been demonstrated to play a primary role in non– and selective-degradation. While non-selective degradation is primarily involved in bulking under stress conditions, selective degradation involves the breakdown of damaged organelles, proteins and even large aggregates to prevent cell damage [50]. However, besides its role in degradation, macroautophagy is involved in a wide range of other crucial processes, such as general cellular homeostasis and induction of programmed cell death type II [51]. Importantly, several neurodegenerative diseases are linked with altered macroautophagy such as Parkinson's, Alzheimer's, Huntington's disease, but also CMT1 [1,52–55]. Macroautophagy encompasses several autophagy-related genes and starts with the formation of a ''*de novo*'' limiting-membrane [56]. This double-membrane further elongates and maturates forming a phagosome. Eventually, it encloses the target cargo, thereby forming the autophagosome [56]. Finally, autophagosomes fuse with the lysosomes that degrade the inner autophagosome membrane and subsequently degrade the enclosed material [56–58].

A second pathway in the lysosomal autophagy system is **chaperone-mediated autophagy**, which specifically recognizes polypeptides with the KFERQ motif and translocates them from the cytosol directly into the lumen of the lysosome via the HSC70 chaperone protein [59–62]. In correctly folded proteins, the KFERQ motif is unreachable. However, in misfolded proteins the KFERQ motif is exposed and proteins are subsequently recognized by cytosolic-HSC70 [63] and translocated to the lysosome via oligomerized lysosomal associated membrane protein 2a (LAMP2a) and lysosomal-HSC70. After entering, proteins are degraded by cathepsins and other lysosomal enzymes [60,62].

Microautophagy, a third pathway of the lysosomal autophagy system, is a complex process in which cytosolic proteins are entrapped starting with the direct invagination at the late endosomal or lysosomal membrane [64–67]. As most research has been performed in yeast, microautophagy in mammalian cells remains poorly understood. Nevertheless, both non-selective and selective degradation has been reported, with the former mainly involved in nutrient sensing and the latter involving selective recognition exercised by HSC70 and several co-chaperones [45,66,68].

4. Safeguarding proteostasis in Schwann cells

Myelination of axons in the PNS is accomplished by Schwann cells. This lipid-rich myelin sheath is crucial for rapid signal propagation of action potentials. Nevertheless, production of this highly dynamic structure requires the production of an enormous amount of myelin proteins, cholesterol and lipids making use of the secretory pathway [9]. This renders Schwann cells particularly sensitive to proteolytic overload [9]. Hence, a tight regulation of Schwann cell proteostasis is essential to

maintain cellular health and to preserve the integrity of the myelin sheath [9]. As in other cells, the ubiquitin-proteasomal system, lysosomal-autophagy system and chaperones are crucial in order to maintain proteostasis, and also Schwann cells have to cope with protein disbalance through the activation of these mechanisms.

As mentioned before, chronic ER stress leads to terminal apoptosis if the UPR response fails to restore the protein balance. Nevertheless, there are strong contradictory data when it comes to Schwann cell apoptosis in peripheral nerve diseases including CMT1. Surprisingly, often there seems to be no clear evidence of apoptotic Schwann cells in peripheral nerve diseases such as CMT1A and B (Table 1) in which proteostasis is perturbed [69–71]. Yet, the disease model as well as the time point of the measurements may be an important factor to consider when studying these progressive neuropathies. For example, Sancho *et al.* showed an increase of apoptosis in CMT1E mice (Table 1) starting from P21 [72]. In contrast, the apoptotic rate was already doubled at P4 in CMT1A compared to wild type mice. Importantly, it must be taken into account that Schwann cell apoptosis occurs naturally in early postnatal development when interpreting apoptotic data. These facts could partly explain the contradictions in the available literature.

Interestingly, recent data suggest that Schwann cells possess particular mechanisms to respond to UPR activation and ER stress upon injury and thereby possibly preventing terminal apoptosis. For example, a crucial role for the phosphoinositol 3 kinase/Akt (PI3K/AKT) pathway was described in the stimulation of Schwann cell survival upon nerve injury by blocking UPR-CHOP-induced apoptosis [73–75]. Although the exact upstream mechanisms are not yet clear, Mantuano *et al.* proposed a role for the low-density lipoprotein receptor-regulated protein-1 (LRP1), which antagonizes the UPR signalling pathway [76]. Schwann cells express the LRP1 receptor in low levels under normal physiological conditions, while it is upregulated in response to injury [76]. The LRP1 receptor recognises injury-induced ligands such as matrix metalloproteinase 9 (MMP-9) and it has been shown that LPR1-ligand interaction is crucial for Schwann cell survival and migration [76–78]. This underlines a novel mechanism for Schwann cells to possibly escape apoptosis caused by terminal UPR [76]. Nevertheless, this pathway has also been reported to be involved in cell survival of neurons [79], macrophages [80] and endothelial cells [81]. Additionally, in CMT1B mice, ablation of CHOP improves the phenotype by primarily reducing demyelination instead of directly impacting cell death. This suggests that CHOP/PERK negatively influences myelination uncoupled from its UPR role [82,83].

A second possible reason for limited Schwann cell apoptosis may be their enormous cellular plasticity, allowing them to respond to stressful situations [84]. During peripheral nerve injury for example, Schwann cells switch to an immature-like phenotype that drives nerve repair. During nerve regeneration, these repair Schwann cells are crucial to promote neuronal repair and survival. In general, this process involves the reduction of promyelinating genes and the upregulation of immature Schwann cell genes, such as Krox20 and c-Jun, respectively [85]. Moreover, in the context of inherited neuropathies such as CMT1A, c-Jun levels are increased in Schwann cells. Interestingly, deleting c-Jun only worseness the phenotype [86–89]. Additionally, Florio *et al.* confirmed that ER-stressed CMT1B Schwann cells increase expression of negative myelin regulators, and thus protect themselves from cell toxicity [90]. This confirms a crucial role for Schwann cell plasticity in axonal protection in CMT1.

Thirdly, Schwann cells have the capacity to clear their own myelin via macroautophagy, a process referred to as **myelinophagy**, which is not seen in oligodendrocytes [86,87,91,92]. Myelinophagy contributes to the extraordinary regenerative potential of the PNS. However, in the context of CMT1 and other neuropathies myelinophagy could contribute to further demyelination [86–89].

5. Dysfunctional proteostasis in CMT1 Schwann cells

As discussed before, CMT1 is the most common demyelinating peripheral neuropathy and is caused by one or several mutated genes expressed by Schwann cells. Although the exact molecular mechanism (s) remain to be elucidated, increasing evidence indicates that a dysfunction in proteostasis could be an overall cause in several forms of CMT1, including CMT1A/E and CMT1B [93–95]. As a consequence, CMT1 appends to the list of well-known neurodegenerative disorders in which a disturbed proteostasis has been shown to be a pathological hallmark of the disease, such as Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease [96–100]. In the following section, we provide a brief overview of the most common CMT1 forms with regard to the existing literature on protein balance defects.

CMT1A is the most common form of CMT1. Approximately 70% of all CMT1 cases are caused by a 1.4 Mb region duplication located on chromosome 17 containing the *PMP22* gene [101,102]. **CMT1E**, on the other hand, is caused by diverse point mutations in the *PMP22* gene, of which $PMP22^{L16P}$ and $PMP22^{G150D}$ are the most well-known that are being studied in the Trembler J (Trj^{L16P}) and Trembler (Tr^{G150D}) mouse models, respectively [103,104]. PMP22 is an aggregation-prone tetraspan membrane protein, located in compact myelin that is produced by Schwann cells. As a consequence, it is not surprising that Schwann cell protein homeostasis is dysregulated upon *PMP22* gene overexpression or alterations causing an overload of misfolded proteins [9,105]. In PMP22-related diseases, a reduced PMP22 turnover and subsequent retention in the secretory pathway was demonstrated. This caused PMP22 aggregation, which has been reported in both CMT1A as well as in CMT1E patients and also in their corresponding disease models [2,106–109]. Although alterations in proteostasis were demonstrated in CMT1A and CMT1E, the exact molecular mechanisms remain to be elucidated. Nevertheless, aggregation-prone proteins are commonly involved in proteasomal overwhelming and even in healthy Schwann cells about 70% of newly synthetized PMP22 is not properly folded and degraded via the proteasomal system [110]. As a consequence, the proteasome could become overloaded in PMP22-related diseases, negatively affecting the normal functioning of this system as demonstrated in several PMP22-related preclinical disease models [2,95,107,108]. For example, Fortun *et al.* have shown that PMP22 aggregates correlates to reduced proteasomal function in C22 mice and Trj^{L16P} mice [2,95]. Moreover, Lee *et al.* confirmed a reduced proteasomal functioning in CMT1A patient-derived fibroblasts [107]. Hence, improper proteasomal degradation is a first hallmark of PMP22-related diseases.

Decades ago, ER-retention has been proposed as a hallmark of dysfunctional protein quality control systems [111]. The exact molecular mechanism remains poorly understood, but several reports demonstrated ER-retention of the mutant $PMP22^{L16P}$ protein, mediated by Cnx, and thereby being a possible pathological process of CMT1E [104,112,113]. The potential involvement of Cnx in demyelinating pathologies has been confirmed in Cnx-deficient mice, showing myelin abnormalities and motor disorders [114,115]. Moreover, Hara *et al.* proposed that the ER sorting receptor 1 (Rer1) was involved in the disease mechanisms of the Trj^{L16P} mouse model [104]. However, neither Cnx nor Rer1 seem to be involved in the retention of the $PMP22^{G150D}$ mutation in the Tr^{G150D} mouse model [104], and although in both the Trj^{L16P} and Tr^{G150D} mouse models ER retention was demonstrated, to date, UPR activation has only been shown in Trj^{L16P} models [10,112,116,117]. This demonstrates that diverse mutations in the same protein can cause different mechanisms of ER-retention [10]. Interestingly, Bai *et al.* have shown increased ER-stress markers in C3 mice, suggesting UPR activation in CMT1A as well [118]. Moreover, treatment with Sephin-1, an eIF2α prolonging agent, significantly improves both the CMT1A as well as the CMT1B phenotype, confirming an important role for UPR activation for both CMT types [118].

Furthermore, WT-PMP22 has the capability to form heterodimers

and hetero-oligomers with mutant PMP22^{L16P} and PMP22^{G150D}. Therefore, this interaction could trap WT-PMP22 and could thereby partly block the transport of WT-PMP22 towards the myelin sheath [103,119,120]. Tobler *et al.* showed a difference in aggregation formation between the two PMP22 mutations, in which PMP22^{G150D} had a higher tendency to aggregate and to sequester more with WT-PMP22 compared to PMP22^{L16P} [120]. Additionally, myelin basic protein (MBP), another crucial myelin protein, has been reported to be entrapped in PMP22 aggregates and thereby further contributing to the instability of the myelin sheath [95].

At the plasma membrane itself, PMP22 physically interacts with MPZ, the most important and prevalent myelin protein of the PNS [121]. Although experimental evidence is missing, alterations in either PMP22 or MPZ have the potential to contribute to destabilization of the myelin sheath and thereby could contribute to the CMT1E/B pathology [121–123].

Apart from Cnx, also other chaperones are involved in PMP22-related diseases such as heat shock factor 1 (HSF1), which is a transcription factor regulating expression of several HSPs (e.g. HSP104, HSP70 and HSP40) [109]. Reduction of HSP90, hence induction of HSF1 [124,125], improved the neuropathy in both CMT1A as well as in CMT1E [126]. Furthermore, HSPs were recruited to tag PMP22 in CMT1A and CMT1E in an attempt to refold or degrade the misfolded proteins [54,127].

Alternatively, the activity of the autophagic-lysosomal system was upregulated in PMP22-related diseases [105,120] and several reports illustrated a colocalization of misfolded or overexpressed PMP22 with phagosomes and lysosomes [2,54]. In addition, mutations involving lysosomal integrity and function cause peripheral demyelination and neuropathy in patients as well as in rodent models [128,129]. The endogenous process of autophagy in Schwann cells does not seem to be sufficient to clear all aggregates, as protein accumulations are seen in nerve biopsies from CMT1A and CMT1E patients as well as in their preclinical animal models [2,106–109]. As mentioned before, Schwann cells are equipped with a remarkable autophagy mechanism, called myelinophagy. The activation of this complex mechanism remains poorly understood, nevertheless recent data suggests activation via c-Jun/JNK signalling [87]. Interestingly, the c-Jun pathway was activated in human demyelinating neuropathies and in C3-PMP22 mice suggesting that myelinophagy contributes to further demyelination in these pathologies [86,87]. Nevertheless, c-Jun is an important key regulator as it also controls Schwann cell morphology and it is strongly upregulated upon nerve injury [88,130]. Hence, further research is needed to unravel the exact role of myelinophagy in CMT1 pathologies.

CMT1B is the second most common form of CMT1. In this case, mutations occur in MPZ, which is a crucial myelin protein. Over >100 mutations in the *MPZ* gene exist and are mostly situated in the extracellular domain of the protein [131,132]. Moreover, MPZ mutations lead to diverse phenotypes ranging from CMT1B to the more severe Dejerine Sottas Syndrome (DSS), for which the MPZ^{S63del} and MPZ^{S63C} mouse models are used, respectively [131].

Several studies demonstrated ER retention and UPR activation of the well-studied MPZ^{S63del} and MPZ^{R98C} mutants suggesting pathology induction via a gain-of-function [93,131,133,134]. Hence, the UPR is with no doubt crucial in CMT1B, but nevertheless contrary data has been demonstrated in literature. Activating the PERK pathway and thereby prolonging eIF2α -phosphorylation leads to overall attenuation of translation and partly rescues the CMT1B phenotype [93,133]. Surprisingly, ablation of the UPR sensor PERK or its downstream factor CHOP partly restored demyelination in MPZ^{S63del} nerves [82,134] despite reduced eIF2α phosphorylation levels. Scapin *et al.* proposed that P-eIF2α prolongation is crucial to maintain Schwann cell differentiation and myelination and further suggested that PERK negatively influences myelination via mechanisms uncoupled from its UPR role [83].

Additionally, the newly produced MPZ^{D61N} mouse model shows partial alteration of mutant protein trafficking, ER retainment and UPR activation in 4-week-old animals [135]. Nevertheless, at the age of 6 months, UPR genes were comparable to WT, suggesting other pathology-inducing factors.

In addition to the gain-of-function pathology, MPZ^{S63del} mutants caused a loss-of-function which resulted in the absence of mutant MPZ in the myelin sheath. This is in contrast to the more severe MPZ mutant form MPZ^{S63C} and the mutant MPZ^{D61N}, where the mutant protein still translocated to the membrane and thereby disrupted the myelin sheath [122,131,135]. Destabilization of the myelin sheath is potentially caused by dysfunctional interactions between mutant MPZ and PMP22, as described previously [122]. Additionally, MPZ has a crucial role in adhesion by forming homo-tetramers in the extracellular domain thereby further ensuring myelin stability. Hence, ablation of mutant MPZ or presence of mutant MPZ in the myelin sheath causes severe destabilization, eventually leading to demyelination. Although most CMT1B mutations are in the extracellular domain, several cytoplasmic mutations were reported [136,137]. Truncation of the MPZ cytoplasmic domain caused conformational changes which disrupted the normal functioning of the extracellular domain and ultimately also a loss of the full-length MPZ protein [138,139]. This led to myelin destabilization and further, contributed to the demyelinating pathology.

CMT1C is caused by mutations in the LITAF gene, also called the small integral membrane protein of the lysosome/late endosome (SIMPLE) protein. LITAF is ubiquitously expressed in multiple cell types such as Schwann cells, mast cells, monocytes and endothelial cells [140,141]. This makes it challenging to understand how LITAF mutations specifically affect the PNS [140]. Although the exact function of LITAF is not known, several reports suggested an important role in *endo*-lysosomal protein trafficking and proteasomal degradation, wherein the latter is proposed to function as a E3 ligase or adaptor [142–144]. Moreover, the intracellular location of LITAF under physiological conditions is still under debate. Moriwaki *et al.* [141] and Saifi *et al.* [143] found its presence in late endosomes and lysosomes. However, Lee *et al.*[142] contradicted this and showed LITAF expression only in early endosomes and observed its degradation via lysosomes rather than being part of it. In either way, in CMT1C, LITAF mutations lead to mislocalization of LITAF from the endosomal/lysosomal membrane to the cytosol, the ablation of LITAFs *endo*-lysosomal function and/or induction of LITAF aggregates. This contributes to the malfunctioning of the Schwann cell protein homeostasis and thereby contributes to the neuropathology of CMT1C [145,146].

A first mechanism by which LITAF mutants can disrupt proteostasis is due to its proposed role in protein sorting and/or degradation. Bennet *et al.* even suggested that the pathological mechanism of mutant LITAF in Schwann cells is highly comparable to what is seen after PMP22 overexpression, due to improper degradation of PMP22 [140]. Moreover, Saifi *et al.* supported these findings by suggesting PMP22 to be a possible target for LITAF-mediated degradation [143]. This could clarify why Schwann cells in particular are susceptible to LITAF-mutations [140]. In line with these data, a case study described a 2-year old patient with both a PMP22 duplication and a LITAF mutation in which a more severe form of CMT1 was observed with an early onset [147].

On the other hand, mutation-induced detachment of LITAF from its membrane exposes its hydrophobic domains, causing aggregation which induces pathology via a toxic gain-of-function. Additionally, endogenous WT LITAF tends to accumulates in aggresomes, suggesting that even WT-LITAF is prone to misfolding and mutations in LITAF possibly lead to overwhelming of protein degradation systems [148]. Moreover, CMT1C related LITAF-mutants promote aggresome formations and thereby also suggest the involvement of autophagy in degrading mutated LITAF [146].

CMT1X used to be classified as a CMT1 form, however, more recently the disease has been considered as an intermediate type of the disease having both axonal degeneration as well as demyelination in the initial stage. While the severity in male patients is rather homogeneous, female patients have large variability in disease symptoms and age of

onset going from mild symptoms to being asymptomatic. This has been shown to be caused by lyonization [149–152], as CMT1X is an X-chromosome-linked disease. CMT1X is caused by diverse mutations in the *GJB* gene encoding for the gap junction protein connexin32 (>450 mutations), which is highly present in myelinating Schwann cells [152,153]. Gap junctions are formed when two hemichannels, each existing of six connexons (Cx), make contact and physically connect adjacent cells or different domains of the same cell as seen in myelinating Schwann cells [149]. This allows transport of small molecules (<1 kDa) [152,154]. In myelinating Schwann cells, gap junctions are formed between adjoining myelin wraps of one Schwann cell at the Schmidt-Lanterman incisures and paranodal loops [155]. Several Cx32 mutant proteins have been demonstrated to disrupt protein trafficking and thereby lead to the loss of Cx32 at the cell membrane [156]. As in other secreted mutant proteins, two possible pathomechanisms were described: (I) disrupted transport towards the plasma membrane but no interference with the degradation pathways, or (II) overwhelming of the protein quality control system [155,157]. An *in vitro* study performed by Vanslyke *et al.* suggested that for three well-known Cx32 mutations (e.g. $Cx32^{E208K}$, $Cx32^{R142W}$ and $Cx32^{E186K}$), the development of the CMT1X pathology occurred via failures in protein transport rather than overwhelming of the proteasome [155]. Efficient degradation of the Cx32 proteins was observed, although, there was a decreased level of functional Cx32 proteins at the plasma membrane. Moreover, Scherer *et al.* confirmed altered protein trafficking in $Cx32^{R142W}$ mice [149,158], and in the $Cx32^{T55I}$ knock-in mouse model disrupted protein trafficking was observed due to ER-retention [159].

Moreover, mutant-Cx32 proteins are assumed to interact with other connexins, causing or worsening the pathology [155]. Bruzzone *et al.* confirmed this by illustrating that Cx32 blocks the activity of other connexins in $Cx32^{E186K}$ and $Cx32^{R142W}$ mutants [160].

Taken together, several CMT1 forms are characterized by a dysfunctional protein householding in Schwann cells (Fig. 2**A**), and an overview is given in Table 2. Nevertheless, details remain unclear and

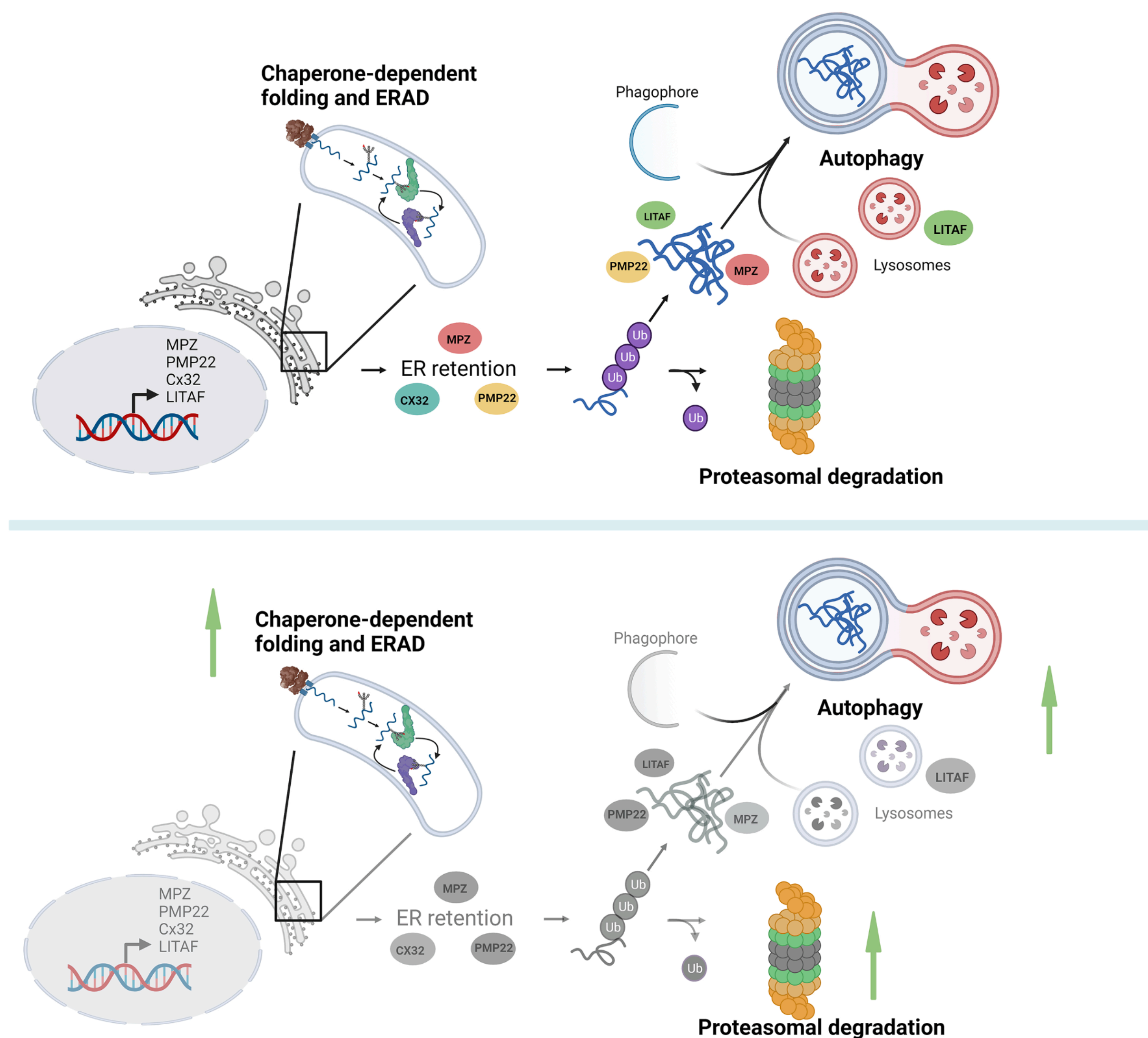

Fig. 2. Illustrating dysregulated proteostasis and potential therapies in CMT1. A: Disrupted SC proteostasis in CMT1A, B, C and X. Frames with CMT1-related proteins illustrate a role in disrupted proteostasis at that particular level. Genes are transcribed in the nucleus after which proteins enter the ER where chaperone-dependent folding is accomplished. After proper folding, further processing is accomplished via the Golgi apparatus, and the proteins are transported towards their proper location. Nevertheless, if refolding fails, the misfolded protein is tagged and degraded via ERAD/proteasomal system. If the ERAD is overloaded, for example by mutated or overexpressed proteins, UPR activation and protein aggregation occurs. Cell proteostasis has to be rebalanced by the protein degradation systems: proteasomal degradation and autophagy. **B:** Potential therapies are based on the restoring of SC proteostasis. Reports demonstrate improvement in phenotype via the inductions of chaperone- and ERAD-related genes, proteostasis activation or autophagy. MPZ: myelin protein Zero, PMP22: peripheral myelin protein 22, Cx32: connexin32, LITAF: Lipopolysaccharide-induced tumor necrosis factor α. Created with BioRender.com.

Table 2
Altered proteostasis in relation to genetic alterations in PMP22, MPZ, LITAF and GJB1.

	Altered proteostasis	Model	Source
PMP22	Reduced proteasomal	C22, Trj^{L16P}	[2,54,95]
	activity	Trj^{L16P}, Tr^{G150D}	[111,120]
	Disrupted protein	C22, Trj^{L16P}, Tr^{G150D}	[2,108]
	trafficking		
	Lysosomal-autophagic	C3	[118]
	upregulation	C22, Trj^{L16P}	[2,54]
	UPR activation		
	Aggregates		
MPZ	Disrupted protein	MPZ^{S63del}, MPZ^{R98C},	[93,135,191]
	trafficking	MPZ^{D61N}	[82,93,100,118]
	UPR activation	MPZ^{S63del}, MPZ^{R98C}	[166,191]
	Aggregates	MPZ^{S63del}, MPZ^{S63Phe}	
LITAF	Defects in endosomes/	LITAF^{W116G},	[145,146]
	lysosomes	LITAF^{P135T}	[145,146]
	sorting/degradation		[146]
	Mislocalization and	LITAF^{W116G},	
	aggregation	LITAF^{P135T}	
GJB1	Disrupted protein trafficking	Cx32^{R142W}, Cx32^{E186K}, Cx32^{T55I}	[155,158,159]

PMP22: peripheral myelin protein 22; MPZ: myelin protein zero; LITAF: lipopolysaccharide-induced tumor necrosis factor α; GJB1: gap junction beta1 gene.

current research attempts aim to decipher the exact molecular mechanisms of these pathways.

6. Restoring Schwann cell proteostasis as a therapy for CMT1

Until now, there is an unmet need for treatments to cure neurodegenerative diseases as several promising therapeutic candidates failed when translated to the clinic [161,162]. This is also the case for CMT1, and no effective treatment for this group of demyelinating pathologies has been reported yet. With a disrupted proteostasis being a hallmark of CMT1, restoring the protein balance has been proposed to be a promising therapeutic approach (Fig. 2**B**).

Potential approaches to restore Schwann cell proteostasis are reducing ER retention, preventing UPR-induced damage, inducing autophagy and hence, improving abnormal protein degradation.

As mentioned previously, chaperones are crucial in protein trafficking and (re)folding and assisting proteolytic systems [4,5]. Therefore, it is not surprising that the **induction of chaperones** is important to restore ER-stress and prevent terminal UPR. These chaperones are thereby proposed as potential therapeutic targets in several CMT1 forms [163].

Although the exact molecular mechanisms of curcumin are not yet defined, Egan *et al.* demonstrated that curcumin improved mutant protein trafficking and processing via the induction of ER calcium-dependent chaperones [164]. Curcumin has been shown to reduce ER-retention [116] and to improve motor coordination and muscle strength in the Trj^{L16P}- CMT1E mice [165]. In addition, curcumin administration resolved protein aggregation-induced apoptosis in the Tr^{G150D} mouse model and mutant MPZ cells [166]. Recently, Caillaud *et al.* have developed curcumin-cyclodextrin/cellulose nanocrystals, which significantly improves the CMT1A-rat phenotype [167]. Moreover, treatment with curcumin was proven to be safe in human trials [168].

Additionally, the use of chaperone-induced rebalancing has also been reported by Rangaju *et al.* showing improved Schwann cell myelination and protein trafficking in C22-CMT1A mice via the inhibition of HSP90, and thus by general chaperone induction (e.g. HSP70 and HSP40) [169]. The positive effect of HSP90 inhibition was confirmed in CMT1A and CMT1E models [126], with an increased amount of properly myelinated nerves. Furthermore, diet-induced enhancement of chaperone expression improved locomotor activity in Trj^{L16P} CMT1E mouse model [170]. In line with these data a recent paper demonstrates that the specific HDAC6 inhibitor CKD-504 improves the CMT1A phenotype by modulating chaperone proteins such as HSP90/70. However, HDAC6 inhibitors also influence other mechanisms such as microtubule stability, cholesterol levels and axonal transport [171]. CKD-504 is now in a phase I Clinical trials, testing its potential as a therapy for CMT1A [171].

Hence, chaperone-induced restoring of proteostasis has great potential to improve myelination as it has been shown to ameliorate the CMT1A, CMT1E and CMT1B pathology.

Another possibility to prevent UPR-induced damage and thereby alleviating the CMT1 phenotype, is to **target UPR downstream mediators**. As mentioned above, PERK activation causes phosphorylation of eIF2α, thereby reduces general protein translation to alleviate the ERAD proteasomal system. Salubrinal is known to block eIF2α dephosphorylation via inhibiting GADD34 an important eIF2α phosphatase [172]. The cytoprotective properties of Salubrinal were shown in animal models of ischemia [173], traumatic brain injury [174] and spinal cord injury [175]. Importantly, Salubrinal administration reduced demyelination and improved signal conduction in nerves of MPZ^{S63del} in CMT1B mice [134]. In line with these data, deletion of GADD34 from CMT1B nerves indeed decreased demyelination and improved myelin thickness [134]. Moreover, depletion of CHOP, another downstream factor of PERK, also partly improved myelination in MPZ^{S63del} nerves [82,134]. Nevertheless, the use of Salubrinal for human diseases is difficult, due to sparse solubility in aqueous solutions and therefore the requirement of a DMSO solvent [176]. Another highly selective GADD34 inhibitor, Sephin-1 (also called IFB-088) has been shown to improve both CMT1A and CMT1B mice models and successfully completed clinical trial phase I, making it a promising therapy for CMT [118].

Furthermore, proteostasis can be restored by directly **improving the activity of the proteasome**. Abnormal proteins in the ER are retrogradely transported towards the cytosol, polyubiquitinated and degraded via the proteasome. Interestingly, inhibition of the proteasomal deubiquitinating enzyme USP14 improved proteasomal degradation in CMT1B and surprisingly also prolonging eIF2 phosphorylation [177]. Several other proteasome inducing agents, such as oleuropein, could be promising in suppressing the CMT1 phenotype but this needs to be explored in experimental models [175,176]. Nevertheless, focusing on improving the activity of the proteasome by itself might not be sufficient to resolve CMT1, due to the fact that the proteasome is inefficient in the degradation of protein aggregates and oligomeric forms [142].

Induction of autophagy was also proposed to alleviate CMT1 pathology. Autophagy plays an important role in the degradation of aggregates of misfolded proteins which have been reported in CMT1A, E, B and C [2,54,119,146]. This suggests that increasing autophagy could be a promising strategy to restore proteostasis and to alleviate the disease [178]. Although autophagy is known as a highly dynamic and complex mechanism, it can be easily influenced via dietary restriction or by rapamycin administration, which were shown to improve CMT1E and both CMT1A and E mouse phenotypes, respectively [127,170]. Additionally, rapamycin increases mutant LITAF protein degradation *in vitro*, which could suggest potential phenotype rescuing in CMT1C [146]. However, prolonged caloric retention is difficult in humans and up to now, no clinical studies with rapamycin have been reported for CMT1.

7. Summary

Safeguarding protein homeostasis in the cell is a highly dynamic process and altered proteostasis can easily lead to unfavourable outcomes as seen in several neurodegenerative diseases. Convincing evidence shows altered proteostasis to be involved in several CMT1 forms and classifies CMT1 as so-called: proteinopathies. This makes restoring protein balance a potential therapy in CMT1. Proteostasis can be rebalanced using several pathways including Chaperon- and UPR rebalancing, autophagy- and, proteasomal induction (Table 3). Although several reports demonstrate the therapeutic potential of targeting proteostasis

Table 3
Overview of potential drug candidates and their mode of action related to CMT1 types.

Potential candidates	Mode of action	Models tested	Potentially benefits	Source
Curcumin	(?)Details remain unclear, presumably via activation of chaperons and anti-oxidant activity	Trj^{L16P} CMT1A-rats *in vitro*	CMT1E CMT1A CMT1B(?)	[165–167]
EC137	HSP90 inhibitor	C22	CMT1A	[169]
AUGY922	HSP90 inhibitor	C22 Trj^{L16P}	CMT1A CMT1E	[126]
CKD-504	HSP90 and HSP70 chaperone modulator	C22	CMT1A	[171]
USP14 inhibition	Promote proteasomal degradation	MPZ^{S63Del}	CMT1B	[177]
Sephin1 (IFB-088)	Blocks eIF2α dephosphorylation	C3 MPZ^{S63Del} MPZ^{R98C}	CMT1A CMT1B	[118]
Salubrinal	Blocks eIF2α dephosphorylation	MPZ^{S63Del}	CMT1B	[134]
Rapamycin	Autophagy induction	C22 Trj^{L16P} *In vitro*	CMT1A CMT1ECMT1C (?)	[127,146]

HSP: Heat shock protein CMT: Charcot-Marie-Tooth, eIF2: Eukaryotic initiation factor 2α Trj: Trembler J, MPZ: Myelin protein zero.

in CMT1, we are only at the beginning to understand the complexity of these processes, and further clarification is necessary to alleviate the disease. Nevertheless, altering the mechanism of proteostasis has strong potential to be targeted in future treatments, not only in CMT1, but also in other neurodegenerative diseases.

Potential conflict of interest

"LVDB is head of the Scientific Advisory Board of Augustine Therapeutics (Leuven, Belgium) and is part of the Investment Advisory Board of Droia Ventures (Meise, Belgium). EW is part of the Scientific Advisory Board of Innoser (Diepenbeek, Belgium)."

CRediT authorship contribution statement

Karen Libberecht: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Investigation. **Tim Vangansewinkel:** Conceptualization, Writing – review & editing, Supervision, Investigation. **Ludo Van Den Bosch:** Funding acquisition, Writing – review & editing, Supervision. **Ivo Lambrichts:** Funding acquisition, Writing – review & editing, Supervision. **Esther Wolfs:** Conceptualization, Funding acquisition, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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