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Human Genome & Diseases: Review

Giant axonal neuropathy

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Abstract. Giant axonal neuropathy (GAN) is a rare autosomal recessive disorder affecting both the central and peripheral nervous systems. Cytopathologically, the disorder is characterized by giant axons with derangements of cytoskeletal components. Geneticists refined the chromosomal interval containing the locus, culminating in the cloning of the defective gene, *GAN*. To date, many distinct mutations scattered throughout the coding region of the locus have been reported by researchers from different groups around the world. *GAN* encodes the protein, gigaxonin. Recently, a genetic mouse model of the disease was generated by targeted disruption of the locus. Over the years, the molecular mechanisms underlying GAN have attracted much interest. Studies have revealed that gigaxonin appears to play an important role in cytoskeletal functions and dynamics by directing ubiquitin-mediated degradations of cytoskeletal proteins. Aberrant accumulations of cytoskeletal-associated proteins caused by a defect in the ubiquitin-proteasome system (UPS) have been shown to be responsible for neurodegeneration occurring in GAN-null neurons, providing strong support for the notion that UPS plays crucial roles in cytoskeletal functions and dynamics. However, many key questions about the disease remain unanswered.

Keywords. GAN, gigaxonin, cytoskeletal network, ubiquitin-proteasome system, neurodegeneration, axonal transport, microtubule, neurofilament.

Clinical features

Giant axonal neuropathy (GAN) is a progressive neurodegenerative disorder first identified by Asbury et al. [1] and Berg et al. [2]in 1972. Age of onset for GAN may vary from soon after birth to up to ten years of age, or older. Affected children may develop symptoms that progress from a 'waddling gait' to a pronounced difficulty to ambulate. Symptoms vary considerably. The typical clinical manifestations of GAN present with evidence of both motor and sensory involvement, including progressive and predominant distal 'clumsiness' and muscle weakness, impaired sensation, absent tendon reflexes, and a pronounced gait disturbance. The cranial nerves can also be affected, especially the third and the seventh. Lesions in the brain and spinal cord can cause mental retardation, dysmetria, seizures, nystagmus, and dysarthria, as well as signs of spasticity. Scoliosis, kyphosis, optic atrophy, ophthalmoplegia, and epilepsy are reported less commonly [2--5]. Sometimes, the symptoms and signs of central nervous system (CNS) involvement can predominate. It is reported that even if there is no clinical sign of CNS involvement, there may be electroencephalogram (EEG) and magnetic resonance imaging (MRI) abnormalities [6]. Curly or kinky hairs are not constant findings, but appear to occur frequently [7, 8]. Patients generally become wheelchair bound before

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the age of 20 and die by their second or third decade [4].

Pathology

Abnormalities in the cytoskeletal network are a minor feature of several neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), infantile spinal muscular atrophy (SMA), and Charcot-Marie-Tooth disease. However, such changes in GAN are unusually striking. Peripheral nerve biopsy revealed enlarged axons with segmental swellings filled with accumulations of disorganized neurofilaments and reduced numbers of microtubules. In fact, disorganization and accumulation of other types of intermediate filaments (IFs) are found in skin fibroblasts, Schwann cells, and muscle fibers. Scanning electron microscopy of the hair revealed an extraordinarily irregular cuticle and longitudinal grooves in the hair, suggesting an aberrant hair keratin network [8, 9]. Demyelination and onion bulb formations of Schwann cells are present [10, 11]. In the CNS, there is a great variability in the amount of fiber loss and in the number of giant axons among different fascicles [12]. The most severely affected areas include the corticospinal tracts, the middle cerebellar peduncles, the posterior columns, and the oligocerebellar connections [13]. Loss of Purkinje cells and other neuronal cells has been reported [14].

Analysis of GAN patient fibroblasts in culture showed apparently normal levels of vimentin with biochemical properties that were indistinguishable from normal, leading to the hypothesis that GAN is the consequence of intermediate filament disorganization [15]. Klymkowsky and Plummer [16] demonstrated that the intermediate filament phenotype in GAN patients can be induced conditionally in cultured GAN fibroblasts, since shifting the cells to low serum reversibly induced many more cells to display vimentin aggregation than normal serum levels. This study was confirmed in GAN fibroblasts with defined mutations, and showed that microtubule depolymerization made the intermediate filament networks collapse in GAN fibroblasts [17].

Genetics

Familial inheritance patterns indicate that GAN is an autosomal recessive disorder [ref. 18 and references therein]. Using homozygosity mapping techniques, the *GAN1* locus has been mapped to chromosome 16q24.1 [19, 20]. However, Koenig and co-workers recently discovered heterogeneity in GAN, reporting an Algerian family with three affected siblings but with no linkage to chromosome 16q24.1 [21]. Nerve biopsy showed a moderate loss of myelinated fibers and several giant axons filled with neurofilaments, no involvement of the CNS, normal MRI, and absence of curly/kinky hairs. Thus, they proposed that defects in a second locus, *GAN2,* underlie the disorder in this Algerian family. It was also suggested that a link may exist between GAN and insulin-dependent diabetes mellitus [22].

As an important step in understanding the pathogenesis of GAN, Koenig and colleagues successfully cloned the *GAN* gene and identified mutations of the locus [23]. The distinct mutations identified in GAN patients in 14 families include all types of mutation, which are scattered throughout the coding sequence of gigaxonin, the protein encoded by *GAN* (Fig. 1). Subsequently, many new mutations have been described in other families [3, 24, 25] (Fig. 1). Notably, an insertion mutation at the 6th amino acid (A6ins) causes a frame shift resulting in premature termination at the 8th amino acid, implicating it as a functionally disruptive mutation. Kuhlenbäumer et al. [3] reported a mutation that caused a change of isoleucine to threonine at A423 that may cause a mild subclinical neuropathy in heterozygous individuals, revealed by abnormal nerve conduction velocities [3].

Animal models

To better understand the disease course and to facilitate rigorously controlled investigations of disease pathogenesis, development of GAN animal models was essential and of general interest. Before the genetic cloning of *GAN*, an experimentally induced animal model of GAN in rat was developed [26, 27]. The neuropathy induced by administration of 2,5-HxD in drinking water featured eversion and inability to extend the hindlimbs, and upper extremity weakness. Ultrastructural studies on the affected animals revealed giant axonal swellings containing masses of neurofilaments. In addition, some German Shepherd dogs were also reported to display phenotypes mimicking peripheral neuropathy of GAN that features giant axons, abnormal curly hairs, and autosomal-recessive inheritance [28].

The identification of *GAN* provided the opportunity to develop an animal model using genetic approaches. Utilizing traditional gene-targeting techniques, our group recently ablated gene expression of *GAN* in mice [29]. A prominent feature of the *GAN*-null mutants is a progressive deterioration in motor function with onset varying individually from 6 to 10 months. The initial sign of neurological abnormalities

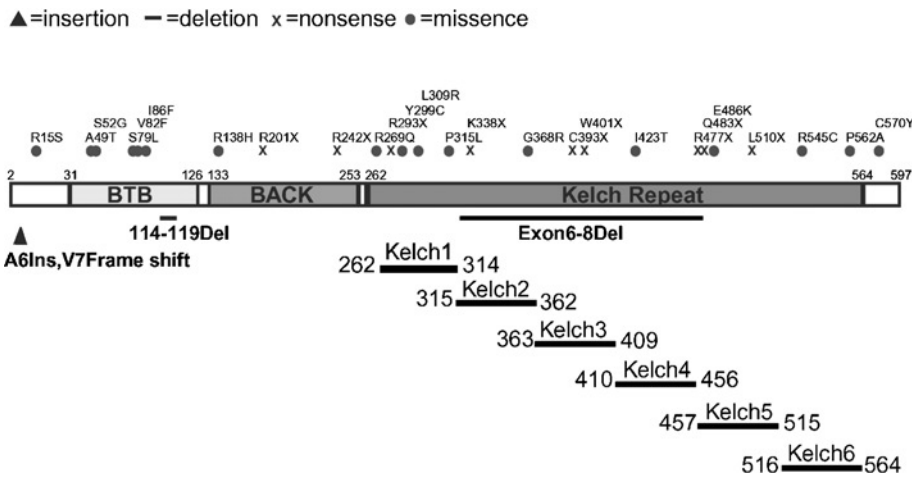

Figure 1. Gigaxonin contains a BTB (Bric-à-brac, Tramtrack and Broad complex), BACK (BTB And C-terminal Kelch), and C-terminal Kelch domain comprised of six repeats. Twenty-eight mutations identified in GAN patients including insertion, deletion, nonsense, and missense mutations, they are distributed throughout the gigaxonin coding sequence, and are found in each functional domain.

is a substantial reduction of spontaneous movement. Sub-clinical motor deficits of GAN-null animals were demonstrated by the footprint test showing compromised motor functions and gait disturbance. The involvement of limb weakness started from the hindlimbs, followed by an increasingly pronounced hindlimb eversion, and progressed into the forelimbs. By the age of 12 months or older, all of the null mice exhibited muscle wasting and progressive weight loss (Fig. 2B), and occasional signs of spasticity and epilepsy. In addition, some of the *GAN*-null mice developed hair phenotypes, including a loss of whiskers and abnormal or fragile hairs (Fig. 2D, right). Despite the phenotypes, the null animals have an almost normal life span, and fertility of both males and females is similar to that of wild-type (WT) littermates. A portion of null mice display no observable or very subtle neurological phenotypes, suggesting that some genetic modifiers may exist.

Pathological lesions found in the null mice include axon loss involving both myelinated and unmyelinated nerve fibers, with variable severity [29]. Pronounced axonal swelling and degeneration with a thin myelin sheath is observed in both the peripheral and central nervous systems. Ultrastructural studies on phenotypic null mice revealed enlarged axons distended with neurofilaments, although giant axons characteristic of the human disorder were never seen. Filamentous accumulations could also seen be outside the nervous system in fibroblasts. Interestingly, cytoskeletal network aberrations were observed prior to other signs of severe neurodegeneration [30], so analysis was performed in pre-phenotypic null animals to eliminate secondary effects from vesicular accumulation. While some axoplasms from the null mice are densely packed with neurofilaments and others are extremely disorganized, the common and consistent feature observed in all areas is a significant reduction of microtubule density. Quantitative analysis in the null animals confirmed the microtubule pathology. Thus, the phenotypic and pathological features of *GAN*-null mice validate that the null mouse is a genetic model of the human GAN condition.

Gigaxonin

GAN encodes a ubiquitously expressed protein, gigaxonin, which is a distant member of the BTB (Bric-à-brac, Tramtrack and Broad complex)/kelch superfamily [31]. Little was known about the potential function(s) of the protein when the gene was first identified. BTB domains in other proteins have been shown to play a role in broadly diverse functions, including transcriptional activation, cytoskeletal associations, ion conductance and, more recently, the ubiquitin-proteasome (UPS) pathway [ref. 32] and references therein]. Cullen et al. demonstrated that the gigaxonin BTB domain dimerizes, as well as associates with the Golgi apparatus [33]. Kelch repeats form a β-propeller structure that plays a role in protein/protein interactions [ref. 34] and references therein]. Recently, the crystal structure of the Keap1 kelch repeat and its cognate substrate, an Nrf2 peptide, has been resolved [35]. Interestingly, the GAN patient mutation, G386R, disrupts a conserved residue shared by Keap1, ENC1, and Mayven, while P315L disrupts a shared residue in Keap1 and ENC1. The sequence alignment and crystal structure analysis suggest that P315L and G386R mutations would flank substrate-contacting sites in Keap1. Along with the BTB/kelch motifs, many such proteins also contain a BACK domain (BTB And C-terminal Kelch) that is comprised of the intervening hundred or so residues between the two motifs, which may play a role in

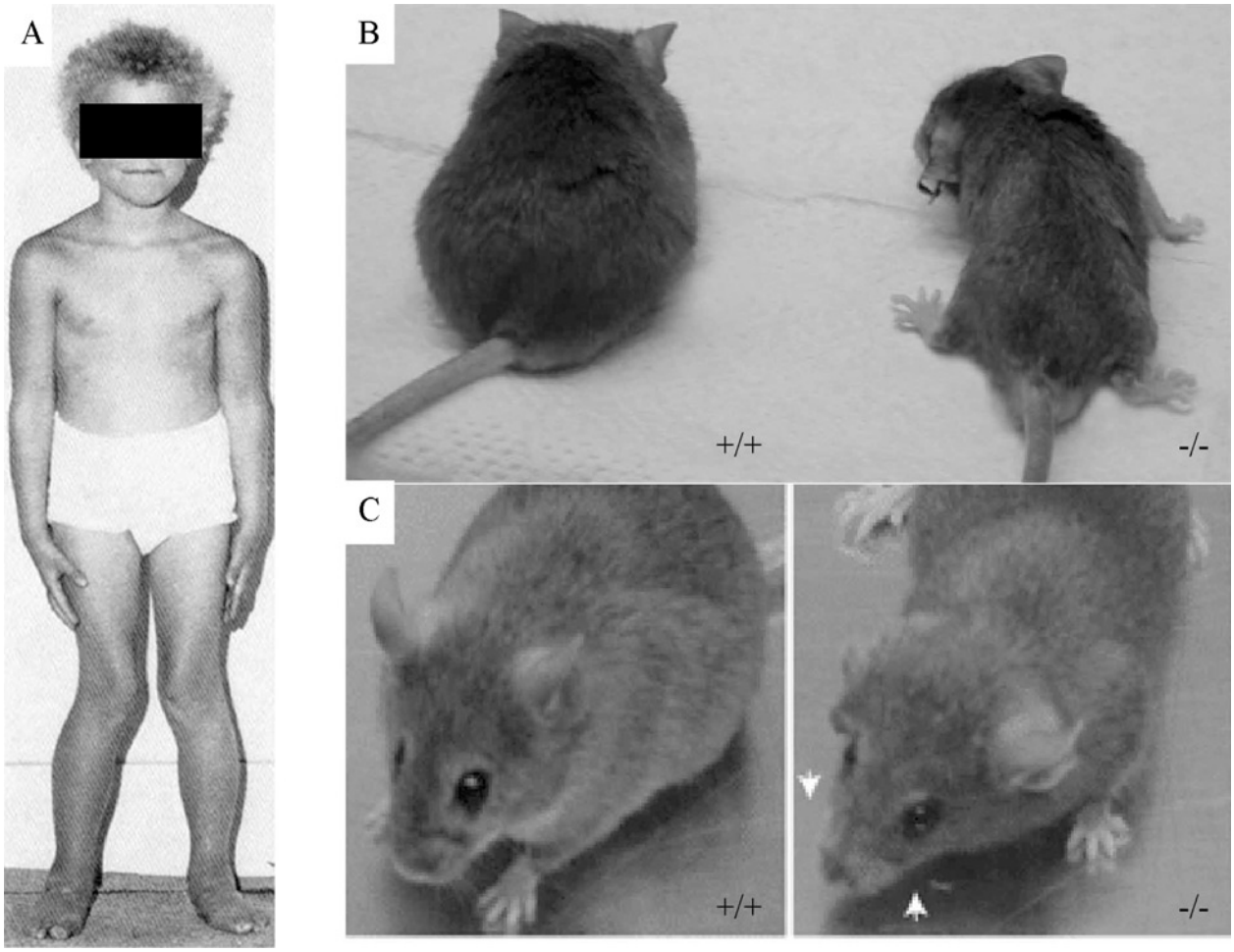

Figure 2. (**A**) A 5 year old GAN patient. (**B**–**C**) Nine- to 14-month-old mice of wild type (left in **B**; **C**) and GAN-null mutants (right in panel **B**; **C**). Note the eversion of the patient's legs (**A**) and the mutant mouse hindlimbs (**B**, right). The arrows indicate out the lack of whiskers.

substrate orientation for protein degradation [ref. 36] and references therein].

The most prominent cytopathological feature of GAN is an aberrant cytoskeletal network. However, early studies of recombinant gigaxonin surprisingly revealed no direct association with the cytoskeletal network [37]. To understand crucial biological pathways involving gigaxonin in cytoskeletal organization and function, a yeast two-hybrid screen was initiated, leading to identifications of several cytoskeletal-associated proteins as neuronal binding partners of gigaxonin. Among the well-characterized binding partners of gigaxonin are the light chain of microtubule-associated protein 1B (MAP1B-LC) [37] and tubulin folding cofactor B (TBCB) [30], as well as a novel microtubule associated protein, MAP8 [29]. The studies strongly suggested that gigaxonin may play a crucial role in the maintenance of a normal cytoskeletal network through its association with cytoskeletal-associated proteins. The finding that these associations could be disrupted by GAN-associated mutations in the kelch-repeat domain pointed again to the physiological importance of the interactions[29, 30 , 37].

Over the last few years, a large number of exciting studies emerged implicating BTB proteins as substrate-specific adaptors that form a new class of SCF-like (Skp1-Cul1-F box) ubiquitin ligases [38]. These proteins complex with a cognate ubiquitin-conjugating enzyme E2 and the ubiquitin-activating enzyme E1 to direct the ubiquitin-mediated degradation of a substrate. Recombinant gigaxonin has been shown to have auto-ubiquitination activity [39] and could be coimmunoprecipitated with Cul3 [39–41] via sequences in the BTB domain. Collectively, these studies support the notion that this large family of BTB-domain proteins may define a new class of Cul3-based E3 ligase complexes, [refs. 41, 42 and references therein]. The direct evidence linking the function of gigaxonin to a role in the UPS came from our study

showing that gigaxonin binds directly to E1. Furthermore, *in vitro* assays indicated that the interaction was impaired by GAN-associated mutations in the BTB domain, V82F and I86F [43]. This finding was unanticipated, and whether it represents a general function of other BTB-domain proteins remains unanswered. In addition, it is not clear whether a complex forms containing E1, cul3, and gigaxonin, or whether binding to the two proteins is mutually exclusive or sequential. The link between gigaxonin and the UPS indicated that gigaxonin may have a role directing ubiquitin-mediated protein degradation. Researchers in our laboratory conducted a series of studies using a combination of *in vivo* and *in vitro* assays to demonstrate that gigaxonin controls the ubiquitin-mediated degradation of MAP1B-LC, MAP8, and TBCB [29, 30, 43]. Consistent with this, gigaxonin ablation results in accumulation of these substrates. The effects on the ubiquitin-proteasome pathway mediated by gigaxonin suggested a novel activity of the ubiquitination cascade and may represent a refinement of the existing model of UPS function. These studies provide the first direct evidence linking cytoskeletal organization and the UPS pathway with a neurodegenerative disorder.

Pathogenesis

Mechanisms underlying the characteristic axonal loss in GAN were unclear. Suggested possibilities included perturbed phosphorylation of intermediate filaments, defective protease activity, disruptions in neurofilament assembly, and metabolic derangement of slow axonal transport [2, 19]. The first possibility is supported by desmin immunostaining of muscle fibers, which shows abnormal aggregation in the subsarcolemmal space and in the middle of the muscle fibers [9]. Disordered thiol metabolism in GAN has also been suggested since the toxins that bind to thiol groups, such as acrylamide and hexacarbons, can cause a similar pathology [44]. These authors have proposed that the condition can result from an inborn error of the metabolism of enzyme-linked sulfhydryl-containing proteins, causing an impaired production of energy needed for the normal organization of intermediate filaments. Studies conducted on optic nerves in the experimentally induced GAN rat model suggested an acceleration of axonal neurofilament transport which may be attributable to the neurofilament accumulations observed in GAN axons [27].

A key step in advancing understanding of the disease pathogenesis was the identification of gigaxonin [23]. Studies linking gigaxonin with the UPS pathway indicated that impaired UPS function may be involved in the pathogenesis of GAN. Defects in the UPS have recently been linked to axonal degeneration, also called Wallerian degeneration (WD) [reviewed in ref. 45]. Overexpression of fusion protein of a ubiquitin regulatory protein (UFD2/Nmnat) in the spontaneous mouse mutant, Wlds, leads to the ability of transected axons to live for weeks (slow Wallerian degeneration) [46, 47], while loss of a ubiquitin hydrolase causes axonal degeneration in the gad mutant [48]. Additionally, *Drosophila* conditional mutants of E1 (UBA) or proteasome subunits display defective axonal pruning leading to a local degeneration of the axon that resembles WD [49], and disruption of the microtubule cytoskeleton network appears to be an early event in this process. Studies of transected axons using pharmacological inhibitors [50] also point to a very early perturbation of microtubule cytoskeleton stability that precedes characteristic neurofilament degeneration. These authors postulate that unidentified MAPs, excluding tau and MAP2, could play a critical role in WD [45, 49, 50]. The *GAN*-null mouse model provides a powerful tool to study the relationship between the UPS, cytoskeletal organization, and neurodegeneration. In *GAN*-null mice, loss of gigaxonin causes an impaired UPS function which resulted in significant accumulations of the cytoskeletal-associated proteins, MAP1B, MAP8, and TBCB [29, 30, 43]. However, the question of whether accumulations of those specific proteins play a role in disease pathogenesis remained.

MAP1B is a large, classical microtubule-associated protein that is cleaved post-translationally to a heavy (243 kDa) and light (27 kDa) chain. It is primarily expressed in neurons where it binds and stabilizes microtubules. MAP1B is the first MAP expressed during development of the nervous system, and its expression in some tissues is dramatically down-regulated post-natally, while that of MAP1A increases. MAP1B is believed to play an important role in microtubule dynamics and axonal extension. Other evidence suggests that disregulated MAP1B expression appears to be linked to disease processes. In cell culture studies, wild-type neurons overexpressing MAP1B-LC displayed fragmented neurites, leading to continuous shortening until they nearly disappeared, and DAPI staining showed extensive nuclear disintegration or condensation 2–4 days post-transfection [43]. Shortly afterwards, an overwhelming majority completely lost any sign of normal morphology, a phenotype mimicking the degeneration and cell death occurring in cultured *GAN*-null neurons. In contrast, green fluorescent protein control and untransfected wild-type neurons were indistinguishable over the entire test period, displaying long axonal processes with marked branching [43]. The finding

that overexpressed MAP1B-LC could cause neurodegeneration and cell death in cultured primary neurons strongly suggested that the aberrant MAP1B accumulation detected in the *GAN*-null mouse contributes to the pathogenesis of GAN. In addition to GAN, abnormal MAP1B expression has also been found in fragile X mental retardation syndrome. The WT fragile X mental retardation protein (FMRP) binds to specific RNA targets to regulate their transcription, and loss of this activity results in an aberrant regulation of many transcripts [51], including MAP1B. The lack of FMRP in knockout mice results in delayed down-regulation of MAP1B in brain, and elevated protein levels lead to abnormally increased microtubule stability [52]. Taken together, these studies provide strong support to the notion that MAP1B expression is under exquisite control.

MAP8/MAP1S is a recently identified MAP containing two microtubule-binding sites [53]. The protein is translated from a single transcript, and cleaved post-translationally into a 100-kDa heavy (HC) and 25-kDa light (LC) chain [53, 54]. It is highly expressed in neuronal tissues and can be detected as early as embryonic day 10. The protein level peaks at approximately post-natal day 20 and persists into adulthood. Like MAP1B, overexpression/accumulation of MAP8 was toxic to neurons and resulted in neuronal cell death. Importantly, when gene-specific interferring RNAs (siRNA) were introduced into *GAN*-null neurons, MAP8 or MAP1B accumulation was prevented. The characteristic axonal degeneration and cell death appeared to be reversed, and the survival rate of the *GAN*-null neurons was significantly improved in culture. These observations provided further evidence indicating accumulated MAPs as causative factors in GAN [29, 43]. Moreover, although tau is not known to play a role in GAN, it is also a classic MAP that has been linked to Parkinson and Alzheimer's disease. Transgenic mouse models of human frontotemporal dementia and parkinsonism overexpressing the four-repeat WT tau isoform in neuronal tissue develop an axonopathy characterized by WD and accumulations of vesicles, mitochondria and cytoskeletal components [55]. It is possible that the toxicity resulting from deregulated levels of cytoskeletal-associated proteins may be a shared pathogenic contributor to many neurodegenerative disorders.

The question regarding how accumulated MAP protein effectuates GAN still needs to be addressed. It is hypothesized that defective axonal transport found in *GAN*-null neurons may directly cause the neurodegeneration characteristic of neurodegenerative diseases. Interestingly, accumulated MAP8 protein was found to alter microtubule organization *in vitro* and to trap dynein motor protein into insoluble aggregates [29]. Other studies indicated that MAP1B may associate with lissencephaly-related protein 1 (LIS1) that is known to bind to the dynein complex [56] and may play a role in retrograde axonal transport of mitochondria [57].

While microtubule-associated proteins control the dynamic properties and stabilities of microtubules, tubulin-folding cofactors are responsible for assembly of tubulin subunit dimers, which are the basic microtubule-building blocks [58]. Polymerization of dynamic microtubules depends on properly folded and dimerized α-/β-tubulin, which requires a group of conserved tubulin-specific chaperone proteins, tubulin-folding cofactors A to E (TBCA, TBCB, TBCC, TBCD, and TBCE) [58]. The amount and isotype composition of available tubulin dimers also affects microtubule function dramatically, and it has been demonstrated that overexpression of TBCD or TBCE destabilizes microtubules [59--61]. The direct involvement of tubulin chaperones in diseases of protein folding is becoming increasingly evident. Mutations in RP2, a homologue of TBCC, are found in patients with a form of X-linked retinitis pigmentosa [62, 63]. Mutations in tubulin chaperone E (TBCE) underly the human disorder hypoparathyroidis, mental retardation and facial dysmorphism (HRD) [64]. Furthermore, mutations in TBCE in mice lead to a progressive motor neuropathy [65, 66]. A feature common to both human HRD patients and TBCE mutant mice is reduced microtubule density. Interestingly, a common and consistent feature found in all tissues of *GAN*-null mice is a significant reduction of microtubules, regardless of neurofilament density or myelination [30]. Like MAP proteins, TBCB also accumulates in the tissues from GAN knockout mice. *In vitro* studies recapitulated the microtubule pathology in *GAN*-null mice by TBCB overexpression in cultured cells, resulting in a marked reduction in the intensity of microtubule staining. The disrupted microtubule network could be restored by coexpression of WT gigaxonin with TBCB [30]. Intriguingly, microtubule recovery appears to be dependent on gigaxonin-mediated UPS function. The GAN-associated mutations, V82F, R293X, and R545C, which disrupt the gigaxonin-UBE1 or gigaxonin-TBCB interaction, fail to promote TBCB poly-ubiquitination and subsequently could not restore microtubule networks in cultured cells. These data provide direct evidence that TBCB is a microtubule-destabilizing factor and strongly suggest that overexpression/accumulation of TBCB in cells is a pathogenic factor responsible for microtubule reduction in the *GAN*-null tissues. Notably, functions of the R15S mutant gigaxonin were

indistinguishable from WT gigaxonin in promoting TBCB ubiquitination and restoring the microtubule network, indicating that there is a yet unknown gigaxonin function that may be disrupted by the R15S mutation. These studies provide another example of the deleterious consequences resulting from overstabilization of cytoskeletal-associated proteins.

Future perspective

The abnormal cytoskeletal organization and disrupted UPS function that are proposed to be involved in multiple neurodegenerative disorders may result from common molecular pathways. Characterization of the molecular defects underlying GAN may shed light on the pathogenesis of these diseases. Gigaxonin plays a unique role by linking cytoskeletal dynamics to the UPS by directing the degradation of excess proteins that control microtubule assembly, stability and dynamics. Preliminary studies suggest that degradation of MAP1B-LC may require GSK-3β kinase activity [unpublished results], implicating this cascade in the disease process as well. Axonal demyelinations observed in *GAN*-null animals remain to be elucidated. The defect could be a secondary consequence of axonal degeneration, but it is also possible that the demyelination pathology is a primary defect due to malfuntions of Schwann cells and oligodendrites. The question of whether the interaction between MAP1B and myelin-associated glycoprotein plays a role in the demyelination pathology in GAN is also of interest [67]. While it is likely that excess TBCB causes a reduced microtubule density in GAN, the critical underlying mechanisms remain to be determined. Also, overexpression/accumulation of microtubule-associated proteins appears to contribute significantly to neuronal degeneration and death, but the question of how these accumulations cause toxicity awaits further investigations. Saturation or positional interference of binding sites on microtubules may impede motor protein binding to the microtubule surface to directly disrupt transport, or alteration of microtubule organization and dynamics may indirectly perturb it. Also reduction of motor protein availabilities for transport could also contribute to the defect. The generalized pathology of intermediate filaments in GAN tissues remains to be elucidated. The question of whether dense accumulations of intermediate filaments (predominantly neurofilaments) in GAN tissues are a compensatory response to reduced microtubule density, or whether gigaxonin controls the stability of a yet unidentified partner that directly regulates intermediate filament organization or function remains unaddressed. Unbalanced neurofilament transport may additionally contribute to the pathology of intermediate filament perturbation. Also, given the ubiquitous expression patterns of gigaxonin, it may control the UPS mediated degradation of molecules outside the nervous system, as well as those that lack a direct link to cytoskeletal organization and dynamics.

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