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# Mechanisms of Granulin Deficiency: Lessons from Cellular and Animal Models

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**Abstract** The identification of causative mutations in the (pro)granulin gene (*GRN*) has been a major breakthrough in the research on frontotemporal dementia (FTD). So far, all FTD-associated *GRN* mutations are leading to neurodegeneration through a "loss-of-function" mechanism, encouraging researchers to develop a growing number of cellular and animal models for GRN deficiency. GRN is a multifunctional secreted growth factor, and loss of its function can affect different cellular processes. Besides loss-of-function (i.e., mostly premature termination codons) mutations, which cause GRN haploinsufficiency through reduction of *GRN* expression, FTD-associated *GRN* missense mutations have also been identified. Several of these missense mutations are predicted to increase the risk of developing neurodegenerative diseases through altering various key biological properties of GRN-like protein secretion, proteolytic processing, and neurite outgrowth. With the use of cellular and animal models for GRN deficiency, the portfolio of GRN functions has recently been extended to include functions in important biological processes like energy and protein homeostasis, inflammation as well as neuronal survival, neurite outgrowth, and branching. Furthermore, GRN-deficient animal models have been established and they are believed to be promising disease models as they show accelerated aging and recapitulate at least some neuropathological features of FTD. In this review, we summarize the current knowledge on the molecular mechanisms leading to GRN deficiency and the lessons we learned from the established cellular and animal models. Furthermore, we discuss how these insights might help in developing therapeutic strategies for GRN-associated FTD.

**Keywords** Neurodegeneration · FTD · Progranulin · Granulins · Cellular model · Animal model · Growth factor · Therapy

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## Introduction

Frontotemporal dementia (FTD) predominantly affects people below the age of 65 years. The average age-at-onset varies between 45 and 65 years with a mean onset age in the 1950s [1]. There is no apparent gender preference and depending on the study, the prevalence varies from 10 to 20/100,000 inhabitants [2]. Therefore, FTD is nowadays recognized as the second most common form of presenile dementia after Alzheimer disease (AD). Clinically, patients with FTD show progressive behavioral changes, language impairment, and/or executive dysfunction caused by an atrophy of the prefrontal and anterior temporal neocortex [3]. Pathologically, most of the patients are characterized by the presence of cellular inclusions of either the microtubule-

associated protein tau or TAR DNA-binding protein-43 (FTD-TDP) in affected brain regions, leaving 10–15 % of patients with a different underlying pathological inclusion protein [4]. The number of FTD patients with a positive family history is high (40–50 %) with just over 10 % of these familial patients belonging to families with an autosomal dominant inheritance of disease [5]. Although recent genetic breakthroughs have increased the number of identified genetic causes for FTD, still >60 % of patients with familial FTD cannot be explained by a mutation in one of the currently known causative genes [6].

In this review, we focus on FTD-TDP caused by mutations in the (pro)granulin gene (*GRN*) and the recent progress realized through the generation and characterization of GRN-deficient cellular and animal models. We discuss the insights in the molecular mechanisms underlying GRN deficiency generated by these models and how current knowledge might be used to design potential therapeutic strategies for GRN-related FTD-TDP.

## Genetics

In the mid-1990s, segregation studies in autosomal dominant families suffering from FTD with parkinsonism linked to chromosome 17 provided the first evidence of a genetic cause located in the chromosomal region 17q21 [7, 8]. A few years later, extensive mutation analyses resulted in the identification of mutations in *MAPT*, a gene located on chromosome 17q21 and encoding the microtubule associated protein tau [9–11]. Today, 44 *MAPT* mutations have been reported in 134 families including deletions, intronic splice site, and missense mutations [12, 13]. Nevertheless, in a large number of autosomal-dominant FTD patients linked to 17q21, no *MAPT* mutations could be identified. Systematic genetic studies in the 17q21 region ultimately led to the identification of mutations in another gene, the (pro)granulin gene (*GRN*) located proximate to *MAPT* and coding for a multifunctional growth factor [14, 15], Today, nearly half of the familial FTD patients can be explained by mutations in *MAPT* and *GRN* [16].

A total of 69 pathogenic *GRN* mutations have been reported in patients with FTD and related disorders [12, 17], the majority of which are loss-of-function mutations, unambiguously suggesting that *GRN* haploinsufficiency is at the basis of the disease pathogenesis. Loss of GRN can be achieved at several levels, either affecting the gene itself, the expression of its transcript or protein [18], the transport, stability, or processing of the mature protein (Fig. 1).

Most mutations are nonsense or frameshift mutations introducing a premature termination codon (PTC) followed by degradation of the mutant transcript by nonsense-mediated mRNA decay (NMD; Fig. 1a; reviewed in [19]). Other mutations lead to genomic deletion of one whole copy of the gene (Fig. 1b) [20, 21], affect a splice donor site (Fig. 1c) resulting in the inclusion of the nuclear retention signal and degradation of the mutant transcript in the nucleus [15, 22, 23] or destroy the translation initiation codon preventing translation (Fig. 1d) [14, 15, 24, 25].

Together, all loss-of-function mutations in *GRN* explain 5–10 % of all FTD patients and near 25 % if the FTD patients have a positive family history of disease [17]. Besides classical loss-of-function mutations, 52 missense mutations have also been described. Twenty-six of those were only observed in patients suggesting their association with FTD and indicate a potential pathogenic effect [12]. The identified missense mutations are scattered over the entire GRN protein (Fig. 2) indicating that they either affect the function of the GRN precursor protein [17] or its proteolysis into functional granulin peptides.

In addition to the chromosome 17q21 locus, another locus on chromosome 9 has been linked in autosomal dominant families to FTD often associated with motor neuron disease [26], and was confirmed by many research groups [25, 27–30]. Nevertheless, it took more than 10 years of collaborative efforts to identify a pathological hexanucleotide repeat expansion in the regulatory region of the C9orf72 gene explaining the linkage to chromosome 9 [6, 31, 32]. In some FTD cohorts, a mutation frequency of 6 % with expansions in *C9orf72* was observed, similar to the *GRN* mutation frequency (7 %) [6], identifying *C9orf72* as another major genetic cause of FTD. Functional analyses showed that the repeat expansion reduced *mRNA* expression in mutation carriers suggesting C9orf72 haploinsufficiency [6, 31]. Conversely, nuclear RNA foci positive for C9orf72 were also detected [31] pointing to multiple biological mechanisms that might contribute to disease in *C9orf72* repeat expansion carriers. Rare mutations in the genes coding for the TAR DNA-binding protein 43 (TDP-43 or *TARDBP*), valosin-containing protein (*VCP*) and charged multivesicular body protein 2B (*CHMP2B*) also contribute to the FTD genetic etiology (reviewed in [33]).

To explore if potential genetic susceptibility factors might contribute to FTD, genome-wide association (GWA) studies were used [34]. The first GWA study was performed on FTD patients with confirmed TDP-43 neuropathology or clinical diagnosed FTD patients carrying mutations in *GRN* that predict TDP-43 pathology. This resulted in the identification of the first FTD risk gene, *TMEM106B*, coding for a type 2 transmembrane protein [35, 36]. Carriers of pathogenic *GRN* mutations showed the strongest association with *TMEM106B* pointing to a possible disease modifying effect of *TMEM106B* in GRN-associated FTD carriers (reviewed in [37]). Initial expression studies suggested a positive correlation with *TMEM106B* mRNA expression [36]; however, this could not be observed in clinically diagnosed FTD patients [38]. Until now, the functional biological link between GRN, TMEM106B and TDP-43 remains unclear. So far, one study showed a significant correlation of *TMEM106B* and GRN

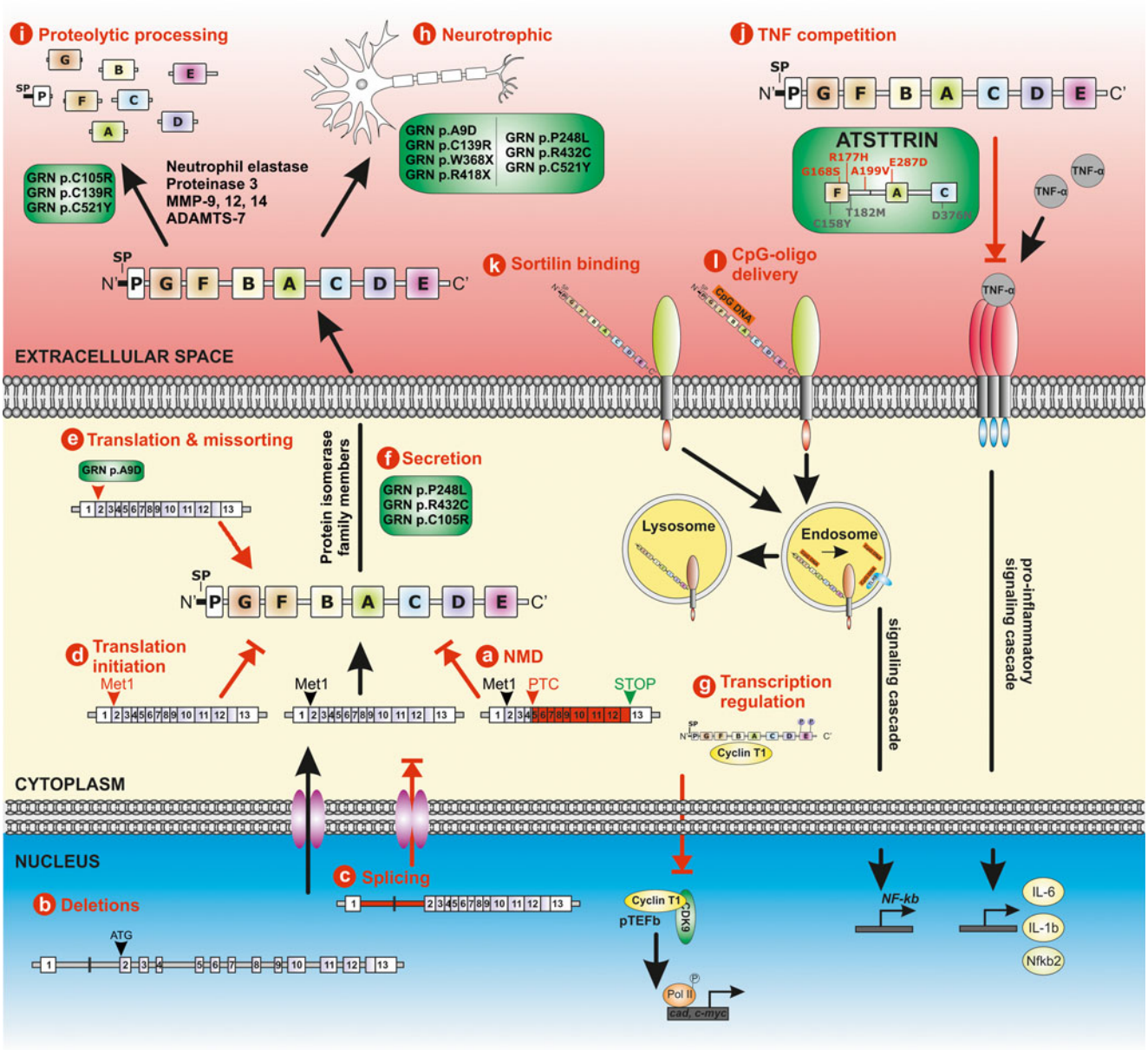


**Fig. 1** Overview of GRN loss-of-function mechanisms and their functional consequences. Loss of GRN can occur on the transcriptional (*a*, *c*), genomic (*b*), and translational level (*d*, *e*). Posttranscriptional mechanisms include cytosolic missorting (*e*), inefficient secretion (*f*), altered proteolytic processing into individual GRN peptides (*i*), and potentially also regulation of transcription via binding to cyclin T1 (*g*). *GRN* missense mutations can affect its neurotrophic properties (*h*) and eventually affect the pro-inflammatory response initiated by TNF-α through alteration of the binding affinity of GRN to the TNF receptor (*j*). Mutations in GRN might also affect binding of GRN to sortilin (*k*) thereby influencing the levels of extracellular GRN or inflammatory signaling cascades stimulated by CpG-DNA (*l*). *SP* signal peptide, *NMD* nonsense-mediated decay, *PTC* premature termination codon

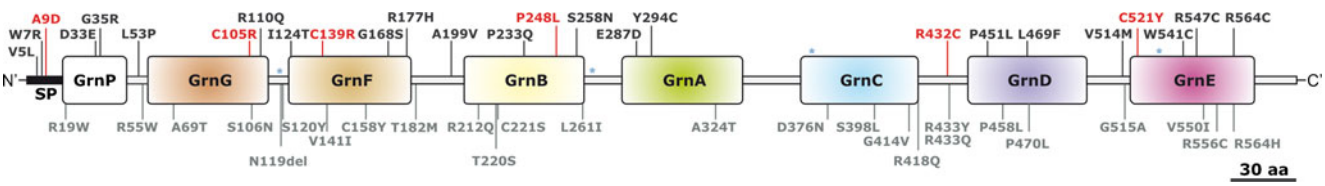


**Fig. 2** Schematic representation of GRN showing the distribution of GRN missense variants within the GRN precursor protein. *Black*, missense mutations detected in patients; *gray*, missense mutations detected in patients and/or controls; *red*, missense mutations with functional evidence of their pathogenicity; *blue asterisk*, predicted glycosylation sites. *SP* signal peptide, *aa* amino acid

levels in plasma [39], and altered expression of microRNAs (miRNA) from the miRNA-132 cluster has been suggested to influence TMEM106B expression levels in FTD patients [40]. However, until now cell culture experiments using TMEM106B overexpression or knockdown showed no consistent effects on GRN levels [35, 40, 41]. Another GWA study indicated an association of FTD with the chromosomal region 1p13 near the sortilin gene as a regulator of plasma GRN levels [42]. Furthermore, on-going efforts using next-generation sequencing technologies might discover additional genetic causal and risk factors contributing to the FTD etiology. Increasing knowledge of the genetic etiology of FTD will likely increase our understanding of the underlying disease mechanisms and might help directing future functional biological studies to unravel disease pathways.

## Clinical and Neuropathological Characteristics

FTD patients carrying a *GRN* mutation clinically present with a large phenotypic variability even within one family segregating the same mutation [43–45]. Despite this variability, patients generally present with behavioral changes, including apathy and social withdrawal as the most prominent clinical symptoms (reviewed in [46]). Some *GRN* mutation carriers also show clinical symptoms characteristic for AD, Parkinson disease (PD) [22], progressive nonfluent aphasia [47] and corticobasal syndrome [48]. Even some clinical overlap with psychiatric disorders was recently described [49]. Due to this clinical heterogeneity and because symptoms can change over time, it can be a challenging task for neurologists to provide patients with the precise clinical diagnosis. The age at onset in *GRN* mutation carriers is also highly variable and ranges from 35 to 89 years with a mean onset of around 60 years [46]. The penetrance of *GRN* mutations is incomplete with approximately 50 % carriers affected at age 60 and 90 % at 70 years [24]. Patients with GRN-associated FTD have considerable frontal atrophy but also temporoparietal atrophy is associated with *GRN* mutations [50]. Mostly, asymmetric distributed hemispheric atrophy can be observed in *GRN* mutation carriers and based on the asymmetric pattern, neuroimaging can differentiate *GRN* and *MAPT* mutation carriers [51]. Further studies also showed that FTD patients with a *GRN* mutation have a faster rate of whole brain atrophy than patients with a *MAPT* mutation resulting in smaller brain volumes in the *GRN* carrier group [51, 52]. Interestingly, a study reported potential compensatory mechanisms of brain plasticity in both presymptomatic *GRN* mutation carriers with normal cognitive and behavioral performances as in FTD patients with *GRN* mutations [53].

Neuropathologically, GRN-associated FTD is predominantly characterized by neuronal and glial cytoplasmic and/or lentiform intranuclear inclusions (NCI or NII, respectively) in the affected cortical regions, which are immunoreactive to ubiquitin and TDP-43 but not to tau and alpha-synuclein [54]. Other consistent neuropathological features caused by GRN deficiency are extensive astrogliosis, loss of myelin in the underlying white matter, hippocampal sclerosis and irregular dystrophic neurites [46]. Biochemically, GRN-associated FTD is characterized by the accumulation of abnormally phosphorylated TDP-43 and both TDP-43 full-length and C-terminal fragments (CTFs) are recovered in detergent insoluble urea fractions from affected brain regions [55–57]. The exact mechanism how GRN haploinsufficiency is linked to the generation of pathological TDP-43 is still not completely understood, but evidence is accumulating that GRN deficiency reduces the efficiency of cellular degradation processes and in turn increases the general susceptibility towards cellular stressors [58].

## Progranulin: A Multifunctional Protein

### *Gene and Protein*

In humans, the gene coding for *GRN* is located on chromosome 17 at cytogenetic band 17q21 and comprises 12 exons [59] (Fig. 3). *GRN* is coding for a 593 amino acid long secreted protein with a predicted molecular weight of 68.5 kDa. GRN contains a signal sequence and seven and a half tandem repeats of a unique 10–12 cysteine-containing motif called granulin domains, a modular organization resembling the precursor of the epidermal growth factor [60]. During maturation of GRN the signal peptide gets cleaved off and mature GRN is secreted as a glycosylated full-length protein with an apparent molecular weight of 90 kDa, which can undergo proteolysis resulting in the generation of individual granulin peptides [60].

The mouse homolog to human *GRN* (*Grn*) is located on chromosome 11 [61], and both mouse and rat *Grn* code for a 589-amino acid protein displaying 75 % overall identity and a similar modular structure [62]. Each granulin domain is encoded by two neighboring exons suggesting the formation of hybrid granulin-like proteins by alternative splicing [59]. Possible alternative spliced transcripts have been observed in some cell lines [63], however, their expression in vivo and thus biological relevance are still unknown. Several independent research groups have purified and characterized individual granulin peptides from different cells and organisms and named them granulins [64] or epithelins [65] due to their association with granulocytes or epithelial cells. The diverse functions of GRN are already reflected in the different designations used in the literature, i.e., proepithelin, GRN, granulin-epithelin precursor, acrogranin, 88 kDa glycoprotein, epithelial transforming growth factor, or PC cell-derived growth factor [66–69]. In this review paper, we use the official designated nomenclature for the human (*GRN* and GRN) and murine (*Grn* and Grn) gene and protein, respectively (http://www.genenames.org/).

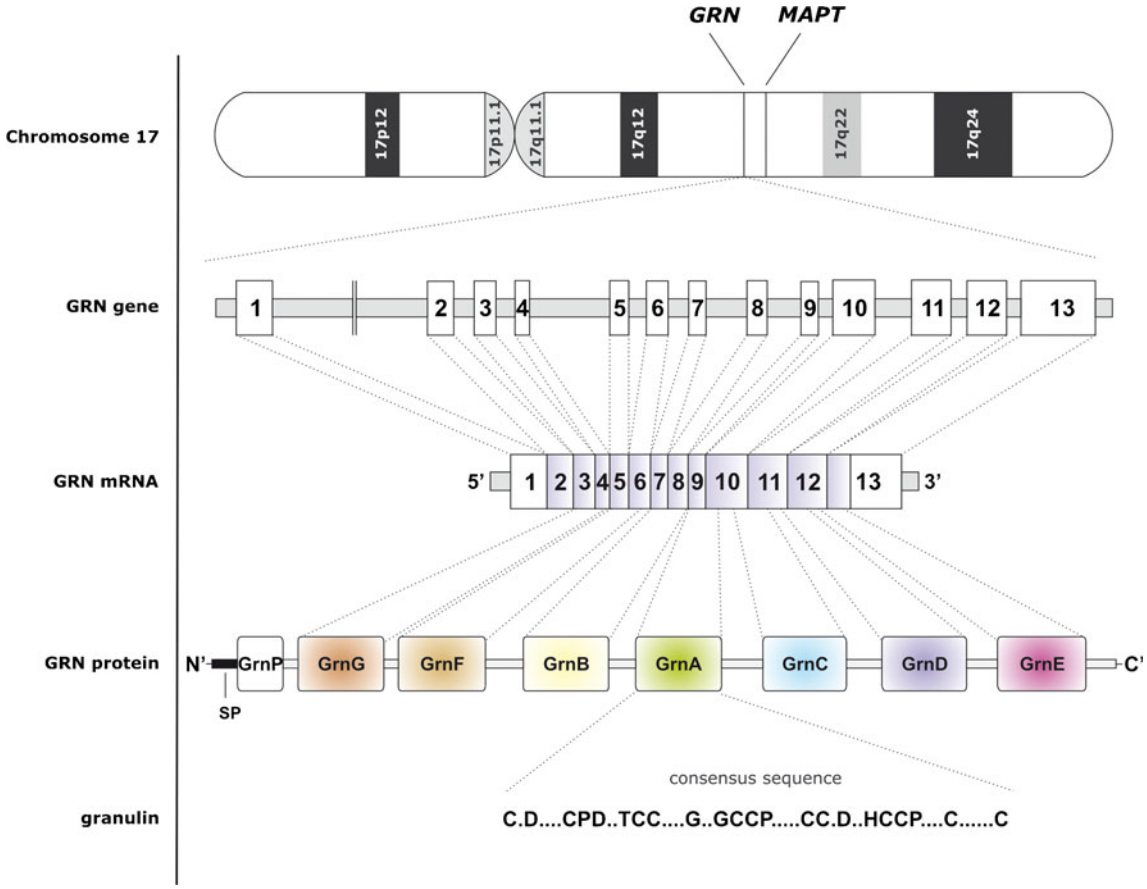


**Fig. 3** Schematic representation of the GRN locus on chromosome 17, the structure of the *GRN* gene, mRNA, and GRN protein as well as the consensus amino acid sequence of a granulin domain

## Gene and Protein Expression

Gene expression can be detected throughout the whole body but *GRN* is predominantly expressed in epithelial and hematopoietic cells [60, 62]. Among the various analyzed tissues, protein expression was particularly high in spleen, placenta, and kidney [60, 70]. In the central nervous system (CNS), GRN is expressed both in neurons and microglia but not in astrocytes as observed in transgenic knock-in mice expressing a reporter gene from the *Grn* locus [71]. Two additional studies evaluating GRN mRNA and protein distribution, showed GRN expression in various brain areas including the cingulate and piriform cortices, the pyramidal cell layer and dentate gyrus of the hippocampus, the amygdala, the ventromedial, and arcuate nuclei of the hypothalamus and the Purkinje cell layer in the cerebellum [72, 73]. Expression of *Grn* mRNA in the hypothalamus is inducible by androgens suggesting an involvement of Grn in the masculinization of the brain [74–76]. GRN expression can also be regulated by miRNAs [77–80], which are posttranscriptional regulators of gene expression, and brains of GRN associated FTD patients show significant alterations in miRNA expression [40, 81]. Furthermore, increased methylation of the *GRN* promoter has recently been suggested to correlate with *GRN* expression in peripheral blood mononuclear cells [82] and further studies are awaited evaluating whether *GRN* promoter methylation can also be correlated with *GRN* expression in brains of FTD patients. In microglia, GRN expression is largely increased upon activation whereas in neurons it increases during maturation [71]. Although baseline levels of GRN expression in astrocytes were below the detection limit in most studies, stimulation with Toll-like receptor (TLR) ligands and Th1 cytokines led to significant upregulation of GRN expression in human primary astrocytes [83].

The reports on the expression of GRN in the brain during normal aging are contradictory. One study, analyzing mice up till 12 months of age, reported decreased *Grn* mRNA levels in an age-dependent manner in the hippocampus and hypothalamus as well as in the cerebral cortex [73], suggesting important functional consequences for the early pathogenesis of GRN-associated FTD. In contrast, a meta-analysis of a large set of expression arrays reported increased *GRN* mRNA levels in association with aging in a large variety of tissues including cortex and hippocampus [84]. It is possible though that region-specific reduction of *GRN* expression contributes to disease pathogenesis and the observed increases of *GRN*

mRNA expression with age might reflect increased microglia reactivity due to chronic low-level neuroinflammation, a feature observed also in the normal aged brain [85].

## GRN Protein Structure

Dissecting the protein structure of human GRN by high-resolution NMR shows that three of the granulin peptides (granulin A, C, and F) contain relatively well-defined three-dimensional structures in solution with a stable stack of two β-hairpins in their N-terminal subdomains [86]. This is in accordance with the previously reported more rigid stacked β-hairpin granulin fold of crap granulin A [87]. In contrast, the C-terminal subdomain of the granulins seems to be more flexible [86]. While granulin A, C and F represent well-folded peptides, the residual granulin peptides (granulins B, D, E, and G) exist as poorly structured disulfide isomers [86]. Whether and how these structural differences are responsible for the biological activity of the individual granulins or even the GRN protein, requests systematic analysis in the future.

## GRN as a Mitogen and Neurotrophic Protein

Since the initial reports on the identification of GRN, a large body of literature has accumulated describing the mitogenic or inhibitory effects of GRN and its proteolytically cleaved granulins on various cell types (for a recent review, see [88]). Further evidence for the activity of GRN as an important growth factor came from the numerous oncological studies reporting increased GRN expression as a negative prognostic factor in many different cancers [89]. The effect of GRN on neuronal cells is less well described. Recently, extracellular administration of GRN was reported to stimulate neurite outgrowth in cultured motor and cortical neurons [90], demonstrating for the first time the neurotrophic properties of GRN. In similar experiments, the putative loss-of-function outcome of some FTD-associated *GRN* missense mutations could be demonstrated [91–93]. Moreover, GRN was able to increase neuronal survival [90] and to protect neurons from neuronal apoptosis caused by Grn deficiency [94] or by toxic insults [95], suggesting that GRN is a neuronal survival factor. Although granulin E showed similar neurotrophic and neuroprotective properties, it would not be surprising if some of the other granulins would have opposing effects, as it was observed on other cell types [86]. Further studies are awaited to determine the actual contribution of the individual granulins on neuronal homeostasis.

## GRN Signaling

GRN is thought to exert its mitogenic effect through the stimulation of both the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase pathways [96–99]. In neurons, GRN was shown to stimulate the phosphorylation of Akt [91, 94, 95] and glycogen synthase kinase (GSK)-3β [91, 95], while the effect on the MAPK pathway was not consistently observed [91, 94, 95]. Furthermore, GRN also affects insulin signaling in adipocytes downstream of the insulin receptor [100], is the only growth factor that can stimulate cells deficient for the insulin-like growth factor 1 (IGF-1) receptor [101] and can substitute for IGF-1 signaling in the regulation of muscle growth [102]. Initial attempts to find the GRN cell surface receptor by chemical crosslinking techniques resulted in the identification of two classes of binding sites on epithelial cells and fibroblasts: a high-affinity site with a relatively low number of receptors per cell and a low-affinity site with a larger number of receptors per cell [103, 104]. One study estimated a size of ≈120 kDa for the receptor [104], while another study reported the interaction of GRN with a receptor of ≈170–175 kDa [105].

Sortilin, a 100-kDa type-1 membrane receptor involved in lysosomal targeting [106] was identified as a high-affinity neuronal receptor of GRN [107]. Sortilin facilitates rapid endocytosis and delivery of GRN to the lysosomes through binding of the GRN C terminus to the beta-propeller region of sortilin [108] and thereby regulating extracellular levels of GRN [42, 107]. Sortilin not only regulates the release of pro-neurotrophins (proNT), but is also implicated, together with p75NTR, in regulating signal transduction by proNTs [109]. A study using hippocampal neurons from sortilin knockout mice showed that the neurotrophic effect of GRN is independent of sortilin since GRN stimulated neurite outgrowth was not affected in these neurons [92]. Hence, the identification of additional cell surface receptors for GRN will be a major step forward in understanding GRN-linked signaling events.

## GRN and Inflammation

Both the human and murine promoter contain several regulatory *cis*-elements that are possibly involved in cytokine and growth factor regulated transcriptional gene expression [110], and the involvement of GRN in inflammatory processes has been well described [111–115]. GRN has a function during wound healing, where it increases the accumulation of neutrophils, macrophages, blood vessels, and fibroblasts in the wound [111]. Mediation of the inflammatory response involves proteolytical processing of anti-inflammatory GRN into pro-inflammatory granulins by the serine proteases neutrophil elastase and proteinase 3 [112, 115]. Some metalloproteinases (MMP-9, MMP-12, MMP-14, and ADAMTS-7) also show substrate specificity for GRN [83, 116–118] and can act as GRN convertases. Secretory leukocyte protease inhibitor (SLPI) is known as a natural regulator of the proteolytic process through binding to GRN and inhibiting the elastase-mediated GRN proteolysis [115]. Accordingly, *SLPI*-deficient

mice show impaired wound healing, most likely due to a reduction of Grn caused by increased elastase activity [119]. Subcutaneous administration of GRN completely restored the proper wound healing process in *SLPI*-deficient mice [115]. Furthermore, GRN was described as a potent inhibitor of the inflammatory cytokine tumor necrosis factor alpha (TNF-α) signaling [115, 120], whereas individual granulins A and B are thought to be pro-inflammatory through the induction of the pro-inflammatory cytokines interleukin-8, TNF-α, and interleukin-1b expression [115, 121]. A further role for GRN as a soluble co-factor for the delivery of CpG-oligonucleotides to TLR 9 was described [122], but how exactly CpG-oligonucleotide-bound GRN meets TLR9 in the endolysosomal compartment is not known. One could speculate that binding of GRN to sortilin and subsequent endocytosis might be involved in this process [123].

In the CNS, GRN can act as a chemoattractant to recruit and/or activate microglia followed by increased endocytosis of extracellular peptides such as amyloid beta [124]. Increased GRN expression, especially in activated microglia, is a consistent feature of human neurodegenerative conditions [125–127] including FTD caused by GRN haploinsufficiency [14, 15, 128]. Grn was also among the top upregulated molecules around amyloid plaques in AD mouse models [129], and in activated microglia of models of motor neuron degeneration [130–132].

Inflammatory mediators like TNF-α and TGF-β are increased in the cerebrospinal fluid (CSF) but not in serum of patients with FTD [133], and FTD patients with *GRN* mutations have significantly higher circulating levels of IL-6 compared with FTD patients without a *GRN* mutation or control individuals [134]. Similarly, *Grn* knockout mice ($Grn^{-/-}$) responded with an exaggerated production of pro-inflammatory cytokines upon LPS stimulation and delayed recovery of bacterial infections [114], suggesting that the GRN-mediated neurodegeneration could be a result of cumulative damage through deregulation of inflammation. This is in line with the sustained neuroinflammatory processes that contribute to neurodegenerative diseases such as AD [135] and PD [136].

## GRN in Energy Homeostasis

Obesity and aging of the human population are two key concerns worldwide with a large social, medical, and economic impact. A link between neurodegenerative diseases and obesity has been suggested and a number of studies associate obesity with cognitive decline and enhanced vulnerability to brain injury [137]. Insulin resistance and type 2 diabetes are associated with the pathogenesis and pathophysiology of some human neurodegenerative diseases [138], and deficits in insulin signaling lead to hyperphosphorylation of tau [139]. In addition, increased GRN serum levels were linked to type 2 diabetes and physical training could significantly reduce GRN levels by about 20 % in these patients [140]. Furthermore, renal function has also been suggested to significantly affect GRN serum levels [141]. Increased GRN levels were also associated with insulin resistance in obese individuals, which had also a tendency for higher IL-6 and MCP-1 serum concentrations [142].

Mouse models of obesity also showed increased Grn levels in blood and adipose tissues, which could be normalized by the insulin-sensitizing agent pioglitazone [100]. High fat diet leads to insulin resistance through the induction of IL-6 and Grn was shown to be a key mediator of this process [100]. Extracellular administration of GRN results in impaired insulin signaling downstream of the insulin receptor and leads to insulin resistance, whereas Grn deficiency enhances insulin sensitivity resulting in reduced deposition of peritoneal fat in GRN-deficient mice [100]. In the hypothalamus, Grn is involved in glucose sensing and GRN levels have been inversely correlated with appetite and food intake [143] and behavioral changes in FTD patients, including *GRN* mutation carriers, include overeating as well as a preference for sweet food [16]. These studies suggest important functions of GRN as an adipokine and one might hypothesize that disturbances in energy homeostasis could contribute to precipitate neurodegeneration in GRN-associated FTD.

## GRN Loss-of-Function Mechanisms

Besides classical loss-of-function mutations, mutations leading to non-synonymous amino acid substitutions have also been described and some of them are predicted in silico to affect protein function [17]. Due to the multifunctional character of GRN, the functional consequences of these missense mutations might be widespread and subtler. Some *GRN* missense mutations are potentially causing a partial haploinsufficiency by affecting protein translation, sorting and GRN secretion (Fig. 1e, f). The first identified missense mutation (p.A9D) was located in the GRN signal peptide [144], and functional analyses showed that the mutant protein was not secreted due to cytoplasmic missorting [145, 146]. Two other missense mutations (p.P248L and p.R432C) affected protein secretion and stability and potentially reduce the amount of available GRN [146]. Intracellular GRN has been shown to bind to cyclin T1 in the cytoplasm and blocking its translocation to the nucleus (Fig. 1g). This interference with the assembly of functional pTEFb complexes leads to inhibition of transcription from cellular promoters like the cad and c-myc promoter [147, 148]. It would therefore be interesting to investigate if *GRN* missense mutations affect gene transcription by altering cyclin T1 binding, ultimately resulting in a distinct molecular phenotype similar to that observed in brains of GRN-associated FTD patients [149]. Furthermore,

missense mutations can affect the neurotrophic properties of GRN (Fig. 1h) as indicated by the reduced ability of mutant GRN to stimulate neurite outgrowth and neuronal survival [91, 93]. The exact mechanism is unknown but it was suggested that missense mutations might affect proper receptor-ligand interaction due to conformational changes in the protein [93]. Serine proteases like neutrophil elastase or proteinase 3 are involved in converting GRN into granulins (Fig. 1i) [112, 115], a process that is important during inflammatory conditions. A set of missense mutations, especially those affecting highly conserved cysteine residues (p.C139R and p.C521Y), were shown to interfere with proper proteolytic GRN processing [93]. This eventually affects the course of an inflammatory response and thus might contribute to neurodegenerative disease. It is also intriguing to consider that missense mutations in *GRN* might affect the regulation of inflammatory processes by affecting the ability of GRN to compete with TNF-α for its cognate receptors [120, 150] and thus attenuating the inflammatory reaction (Fig. 1j). A recombinant GRN peptide called antagonist of TNF/TNFR signaling via targeting to TNF receptors (ATSTTRIN) that includes parts of granulins F, A, and C, proved even more potent than GRN in attenuating the response to TNF-α [120]. Of note is that eight *GRN* missense mutations affect amino acids inside this region [12]. Moreover, it would also not be surprising if some missense mutations would affect binding of GRN to sortilin (Fig. 1k) and by this either alter the levels of extracellular GRN or influence the innate immune response by affecting the, until now hypothetical, model of CpG-oligonucleotide delivery to TLR9 in the endosomes (Fig. 1l) [122, 123]. Detailed information on how *GRN* missense variants affect GRN function and/or processing is still scarce and therefore further functional studies will be tremendously important to group the missense mutations into functional clusters depending on the biological process they are affecting.

## Modeling Progranulin Loss in Cells

Keeping the balance of bioavailable GRN seems to be crucial for maintaining cellular homeostasis as both GRN overexpression and deficiency are linked to the development and progression of cancers and neurodegeneration, respectively. Increased GRN levels are correlated with significantly increased tumorigenicity in several types of cancer and reducing the levels by RNA interference or neutralizing antibodies generally reduces cell proliferation and tumorigenicity [151]. Downregulation of GRN expression causes alterations in cell cycle progression due to reduction of cyclin D1, CDK4, and alpha-tubulin [152–154] and leads to caspase-mediated apoptosis or increased susceptibility to it, depending on the cell type [91, 94, 155–158].

Following the identification of *GRN* loss-of-function mutations in FTD patients [14, 15], and the proposed role of GRN as a modifying factor in other neurodegenerative diseases [159, 160], the neuroscience community has put increasing efforts in understanding and characterizing the role of GRN in the CNS. One of the key questions to answer is how GRN deficiency can lead to neurodegeneration. GRN is widely expressed in the CNS [72, 73] and its expression and secretion was confirmed in neuronal progenitor cells (NPCs), cultured primary neurons, motor neuron cell lines, and neurons derived from induced pluripotent stem (iPS) cells [94, 161–164], making those valuable tools to further study GRN function. The addition of exogenous GRN proved beneficial for neurite outgrowth and neuronal survival [90] and supported neuronal survival of motor neuron cell lines even under serum deprivation [164]. Both GRN and granulin E showed neurotrophic properties in vitro [90, 92] and three studies using mutant GRN proteins showed that the missense mutations interfere with the neurotrophic functions of GRN [91–93]. However, the exact mechanism how GRN promotes neurite outgrowth and neuronal survival is still not completely understood. The first study using neurons derived from iPS cells, generated from an FTD patient with a *GRN* nonsense mutation, suggests defects of GRN-deficient neurons in the PI3K/Akt and MAPK signaling pathways [161]. Furthermore, GRN preferentially activates the PI3K/Akt signaling pathway in cortical neurons derived from $Grn^{-/-}$ mice and Grn deficiency in these cells leads to a subtle reduction in phosphorylated Akt [94]. In NPCs [162] and primary neurons [91] GRN stimulated GSK3-Beta (GSK3β) phosphorylation, which could be abolished by PI3K inhibitors [162], supporting the involvement of the PI3K/Akt pathway in GRN-mediated survival signaling. While GRN stimulation results in significantly increased neurite outgrowth, siRNA-mediated GRN knockdown has the opposite effect [165] and causes impaired retinoic acid-induced neuronal differentiation of neuroblastoma cells possibly through reduced phosphorylation of GSK3β [91]. Together these data suggest an essential role of PI3K/Akt signaling and regulation of GSK3β in GRN-mediated neuronal integrity. GSK3β is known to be critically involved in the canonical Wnt-signaling pathway [166], but a recent study using inducible GRN knockdown in human NPCs also identified a major adaptive role for the noncanonical Wnt signaling pathway in GRN-associated FTD [163]. The amount of data and their functional impact further strengthen the validity of using such cellular models to find key molecular mechanisms that are affected by GRN deficiency.

Proper neuronal connectivity is crucial for maintaining brain homeostasis and Grn knockdown [165] or knockout [167] in primary hippocampal cultures has been associated with reduced neural connectivity. In these experiments, Grn loss led to decreased neuronal arborization and length as well as spine and synapse density [165, 167], which could be responsible for alterations in the synaptic output. Although Grn knockdown resulted in a significant reduction

in synapse density, the number of synaptic vesicles per synapse was increased, a phenomenon also observed in postmortem brain sections of GRN-associated FTD patients [165]. In this study, the authors also reported an increased frequency of spontaneous glutamatergic transmission upon Grn knockdown in hippocampal neurons [165], thereby supporting the theory that GRN deficiency increases the probability of release at remaining synapses due to increased vesicle density. This might be directly caused by GRN deficiency or could be reflecting a compensation of the reduced number of synapses [165]. However, in hippocampal slice cultures the release probability was comparable between slices from *Grn*$^{-/-}$ and wild-type (wt) mice, supporting the theory that the decreased synaptic output might rather be caused by the reduced number of functional synapses [167]. Further studies are highly encouraged as the observed synaptic dysfunction significantly precedes most indications of neuropathological changes in this model [167].

Loss of GRN is ultimately associated with degeneration of cortical and hippocampal neurons [168] and the first cellular studies are highlighting the important role of GRN expression on neuronal survival. Complete loss of Grn [94] or persistent GRN knockdown in mouse primary cortical neurons [155, 169] and human NPCs [163] or GRN haploinsufficiency in patient derived iPS cells [161] led to significantly increased caspase activation and reduced neuronal survival, however, no effect on neuronal survival of rat hippocampal neurons was observed upon Grn knockdown in another study [165]. Furthermore, GRN deficiency was priming neuroblastoma cells for staurosporine-induced apoptosis [91] and increased the susceptibility of cultured neurons or hippocampal slice cultures to cellular stressors such as inhibiting proteasomal proteolysis [94], NMDA-mediated excitotoxicity, oxidative stress [155] glucose, and oxygen starvation [114] or kinase inhibitors [161]. Overexpression of GRN or addition of extracellular GRN rescued these effects [95, 155, 161] due to the activation of cell survival signaling pathways [95], suggesting that the increased susceptibility of neurons is specific for GRN deficiency.

Inadequate responses to inflammatory insults are also likely contributing to FTD pathogenesis and cellular studies using macrophages or microglia from Grn-deficient mice indicated a critical involvement of Grn in regulating TLR9 signaling [122]. Furthermore, Grn deficiency resulted in an exaggerated inflammatory response defined by an increased production of pro-inflammatory cytokines and reduced production of anti-inflammatory IL-10. Grn-deficient microglia were also more cytotoxic compared with wt controls [114, 163].

Lastly, the major biochemical feature of GRN-associated FTD is the redistribution of TDP-43 from the nucleus to the cytoplasm, increased TDP-43 phosphorylation and generation of aggregation-prone TDP-43 CTFs that accumulate in neuronal intranuclear or cytoplasmic inclusions [55, 57]. The first study investigating the cellular link between GRN and TDP-43 reported increased accumulation of TDP-43 CTFs, with no obvious redistribution of TDP-43 from its predominant nuclear localization in non-neuronal cells [158]. However, subsequent attempts to reproduce this effect in similar cell lines failed to detect increased caspase activation and TDP-43 fragmentation upon GRN knockdown in non-neuronal cells [94, 146, 170]. Moreover, N-terminal sequencing of the TDP-43 CTFs isolated from brains of FTD patients indicated that these CTFs are different from those generated upon caspase cleavage [171]. Interestingly, increased redistribution of TDP-43 from the nucleus to the cytoplasm was observed in cortical neurons upon persistent knockdown of Grn [155] and neurons derived from GRN-deficient iPS cells [161]. Additional stress by disturbing the proteasomal machinery also resulted in increased accumulation of phosphorylated full-length TDP-43 in primary cells derived from *Grn*$^{-/-}$ mice [94].

The established cellular models of GRN deficiency have already improved our understanding on the multiple cell biological functions of GRN. However, so far, no GRN-deficient cell-culture model could recapitulate all pathological hallmarks of FTD including the ubiquitinated and TDP-43 positive nuclear and/or cytoplasmic aggregates. As some of the FTD characteristic features might be difficult to completely recapitulate in vitro, animal models of GRN deficiency have been developed to further deepen our understanding of the GRN biology on the level of a whole organism.

## Progranulin-Deficient Animal Models

The granulin/epithelin motif defines a family of structurally unique proteins, of great evolutionary antiquity [60, 172]. Granulin motif encoding genes are present in most commonly used laboratory animals including *Caenorhabditis elegans* [173], *Danio rerio* [172], *Xenopus leavis* [174], and *Mus musculus* [61], making them valuable tools to study the effect of GRN deficiency in an in vivo setting.

## Nonrodent Models of GRN Deficiency

In the nematode *C. elegans*, the *GRN* gene encodes for a secreted protein with three predicted granulin domains, which is expressed in intestinal cells and selected neurons, but not in muscle cells [173]. *GRN* deletion mutants appeared grossly normal with a normal lifespan but they produced approximately 20 % less progeny. GRN-deficient *C. elegans* showed significantly fewer apoptotic bodies, a phenomenon that was attributed to an increased clearing of apoptotic cells [173]. Based on these results, Kao et al. proposed a speculative model suggesting that cells of GRN-deficient organisms do not have enough time to recover from sub-lethal stress ultimately leading to cumulative cellular loss over time [173]. Table 1 gives an overview of the currently described nonrodent GRN-deficient animal models.

**Table 1** Overview of nonrodent models of GRN deficiency and primary phenotypes

| | Kao et al. [173] | Shankaran et al. [146] | Li et al. [175] | Chitramuthu et al. [176] | Laird et al. [177] |
|---|---|---|---|---|---|
| Model organism | *Caenorhabditis elegans* | *Danio rerio* | *Danio rerio* | *Danio rerio* | *Danio rerio* |
| GRN genes | *pgrn-1* | | *zfGRN-1*, *zfGRN-2* (precursor); *zfGRN-A*, *zfGRN-B* (shorter peptides) | | |
| Structure | 3 granulin domains | | zfGRN-A, 10 granulin domains; zfGRN-B, 9 granulin domains<br>zfGRN-1, 1.5 granulin domains; zfGRN-2, 1.5 granulin domains | | |
| Modification | Deletion mutant | zfGRN-B knockdown (antisense gripNA) | zfGRN-A knockdown (morpholino based) | zfGRN-A knockdown | zfGRN-A and zfGRN-B knockdown (morpholino based) |
| | 347-bp deletion including part of GRN promoter, exon 1, and part of first intron | | | zfGRN-B knockdown (morpholino based) | |
| Phenotype | Normal life span | No morphological phenotype | Reduced proliferation and increased apoptosis in hepatocytes | Truncated motor neurons (zfGRN-A) | Truncated motor neurons; more pronounced with zfGRN-A knockdown |
| | 20 % less progeny | No effects on TDP-43 localization | Decreased liver size | Inappropriate early branching (zfGRN-A) | No effects on TDP-43 localization |
| | Fewer apoptotic bodies during development, but no defects in cell death | | Reduced expression of hepatic MET | Swimming deficit, but normal touch response (zfGRN-A) | |
| | Altered kinetics of cell death; faster clearing of apoptotic cells | | | Severe phenotype | |
| | | | | Reduction in head size 24 hpf (zfGRN-B) | |
| Rescued by overexpression | Yes | n.d. | Yes | Yes | Yes |

In zebrafish (*D. rerio*), a useful model of vertebrate development and disease, four *GRN* paralogues were identified that are coding for two precursor proteins (zfGRN-A and B) and two shorter forms of GRN (zfGRN-1 and zfGRN-2) [172]. A noncoding RNA gene with antisense complementarity to both *zfGRN1* and *zfGRN2* has also been identified, with a possible function in regulating gene dosage [172]. Both *zfGRN-A* and *zfGRN-B* transcripts are expressed in a wide variety of tissues including the gills, heart, multiple visceral organs and at modest expression levels in the brain [172]. Knockdown of zfGRN-A using antisense morpholinos led to reduced proliferation and increased apoptosis in hepatocytes and zfGRN-A-deficient zebrafish had a decreased liver size. Impaired liver morphogenesis was linked to reduced expression of hepatic MET, a receptor tyrosine kinase known to have functions controlling liver size [175]. zfGRN-A expression can also be found within the peripheral and CNS and knockdown by antisense morpholinos resulted in truncated motor neurons (MNs) and inappropriate early branching [176]. In contrast, overexpression of zfGRN-A or human GRN caused increased MN branching and rescued the truncation defects caused by zfGRN-A deficiency, survival of motor neuron 1 (smn1) deficiency [176] or overexpression of mutant TDP-43 [177]. The effect of zfGRN knockdown on MN axonal growth was confirmed by another study where knockdown of zfGRN-A produced a greater decrease in axonal length than zfGRN-B knockdown [177]. Additional to the observed MN defects, and most likely as a consequence, zfGRN-A morphants showed a marked progressive swimming defect although the touch response was unaltered [176]. Cytoplasmic redistribution of TDP-43 or proteolytic processing into aggregation-prone CTFs are characteristic disease features of GRN-associated FTD, but downregulation of zfGRN did not cause any of these alterations [146, 177].

## Rodent Models of Grn Deficiency

Rodent models are the most frequently used animal models in biomedical research for several reasons including their anatomical similarities to humans as well as the possibility to generate disease models through targeted gene manipulation such as gene knockout.

Five independent $Grn^{-/-}$ mouse models have been established to date [75, 114, 167, 173, 178], of which a general overview of their characteristics is included in Table 2. So far, most studies used all-tissue knockout of *Grn*, yet some of the models also allow tissue-specific knockout [114, 167, 173, 178]. Utilizing one of these conditional *Grn* knockout lines [173], Martens et al. report deregulated microglial activation in microglia-specific $Grn^{-/-}$ mice leading to increased neuron loss in a model of neuronal injury [179]. All described $Grn^{-/-}$ mice are viable, fertile and reproduce with a normal Mendelian pattern of inheritance. However, one follow up-study on the mice established by Kayasuga et al. [75] suggested a decreased generation frequency of $Grn^{-/-}$ mice and postnatal sensitivity to handling [180]. This was not observed in another follow-up study by Ghoshal et al. on the same $Grn^{-/-}$ mice [181]. Two further studies observed

**Table 2** Generated $Grn^{-/-}$ mouse models and primary characterization

| | Kayasuga et al. [74] | Yin et al. [114] | Kao et al. [173] | Petkau et al. [167] | Wils et al. [178] |
|---|---|---|---|---|---|
| Type | Constitutive | Constitutive | Constitutive | Constitutive | Constitutive |
| Conditional option | No | Yes | Yes | Yes | Yes |
| Modified region | Deletion of exons 2–13 | Deletion of exons 1–4 | Deletion of exon 2-13 | Disruption of Grn gene by insertion of lacZ/neomycin fusion protein between exons 4 and 5 | Deletion of exons 2–4 |
| Genomic clone | 129 SvJ | Not specified | 129/SvJae | 129S1/ SvImJ | 129/Sv |
| Blastocysts | C57BL/6×DBF1 | C57BL/6 | C57BL/6J | C56BL/6J | C56BL/6J |
| Background | Backcrossed to C57BL/6J | Backcrossed to C57BL/6 | n.d. | Backcrossed to C57BL/6J | Mixed Bl6/129Sv |
| Tissue | All | All | All | All | All |
| Viable | Yes | Yes | Yes | Yes | Yes |
| Fertile | Yes | Yes | Yes | Yes | Yes |
| Pattern of inheritance | Mendelian [74, 181][a] | Mendelian | n.d. | Mendelian | Mendelian |
| Survival of aged mice | Increased adult onset mortality [180][a] | n.d. | n.d. | n.d. | Increased adult onset mortality |

[a] Ahmed et al. reported reduced frequency of $Grn^{-/-}$ mice and increased postnatal sensitivity to handling of $Grn^{-/-}$ mice [180] established by Kayasuga et al. [74]

*n.d.* not determined

increased age related mortality with differences appearing from 10 months onwards [178, 181]. In contrast, no decreased survival was reported in two other independent mouse models [114, 167]. Although in humans, GRN haploinsufficiency is sufficient to lead to neurodegeneration, none of the *Grn*$^{-/-}$ mice studies identified any obvious defects yet, mimicking the condition in human disease. Therefore, all studies focused on characterizing the behavioral (Table 3) and neurohistological (Table 4) consequences of Grn loss in *Grn*$^{-/-}$ mice.

Initial studies, using siRNA-mediated knockdown or administration of neutralizing Grn antibodies, have suggested that hypothalamic Grn exerts anorexigenic effects affecting weight gain and loss, indicating a potential role of Grn in hypothalamic glucose sensing [143]. However, consistent with another report [182], no effect on body weight was yet described in *Grn*$^{-/-}$ mice [75, 114, 167, 178].

In rodents, *Grn* has been shown to be an androgen-inducible gene in the neonatal hypothalamus that is expressed at high levels in males, throughout the critical period for the sexual differentiation of the brain, while in females the Grn expression levels drop during this critical period [183, 184]. Accordingly, infusion of *Grn* antisense oligonucleotides into the third ventricle of neonatal male rats significantly suppressed male sexual behaviors, like frequency of mount, intromission and ejaculation in the adulthood [182]. Alterations in male sexual behavior, aggression, and anxiety were also observed in the first *Grn*$^{-/-}$ mouse model [75]. In the open-field test, wt females generally show higher levels of anxiety than males and Grn loss raises the anxiety level of males significantly to similar levels of females [75]. A follow up-study on the same mice linked increased anxiety in *Grn*$^{-/-}$ males to an increase in the volume and number of cells in the locus ceruleus [185], a nucleus involved in physiological responses to stress and anxiety. Furthermore, *Grn*$^{-/-}$ males exhibited enhanced aggressiveness towards females and increased frequency of biting attacks in the resident-intruder test, which was ascribed to alterations in the brain serotonergic system of *Grn*$^{-/-}$ mice [75]. However, diminished social interaction and passive disinterested behavior, rather than increased aggression was observed in two other studies [167, 181]. Yin et al. reported increased signs of depression in *Grn*$^{-/-}$ mice using a tail suspension and forced swimming test [186], while Petkau et al. did not observe any abnormalities in their *Grn*$^{-/-}$ model [167].

Perhaps the most consistent behavioral phenotype in Grn$^{-/-}$ mice is a reduction in social interactions [167, 181, 186], a feature observed already at very young age [186]. Assessment of spatial memory functions by Morris water maze was less consistent between the individual publications. While Petkau et al. did not observe any deficits of *Grn*$^{-/-}$ mice in the Morris water maze [167] other studies report subtle impairments in the oldest mice using the same test parameters [178, 181, 186]. Generally, motor functions seem not to be affected largely by Grn deficiency, however, *Grn*$^{-/-}$ mice tended to swim somewhat slower [178, 181], took longer to learn the rotarod task [167] and showed reduced performance in the inverted screen test at old ages [181]. Such subtle locomotor deficits might be explained by increased inflammation in joints as *Grn*$^{-/-}$ mice were recently shown to have increased susceptibility to collagen-induced arthritis [113]. Although all *Grn*$^{-/-}$ mouse models have been maintained in similar backgrounds (Table 2), subtle differences in background strain or in the applied test protocols, two important variables in behavioural studies [187, 188], might account for some of the observed phenotypic differences in *Grn*$^{-/-}$ mice.

Reports on the neuropathology of *Grn*$^{-/-}$ mice are more consistent (Table 4). All studies report a pronounced microgliosis and astrogliosis in brain areas including the cortex, hippocampus, thalamus and brainstem [114, 167, 178, 180, 181, 186]. Microgliosis tends to be detectable at earlier time points, with significant differences emerging from 12 months onwards, and both microgliosis and astrogliosis show a progressive worsening [178, 180, 186]. Accumulation of ubiquitinated proteins in the same brain areas is another consistent feature of all *Grn*$^{-/-}$ models [114, 167, 178, 180, 181, 186], suggesting either an overproduction of a ubiquitin target protein or more likely a perturbation in the functioning of the ubiquitin-proteasomal and/or autophagy-lysosomal degradation machineries. Furthermore, a robust increase in the accumulation of the aging pigment lipofuscin in the brains of *Grn*$^{-/-}$ mice [167, 178, 180], associated with vacuolation in the habenula and hippocampus [180], was observed. Interestingly, a homozygous GRN mutation has recently been identified in two siblings with neuronal ceroid lipofuscinosis (NCL) [189], a lysosomal storage disorder with prominent accumulation of lipofuscin [190], calling for future studies to determine whether the same pathways could be affected in FTD and GRN-related NCL.

Together with increased accumulation of the autophagy-related receptor p62 as well as lysosomal proteases such as cathepsin D [178], this points towards an involvement of the autophagy-lysosome degradation system in Grn-mediated neuropathology.

Although cell culture experiments suggested a link between GRN deficiency and caspase activation [94, 155, 158], no obvious signs of apoptosis were observed in the brains of *Grn*$^{-/-}$ mice [178, 180]. Neuronal loss is not a prominent feature of *Grn*$^{-/-}$ mice [178, 180], although some focal neuronal loss in the CA2-3 region of the hippocampus and small nonsignificant reduction in neuron density in the dorsal thalamus were observed in very old mice [180, 181]. While such changes might be very subtle and only detectable in old mice, impaired neuronal function is predicted to manifest much earlier. Accordingly, reduced hippocampal synaptic connectivity and impaired synaptic plasticity (e.g.,

**Table 3** Behavioral phenotypes of *Grn*$^{-/-}$ mouse models

| Behavioral phenotyping | | Kayasuga et al. [74] | Chiba et al. [185] | Ahmed et al. [180] | Yin et al. [186] | Ghoshal et al. [181] | Petkau et al. [167] | Wils et al. [178] |
|---|---|---|---|---|---|---|---|---|
| General | Age at analysis | 1 age group (7–11 weeks) | 1 age group (7 weeks) | – | 4 age groups (1–2, 4–7, 12, and 18 months) | 3 age groups (9–12, 13–16, and 16–19) | 1 age group (8 months) | 2 age groups (13–15 and 20–22 months) |
| | Gender | Males | Males and females (separate) | Not specified | Not specified | Males and females (pooled) | Males and females (separate) | Males (13–15 months) and females (20–22 months) |
| | Reactivity to handling | Not specified | Not specified | Sensitive | Not specified | Normal | Not specified | Normal |
| Sexual behavior | Ejaculation frequency | Reduced | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| | Latency and frequency of mount and intromission | No difference | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| Anxiety | Open field test | Less time in center, no change in distance traveled and rearing | *Grn*$^{-}$ males spend less time in center | n.d. | Normal | n.d. | Less center crossings of *Grn*$^{-/-}$ males | Normal |
| | Elevated plus maze | n.d. | *Grn*$^{-}$ males spend less time in open arms | n.d. | Longer in open arms | n.d. | n.d. | n.d. |
| Spatial learning | Morris water maze | n.d. | n.d. | n.d. | Increased latency to find hidden platform (18 months)<br>Reduced spatial memory (18 months) | Normal escape path length or latency<br>Reduced acquisition of the learning set task<br>Impaired retention and acquisition performance | Normal average latencies to find hidden platform<br>No difference in spatial memory | Less efficient in finding hidden platform |
| Motor performance | Swim speed | n.d. | n.d. | n.d. | n.d. | Reduced | Normal | Reduced |
| | Accelerated rotarod | n.d. | n.d. | n.d. | Normal | n.d. | *Grn*$^{-/-}$ males took longer to learn rotarod task | Normal |
| | Locomotor activity and exploratory behavior | No difference in duration of walking and number of rearing (resident-intruder test) | n.d. | n.d. | Normal | Normal | *Grn*$^{-/-}$ males hypoactive at first day of testing | Normal |
| | Grip strength | n.d. | n.d. | n.d. | n.d. | n.d. | Normal | n.d. |
| | Sensorimotor test | n.d. | n.d. | n.d. | n.d. | Normal except for inverted screen test (oldest age group) | n.d. | n.d. |
| Aggression, depression, and social behavior | Resident-intruder | Increased biting attacks | n.d. | n.d. | n.d. | Diminished social interaction, rather than increased aggression | No attacks of *Grn*$^{-/-}$ males, longer latency to first interaction; passive and disinterested | n.d. |
| | Tail suspension test | n.d. | n.d. | n.d. | Longer immobile | n.d. | Normal | n.d. |
| | Forced swimming test | n.d. | n.d. | n.d. | Longer immobile | n.d. | Normal | n.d. |
| | Social behavior test | Female-directed aggression | n.d. | n.d. | Progressive deficit starting from 1 month | Spent more time alone and follow intruder shorter | Reduced social interactions | n.d. |
| | Novel object recognition | n.d. | n.d. | n.d. | n.d. | n.d. | Absent in *Grn*$^{-/-}$ males | n.d. |
| | Passive avoidance | n.d. | n.d. | n.d. | n.d. | n.d. | Normal | normal |
| | Olfactory sensitivity test | n.d. | n.d. | n.d. | Normal (2 and 6 months)<br>Reduced (18 months) | Normal | n.d. | n.d. |

*n.d.* not determined

**Table 4** Neuropathological characteristics of *Grn*$^{-/-}$ mouse models

| Neuropathological characteristics | | Ahmed et al. [180] | Ghoshal et al. [181] | Yin et al. [114] | Yin et al. [186] | Petkau et al. [167] | Wils et al. [178] |
|---|---|---|---|---|---|---|---|
| General | Age groups | 1, 7, 12, and 23 months | 24 months | 18 months | 3, 12, and 18 months | 8 and 18 months | 6, 12, 16–18, and 21 months |
| | Neuronal loss | Not overt, but focal neuronal loss at 23 m | Nonsignificant reduction in neuron density in the dorsal thalamus | n.d. | n.d. | n.d. | Not observed |
| | Histological alterations | Vacuolation (habenular nucleus, CA2–3) | n.d. | n.d. | n.d. | Decreased dentritic length and spine density (apical dendritic arbor) | Morphological liver changes |
| | Apoptosis | No differences | n.d. | n.d. | n.d. | n.d. | No differences |
| Microgliosis | Effect | Progressive increase | Increased | Increased | Progressive increase | Increased | Progressive increase |
| | Significant from | 12 months | 24 months | 18 months | 12 months | 18 months | 12 months |
| | Brain area (s) | Cortex, hippocampus, thalamus, and brainstem | Hippocampus, cortex, and thalamus | Hippocampus, cortex, and thalamus | Hippocampus, cortex, and thalamus | Hippocampus | Hippocampus, cortex, and thalamus |
| | Marker | Iba-1 | Iba-1 | CD68 | CD68 | Iba-1 | Iba-1 and Ferritin |
| Astrogliosis | Effect | Progressive increase | Increased | Increased | Progressive increase | Increased | Progressive increase |
| | Significant from | 12 months | 24 months | 18 months | 12 months | 18 months | 12 months |
| | Brain area (s) | Cortex, hippocampus, thalamus, and brainstem | Hippocampus, cortex, and thalamus | Hippocampus, cortex, and thalamus | Hippocampus, cortex, and thalamus | Hippocampus | Hippocampus, cortex, and thalamus |
| | Marker | GFAP | GFAP | GFAP | GFAP | GFAP | GFAP |
| Ubiquitin | Effect | Progressive increase | Increased | Increased | Progressive increase | Qualitative increased | Progressive increase |
| | Significant from | 7 months | 24 months | 18 months | 12 months | n.d. | 6 months |
| | Brain area (s) | Hippocampus, thalamus, midbrain, and brainstem | Hippocampus and thalamus | Hippocampus and thalamus | Hippocampus | n.d. | Hippocampus, cortex, and thalamus |
| Lipofuscin | Effect | Increased | n.d. | n.d. | n.d. | Increased | Increased |
| | Significant from | 7 months | | | | 8 months | 16–18 months |
| | Brain area (s) | Hippocampus | | | | Hippocampus | Cortex, thalamus, and hippocampus |
| p62 | Effect | n.d. | n.d. | n.d. | n.d. | n.d. | Increased 7 months cortex, thalamus |
| | Significant from | | | | | | |
| | Brain area (s) | | | | | | |
| TDP-43 | Cytoplasmic TDP-43 | Negative | n.d. | n.d. | n.d. | Negative | Negative |
| | Aggregates (IHC) | Negative | Negative | n.d. | n.d. | Negative | Negative |
| | pTDP-43 IHC | Negative | Negative | Increased cytoplasmic reactivity | Progressive increased cytoplasmic reactivity | n.d. | Occasional |
| | pTDP-43 WB | n.d. | n.d. | n.d. | n.d. | n.d. | Increased insoluble pTDP-43 from 12 months |
| Other | β-amyloid, tau, α-synuclein, FUS | n.d. | Negative | n.d. | n.d. | n.d. | Negative |
| | Additional phenotypes | Decreased expression of serotonergic receptors after aggressive encounters [74] | None reported | Normal phagocytic capacity of BMDMS[a] | None reported | Reduced hippocampal synaptic connectivity | Abnormal regenerative and degenerative liver changes |
| | | | | Increased proinflammatory and decreased anti-inflammatory cyto- and chemokines | | Impaired long term potentiation | Increased expression of lysosomal proteases (brain and liver) |
| | | | | | | | Increased *IGF-1* mRNA (brain) |

*n.d.* not determined

[a] Kao et al. reported an increased phagocytic activity of *Grn*$^{-/-}$ macrophages [173]

reduced long-term potentiation) were reported in 10- to 12-month-old *Grn*$^{-/-}$ mice [167].

Another disease feature of GRN-associated FTD is the cytoplasmic mislocalisation of TDP-43 with concomitant nuclear clearing, abnormal phosphorylation and formation of nuclear and cytoplasmic TDP-43 positive aggregates [55, 57]. Histological studies were so far unsuccessful in detecting TDP-43 positive aggregates in brains of *Grn*$^{-/-}$ mice. While Yin et al. observed increased cytoplasmic reactivity to phosphorylated TDP-43 in hippocampal and thalamic areas [114, 186], other studies did not [167, 178, 180, 181]. Biochemically, *Grn*$^{-/-}$ brains did not show increased generation of TDP-43 CTFs [170, 178], but full-length phosphorylated TDP-43 was significantly increased from 12 months onwards in the insoluble fraction prepared from *Grn*$^{-/-}$ brains [178]. Absence of major pathological TDP-43 alterations like fragmentation and aggregation in *Grn*$^{-/-}$ mice suggests that these events might merely be a late event in the pathogenesis of FTD.

Besides consistent neuropathological changes in the brains of *Grn*$^{-/-}$ mice, other vital organs and processes are also negatively affected by Grn deficiency. For example, *Grn*$^{-/-}$ mice react with an exaggerated immune response to foreign pathogens and are less efficient in clearing bacterial infections resulting in prolonged inflammation [114]. While young *Grn*$^{-/-}$ mice did not show any morphological, hematological or biochemical abnormalities [114], the liver of aged *Grn*$^{-/-}$ mice showed increased signs of cellular ageing with abnormal hepatic and ductal morphology and significant upregulation of lysosomal proteases, like cathepsin D, in lysosomes within sinusoidal foamy histiocytes [178]. These findings, together with the recently reported biological role of Grn in energy homeostasis [100], call for the investment into more holistic approaches especially when studying deficiencies of multifunctional proteins such as GRN.

## Restoring GRN Function: A Way to a Successful Therapy

Recent advances in functional genomics have brought us a few steps closer to understanding the biological mechanisms involved in the pathogenesis of FTD. Cellular and animal models for GRN-associated FTD, have produced a tremendous amount of information, nourishing our hope that, if effectively translated into treatment opportunities, we would be able at some stage to delay or cure this devastating disease. However, despite recent advances, treatments for FTD are still lacking and only limited symptomatic treatment options are available [191].

## Targeting GRN Expression

Reduced GRN levels in biofluids like serum or CSF can be used as a reliable biomarker for the diagnosis and early detection of FTD caused by *GRN* mutations [192–194]. Because of the underlying haploinsufficiency mechanism, targeting or modifying GRN expression is assumed to be beneficial in preventing neurodegenerative diseases. Increasing Grn expression has already been shown to be advantageous in several animal models [173, 175–177, 195]. Modulating GRN expression by boosting the expression from the mutant or the wt allele might proof beneficial in delaying disease pathogenesis and could be a valid future therapeutic strategy. Stimulation of the mutant *GRN* allele by ribosomal read-through has been suggested as a worthwhile approach to be pursued in future FTD clinical trials [196]. Several compounds including ataluren (PTC124), aminoglycosides (e.g., gentamicin) and non-aminoglycosides have proven premature termination codon read-through activity in vitro and in vivo [197–199]. PTC124 has been shown to be safe and tolerable [198] and clinical trials for other diseases with genetic deficiency, like Duchenne muscular dystrophy and cystic fibrosis [200, 201] have been started.

An alternative strategy to normalize the levels of GRN could be to increase the expression and production from the wt allele. Increased transcription might be achieved by androgens as shown in the hypothalamus of neonatal rats [184], and administration of estrogen or selective stimulation of estrogen receptors in the brain could be considered a potential strategy for increasing GRN levels in the brain. A high-throughput screen of 1200 FDA approved drugs identified suberoylanilide hydroxamic acid (SAHA), a histone deacetylase (HDAC) inhibitor, as potent activator of GRN expression [202]. Other pan-HDAC inhibitors showed similar GRN stimulating effects and administration of SAHA was able to normalize the expression of GRN in cells derived from *GRN* mutation carriers [202]. Posttranscriptional mechanisms might also be involved in regulating GRN expression, and inhibitors of vacuolar ATPase and the FDA-approved alkalizing drugs chloroquin, bepridile, and amiodarone were shown to increase intracellular and secreted GRN protein levels via a translational mechanism independent of lysosomal degradation, autophagy or endocytosis [203]. Identification of protein disulfide isomerase family members as GRN interactors and modulators of GRN secretion [204], together with the observed inefficient posttranslational processing and secretion of GRN in neurons and microglia, suggested that modulation of the endoplasmatic reticulum chaperone network might be another potential therapeutic target to increase GRN expression [204].

Rediscovery of already approved drugs with the potential to modify GRN expression and production are considered valuable approaches to accelerate the process of establishing first generation drugs for FTD and related disorders. However, because of the role of GRN in promoting tumor growth, future studies will need to delineate the tolerated GRN levels and the potential adverse effects of increasing or administering GRN over a longer period of time [196].

## Targeting GRN Receptors

GRN is a secreted growth factor stimulating both growth and survival signals in various cell types. Recently, sortilin has been identified as the first neuronal receptor for GRN [107] and is thought to be involved in controlling the extracellular levels of GRN via receptor-mediated endocytosis [42, 107]. Molecules or compounds that could selectively interfere with the GRN/sortilin interaction and thus raise the levels of extracellular GRN could therefore be considered as potential candidates for future clinical and preclinical trials for FTD. Although the idea of raising GRN levels through compounds that modify GRN/sortilin interaction is intriguing, first the question needs to be addressed whether sortilin is only involved in GRN clearance or also in survival signaling.

In addition, GRN can bind with high affinity to the TNF receptors (TNFR1 and TNFR2) [113] and hence acquired a therapeutic potential in inflammatory conditions such as rheumatoid arthritis [113] or acute respiratory distress syndrome [150]. A modified hybrid granulin peptide (named ATSTTRIN) was even more effective than full-length GRN in diminishing the pro-inflammatory signaling cascade elicited by TNF-α. Neurodegenerative diseases, including FTD, have a strong neuroinflammatory component and it will therefore be important to test if administration of GRN or hybrid granulin peptides are beneficial in delaying the disease pathology. It is likely that additional receptors or combination of receptors are involved in GRN-mediated prosurvival signaling and their identification will hopefully provide additional points of entry for possible therapeutic interventions.

## GRN and the Serotonergic System

The serotonergic system is important for behavioral modulation [205] and decreased serotonin receptor binding has been reported in affected brain areas in autopsied FTD patients [3]. Alterations in the serotonergic system were also recapitulated in *Grn*$^{-/-}$ mice, which have reduced expression of the serotonergic receptor 5-HT1A in the hippocampus after an aggressive encounter [75]. The exact role that GRN plays in modulating the serotonergic system is not well characterized, but it has been suggested to relate to the organization and/or activation of the serotonergic system in the brain [75]. In line with this, some studies using selective serotonin-reuptake inhibitors or 5-HT receptor agonists to treat behavioral deficits in FTD patients showed efficacy of the treatment [206, 207].

## GSK3β and Wnt Signaling

GSK3β is an enzyme regulating many cellular functions including cellular structure and survival [166]. Deregulation of GSK3β is linked to several common pathological conditions, including diabetes and AD [166]. GSK3β is known to be involved in phosphorylating tau and clinical studies are evaluating the effects of GSK3β inhibitors like lithium chloride and valproic acid on AD pathogenesis [208]. Data obtained from the cellular models of GRN deficiency suggest that GRN is, at least to some extent, exerting its neurotrophic and neuroprotective function via regulating GSK3β phosphorylation [91, 162]. Interestingly, hnRNPs have also been shown to belong to the many substrates of GSK3β [166], but whether GSK3β also contributes to pathological TDP-43 phosphorylation is not known. While further data on this subject are awaited, *Grn*$^{-/-}$ mice showed a general increase in the phosphorylation state of proteins [178], and one could hypothesize that this could be caused by alterations in the activity of GSK3β.

Additionally, GRN loss has recently been implicated in altering the expression of FZD2, a receptor involved in the noncanonical Wnt signaling pathway [163]. This upregulation of FZD2 is thought to be neuroprotective and thus compensating for the lost GRN function. Manipulation of the expression of the FZD2 receptor or one of its downstream effectors through compounds with agonistic functions could therefore be considered as a potential future therapeutic strategy for GRN-associated FTD [163].

## Modulating Conversion of Grn to Granulins

Many neurodegenerative diseases, including FTD, are characterized by considerable neuroinflammation [209]. GRN and its granulin peptides are critically involved in regulating inflammatory reactions through the conversion of anti-inflammatory GRN to pro-inflammatory granulins. Several proteinases such as neutrophil elastase, proteinase-3 and some metalloproteinases are involved in this process, which can be inhibited for example by the binding of SLPI to GRN [115]. *Grn*$^{-/-}$ mice show a predominant proinflammatory response [114] and micro- and astrogliosis are one of the most consistent pathological features of *Grn*$^{-/-}$ mice (see Table 4), most likely caused by the loss of full-length GRN. Pathogenic cysteine mutations also affect the processing of GRN into granulins [93] thereby potentially shifting the balance towards a proinflammatory response. Modulating the activity of GRN converting enzymes or increasing the expression of inhibitory proteins might therefore be considered as potential strategy to increase the GRN/granulin ratio. While topically applied SLPI peptides have already been tested to treat impaired wound healing in elderly people, systemic administration of proteinase inhibitors like SLPI might cause severe side effects as overexpression of SLPI has been associated with several forms of cancers [210]. The few existing studies evaluating the effect of proteinase inhibitors on neuronal survival report beneficial effects of a neutrophil elastase inhibitor in attenuating MN death or hippocampal neuronal damage after ischemic insults [211–213]. Whether these neuroprotective effects could be driven by stabilization of GRN was however not

investigated. These initial results clearly encourage further studies investigating the neuroprotective effects of proteinase inhibitors and their potential for attenuating the neuropathology associated with GRN deficiency.

## Conclusions

Deregulation of GRN is critically involved in cancer and neurodegeneration, two of the major pathological conditions our aging population has to face today. GRN deficiency is associated with FTD, a neurodegenerative condition for which currently no pharmacological treatment to cure or delay its progression is available. The generation and characterization of GRN cellular and animal models, as highlighted in this review, has been essential in increasing our knowledge about the diverse biological functions of GRN over the last couple of years. However, many biological functions of GRN are still poorly understood and the described models will help us to gain further insight into the GRN biology. Generating novel and ongoing characterization of the existing GRN models will hopefully provide us soon with enough mechanistic information to enable us to translate our findings into novel therapeutic strategies for neurodegenerative diseases related to GRN deficiency.

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