Missense Mutations in the Progranulin Gene Linked to Frontotemporal Lobar Degeneration with Ubiquitin-immunoreactive Inclusions Reduce Progranulin Production and Secretion*

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Loss of function mutations in progranulin cause tau-negative frontotemporal lobar degeneration with ubiquitin-positive inclusions. A major protein component of these inclusions is TDP-43, which becomes hyperphosphorylated, ubiquitinated, and cleaved to generate C-terminal fragments, which apparently translocate from nuclei to the cytoplasm. Most progranulin mutations are nonsense mutations resulting in nonsense-mediated mRNA decay and consequently reduced progranulin protein levels. However, some missense mutations are described that occur within the signal sequence and mature progranulin. Thus apparently all progranulin mutations cause reduced protein expression or secretion, although by different cellular mechanisms. To investigate a putative relationship between reduced expression of progranulin and TDP-43 relocalization and deposition, we down-regulated progranulin in human cell lines and in zebrafish. Upon reduction of progranulin, neither a major redistribution of TDP-43 nor proteolytic processing to disease-characterizing C-terminal fragments could be observed.

Dementias are a major health problem in our aging society. The most frequent forms of dementia, namely Alzheimer disease, frontotemporal lobar degeneration (FTLD), as well as dementia with Lewy bodies and related disorders are associated with selective neuronal cell loss. In these neurodegenerative disorders, proteins, which are normally soluble are known to misfold because of proteolytic processing and/or abnormal posttranslational modifications. Such insoluble amyloidogenic proteins are often deposited and may form reservoirs for neurotoxic oligomers (1). FTLD, which accounts approximately for 15% of all dementias, is characterized by two different types of cellular inclusions. About 40% of FTLD cases have tau-positive inclusions (2, 3). Genetic linkage led to the identification of more than 40 different mutations in the microtubule-associated protein tau gene locus on chromosome 17 (4). However, a number of familial FTLD cases failed to exhibit mutations in the tau gene, although strong linkage to chromosome 17 was observed (5). These cases were characterized by tau- and α-synuclein-negative, ubiquitin-positive cytoplasmic and intranuclear inclusions (3). The inclusions are observed in the frontotemporal cortex, the temporal neocortex, and the hippocampus and define the frontotemporal lobar degeneration with ubiquitin-immunoreactive inclusions (FTLD-U). FTLD-U is the most frequent neuropathological form of FTLD and presents with progressive social, behavioral symptoms, and language dysfunction. Patients may also develop typical symptoms of motoneuron diseases. Most recently, the major protein component of the ubiquitin-positive inclusions was identified as TDP-43 (the TAR DNA-binding protein of 43 kDa) (6). Insoluble, hyperphosphorylated, and proteolytically processed C-terminal fragments of the nuclear TDP-43 were found as the disease-characterizing signature and shown to accumulate in most FTLD-U cases within the cytoplasm of neurons (7–11). TDP-43 has initially been identified as a DNA-binding protein (12). In addition TDP-43 contains two RNA recognition motifs and is able to bind UG repeats in RNA (13). Consistent with its putative RNA binding properties TDP-43 may also be part of a protein complex involved in RNA splicing (13–16). Based on

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3 The abbreviations used are: FTLD, frontotemporal lobar degeneration; FTLD-U, FTLD with ubiquitin-immunoreactive inclusions; PBS, phosphate-buffered saline; siRNA, small interfering RNA; RT, reverse transcription; wt, wild type; dpf, day(s) post-fertilization; RNAi, RNA interference; TDP-43, TAR DNA-binding protein of 43 kDa; PGRN, progranulin.
these putative nuclear functions, the deposition of TDP-43 within the cytoplasm in FTLD-U and its corresponding absence within nuclei appears to be rather surprising, raising the possibility that some essential normal function of TDP-43 may be lost in FTLD-U.

Chromosome 17-associated familial FTLD-U cases are linked to PGRN (progranulin), a gene located close to the microtubule-associated protein tau gene locus (2, 17). PGRN is a secreted protein that probably serves as a growth factor (18, 19) but is not a component of the pathological inclusions in FTLD-U. It contains seven and a half granulin domains composed of 12 cysteine-rich tandem repeat motifs (see also Fig. 2A). PGRN exists as a secreted full-length protein, which can be converted into several granulins via proteolytic processing by elastase (20). PGRN may act as a wound-healing factor, although the granulins and the full-length PGRN may have opposite functions in wound repair and inflammation (21). Interestingly, in Alzheimer disease, PGRN is up-regulated in defense response to neuritic degeneration. Most of the PGRN mutations apparently cause a loss-of-function because of nonsense-mediated mRNA decay (2, 17). Some missense mutations occur within the native PGRN (22, 23) and one mutation within its signal sequence (24). Whether these mutations affect PGRN function, and expression is currently not known. Patients with PGRN mutations develop TDP-43-positive inclusions (6, 11, 25, 26). Because the PGRN gene was associated with many cases of FTLD-U, and all of these cases show TDP-43-positive inclusions (6, 11, 25, 26), it is tempting to propose that TDP-43 and PGRN deficiency may be responsible for TDP-43 mislocalization, deposition, and proteolytic fragmentation. To prove this hypothesis, we first investigated the consequences of three missense mutations on PGRN expression and transport. In addition we down-regulated PGRN in human cell lines and in zebrafish and searched for a change in subcellular localization and processing of TDP-43.

**MATERIALS AND METHODS**

**Antibodies**—For the detection of TDP-43 and PGRN by immunoblotting, the following antibodies were used: a rabbit polyclonal anti-TDP-43 antibody (TARDBP; Proteintech Group, 1:2000) and a rabbit polyclonal anti-PGRN antibody (PCDF antibody 40–3400; Zymed Laboratories, 1:250). Myc-tagged PGRN was detected with the anti-Myc antibody 9E10 (Santa Cruz). β-Actin was detected using the mouse monoclonal antibody (A-5316; Sigma, 1:2000). Immunocytochemistry was performed with the following antibodies. For the detection of TDP-43, a mouse monoclonal (TARDBP 2E2-D3; Abnova, 1:200) and a rabbit polyclonal (Proteintech Group, 1:200) antibody were used. PGRN was detected by a rabbit polyclonal (PCDF antibody 40–3400; Zymed Laboratories, 1:100) and a mouse monoclonal (MAB2420; R & D Systems, 1:500) anti-PGRN antibody. For endoplasmic reticulum labeling a monoclonal anti-BIP antibody was used (SPA-827; StressGene, 1:200) and for Golgi staining, an anti-giantin antibody was used at 1:1000 as described before (27).

**Cell Culture and Immunoblotting**—Human cervical carcinoma (HeLa) cells were cultured in Dulbecco’s modified Eagle’s medium with Glutamax I (Invitrogen). All of the media were supplemented with 10% fetal calf serum (Invitrogen) and penicillin/streptomycin. The transfections were carried out as described by the manufacturer using Lipofectamine 2000 (Invitrogen). 48 h post-transfection, the cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 1% Nonidet P-40, 0.05% Triton X-100, 0.5% sodium deoxycholate, 2.5 mM EDTA) supplemented with 1× protease inhibitor mix (Sigma) and phosphatase inhibitors (100 mM NaF, 10 mM sodium pyrophosphate, and 1 mM sodium vanadate). The insoluble radioimmune precipitation assay pellets were subject to urea extraction as described (6). For SDS extracts cell pellets were directly lysed in SDS-PAGE sample buffer.

The proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. For detection, the indicated antibodies were used. Bound antibodies were visualized by horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence technique (Amersham Biosciences).

**Tunicamycin Treatment**—To block the glycosylation of progranulin, the cells were treated with 10 μg ml−1 tunicamycin for 5 h. The samples were separated on an SDS gel and analyzed by immunoblotting using the monoclonal anti-progranulin antibody.

**Metabolic Labeling and Immunoprecipitation**—To analyze expression and maturation of PGRN, HeLa cells were starved for 1 h in methionine-, cysteine-, and serum-free minimal essential medium (Invitrogen) and subsequently metabolically pulse-labeled with 300 μCi of [35S]methionine/cysteine (Pro-mix; Amersham Biosciences) in methionine-, cysteine-, and serum-free medium for 15 or 30 min as indicated and chased in the presence of excess amounts of unlabeled methionine for the indicated time points. PGRN was immunoprecipitated from medium and cell lysates using the monoclonal anti-progranulin antibody. Quantification was performed by phosphorimaging (PhosphorImager; Molecular Dynamics).

**Immunohistochemistry**—HeLa cells were grown on polylysine-coated coverslips, fixed for 20 min with 4% paraformaldehyde and 4% sucrose in PBS, permeabilized for 10 min with 0.2% Triton in PBS with 50 mM NH₄Cl, and subsequently blocked for 1 h with 5% bovine serum albumin/PBS. The cells were then double stained with indicated mouse monoclonal and rabbit polyclonal antibodies for 2 h at 37°C. After washing repeatedly with PBS, the cells were incubated with Alexa 488 and Alexa 555 (Invitrogen) coupled secondary anti-mouse or anti-rabbit antibodies as indicated at 1 h at 37°C. Subsequently the cells were washed with PBS, and the nucleus was labeled using TopRo-3 (monomeric cyanine nucleic acid stain, 1:1000; Invitrogen). The coverslips were mounted on glass slides using Mowiol (Hoechst) supplemented with 0.5% DABCO (Sigma), and the images were obtained using Zeiss confocal laser scanning microscope. Fish embryos were fixed in 4% paraformaldehyde for 24 h at 4°C and then paraffin-embedded. 5-μm sections were cut and used for hematoxylin-eosin staining and immunohistochemistry. For TDP-43 immunohistochemistry using rabbit polyclonal anti-TDP-43 antibody (1:2000), antigen retrieval was performed by boiling the sections in 10 mM citrate buffer (pH 6.0) in a microwave oven (3 ×
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5 min. Immunohistochecmistry was carried out using the avidin-biotin complex detection system (Vector Laboratories) and 3,3-diaminobenzidine as chromogen.

**cDNA Constructs**—cDNA constructs encoding human PGRN (clone IRATp970H1136D6, from RZPD) were subcloned into the EcoRV and Not1 sites of the pcDNA 3.1 (+) hygro expression vector (Invitrogen). Myc-tagged PGRN wt or PGRN A9D was generated by PCR and subcloned into the BamHI and XhoI sites of pcDNA 4 Myc-his (B) expression vector (Invitrogen). The PGRN point mutations were introduced by site-directed mutagenesis (Stratagene) and verified by DNA sequencing.

**Transient siRNA Knockdown of PGRN**—The PGRN knockdown in HeLa cells was achieved using Ambion Silencer pre-designed siRNAs 144869, 10849, and 11032. Nontargeting siRNA negative control 7 was used to assess unspecific effects of designed siRNAs 144869, 10849, and 11032. Nontargeting siRNA using Lipofectamine 2000 and analyzed 48 h post transfection.

**Animal Husbandry**—All of the experiments were performed in compliance with the guidelines of the German Council on Animal Care. Zebrafish were maintained, mated, and raised as described (28). The embryos were kept at 28 °C and staged as described (29). The wild type line AB was used for all of the experiments.

**In Situ Hybridization**—In situ hybridizations were carried out as described elsewhere (30) with slight modifications. The single-stranded RNA probes were labeled with digoxigenin-UTP (Roche Applied Science). All of the washing steps were carried out at 65 °C. The washes are as follows: twice for 30 min in 50% formamide, 2× SSCT (standard saline citrate + 0.1% Tween); once for 15 min in 2× SSCT, twice for 30 min in 0.2× SSCT. To reduce endogenous phosphatase activity, 0.25 mg ml−1 levamisole was added to the staining buffer.

**Design of Antisense gripNAs and Microinjection of Zebrafish Embryos**—Rhodamine-labeled antisense gripNA of 18 nucleotides in length targeting zebrafish granulinB was obtained from active motif. The grip was designed against the exon-intron junction between exon 6 and intron 6–7. The underlined nucleotides refer to the start GT of intron 6–7; granulinB GT-grip, 5′-TATGTTGATGCACCTTTGG-3′. The embryos were microinjected using a Femto Jet Microinjector (Eppendorf) at the one-cell stage with gripNA concentration of 1.0 μM. The injected volume was 2–4 pl.

**RT-PCR**—Zebrafish embryos injected with granulinB GT-grip and wt controls were fixed in liquid nitrogen after the following time points: 14–15 hours post-fertilization and 1, 2, 3, 4, 5, and 6 dpf. Total RNA was prepared using the RNAeasy Kit (Qiagen). cDNA was prepared from the RNA using the high capacity cDNA reverse transcription kit (Applied Biosystems). PCRs were carried out using the cycle conditions starting with 94 °C for 2 min, followed by 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min in 35 cycles, and a final extension at 72 °C for 5 min. The following primers were used: zfGRNBex4For (P1), 5′-GTGTGCTGTGATAGGAAACACT-3′; zfGRNBex6AFor (P2), 5′-TCCAGATAAGAATTTCCAATGTTCC-3′; zfGRNBex7Arev (P3), 5′-AGAACAGTGCAGCTGGGATTT-3′; zfActinFor, 5′-TATGTGATGCACCTTTGG-3′; zfActinrev, 5′-TTCTCCTTGATGTCATCACGGAC-3′; zfTDP-43exon3F1, 5′-GCCAGATATAAAGAGGAAGATGGA-3′; and zfTDP-43exon4R1, 5′-CCGCCTGTGTCATCACTCTTAC-3′.

**RESULTS**

**Expression of Endogenous PGRN and TDP-43 in Cultured Cells**—We first investigated the expression of endogenous wt PGRN and TDP-43 in HeLa and HEK 293T cells. In cell lysates PGRN was found predominantly to be an immature species (Fig. 1A). Consistent with this finding, the secreted PGRN species have a higher molecular weight (Fig. 1A), reflecting mature glycosylated PGRN (31). The low abundance of mature PGRN within cell lysates suggests rapid and efficient secretion. In the same lysates endogenous TDP-43 was detected as a 43-kDa protein (Fig. 1B). As expected no TDP-43 was found in cell lysates. 50 μg of protein was loaded for each sample.

**FIGURE 1. Cellular expression of endogenous PGRN and TDP-43.** A, lysates and conditioned media of indicated cell lines were investigated for PGRN expression by immunoblotting. Note that PGRN occurs as two variants, probably reflecting immature (arrowheads) and fully mature glycosylated species (arrow). The mature species accumulates in conditioned media. The bottom panel shows a lower exposure of the upper panel. B, TDP-43 is expressed as a protein with an apparent molecular mass of 43 kDa. As expected, TDP-43 is only found in cell lysates. 50 μg of protein was loaded for each sample.

|    | 293T | HeLa |
|----|------|------|
| L  | 98   | 98   |
| M  | 64   | 64   |

**TDP-43**

TGGTTTCCCTCATTGTGGG-3′; zfActinrev, 5′-TTCTCCTTGATGTCATCACGGAC-3′; zfTDP-43exon3F1, 5′-GCCAGATATAAAGAGGAAGATGGA-3′; and zfTDP-43exon4R1, 5′-CCGCCTGTGTCATCACTCTTAC-3′. Primers P1/P3 are located on exons 4 and 7, respectively, and amplify a PCR product of 404 bp (base pairs). Primers P2/P3 are located on exon 6 and 7, respectively, and amplify a PCR product of 214 bp. zfActin primers amplify a PCR product of 550 bp, whereas zfTardbp (zfTDP) amplify a PCR product of 262 bp on cDNA and 457 bp on genomic DNA.
exchange only single amino acids (22–24). We now investigated the consequences of three such missense mutations on PGRN expression and cellular transport. These mutations included the A9D mutation within the signal sequence (24), the P248L and the R432C mutations in mature PGRN (22) (see schematic presentation in Fig. 2A). cDNAs encoding wt PGRN or the three mutations were transiently transfected into HeLa cells. The cells were pulse-labeled with [35S]methionine for 30 min and then chased in excess amounts of unlabeled methionine for 1 h. The cell lysates and conditioned media were then immunoprecipitated with a monoclonal antibody directed against PGRN. The PGRN P248L and R432C mutations were expressed during the pulse and detected predominantly as immature proteins like the wt PGRN species (Fig. 2B). However, after a 1-h chase period significantly less mature PGRN P248L and R432C was observed in conditioned medium as compared with wt (Fig. 2B). Quantification of three independent experiments revealed an ~70% decrease of secretion for PGRN P248L and an ~45% reduction of secretion for PGRN R432C in comparison with wt PGRN (Fig. 2C). In contrast, the cells expressing PGRN A9D showed little protein expression. Only upon a long exposure immature PGRN (probably corresponding to endogenous PGRN) and a faster migrating protein species (probably corresponding to PGRN A9D) could be detected (Fig. 2B, for further analysis of PGRN A9D, see Fig. 3).

The cellular mechanisms of reduced PGRN secretion upon expression of PGRN P248L and PGRN R432C were further investigated in pulse-chase experiments. To label a defined PGRN population, the pulse period was reduced to 15 min, and multiple chase points were analyzed to monitor PGRN release into the medium. This revealed a delay and significant decline in secretion of mutant PGRN over time as compared with wt PGRN (Fig. 2, D and E). Moreover, mutant PGRN was not only secreted at a much slower rate, but cellular mutant PGRN compared with wt PGRN was also subject to enhanced degradation (Fig. 2F).

The PGRN A9D Missense Mutation Affects Its Cellular Localization and Expression—The above described results may suggest that the PGRN A9D mutation, which occurs within the signal peptide, affects the subcellular localization and expression of the mutant protein. To prove whether the lower molecular weight species detected in Fig. 2B (long exposure) represents low abundant PGRN A9D, which may fail to be glycosylated because of missorting to the cytosol, cells expressing endogenous PGRN or PGRN A9D were treated with tunicamycin. Tunicamycin inhibits N-glycosylation by blocking the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichyl-P. In cells expressing PGRN A9D, an additional low molecular weight band was observed as compared with vector transfected cells (Fig. 3A), which most likely reflects a low abundant PGRN A9D-derived protein species. Tunicamycin treatment had no effect on the migration behavior of this protein in SDS gels. In contrast, following tunicamycin treatment, the higher molecular weight endogenous PGRN observed in vector control transfections and in the PGRN A9D-transfected cells migrated to a lower molecular weight similar to that of the PGRN A9D (Fig. 3A). Thus PGRN A9D appears to be mis-sorted to the cytosol, where it fails to undergo N-glycosylation. To facilitate the selec-

![FIGURE 2. PGRN missense mutations are impaired in secretion.](image-url)
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detective detection of PGRN A9D, the mutant protein was Myc-tagged. Upon transfection of PGRN A9D<sup>myc</sup>, only one protein band migrating at lower molecular weight than wt<sup>myc</sup> PGRN was observed by immunoblotting with an anti-Myc antibody (Fig. 3B). Tunicamycin treatment did not affect the migration of this species, whereas tunicamycin treatment of cells transfected with wt<sup>myc</sup> PGRN resulted in the expected molecular weight shift (Fig. 3B). Note that the molecular weight of PGRN A9D<sup>myc</sup> is slightly higher than deglycosylated wt<sup>myc</sup> PGRN, probably because of the lack of signal peptide removal. Thus PGRN A9D indeed fails to be N-glycosylated, suggesting that it is missorted into the cytosol. Furthermore, the extremely low expression levels suggest that PGRN A9D also fails to be efficiently translated and/or is rapidly degraded.

**Subcellular Localization of Mutant and wt PGRN and TDP-43**—To determine the precise subcellular localization of TDP-43 and PGRN and to investigate whether PGRN mutations affect TDP-43 localization, we performed an immunocytochemical analysis. Endogenous TDP-43 was observed within nuclei (Fig. 4A, top panel). Nucleoli apparently lack TDP-43 (Fig. 4A, top panel). In contrast PGRN was observed within a reticular- and Golgi-like compartment (Fig. 4A, top panel). Double immunocytochemistry using maker antibodies for the endoplasmic reticulum (Fig. 4A, middle panel) and the Golgi (Fig. 4A, bottom panel) revealed co-localization of PGRN with both marker proteins. The PGRN mutants P248L and R432C showed a very similar cellular distribution like wt PGRN (Fig. 4, B and C). Consistent with the Western blot analysis (above described data) (Figs. 2 and 3), expression of PGRN A9D was below the detection limit by immunocytochemistry (data not shown). The subcellular localization of TDP-43 was not affected upon transfection of the PGRN mutants P248L (Fig. 4B, top panel) or R432C (Fig. 4C, top panel).

Taken together with the biochemical analysis, these data demonstrate that wt and mutant PGRN and TDP-43 are not co-expressed in any subcellular compartment excluding a direct interaction. Moreover, at least in the background of endogenous PGRN, the PGRN mutants do not affect the nuclear localization of TDP-43.

**Acute Down-regulation of PGRN Does Not Selectively Affect Proteolytic Processing of TDP-43 in Cultured Cells**—Because familial FTLD-U is characterized by a loss of PGRN and a concomitant accumulation of TDP-43-positive cytoplasmic deposits.

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**FIGURE 3.** PGRN A9D fails to undergo maturation. A, cells transiently transfected with PGRN A9D and vector control were treated with tunicamycin at 10 μg/ml for 5 h. The lysates of treated and untreated cells were analyzed for PGRN expression by immunoblotting. Upon tunicamycin treatment endogenous PGRN shifts to a lower molecular weight, whereas no change of the additional lower molecular weight species occurring in PGRN A9D transfected cells can be observed. 25 μg of protein was loaded for each sample. B, to verify the lower migrating species as PGRN A9D and to analyze selectively its maturation PGRN A9D was Myc-tagged. Cells transiently transfected with PGRN A9D<sup>myc</sup>, wt<sup>myc</sup> PGRN, and vector control were treated with tunicamycin as described above. Because of the low expression level of PGRN A9D 50 μg of protein was loaded as compared with 10 μg of wt PGRN. Note that PGRN A9D<sup>myc</sup> is expressed as a single tunicamycin-resistant protein, marked with an asterisk. Note that the molecular weight of A9D is lower than that of glycosylated wt PGRN but higher than nonglycosylated (non-glyc.) wt PGRN, probably because of the attached signal peptide.

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**FIGURE 4.** Subcellular localization of PGRN, PGRN variants, and TDP-43. Immunofluorescence was carried out using HeLa cells expressing endogenous TDP-43 and transfected wt PGRN (A), PGRN P248L (B), and PGRN R432C (C). The top panels show endogenous TDP-43 staining with the monoclonal anti-TDP-43 antibody and PGRN staining detected with the rabbit polyclonal anti-PGRN antibody. wt PGRN and mutant PGRN are localized within a reticular cytoplasmic compartment and do not affect the nuclear localization of TDP-43. For a more exact determination of the wt and mutant PGRN distribution, co-staining with an antibody against the endoplasmic reticulum marker BIP (middle panel) or a Golgi staining with an anti-giantin antibody (bottom panel) was performed. Nuclei are labeled with ToPro-3 in blue (A). Scale bar, 10 μm.
its, we next down-regulated endogenous PGRN using three independent RNAi. Immunoblotting revealed that all three efficiently reduced PGRN expression (Fig. 5A). Efficient down-regulation of PGRN may mimic its loss of function in FTLD-U and result in the formation of insoluble TDP-43 and its disease-defining C-terminal fragments. We therefore extracted the cell lysates with radioimmune precipitation assay buffer, urea, and SDS. As an additional control we loaded urea extracts from brain lysates of a FTDL-U patient (6). Specifically in the SDS extracts but also within the urea extract, we obtained an ~36-kDa fragment of TDP-43. However, this fragment was observed in both, control cells and RNAi (144869)-treated cells (Fig. 5B). In addition small amounts of a lower molecular weight fragment were also detected in the SDS extract; however, these were found again independent of the down-regulation of PGRN (Fig. 5B). These fragments may therefore present breakdown products that are not related to the disease-defining proteolytic fragments. This may also be supported by their failure to selectively accumulate within the urea-extractable material (Fig. 5B).

Acute Down-regulation of PGRN Does Not Affect the Subcellular Localization of TDP-43 in Cultured Cells—To prove whether down-regulation of PGRN causes a redistribution of TDP-43 into the cytoplasm as observed in FTLD-U cases, immunocytochemical experiments were performed. As shown in Fig. 5C, cells mock-transfected, transfected with a negative control RNAi or transfected with the most efficient RNAi (#144869) showed no difference in the subcellular localization of TDP-43. In all cases TDP-43 was found within nuclei (Fig. 5C).

Developmental and Subcellular Expression of the TDP-43 Orthologue in Zebrafish—We next investigated whether a reduction of PGRN in an animal model is sufficient for redistribution and deposition of TDP-43. We chose zebrafish to efficiently down-regulate PGRN and to follow the subcellular distribution of TDP-43. In zebrafish, there are two orthologues of human TDP-43, referred to as Tardbp (TAR DNA-binding protein) and Tardbpl (TAR DNA-binding protein-like). Tardbp displays 72% identity on the amino acid level to human TDP-43, whereas Tardbpl only shows 56% identity (Fig. 6A). Interestingly, Tardbpl is lacking the C-terminal domain of the protein reminiscent of splice variants of human TDP-43 (Fig. 6A) (32). RT-PCR analysis of Tardbp and Tardbpl

![Image](https://example.com/image.png)
void of Tardbp/Tardbpl, no cytoplasmic Tardbp/Tardbpl staining was observed (Fig. 7).

**Acute Down-regulation of PGRN Does Not Affect the Localization of Tardbp**—Mammalian PGRN is represented in zebrafish by two co-orthologues, granulinA and granulinB (31). Whole mount in situ hybridization revealed that granulinB is the orthologue expressed in the zebrafish brain, whereas granulinA is restricted largely to the hematopoetic lineage during early embryonic development (31). GranulinB and Tardbp/Tardbpl are co-expressed in zebrafish brain during development (33). We have targeted granulinB for knockdown in zebrafish using antisense gripNA technology and asked whether we can observe changes in Tardbp/Tardbpl nuclear distribution (Fig. 8, A–C). GranulinB in zebrafish consists of 13 exons. The rhodamine-labeled granulinB GT-grip (referred to as grnB GT), targets the exon-intron junction between exon 6 and intron 6–7 (Fig. 8D). No obvious morphological phenotype was observed in grnB GT-injected embryos, despite the uniform distribution of the rhodamine label of the grip within the embryo (Fig. 8A). Effectivity of the grnB GT-mediated knockdown was monitored by RT-PCR and showed a clear interference with proper splicing of the granulinB pre-mRNA. RT-PCR from grnB GT injected embryos showed the appearance of new bands, reflecting inappropriate splicing at the splice donor site.
which is masked by the GT-grip (Fig. 8E). The grnB GT leads to a partial knockdown in zebrafish embryos, because a weaker wild type RT-PCR band is still visible in the grip-injected embryos. However, a partial reduction of PGRN secretion is sufficient to cause the disease as suggested above (Fig. 2E). Brains of grnB GT-injected fish were further analyzed for subcellular localization of Tardbp/Tardbpl at 3 dpf (Fig. 9, A–D). A clear overlap of brown Tardbp/Tardbpl staining and the blue-stained nuclei was observed in numerous nuclei. We never observed Tardbp/Tardbpl staining in the cytoplasm nor a marked reduction of the nuclear staining in the knockdown embryos. This suggests that acute reduction of granulinB does not affect the subcellular localization of Tardbp/Tardbpl in zebrafish, a result consistent with our data in cell culture (Fig. 5).

**DISCUSSION**

PGRN and TDP-43 are two proteins associated with FTLD-U (2, 6, 17). Full-length TDP-43 and its proteolytic fragments, which are hyperphosphorylated and translocated to the cytosol, are deposited and appear to be the proteinaceous disease signature (6). Aberrant translocation, processing, and abnormal phosphorylation of TDP-43 appears to occur in all FTLD-U cases including those with mutations in PGRN and the valosin-containing protein (6, 7, 26). Whether mutations in PGRN drive TDP-43 translocation and abnormal post-translational processing is currently unknown. Similarly, a major question is whether all PGRN mutations generally result in a loss of function.

In this study we addressed these two major questions. First we focused on the expression of PGRN missense mutations. Most PGRN mutations cause frame shifts or premature terminations or affect initiation of translation in case of mutations within the Kozak consensus sequence (2, 17, 25, 34–36). These mutations result in a reduction of the PGRN protein levels because of nonsense-mediated mRNA decay (2, 17). Haploin-
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sufficiency is therefore discussed as a major pathogenic mechanism caused by PGRN mutations. However, several missense mutations were identified as well (22–24) (Fig. 2A). It has been speculated that these mutations may change the structure and function of PGRN (22), but in vivo consequences were unknown. It is very important to address this question by providing direct experimental evidence, because a common mechanism of all PGRN mutations would shed light on the pathological pathways of FTLD-U. We therefore selected the PGRN A9D (24), P248L, and R432C (22) mutations and analyzed their expression, maturation, and cellular trafficking. The PGRN A9D mutation occurs within the hydrophobic signal sequence of PGRN and may therefore affect its co-translation into the endoplasmic reticulum (24). Indeed we found that this protein fails to undergo N-glycosylation, suggesting its mislocation to the cytosol. Moreover, the nonglycosylated PGRN A9D protein is expressed at extremely low levels, probably because of inefficient translation or degradation. In contrast, the two other PGRN variants containing missense mutations in the mature protein showed significant expression levels upon transient transfection in HeLa cells. However, both mutant variants failed to be efficiently transported through the secretory pathway as compared with wt PGRN. In addition both PGRN mutants are also degraded during their passage through the secretory pathway. Consequently a significant reduction of their secretion was observed. Interestingly, both mutants may differ in their reduction of PGRN secretion. The PGRN P248L mutant reduces secretion by about 70%, whereas the PGRN R432C mutant shows a weaker effect and reduces secretion only by about 45%. Moreover, at least the mutations, which affect the native protein, show a weaker effect on secretion than all nonsense mutations and probably the A9D mutation, which which reduce expression and therefore secretion of PGRN by almost 100% (per affected allele). Whether this difference affects the age of onset of these patients with nonsense or missense mutations remains to be investigated. However, this finding may indicate that a rather modest restoration of PGRN levels in patients could be sufficient to prevent disease onset or progression. The fact that PGRN P248L and PGRN R432C are expressed but fail to be secreted efficiently suggests that specifically the secreted species of PGRN is physiologically important, although we cannot exclude the possibility that misfolded PGRN lost an intracellular function. Our hypothesis is supported by the findings that PGRN may have an important function upon secretion (21). Secreted PGRN is involved in wound healing (19–21). PGRN may therefore be required to stabilize neurons, which are injured by deposited proteins, such as TDP-43. In fact, PGRN was shown to be up-regulated in activated microglia around amyloid plaques (2), again suggesting a protective function. Furthermore PGRN promotes growth and survival of PC12 cells (37) and is involved in estrogen-induced neurogenesis (38), which also suggest a function as a growth factor. A growth promoting function of secreted PGRN is also supported by the involvement of PGRN in tumor progression and invasion (20, 39–42). One may therefore suggest a more general protective role of secreted PGRN for stressed neurons, which are injured by cytoplasmic depositions of not only TDP-43, but also other proteins such as tau, α-synuclein, or extracellular amyloid β-peptide. In that regard it may be interesting to investigate whether the loss of PGRN may be a risk factor for neurodegenerative disorders other than FTLD-U. Based on our findings all PGRN mutations including nonsense and missense mutations may lead to a reduced secretion of PGRN, which is not sufficient to cause an acute translocation of TDP-43. We would rather argue that secreted PGRN may be required as a survival factor for vulnerable neurons, which accumulate cytoplasmic TDP-43 fragments because of other reasons. Lack of PGRN may then further increase vulnerability of these neurons and facilitate cell death. However, whereas this manuscript was under consideration Zhang et al. (43) reported that proteolytic fragments of 35 and 25 kDa of TDP-43 are selectively produced by caspase-mediated processing upon suppression of PGRN. We found similar fragments of TDP-43, but these were generated independent of the PGRN knock-down, because these fragments were also observed in control cells expressing physiological levels of PGRN (Fig. 5B). However, currently we cannot exclude the possibility that a permanent knock-out of PGRN in contrast to the acute knockdown investigated here may affect TDP-43 localization and deposition.

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