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Frontotemporal dementia nonsense mutation of progranulin rescued by aminoglycosides

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Abstract

Frontotemporal dementia (FTD) is an early onset dementia and is characterized by progressive atrophy of the frontal and/or temporal lobes. FTD is highly heritable with mutations in *progranulin* accounting for 5-26% of cases in different populations. Progranulin is involved in endocytosis, secretion and lysosomal processes, but its function under physiological and pathological conditions remains to be defined. Many FTD-causing nonsense progranulin mutations contain a premature termination codon (PTC), thus progranulin haploinsufficiency has been proposed as a major disease mechanism. Currently, there is no effective FTD treatment or therapy.

Aminoglycosides are a class of antibiotics that possess a less known function to induce eukaryotic ribosomal readthrough of PTCs to produce a full-length protein. The aminoglycoside-induced readthrough strategy has been utilized to treat multiple human diseases caused by PTCs. In this study, we tested the only clinically approved readthrough small molecule PTC124 and eleven aminoglycosides in a cell culture system on four PTCs responsible for FTD or a related neurodegenerative disease amyotrophic lateral sclerosis. We found that the aminoglycosides G418 and gentamicin B1 rescued the expression of the progranulin R493X mutation. G418 was more effective than gentamicin B1 (~50% rescue vs <10%), and the effect was dose and time-dependent. The progranulin readthrough protein displayed similar subcellular localization as the wild-type progranulin protein. These data provide an exciting proof-of-concept that aminoglycosides or other readthrough-promoting compounds are a therapeutic avenue for familial FTD caused by progranulin PTC mutations.

Introduction

Frontotemporal dementia (FTD, also known as frontotemporal lobar dementia or FTLD) is a clinically and pathologically heterogeneous group of non-Alzheimer dementias characterized by progressive atrophy of the frontal and/or temporal lobes (1). FTD is characterized by gradual impairment of cognitive and language skills as well as personality and behavioral changes. It is the second most common dementia after Alzheimer's disease (2). FTD is highly heritable with approximately 35-50% familial cases (3, 4). Several genetic mutations have been identified that cause FTD or related disorders, including mutations in the *C9ORF72* (5), *Fused in Sarcoma (FUS)* (6), *microtubule-associated protein tau (MAPT)* (3), and *progranulin (GRN)* genes (3, 5, 7). Pathogenic mutations in *progranulin* were detected in ~10% of FTD cases and ~22% in familial FTD cases (8).

Human *progranulin* encodes a 593 amino acid protein involved in many biological processes including development (9), wound repair (10), and neuroinflammation (11-13). Progranulin is localized in endosomes, Golgi (14) and lysosome, and it likely participates in endocytosis, secretion and lysosomal functions (15, 16). However, the molecular function of progranulin under physiological and pathological conditions remain to be defined. Many FTD-causing mutations in progranulin are nonsense mutations with a premature termination codon (PTC) that result in a truncated protein. Consequently, haploinsufficiency of functional progranulin has been proposed as a major contributor to FTD. Knockout (17) and knock-in (18) animal models demonstrated that progranulin haploinsufficiency cause FTD pathology. Additionally, restoring progranulin levels improved preexisting FTD pathology in progranulin deficient mice (19). The homozygous knockout caused lysosomal dysfunction similar to those observed in the human lysosomal storage disease neuronal ceroid lipofuscinosis (NCL), which was also partially rescued by restoring progranulin levels (19). Together, these results support the notion that restoring progranulin expression can be an effective therapeutic approach.

Aminoglycosides are a class of gram-negative bacilli antibiotics that function by binding to bacterial ribosomes and interfering with protein translation (20). A lesser known function of aminoglycosides is to induce the eukaryotic protein translation machinery to readthrough PTC mutations

and yield a full-length protein. The aminoglycoside-induced readthrough strategy has been utilized in multiple diseases caused by PTC mutation, including cystic fibrosis (21, 22), Duchenne muscular dystrophy (DMD) (23), ataxia-telangiectasia (24), Rett's syndrome (25, 26), and most recently junctional epidermolysis bullosa (27). Partial restoration of protein expression resulting from PTC readthrough has been demonstrated in *in vitro* assays, cell culture systems, mouse models, and human patients (28, 29). Although aminoglycosides have some potential side effects, such as impairment of mitochondrial translation in eukaryotic cells, their ability of inducing PTC readthrough has raised the possibility of treating human diseases caused by PTCs. Moreover, the new readthrough compound PTC124 (or ataluren) displayed beneficial effects in clinical trials (30) and was clinically approved to treat DMD in Europe in 2014.

We screened eleven aminoglycosides and PTC124 in a cell culture system to determine whether any compound can induce readthrough of *progranulin* PTC mutations in FTD or a *FUS* PTC mutation in the related neurodegenerative disease amyotrophic lateral sclerosis (ALS). The aminoglycosides gentamicin B1 and G418 (also known as geneticin) specifically rescued expression of the R493X mutation of progranulin but not other progranulin or *FUS* PTC mutations. G418 rescued R493X expression to nearly 50% of wild-type (WT) progranulin while gentamicin B1 rescued less than 10%. The readthrough effect was dose and time-dependent. Strikingly, the readthrough protein displayed similar subcellular localization patterns as WT progranulin. These results provide a proof-of-principal that aminoglycosides, or other compounds promoting progranulin PTC readthrough, could be an exciting therapeutic avenue for familial FTD caused by progranulin nonsense mutations.

Result

1. G418 and gentamicin induce readthrough of the progranulin R493X nonsense mutation.

We constructed WT *progranulin* and three plasmids each with a single nonsense mutation (Q125X, Y229X, or R493X) and a C-terminal Flag tag (Figure 1A). A full-length protein must be generated for the FLAG to be detected, thus a truncated progranulin will not be detected by FLAG

Western blotting. If readthrough of the *progranulin* PTC occurs, then progranulin will be detectable by FLAG Western blotting.

We chose R493X, which is the most common nonsense mutation in progranulin-mediated familial FTD (8, 31), to test whether aminoglycosides could induce readthrough. N2A cells were transfected with the indicated plasmid, allowed to recover for 6 hours, and fresh media containing the aminoglycoside at the indicated concentrations was added to the cells for 24 hours. Cells were lysed and analyzed by Western blotting to determine the amount of progranulin protein. Among eleven commercially available aminoglycosides and PTC124, only gentamicin and G418 induced readthrough of R493X as evidenced by positive bands in FLAG Western blot (Figure 1B). The other nine aminoglycosides (kanamycin, streptomycin, amikacin, tobramycin, apramycin, neomycin B, netilmicin, paromomycin and sisomicin) and PTC124 did not have any detectable readthrough effect as no FLAG-positive bands were observed. No signal was detected in lysate from cells transfected with R493X in the absence of any compounds, serving as a negative control. The FLAG-tagged WT progranulin was included as a positive control to define the expected progranulin-FLAG amount. In addition, Western analysis using a progranulin antibody that detects full-length protein and the R493X protein also demonstrated the expression of full-length progranulin from R493X plasmid in the presence of gentamicin or G418 (Figure 1B). It is noted that G418 induced a stronger effect than gentamicin.

We next tested whether G418 or gentamicin could induce readthrough of two additional progranulin nonsense mutations (Q125X or Y229X) and a FUS nonsense mutation R495X that has been identified in juvenile patients of the related neurodegenerative disease ALS. Interestingly, G418 did not have any readthrough effect on the progranulin Q125X or Y229X mutation as no full-length readthrough protein was detected by the FLAG antibody (Figure 1C). G418 did not induce readthrough of the FUS R495X mutation either since the full-length protein was not observed (Figure 1D). Similarly, gentamicin did not induce readthrough of Q125X, Y229X, or R495X either (Supplemental Figure S1). The above data collectively support that G418 and gentamicin specifically induced readthrough of the progranulin

R493X mutation. Thus, we focused on these two compounds and the R493X mutation in the rest of the study.

2. G418 and gentamicin induce the readthrough of progranulin R493X in a dose- and time-dependent manner.

We tested a series of G418 and gentamicin concentrations to examine the dose response of the readthrough effect. N2A cells were transfected with the R493X progranulin plasmid, allowed to recover for 6 hours, fresh media containing either G418 or gentamicin at 100, 500, 1000 or 2000 $\mu\text{g/ml}$ was added to cells for 24 hours. Cells were then lysed and lysates analyzed by Western blotting. The readthrough was dose-dependent with the highest progranulin-FLAG signal corresponding to the highest concentration of compound (Figure 2A). Band intensity of the FLAG-tagged readthrough proteins were quantified as a percentage of the WT progranulin (the last lane in Figure 2A). A maximum readthrough of 33.8% was observed when cells expressing R493X progranulin were treated with 2000 $\mu\text{g/ml}$ of G418 (Figure 2B). While G418 yielded >30% readthrough, the maximal concentration of gentamicin produced 8.6% readthrough.

We next examined the readthrough effect of G418 and gentamicin with respect to time. In this experiment, 1000 $\mu\text{g/ml}$ of G418 or 2000 $\mu\text{g/ml}$ of gentamicin was added to cells for the indicated number of hours before cells were harvested for analysis. Readthrough of full-length progranulin was detected by both FLAG and the progranulin antibody after 12 hours of G418 treatment. After 12 hours of G418 treatment, the readthrough was $\approx 5\%$ of the WT control and it increased up to 47.3% after 48 hours (Figure 3A-B). Treatment with gentamicin yielded a similar time-dependent readthrough though the maximum readthrough was less than 10% (Figure 3C).

3. G418-induced readthrough protein displays similar subcellular localization as WT progranulin.

Aminoglycoside-induced readthrough is predicted to introduce a near-cognate amino acid into the PTC site (32), thus it is necessary to evaluate whether the readthrough protein exhibits similar function as

WT progranulin. Since the function of progranulin is complex and there is no established progranulin activity assay, we examined the subcellular localization of the readthrough progranulin to determine if it is similar as WT. We co-transfected both N-terminal HA-tagged and C-terminal FLAG-tagged progranulin plasmids (Figure 4A) into N2A cells and determined their subcellular localization using immunofluorescence microscopy. Confocal images demonstrate that the HA-tagged and FLAG-tagged WT progranulin were largely co-localized (Figure 4B, top row). Analysis using NIS-Elements AR software (Nikon, v3.2) demonstrates a Mander's overlap coefficient (MOC) of 0.94, meaning the sum of the intensities of red pixels that also have green component divided by the total sum of red intensities is 94% (33). Conversely, when HA-WT progranulin was co-expressed with R493X-progranulin-FLAG in the absence of G418, there was no FLAG staining (Figure 4B, middle row). Strikingly, the readthrough protein of R493X-progranulin-FLAG in the presence of G418 yielded robust FLAG signal that co-localized with HA-tagged WT progranulin (Figure 4B, bottom row). The degree of co-localization as assessed by MOC is 0.97, comparable to that of the WT controls.

Progranulin is reported to play a role in endocytosis, secretion, and lysosomal pathways (34). Therefore, we examined the co-localization of R493X-progranulin readthrough protein with the lysosome marker Lamp1. Transiently expressed WT progranulin (Figure 4C, top row, MOC of 0.59) and the G418-induced R493X-progranulin readthrough protein (Figure 4C, third row, MOC of 0.67) shared a similar pattern, i.e. both were partially co-localized with Lamp1. As a positive control, endogenous progranulin was examined in N2A cells and observed to largely co-localize with Lamp1 (Figure 4C, bottom row, MOC of 0.97). There was no FLAG signal from cells transfected with R493X-progranulin in the absence of G418 (Figure 4C, second row), serving as a negative control. Similarly, WT progranulin (Figure 4D, top row, MOC of 0.63) and the G418-induced R493X readthrough protein (Figure 4D, third row, MOC of 0.53) were partially co-localized with the Golgi apparatus marker GM130. As a positive control, the endogenous progranulin was also partially co-localized with GM130 (Figure 4D, bottom row, MOC of 0.87). As a negative control, there was no FLAG signal in the absence of G418 (Figure 4D, second row). These results demonstrate that the G418-induced readthrough protein shared a similar sub-cellular

localization as the overexpressed WT progranulin in N2A cells, i.e. partial co-localization with lysosomal and Golgi markers. The endogenous progranulin showed a higher degree of co-localization with Lamp1 and GM130. The results suggest that the induced readthrough protein possesses a similar function as WT progranulin.

4. G418 treatment stabilizes full-length progranulin mRNA in cells.

In addition to inducing readthrough, it has been reported that aminoglycosides can also stabilize mRNAs, which could enhance the readthrough effect (35, 36). Therefore, we employed qPCR to determine the mRNA levels in the absence and presence of G418. First, we observed a significant decrease of the full-length mRNA containing R493X PTC mutation as compared to WT progranulin, indicating that the R493X mutant mRNA was turned over more rapidly. In the presence of different doses of G418, the full-length mRNA containing R493X mutation increased up to two-fold as compared to that in the absence of G418 (Figure 5), suggesting that G418 indeed stabilized R493X mutant mRNA. These results indicate that the higher level of full-length progranulin mRNA in G418 treated cells may also contribute to the G418-induced readthrough.

Discussion

We examined whether aminoglycosides can induce readthrough of nonsense mutations in progranulin and FUS, two genes implicated in two related neurodegenerative diseases. R493X, Y229X and Q125X mutations in progranulin have been reported in familial FTD. R495X mutation has been found in juvenile familial ALS patients (37). Among 12 compounds tested (11 aminoglycosides and PTC124), we identified two aminoglycosides, G418 and gentamicin, that specifically induced the readthrough of R493X progranulin (Figure 1) in a dose- and time-dependent manner (Figures 2-3). G418 displayed dramatically better efficacy than gentamicin with respect to both time and dose. Importantly, the induced readthrough protein shared similar lysosome and Golgi apparatus localization as the WT progranulin (Figure 4).

Among 14 nonsense progranulin mutations reported to be associated with FTD, R493X is the most frequent, accounting for ~20% of progranulin-mediated FTD cases (8, 31, 38). In contrast, Q125X and Y229X are rare (39, 40). ALS and FTD are highly related as they share a wide spectrum of clinical, pathological and genetic features (41), thus we included a FUS nonsense mutation implicated in ALS in this study. FUS R495X is a particularly severe mutation that leads to clinical manifestation in juvenile ALS patients (37). Among the four PTCs tested, readthrough was observed only for progranulin 493X upon G418 or gentamicin treatment whereas no readthrough was detected for Q125X or Y229X progranulin or R495X FUS (Figure 1). Previous studies have demonstrated that readthrough is mainly influenced by two factors: the nucleotide sequence of the PTC and the flanking nucleotides (42, 43). It is reported that the difficulty of reading-through a PTC increases from TGA to TAG to TAA (44). In addition, better readthrough efficiency was observed with a C or T at the upstream -1 position and C at the downstream +4 position (42). The sequences of these four nonsense mutations are compiled in Table 1. The R493X mutation in human FTD patients has TGA as a PTC along with T at both upstream -1 nt and downstream +4 nt positions (Table 1). The combination of a favorable TGA PTC and a T at the -1 position are possible reasons that G418 and gentamicin induced R493X readthrough. The PTC for Y229X is TAA, which is the most difficult stop codon for readthrough. Indeed, we did not observe any readthrough product from progranulin Y229X by either G418 or gentamicin. The PTC for Q125X is TAG and the flanking sequence is C at the -1 and T at +4 position, respectively. The TAG PTC is less optimal, C at -1 position is favorable but T at +4 position is less favorable. Consequently no readthrough was observed for Q125X. Similarly in the case of R495X FUS, the TAG PTC is less favorable, C at -1 position is favorable, and G at +4 position is less favorable. These are likely factors explaining no detectable readthrough for R495X FUS.

Aminoglycosides have been reported to exhibit readthrough effects on different PTC mutations in multiple genes including *TP53*, cystic fibrosis transmembrane conductance regulator (*CFTR*), *DMD*, and survival motor neuron (*SMN1* and *SMN2*) (45). Gentamicin and G418 are the two most commonly used aminoglycosides in other studies, with G418 promoting readthrough at lower concentrations and with

higher efficiencies than gentamicin and other aminoglycosides such as amikacin, paromomycin and tobramycin (45). In this study, G418 also exhibited higher efficacy (~47.3% after 48 hours) on progranulin R493X mutation than gentamicin (no more than 10%) (Figure 1B, 2 and 3). The nearly 50% readthrough efficiency of R493X by G418 was better than the reported ~10% readthrough of the dystrophin PTC by G418 (23) and ~20-35% readthrough of the *CFTR* PTC by G418 (21, 22), and comparable to ~30-50% readthrough of the *LAMB3* PTC by gentamicin (27). Conversely, nine other aminoglycosides or PTC124 showed no detectable effect (Figure 3). It is suggested that the interactions of aminoglycosides with 80S eukaryotic ribosomes are critical for the readthrough effect. Aminoglycoside-ribosome interactions allows errors in tRNA selection and consequentially leads to the readthrough of PTCs. (20). In this study, among 12 compounds we tested (eleven different aminoglycosides and PTC124), only gentamicin and G418 showed readthrough effect. It is likely that the structure of these aminoglycosides differ in a way to affect their binding modes to eukaryotic ribosomes, thus producing different readthrough efficiency. It is noted that PTC124 is not an aminoglycoside but has similar effect on ribosome to induce readthrough of PTCs (46). PTC124 is the only readthrough compound approved clinically to treat DMD in Europe (47). However PTC124 did not have any detectable effect on R493X progranulin. The results provide initial insights into the structure-activity relationship and will help future studies to design and develop novel compounds with better efficacy and specificity.

The aminoglycoside-induced readthrough inserts a near-cognate amino acid at the PTC position. It was reported that Gln, Tyr or Lys was inserted at UAA and UAG and that Trp, Arg or Cys was inserted at UGA (32). The frequency of insertion of individual amino acid was distinct for specific PTC codons and readthrough-inducing agents (32). Because of the unknown amino acid at the R493 position, it was necessary to examine whether the readthrough protein functions the same as WT progranulin.

Multiple studies reported that progranulin plays a role in lysosome (19, 48, 49). It has been reported to regulate the maturation of lysosomal hydrolases (50) and homozygous progranulin mutation leads to a lysosomal storage disease NCL (15, 51). In addition, progranulin itself is processed into granulin peptides (52, 53). Progranulin has been reported to be partially co-localized with lysosome

markers in multiple studies (18, 52). Indeed, we found that both endogenous and overexpressed WT progranulin co-localized with lysosome marker Lamp1 (Figure 4C). More importantly, the G418-induced readthrough protein from R493X was also partially co-localized with Lamp1, in a similar fashion as WT progranulin (Figure 4C). In addition, the R493X readthrough protein shared a similar pattern with WT progranulin as both were partially co-localized with the Golgi apparatus marker GM130 (Figure 4D). It is necessary to place an epitope tag at the C-terminus of the R493X mutant to allow specific detection of the G418-induced readthrough protein in confocal imaging studies as evidenced by the lack of FLAG signals in the absence of G418 (Figures 4B-4D). Progranulin can be targeted to lysosomes by two independent mechanisms that are mediated by sortilin (34) and prosaposin (54, 55), respectively. It is noted that the C-terminal tagging may interfere with the sortilin-dependent trafficking since its C-terminus is critical to its interaction with sortilin (56). The partial co-localization of the FLAG-tagged WT progranulin and G418-induced readthrough protein with the lysosomal marker Lamp1 (Figure 4C) is consistent with the previous studies. More importantly, the subcellular localization of the G418-induced readthrough protein is highly similar to that of WT progranulin (Figure 4). Thus, we suggest that the G418-induced readthrough protein likely functions similarly as WT progranulin. A definitive assay is needed in the future to determine the readthrough protein truly functions as the WT protein.

Multiple studies suggest that, in addition to the readthrough effect, G418 could also stabilize mRNA by antagonizing nonsense-mediated decay (NMD) in mammalian cells (22, 35, 36). Treatment with G418 treatment increased the level of Xeroderma pigmentosum complementation group C (XPC) mRNA containing nonsense mutations to about 20%-70% of normal level, which exerts a smaller but similar effect as NMD inhibitor cycloheximide (57). Here, we also observed that the mRNA level of R493X increased from ~30% of WT progranulin in the absence of G418 to ~60% with G418 treatment (Figure 5). We suspect that the binding of G418 to the eukaryotic ribosome, which triggers the translation machinery to continue translation until the real stop codon is reached, would be an effective way to avoiding the activation of NMD. While the absence of NMD activation would in turn make it possible for

cells to maintain a higher level of PTC-containing mRNA, leading to a higher efficiency in readthrough. It is noted that both mechanisms can be leveraged in future translational studies.

In summary, we discovered that gentamicin and G418 can induce readthrough of progranulin 493X mutation to produce full-length progranulin protein in an *in vitro* cell culture model. Our study provides a proof-of-principle that gentamicin and G418 hold therapeutic potential for FTD patients harboring progranulin nonsense mutations. Future studies include the development of new analogs of gentamicin and G418 with higher readthrough efficiency and lower toxicity. We also plan future studies to test whether G418 can induce readthrough, correct the haploinsufficiency, and mitigate the pathological phenotype in progranulin-mediated FTD animal models such as R493X knock-in mice.

Material and Methods

Plasmids

The WT progranulin plasmid with a C-terminal FLAG tag (pC-Flag-PGRN-WT) was purchased from Sino Biological Inc. (Cat.HG10826-CF). Three nonsense mutations, Q125X, R229X and R493X, were generated using Q5 site-directed mutagenesis kit (New England Biolabs Inc., Cat.E0554S) to introduce a single nucleotide substitution in the WT progranulin gene (Figure 1A). The WT progranulin with an N-terminal HA tag (HA-PGRN) was also generated by subcloning using the p3xHA vector (58) and HindIII and BamHI sites.

Antibodies

The primary antibodies for Western analysis and immunofluorescence microscopy were mouse anti-Flag (Sigma, F3165), rabbit anti-Actin (Cell Signaling Technology, Cat. 8457), mouse anti-Flag (Sigma, A8592), rabbit anti-PGRN (Novus, NAP1-87324), goat anti-PGRN (R&D, AF2420), sheep anti-progranulin (R&D, AF2557-SP), rabbit anti-HA (Santa Cruz, sc-805), mouse anti-HA (Santa Cruz, sc-7392), rabbit anti-Lamp1 (Cell Signaling Technology, 9091S), goat anti-Lamp1 (R&D, AF4320), rabbit anti-GM130 (Cell Signaling Technology, 12480P) and rabbit anti GM130 (Novus, NBP2-53420SS). The

secondary antibodies were Alexa Fluor 488 donkey anti-mouse (Life Technologies, A-21202), Alexa Fluor 568 donkey anti-rabbit (Life Technologies, A-10042), and Alexa Fluor 568 donkey anti-goat (Life Technologies, A-11057).

Cell culture, transfection and drug treatment

N2A cells were maintained in DMEM (Sigma, D5796) supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, and 100 µg/mL streptomycin. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Life Technologies, Grand Island, NY, USA). 2 µg of total plasmid was used for each well of 6-well plate unless otherwise described. After 6 hours transfection, fresh medium containing aminoglycoside at indicated concentrations was added to cells. Cells were applied to following experiments after exposed to drugs at certain time points. All cells were kept in a humidified incubator at 37 °C under 5% CO₂/95 % air.

Eleven aminoglycosides were tested in this study: G418 (Sigma-Aldrich, Cat.4727878001), Gentamicin (Sigma-Aldrich, Cat.G1397), Kanamycin (Gold Biotechnology, Cat.K-120-25), Streptomycin (Sigma-Aldrich, Cat.S9137), Amikacin (Alta Aesar, Cat.J67496), Tobramycin (Alta Aesar, Cat.J62995), Apramycin (Sigma-Aldrich, Cat.A2024), Neomycin B (Sigma-Aldrich, Cat.N6386), Netilmicin (Alta Aesar, Cat.J66302), Paromomycin (Alta Aesar, Cat.J61274), Sisomicin (Sigma-Aldrich, Cat.S7796). PTC124 was purchased from MedChemExpress (Cat. 775304-57-9).

Western Blots

Cells were lysed in RIPA buffer (Millipore Sigma, Cat.20-188), centrifuged at 1000 g for 10 mins to remove debris, and then subjected to SDS electrophoresis. After electrophoresis, gels were proceeded for transferring onto nitrocellulose membranes. The membranes were then blocked with 5% milk in TBST (100 mM TRIS-HCl, pH7.5, 0.9% NaCl, 0.1% Tween-20) and incubated with indicated primary antibodies in the same solution. All immunoblotting images were acquired using a BioRad ChemiDoc MP system.

Immunofluorescence Microscopy

Cells were seeded on gelatin coated glass coverslips and transfected with progranulin constructs. Twenty-four hours later with or without drug treatments, cells were rinsed with 1× PBS, fixed with 4 % formaldehyde in 1× PBS, and permeabilized with 0.25 % Triton-X100 in 1× PBS. The samples were mounted by applying Vectashield Mounting Medium (Vector Laboratories) and visualized using a Nikon A1 confocal microscope with a 60× objective. Mander's overlap coefficients (MOE) were calculated using NIS-Elements AR (Nikon, v3.2, 64 bit) to assess protein colocalization.

Quantitative PCR

Total RNA was extracted with Aurum total RNA mini kit (BioRad, Cat.732-6820), and cDNA was generated with SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Cat. 18080-051). Quantitative PCR was performed using SYBR Green (ThermoScientific, Cat.4309155). Beta-actin primers: forward 5'-AGA GCT ATG AGC TGC CTG AC-3'; reverse 5'-GGA TGT CAA CGT CAC ACT TC-3'. Primers used for full length progranulin mRNA (including flag encoding sequence) is: forward 5'-CGT GAA GGC TTG ATC CTG CGA GA-3', reverse 5'-CTT ATC GTC GTC ATC CTT GTA ATC-3'. Annealing temperature for both beta-actin and progranulin qPCR reaction were 60 °C.

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Conflict of Interest Statement

H.Z. declares that a provisional patent application has been filed based on the results in this manuscript. Other authors declares no conflict of interest.

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Figure Legends

Figure 1. G418 and gentamicin induced readthrough of progranulin PTC mutation R493X. (A)

Schematic diagram of WT progranulin and PTC mutations examined in this study. Each construct has a C-terminal FLAG tag. (B) Examination of potential readthrough effect of 11 aminoglycosides and PTC124 on R493X progranulin. N2A cells were transfected with WT or R493X progranulin, allowed to recover, and treated with two different concentrations of each compound for 24 hours. Cell lysates were generated, separated by SDS-PAGE, and analyzed by Western analysis using anti-FLAG (top), anti-progranulin (middle), and anti-actin (lower) antibodies. Among 12 compounds tested, only G418 and gentamicin induced FLAG bands, demonstrating positive readthrough effect. WT progranulin was included as a positive control. No transfection or no compound treatment were included as negative controls. For the anti-progranulin blot, the lower band is the R493X truncated protein and the higher band is full-length progranulin. (C) G418 had no readthrough effect on two other FTD mutations Q125X and Y229X. No full-length readthrough protein was observed in the FLAG blot. In the anti-progranulin blot, the higher band is WT progranulin and the lower band is the Q229Y truncated protein. The Q125X truncated protein was visible with longer exposure. (D) Examination of G418 on the R495X mutation of FUS responsible for familial ALS. WT or R495X FUS was tagged with FLAG at the N-terminus. N2A cells were transfected with WT or R495X FUS, allowed to recover, and treated with three concentrations of G418 for 24 hours. Cells were harvested and cell lysates were subjected to SDS-PAGE and Western analysis using anti-FLAG (top) and anti-actin (lower) antibodies. The slightly higher band is WT FUS and the lower band is R495X truncated FUS. G418 did not induce readthrough of R495X FUS.

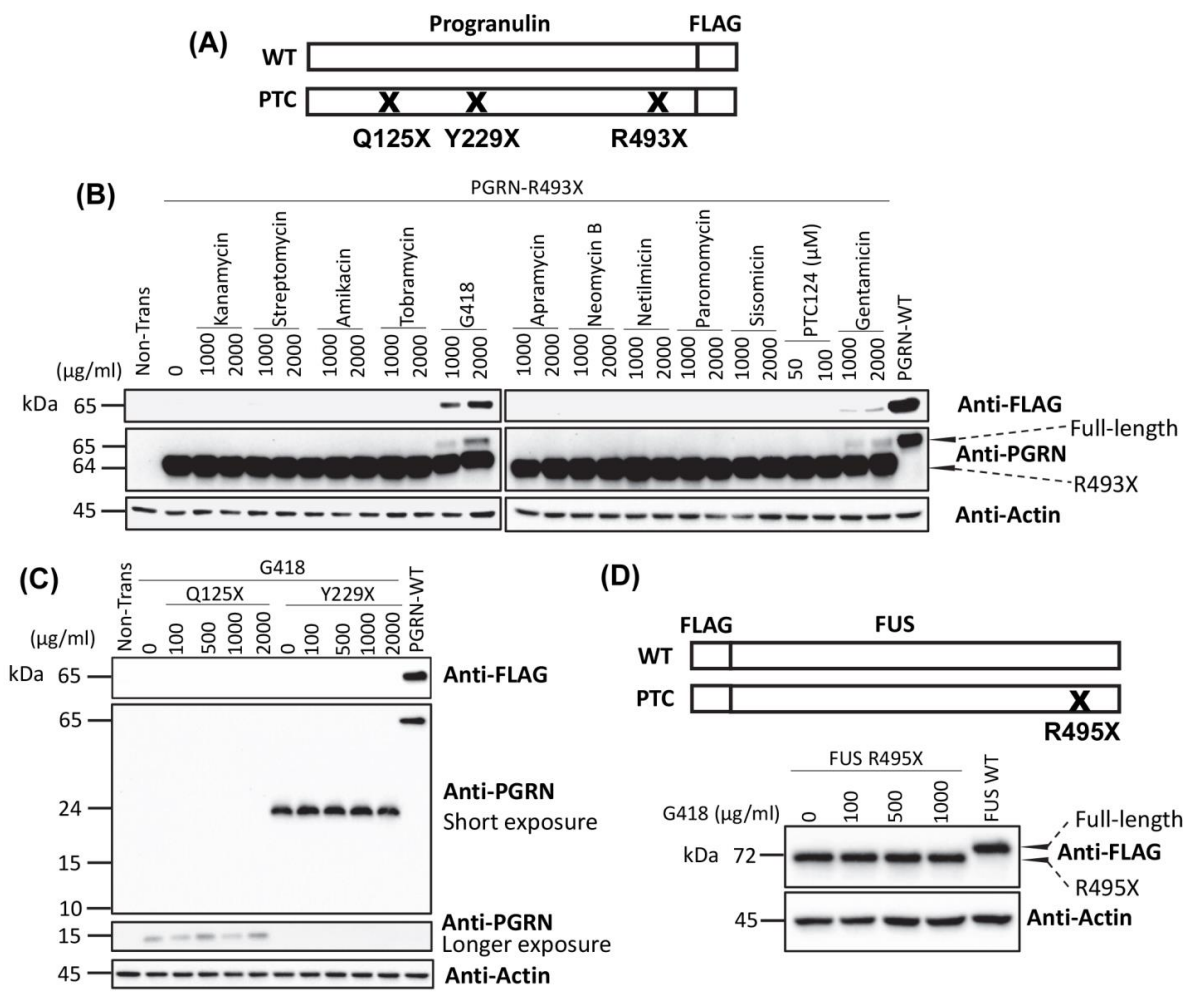


Figure 2. G418 and gentamicin induced dose-dependent R493X progranulin readthrough. (A)

Gentimicin and G418 induced readthrough of progranulin R493X in a dose-dependent manner. N2A cells were transfected with WT or R493X progranulin, allowed to recover, and treated with increasing concentrations of G418 or gentamicin for 24 hours. Cell lysates were generated, separated by SDS-PAGE, and analyzed by Western analysis using anti-FLAG (top), anti-progranulin (middle), and anti-actin (lower) antibodies. For the anti-progranulin blot, the lower band is the R493X truncated protein and the higher band is full-length progranulin. (B) Band intensities were quantified to determine the dose response of readthrough efficiency. All FLAG bands were normalized against corresponding actin bands and the individual readthrough band was subsequently compared to the WT progranulin (the last lane in A). *, $p < 0.1$; **, $p < 0.01$; ***, $p < 0.001$.

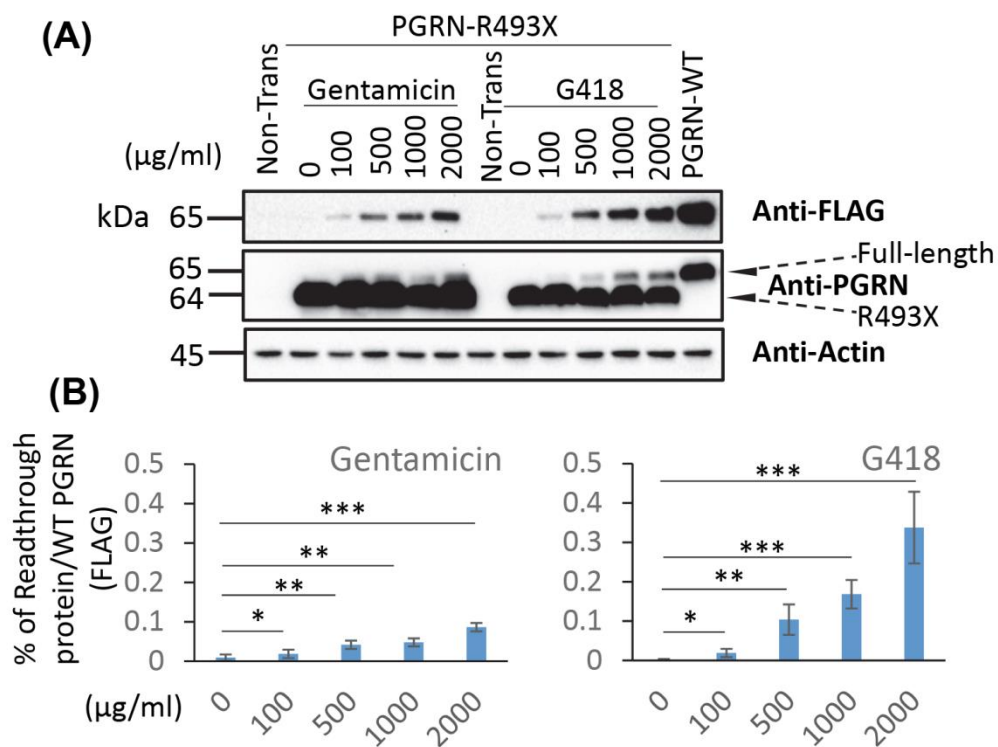


Figure 3. Time dependent readthrough effect by gentamicin and G418 on progranulin R493X. (A) G418 induced readthrough of R493X mutation of progranulin in a time-dependent manner. Cells were treated as in Figure 1 and lysates analyzed by Western analysis using an anti-FLAG (top blot), anti-progranulin (middle blots), and anti-actin (lower blot) antibodies. The two middle blots are a short and long exposure of the anti-progranulin analysis. (B) Band intensities were quantified to determine readthrough efficiency of the time-course. All FLAG bands were normalized against corresponding actin bands and the individual readthrough band was compared to the WT progranulin (the last lane in A). **, $p < 0.01$; ***, $p < 0.001$. (C) The time-course of gentamicin-induced readthrough effect on R493X.

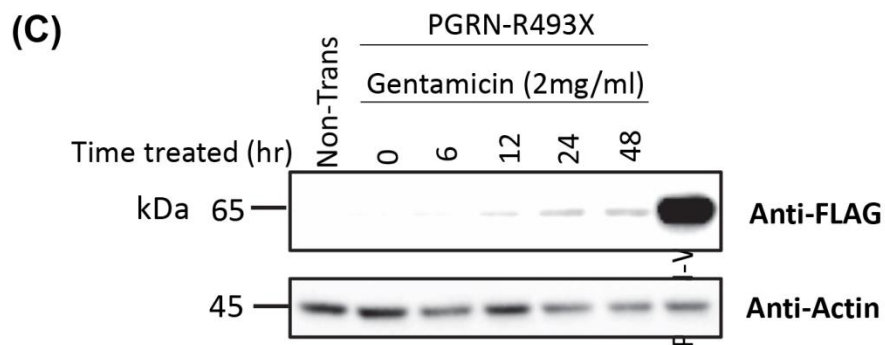
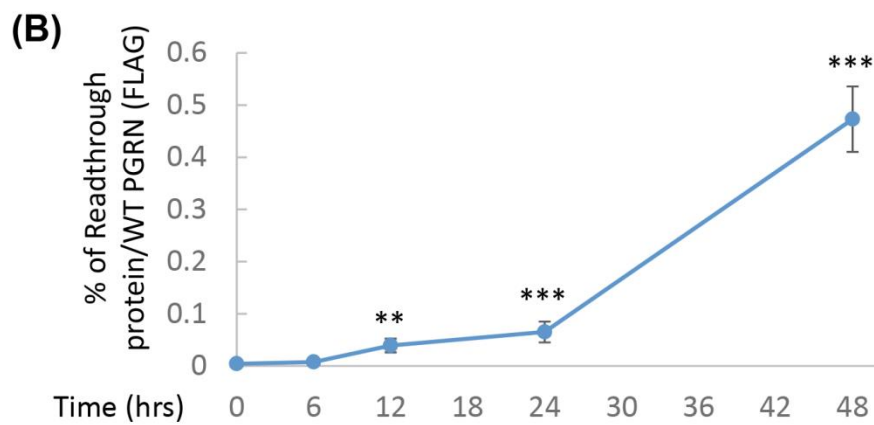
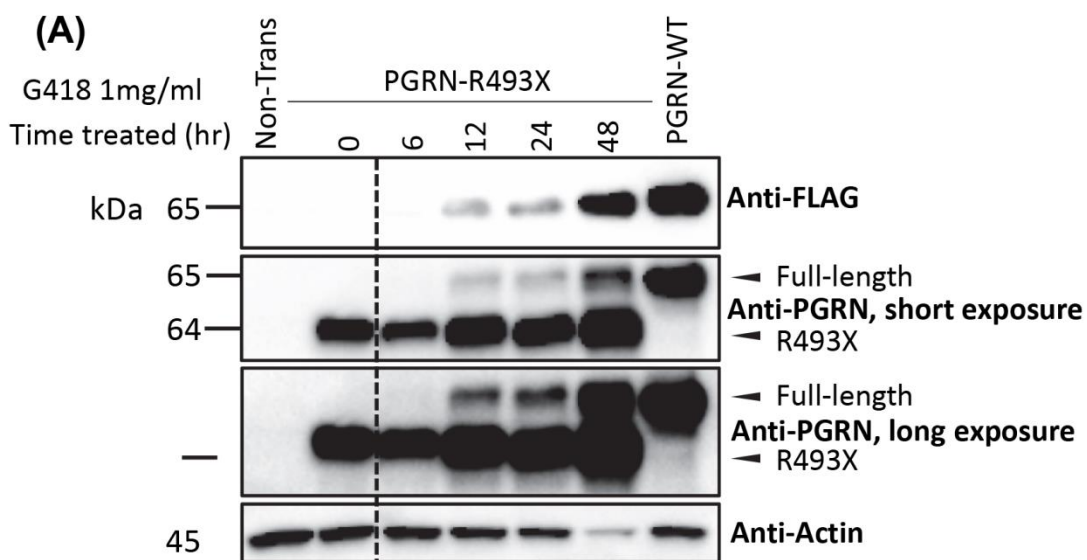


Figure 4. Subcellular localization of the G418-induced R493X readthrough and WT progranulin.

(A) A schematic of N- and C-terminal tagged WT progranulin and expected proteins in the absence and presence of G418. All G418 treatment was 1000 µg/ml for 24 hours in this figure. (B) Top: The N-terminal HA-tagged and C-terminal FLAG-tagged WT progranulin are largely co-localized in N2A cells. Middle: N2A cells expressing HA-tagged WT progranulin and FLAG-tagged R493X-progranulin in the absence of G418. No FLAG signal was observed in the absence of G418. Bottom: N2A cells expressing HA-tagged WT progranulin and FLAG-tagged R493X-progranulin in the presence of G418. The FLAG-tagged R493X readthrough protein co-localizes with the HA-tagged WT progranulin. A histogram shows green (FLAG) and red (HA) signals along the cross section line drawn in the zoom view for each row. The concurrence of green and red signals demonstrates the co-localization of FLAG- and HA-tagged proteins. (C) The overexpressed WT progranulin (top), the G418-induced readthrough full-length protein (third row), and the endogenous progranulin (bottom) are partially co-localized with lysosome marker Lamp1. No FLAG signal was observed in the absence of G418 (second row). (D) The overexpressed WT progranulin (top), the G418-induced readthrough full-length protein (third row), and the endogenous progranulin (bottom) are partially co-localized with Golgi marker GM130. No FLAG signal was observed in the absence of G418 (second row). Scale bars, 20 µm.

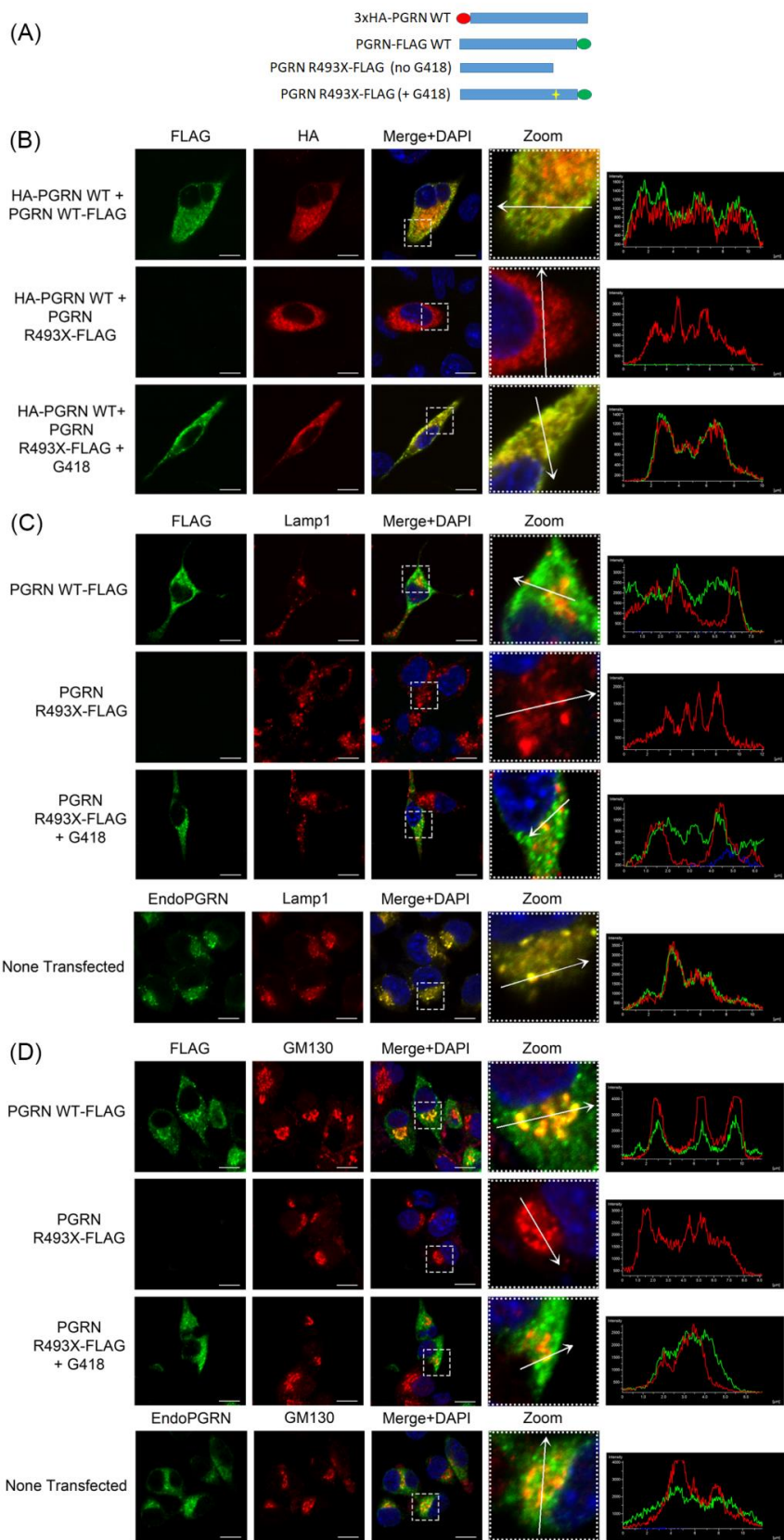


Figure 5. mRNAs levels of WT and R493X progranulin in the absence and presence of G418. N2A cells were exposed to G418 treatment for 24 hours with indicated concentrations after transfection. Primers specific for full length PGRN mRNA were used for qPCR. All results were normalized to full length WT progranulin in non-treated cells. N.S.: no significance. **, p<0.01; ***, p<0.001.

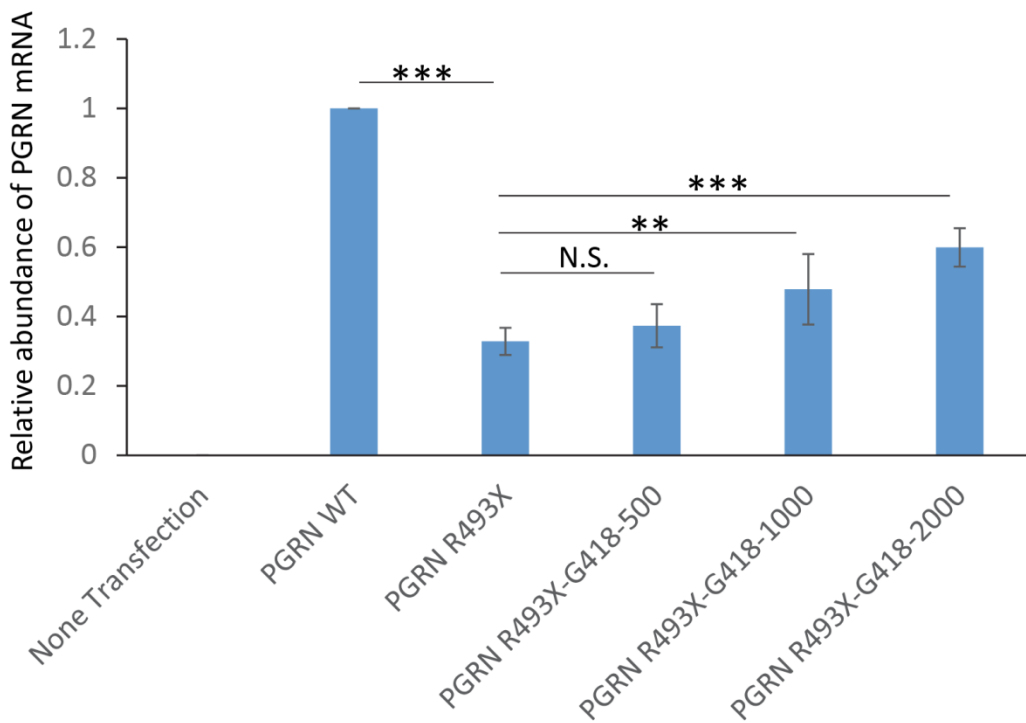


Table 1. PTC and flanking sequences of the nonsense mutations.

	WT sequence	PTC sequence	Flanking sequence
			-1 +4
R493X progranulin	CGA	TGA	CGTGAAGGCT TGAT CCTGCGAGA
Y229X progranulin	TAT	TAA	CAGTGGGAAG TAAG GCTGCTGCC
Q125X progranulin	CAG	TAG	GGGTGCCAT CTAGT GCCCTGATA
R495X FUS	CGA	TGA	TGGAGGCTT CTAGG GGGGCCGGG
Favorable factors		TGA>TAG>TAA	C or T @-1 C @+4

Abbreviations

FTD: frontotemporal dementia

PTC: premature termination codon

ALS: amyotrophic lateral sclerosis

NCL: neuronal ceroid lipofuscinosis

DMD: Duchenne muscular dystrophy

NMD: nonsense-mediated decay