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Functional Genomic Analyses Identify Pathways Dysregulated by Progranulin Deficiency Implicating Wnt Signaling

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Summary

Progranulin (GRN) mutations cause frontotemporal dementia (FTD), but GRN's function in the CNS remains largely unknown. To identify the pathways downstream of GRN, we used weighted gene co-expression network analysis (WGCNA) to develop a systems-level view of transcriptional alterations in a human neural progenitor model of GRN-deficiency. This highlighted key pathways such as apoptosis and ubiquitination in GRN deficient human neurons, while revealing an unexpected major role for the *Wnt* signaling pathway, which was confirmed by analysis of gene expression data from postmortem FTD brain. Furthermore, we observed that the *Wnt* receptor Fzd2 was one of only a few genes up-regulated at 6 weeks in a GRN knockout mouse, and that FZD2 reduction caused increased apoptosis, while its upregulation promoted neuronal survival *in vitro*. Together, these *in vitro* and *in vivo* data point to an adaptive role for altered *Wnt* signaling in GRN deficiency-mediated FTD, representing a potential therapeutic target.

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Keywords

Progranulin; Frontotemporal Dementia; Wnt; Fzd2; WGCNA

Introduction

Frontotemporal dementia (FTD), the second most common cause of presenile dementia (Ratnavalli et al., 2002), is also highly heritable (Chow et al., 1999; Rohrer et al., 2009). Several classes of dominant causal mutations have been identified in the genes for *MAPT*, *CHMP2B*, and most recently, *GRN* (Baker et al., 2006; Cruts et al., 2006), which codes for the protein progranulin (GRN). The pathology of GRN+ FTD is characterized by ubiquitin positive TDP-43 inclusions and absence of tau pathology (Eriksen and Mackenzie, 2008; Josephs et al., 2007; Mackenzie et al., 2006; Neumann et al., 2006). *GRN* mutations are dominantly inherited and the disease mechanism is postulated to be haploinsufficiency (Ahmed et al., 2007; Cruts and Van Broeckhoven, 2008), as most *GRN* mutations lead to an approximately 50% reduction in GRN levels (Baker et al., 2006; Coppola et al., 2008; Cruts et al., 2006).

Unlike MAPT, GRN's role in CNS function was previously not well-recognized prior to the identification of mutations in the *GRN* gene. *GRN* was not a candidate gene based on hypothesis driven work; it was found following exhaustive search by classic positional cloning following linkage (Baker et al., 2006; Cruts et al., 2006). *GRN* was first identified as a gene that was over-expressed in epithelial tumors and it was further found to be a player in wound healing and inflammation (Zhu et al., 2002). Progranulin and granulins have been known to function as growth factors, exerting opposing effects on cell growth and neurite outgrowth (Van Damme et al., 2008). But, the function of GRN in the CNS is poorly understood and it has only been sparsely studied (Neumann et al., 2009; Rohrer et al., 2009). Given its previously unknown role in neurodegenerative processes and disease, a broader understanding of its function in the nervous system would be of great value as a starting point for future therapeutic development.

Here we use a step-wise approach to gain a systems level view of the molecular consequences of GRN deficiency. Given the neuronal specificity of GRN deficiency, we developed an inducible *in vitro* model of GRN haploinsufficiency using shRNA in primary human neural progenitor cells (hNPCs) (Svendsen et al., 1998) and their differentiated progeny to faithfully model GRN deficiency. We next performed genome-wide transcriptome analysis to provide an unbiased and broad view of pathways directly downstream of GRN loss. By applying weighted gene co-expression network analysis (WGCNA), we visualized the network structure of acutely dysregulated genes downstream of GRN loss. We validate the *in vitro* results using expression data from postmortem FTD brain, identifying *Wnt* signaling as one of the major signaling pathways altered both during acute GRN loss in cell culture, and in human brain samples from patients with GRN mutations (GRN+). Functional analysis in human neural progenitors confirmed the predicted relationship between altered *Wnt* signaling and apoptosis observed *in vitro*. These data suggest that the pro-apoptotic effect of GRN knockdown may be mitigated by an alteration in *Wnt* signaling, which may represent a possible target for treatment of FTD.

Results

GRN Knockdown in Human Neural Progenitors and Progeny

The major effect of GRN deficiency in humans primarily involves the loss of neurons, despite nearly ubiquitous GRN expression in most cells and tissues (Daniel et al., 2000). We

therefore developed an *in vitro* model of GRN deficiency using primary human neural stem cells in which shRNA was used to diminish GRN levels by at least 50%, as is observed in cells from patients with GRN mutations (Baker et al., 2006; Cruts et al., 2006; Finch et al., 2009; Ghidoni et al., 2008). We observed robust gene expression changes with GRN knockdown, including enrichment of gene ontology categories pertaining to the cell cycle and ubiquitination (Table S1). This was encouraging, given the presence of ubiquitinated inclusions including TDP-43 as a major pathological feature in patient brains (Liu-Yesucevitz et al., 2010; Neumann et al., 2006). However, we also observed that viral infection itself (Supplemental Methods) led to acute changes in GRN levels, consistent with its upregulation during the acute phase of inflammation (Guerra et al., 2007; He et al., 2003). So, to avoid the potential confound of the acute GRN changes associated with infection, we developed a tetracycline inducible system (Gossen and Bujard, 1992) that enables the study of GRN loss in NHNPs during proliferation, differentiation, and post-differentiation. To control for off-target effects, two hairpins against GRN were used, and a scrambled hairpin was used as a control.

hNPC expression of shRNA was verified by robust RFP expression (Fig. 1A). GRN knockdown was confirmed by western blotting (Fig. 1B), and at the RNA level via analysis of the *GRN* probes on the microarray (Fig. S1). The two *GRN* probes on the array demonstrated robust and statistically significant knockdown (60–74%, p<10E-6). Although the two hairpins had slightly different efficacy of knockdown, each resulted in GRN levels equivalent with GRN levels in patients, which typically range between a 50 and 75% reduction (Finch et al., 2009). Furthermore the cohort of genes differentially expressed with both hairpins was highly significantly overlapping (see below), providing further confidence in the robustness of the results.

Given its role as a mitogen, we first explored GRN's effects on NHNP cells in their proliferative state. We found that GRN has little effect on progenitors, as only 6 genes were differentially expressed between the targeting and scrambled hairpin conditions (Table S1, Bayesian *t*-test, p<0.05, log ratio>0.2). We next investigated cell number and proliferation, observing no change in cell number or in proliferation rate in the face of GRN knockdown (Fig. 2A). This is consistent with the absence of an obvious developmental phenotype in GRN knockout mice (Ahmed et al., 2010; Yin et al., 2010) and the phenotype of patients with GRN mutations, who suffer loss of post-mitotic neurons after several decades of life.

As neurodegeneration primarily affects mature cells, we then studied the effects of GRN knockdown in non-dividing, differentiated hNPC cells. hNPCs were differentiated for four weeks in the presence of doxycycline, which induced shRNA expression. We confirmed cellular differentiation, first showing that nestin staining one month post-differentiation is lost (Fig. S2A–B). We then confirmed the upregulation of markers of early neuronal differentiation and maturation. Differential expression analysis showed clear upregulation of neuronal markers (Table S1) such as *DCX* (p<10E-12) and *TUBB3* (also named *Tuj1*, p<1E-2), and also of glial markers such as *GFAP* (p<5E-3) at the RNA level and MAP2, TUJ1, and GFAP staining at the protein level (Fig. S2C–D). GO analysis provides a more global view of the changes, yielding obvious enrichment in DE categories such as neuron differentiation, axonogenesis and neural projection development, apoptosis, and regulation of cell size (Table S1, EASE Score, p < 10E-6), further confirming neuronal differentiation.

Given that the ultimate consequence of GRN insufficiency (GRNi) in humans is neuronal death, we determined the effect of GRN deficiency on neuronal survival. In GRNi neural progenitor cultures, we observed increased pyknotic nuclei (Fig. 2B) relative to the scrambled hairpin control condition, consistent with increased cell death. To confirm this observation, we assessed activated CASP3 staining, which confirmed the initial observations

of increasing apoptosis with GRN reduction (Fig. 2C). To show that this apoptotic phenotype is not an artifact of progenitor proliferation or differentiation state, we also reduced GRN levels in differentiated cells via doxycycline application after differentiation of the progenitors over a course of three weeks, so that there were no remaining mitotically active cells. We saw a similar increase in CASP3 staining in this condition as well (Fig. 2C).

To determine whether GRN loss preferentially affects neurons or glia, we performed immunostaining, observing loss of Tuj1+ positive cells, but similar numbers of GFAP+ cells, indicating that sub-acute GRN loss leads to neuronal apoptosis (Fig. 2D–E). Additionally, when inducing GRNi post-differentiation, GRNi cultures demonstrated fewer, but not statistically significantly less MAP2 positive cells at a 6 week time point (Fig. S3), further confirming that the loss of neurons is not due to an effect on progenitor cell differentiation. Thus, GRN down-regulation in primary neuronal cells *in vitro* also leads to reduced neuronal survival, as it does *in vivo*.

Differential Gene Expression Analysis Highlights the Wnt Pathway

We next assessed gene expression changes associated with GRNi that might contribute to the apoptotic phenotype. We took the union of the changes observed in both GRN hairpins relative to the scrambled condition, so as to identify a conservative and highly robust group of 58 upregulated and 95 down-regulated genes (Bayesian *t*-test, $p<0.005$; Fig. 1C, Table S3). The intersection of differentially expressed genes between the two hairpins is highly significant (hypergeometric probability of 10E-67). Furthermore, virtually all (>99%) of those identified in these two independent experiments with different targeting hairpins were differentially expressed in the same direction relative to control, a high degree of internal consistency. These data provide evidence of robust alterations in gene expression specifically caused by GRN loss.

As a first step in organizing and categorizing the function of the differentially expressed genes with GRN loss, we conducted gene ontology (GO) analysis using David (http://david.abcc.ncifcrf.gov/) to determine enrichment of GO categories (Table S2). Several potentially important observations emerged (Fig. 3A), including cell death and apoptosis, consistent with the cell death observed by GRN loss in differentiating neural progenitor cells. To further confirm these gene ontology categories, we create a custom network via Ingenuity, in which the top network is significant for cell death (Fig. S4).

Notably, within the GO analysis there was only one KEGG signaling pathway whose members were over-represented with GRN knockdown, the *Wnt* signaling pathway. Members of the *Wnt* signaling pathway with significant alterations in expression included: *CD24*, *WNT1*, *SFRP1*, *NKD2* and the *Wnt* receptor *FZD2*. Other Wnt genes that were nominally significant include *GSK3B*, *PPP2R2B*, *APC2*, and *CER1*. To provide independent validation, gene expression changes of key Wnt signaling pathway members are additionally validated by qRT-PCR (Fig. 3B). These changes follow a clear pattern: genes that typically activate canonical *Wnt* signaling are upregulated (*WNT1*, *FZD2*, *APC2*), whereas genes that normally inhibit *Wnt* signaling are down-regulated (*GSK3B*, *SFRP1*, *NKD2*, *CER1*) (Fig. 3C). This indicates that an early consequence of GRN loss *in vitro* in human neural cells is an increase in *Wnt* signaling components that increases pathway activity. To test this prediction, we performed a direct experimental assay of *Wnt* activity in this model using the canonical LEF/TCF reporter (Methods). LEF/TCF signaling was increased in the GRN knockdown condition (Fig. S5), confirming that indeed *Wnt* signaling is altered. Moreover, non-canonical *Wnt* signaling pathways AP1, cJun, and NFAT assayed by the same reporter system (Methods; Fig. S5) show no significant changes, indicating that the alterations in *Wnt* signaling converge on the canonical pathway.

WGCNA highlights Cell Cycle, Ubiquitin, and Wnt Signaling Pathways

Although the GO analysis points to several potential key alterations in biological and molecular functions coupled with GRN deficiency, GO analysis is considered only a first step, since the function of many genes is not well-annotated. Recently, we have shown that WGCNA (Zhang and Horvath, 2005) provides a system level framework for the understanding of transcriptional profiles in many distinct cellular and tissue contexts (Geschwind and Konopka, 2009; Miller et al., 2008; Oldham et al., 2008; Winden et al., 2009; Voineagu et al., 2011). WGCNA has the power to reveal the underlying organization of the transcriptome of a system under study based on the degree of gene neighborhood sharing, which is defined based on co-expression relationships. This facilitates the identification of modules of functionally related, highly co-expressed genes, as well as the most central or hub genes that are of prime importance to module function (Geschwind and Konopka, 2009; Miller et al., 2008; Oldham et al., 2008; Winden et al., 2009). We condense the gene expression pattern within a module to a “module eigengene”(ME) which is a weighted summary of gene expression in the module (Oldham et al., 2008). Correlation between the ME and a given state, such as GRNi is indispensible as a metric to determine how a module may be relevant to that state.

We performed WGCNA (Methods and Supplemental Methods) identifying 24 modules, five of which (correlation > 0.50, $p<0.05$) were significantly correlated with GRN knockdown (Table S3). Two of these modules were of particular interest: the green module that contained GRN and the yellow module whose module eigengene was most correlated with GRN knockdown.

The green module contains 902 genes, and this module was then further divided into submodules (Supplemental Methods). We focused on the submodule containing GRN (Fig 4A–B, left). GO of this submodule, containing 167 genes, revealed that it is primarily comprised of genes related to mitochondrial function (Table S5, EASE Score $p<0.001$), the majority of which decrease with GRN loss. Mitochondria have been implicated as pivotal organelle in many neurodegenerative diseases, including AD and PD (Morais and De Strooper, 2010; Swerdlow, 2009). These data indicate that alteration in mitochondrial function is a primary effect of GRN deficiency in the CNS and support a role for mitochondrial dysfunction in GRN-related FTD as well.

The key module that may represent a cellular response to GRN loss is the yellow module, which contains 517 genes and was most correlated with GRN deficiency. We observed that this module contained a submodule with even higher correlation to GRN deficiency, so we chose to analyze this submodule as it represents the group of genes with highest correlation to GRNi (Fig. 4A–B, right). The yellow submodule contains 241 genes, and more than 95% of these 241 genes increase with GRNi (Table S4). GO analysis of this module (Table S4) demonstrated that it was enriched in the categories of ubiquitin-mediated proteolysis ($p<0.03$), *Wnt* signaling ($p<0.05$), and apoptosis ($p<0.02$). Notable highly connected (“hub”) genes in this module include *Wnt* signaling genes, such as *FZD2*, but also upregulation of pro-apoptotic genes like *CASP9* and *MGRN1*, the latter a ubiquitin ligase whose depletion has been shown to cause neurodegeneration (Chakrabarti and Hegde, 2009). Other *Wnt* signaling genes such as *WNT1*, *CTNNBL1*, and *VANGL2* are also highly connected. Ubiquitin positive inclusions containing TDP-43 are a hallmark of GRN positive FTD (Neumann et al., 2006), and upregulated genes within this module that are related to this pathway include the ubiquitin conjugating enzymes, *UBE2C* and *UBE2D3*. To test for up-regulation of ubiquitination within this model we performed western blotting with an anti-polyubiquitin polyclonal antibody, demonstrating a significant increase in poly-ubiquitinated proteins with GRN knockdown (Fig. S6). The up-regulation of these pathways here further

confirms an early increase in the ubiquitin protein stress pathways concomitant with GRN loss.

This analysis clearly connects GRN function to mitochondria and protein degradation pathways, and places multiple members of the *Wnt* signaling pathway in central roles downstream of GRN deficiency. These network data also bolster the suggestion, based on the initial analysis of differential expression, that *Wnt* signaling may be integral to GRN function and regulation, as both a supervised analysis using differential expression and an unsupervised analysis using WGCNA highlight *Wnt* signaling as a major pathway associated with GRN loss.

Gene expression related to postmortem human brain

Next, we sought to extend these in vitro observations to *in vivo* human data and provide independent validation of their relevance to human FTD. Although *in vitro* derived expression data have the power to demonstrate which expression changes observed in brain are direct effects of GRN loss, and not postmortem confounders (Mirnics and Pevsner, 2004), the optimal translational value lies in extending these observations to human patient material (Karsten et al., 2006). In this framework, first we determine causality in the absence of post-mortem confounders *in vitro*, and we then use the postmortem tissue to confirm the relevance of the *in vitro* findings to human FTD pathophysiology (Karsten et al., 2006).

We performed WGCNA on a dataset provided by Chen-Plotkin et al. (Chen-Plotkin et al., 2008) where microarrays were run on three brain regions (cerebellum, hippocampus, and frontal cortex) from three subject groups (controls, sporadic FTD, and GRN+ FTD). Following quality control to remove technical outliers (Methods; Oldham et al., 2006), 52 arrays remained. WGCNA identified 29 modules (Fig. 5A), 14 of which were related to brain region (e.g. cortex or cerebellum; Oldham et al., 2006), 8 were significantly correlated with GRN+ FTD (correlation>0.50, $p<0.05$, Table 1), and 2 of which were significantly correlated with sporadic FTD (Table S5, Methods). Lastly, some modules are driven by individuals, and may be related to factors such as cause of death or agonal state, as has been previously reported (Oldham et al., 2008). The eight modules whose ME is significantly correlated with GRN+ FTD show that there is a specific gene network associated with GRN + disease state. Given the similarity in pathology of GRN+ and sporadic FTD, this is remarkable in showing that despite the chronic inflammation and microgliosis present in both forms of FTD, GRN loss produces a specific set of altered gene networks that is preserved even late in disease (Figs. S7, A–G). Of note, there is nearly total agreement between the ME correlations observed in two brain regions, hippocampus and frontal cortex, consistent with the notion that the ME is a robust measure, as has been previously demonstrated in several settings (Konopka et al., 2009; Oldham et al., 2008; Winden et al., 2009; Voineagu et al., 2011).

GO analysis of the GRN+ associated modules (Methods) revealed some pathways previously linked to neurodegeneration, such as those relating to inflammation, mitochondria, synaptic transmission, neural development, and cell loss. At the same time, other modules with significant correlation to GRN+ disease status revealed unexpected GO categories, such as myelination, gliogenesis, and protein kinase and nucleotide binding (Table 1). These data complement the data already provided in (Chen-Plotkin et al., 2008), providing a systems level framework in which to delineate the GRN+ FTD molecular signature identified using differential expression analysis in the original paper. WGCNA allows for separation of distinct factors that may be related to GRN+ FTD, and facilitates the focus on the gene expression changes most relevant to disease pathogenesis.

The GRN Co-expression Module Implicates Apoptosis and Wnt Signaling Alterations

To further explore the relationship of the genes identified in vitro with GRN downregulation *in vivo*, we analyzed the GRN containing module, the blue module. The blue ME is highly specific to GRN+ FTD affected brain regions (Fig. 5B), indicating that genes in this module are specifically upregulated in these brain areas. GO analysis identified *Wnt* signaling to be significantly enriched within this module (Table S6), including canonical *Wnt* pathway transcription factors *LEF1*, *TCF7L1*, and *TCF7L2*. To probe the genes most associated with chronic GRN deficiency *in* vivo, we again examined the submodule containing *GRN* within this larger module. Remarkably, this module is centered around two hub genes that are both upregulated in disease (Fig. 5C): Annexin-V (*ANXA5*), a known mediator of apoptosis (Vermes et al., 1995), and *LRP10*, a newly discovered inhibitor of the canonical *Wnt* signaling pathway (Jeong et al., 2010). This module also contains *FZD2*, which is up-regulated and negatively correlated with GRN levels *in vivo*, consistent with the *in vitro* data. Analysis of these human brain samples revealed that *FZD2* is significantly up-regulated only in frontal cortex of GRN+ FTD samples, underscoring its potential role in disease pathogenesis.

Target Validation in Mouse

The up-regulation of multiple *Wnt* pathway activating components and down-regulation of negative regulators both *in vitro* and *in vivo* showed a remarkable degree of consistency. These data not only support the relevance of the *Wnt* pathway changes observed in cell-culture and in human FTD *in vivo*, but conversely indicate which of the changes observed in brain are a direct effect of GRN loss, and are not due to postmortem confounders, such as a change in cell composition (due to inflammation or cell loss) during the neurodegenerative process. We were particularly intrigued by the consistent upregulation of *FZD2*, since it is one of the most proximal pathway members, acting as a *Wnt* receptor (Chan et al., 1992; Slusarski et al., 1997). To follow *Fzd2 in vivo* at a time prior to neuropathological alterations or overt neurodegeneration, we analyzed independent gene expression data from cerebellum, cortex, and hippocampus of 6-week old GRN knockout mice, at a time point before overt cell loss or neuropathology. This analysis demonstrated only 25 differentially expressed genes in cortex (Table S7, $p<0.005$, log ratio > 0.2) and no significantly enriched KEGG pathways. A comparison to the human data showed that human GRNi stem cells have a greater number of genes in common with patient GRN+ brain than the knockout mice at this time point (Fig. 6A). But, of the few changes observed in mice, *Fzd2* upregulation is one of the most consistently up-regulated targets at this early stage in all brain regions (Fig. 6B). *Fzd2* is also the second most upregulated gene in cortex (Table S7) and it is upregulated in proportion to GRN loss; *Grn* −/− mice have twice the *Fzd2* increase as the *Grn* +/− mice.

In mouse, *Fzd2* remains upregulated at 6 and 9 months of age (Fig. 6C). But far more gene expression changes are detected at these later ages, consistent with progression of the disease (Table S7, 1203 and 813 genes, respectively, Bayesian *t*-test $p<0.05$, log ratio > 0.2). At 9 months "*lysosome*" is the most statistically significant gene ontology category, which is notable because progranulin is endocytosed and delivered to lysosomes by sortilin (Hu et al., 2010). Additionally, at 6 and 9 months the *Wnt* signaling pathway becomes enriched in cortex of *Grn* −/− mice (Table S8, $p<0.05$). These mouse data confirm that *Fzd2* is upregulated with GRN loss, but that it is one of the earliest features of GRN deficiency, confirming the human data that it is not an *in vitro* artifact, or the result of chronic degenerative or postmortem changes.

Experimental Validation of FZD2 In Vitro

Given the role of both the canonical and non-canonical *Wnt* signaling pathway in cell death and survival in many contexts from cancer to the nervous system, we set out to provide a proof of principle experimental test of our analyses. Since FZD2 is a *Wnt* receptor and its expression showed early and robust upregulation with GRN knockdown, we sought to manipulate FZD2 expression *in vitro* (Fig. S8), and to test one of two models based on our data. The first is that *FZD2* upregulation reflects a protective or compensatory response, and the second that it is part of the neurodegenerative process. In the first case, knocking down FZD2 would be pro-apoptotic and in the second knocking down FZD2 would be protective. FZD2 deficient differentiating neuronal progenitors (Methods; Fig. 6D, F; Fig. S7) had fewer total cells (Fig. 6E) relative to the control condition and demonstrated an increase in activated CASP3 staining, indicating apoptotic cell death. Dual FZD2/GRN knockdown cells demonstrated similar numbers of apoptotic cells relative to FZD2 knockdown alone (Fig. 6D), suggesting that upregulation of FZD2 is directly downstream of GRN down-regulation, since there was no additive effect.

We next verified that upregulation of FZD2 increases cell survival in the context of GRN loss in post-differentiated, non-proliferating GRNi cells that subsequently overexpress FZD2. Upregulation of FZD2 in these GRNi cells increases total cell number (Fig. 7A), and decreases cell death (Fig. 7B). Overexpression of FZD2 does not cause an increase in proliferation as measured by PCNA immunoblotting (Fig. 7C) and by brdU immunocytochemistry (Fig. 7D), indicating that this is not via an effect on proliferation, but rather cell survival. The induction of cell death by FZD2 down-regulation suggests that the observed consistent upregulation of FZD2 in neurons *in vitro* and *in vivo* in patient brain reflects a potential neuroprotective response to GRN loss.

Discussion

The present study was designed to identify the systems-level changes that accompany GRN reduction in human neurons. Initially, we examined the effects of progranulin deficiency in human fetal neural progenitors, a well-controlled *in vitro* model of GRN-haploinsufficiency. An important strength of this model is that it avoids potential confounding effects, such as chronic inflammation, that plague postmortem studies of dementia. Using this model, we demonstrate that progranulin deficiency selectively compromises neuronal survival and engages the canonical *Wnt* signaling pathway, the latter by way of both gene expression and a direct measure of signaling activity. The canonical *Wnt* signaling pathway is classically understood to increase cell survival, which is likely its role here in the context of GRN loss. Moreover, this association with *Wnt* is robustly preserved in the higher-order network architecture of transcriptional changes in the frontal cortex of FTD patients with progranulin mutations, and in *Grn* −/− mouse, proving its *in vivo* relevance. These data show that the combination of *in vitro* data, which can prove causality, and *in vivo* data, which confirms relevance to human disease, is a powerful approach.

Given the potential divergence of mouse and human transcriptional networks, this parallel between mouse and human systems was not a forgone conclusion (Miller et al., 2010), but provides another key line of evidence supporting the role of *Wnt* signaling and *FZD2*. Since these changes are evident well before the onset of observable inflammation, microgliosis, or overt neurodegeneration, these data suggest that FZD2 may prove useful as an early biomarker of disease progression. We show that loss of FZD2 is sufficient to cause cell death, but gain of FZD2 increases cell survival, suggesting that the upregulation of FZD2 is likely neuroprotective, consistent with its role as a growth factor. Together these data suggest that FZD2 plays a compensatory, potentially neuroprotective role in GRN deficient cells.

Dual Role of Wnt Signaling in Neurodegeneration

A general association exists between Wnt-Frizzled signaling and cell survival in many cellular systems (Chen et al., 2001; Rawal et al., 2009). A more specific link between aberrant *Wnt* signaling and FTD or Alzheimer's Disease (AD) was first postulated because the canonical messenger GSK-3β is responsible for phosphorylation of tau, an initial step in the formation of neurofibrillary tangles (Behrens et al., 2009; Boonen et al., 2009; Jackson et al., 2002; Karsten et al., 2006). Subsequently, animal models of AD and FTD further implicated *Wnt* signaling in the etiology of neurodegeneration by showing (1) disease causing mutated presenilin alters *Wnt* signaling, increasing apoptosis (Soriano et al., 2001; Weihl et al., 1999), and (2) Aβ can both directly and indirectly interfere with canonical Wnt signaling and that this interference compromises neuronal survival (Caricasole et al., 2004; Scali et al., 2006). *Wnt* signaling therefore may provide a bridge between neurodevelopment and neurodegeneration (Geschwind et al., 2001).

Implications for Disease Pathophysiology

It is still an open question as to whether loss of GRN causes FTD pathology through a cell autonomous or non-cell autonomous mechanism. Loss of GRN may increase neuronal vulnerability, conferring an intrinsic property in which neurodegeneration is more likely. Alternatively, microglia lacking GRN may become hyperactive, creating a poor extrinsic environment that leads to neuronal death. This is a complex issue because GRN is a secreted protein, the form of GRN that is clinically relevant to FTD is currently unknown, and GRN transcripts are decreased in blood, but are paradoxically increased in GRN+ diseased brain (Chen-Plotkin et al., 2009).

The data that we present here, in which GRN loss is sufficient to produce cell death in the absence of microglia, shows that neuronal GRN deficiency is sufficient to significantly reduce neuronal survival, a finding important for potential therapeutics development. While these data argue that GRN loss can indeed increase neuronal vulnerability, they do not preclude the compounding involvement of microglia in the pathophysiology of FTD in patient brain, which may form a vicious cycle (Pickford et al., 2011; Yin et al., 2010).

FZD2 and Non-Canonical Wnt Signaling, and Clinical Significance

Here we provide data from multiple systems showing that FZD2 expression increases with GRN loss. We then performed a proof of principle experiment, indicating that this increase may be protective. Classically, signaling through the FZD2 receptor activates the non-canonical *Wnt* signaling pathway (Oishi et al., 2003). This suggests that modulation of this pathway may have therapeutic relevance; previous work has shown that non-canonical Wnt agonists can be protective in other forms of dementia (Inestrosa and Toledo, 2008). As opposed to canonical Wnt signaling, which has already been established as a major player in neurodegeneration (Hooper et al., 2008; Toledo et al., 2008), these data indicate a role for *Wnt* signaling independent of GSK-3β and β-catenin in neurodegenerative pathology. Additionally, FZD2 is the initial change that precedes alterations in other *Wnt* pathway members in mouse *in vivo* at stages well prior to neurodegeneration or neuronal loss. Thus, FZD2 represents a primary target in that it is a consistent and early upregulated gene in the context of GRN loss.

Neuroprotection via upregulation of FZD2 is consistent with the characteristically understood role of *Wnt* signaling as pro-growth and anti-apoptosis in multiple systems (Chen et al., 2001; Lie et al., 2005; Willert et al., 2003; You et al., 2002). Further, disease onset and rate of progression may correlate with endogenous baseline activity of neuroprotection provided by the *Wnt* signaling pathway (De Ferrari et al., 2007). The manipulation of FZD2 and other members of its pathway is thus a potential therapeutic

target worthy of further investigation. Thus it is reasonable to consider that small molecule agonists of the non-canonical Wnt signaling pathway are potential new targets in GRN+ FTD. Since *Wnt* signaling is cell context and receptor dependent, gaining a full understanding of the other players in the pathway that may interact with FZD2 is crucial. This work provides the impetus for further in depth exploration of Wnt signaling in FTD and suggests the potential use of *Wnt* agonists to assuage the neurodegenerative phenotype of GRN+ FTD.

Experimental Procedures

Microarray Data and WGCNA Analysis

Human neural progenitor cells were infected, selected with puromycin, and differentiated for 4 weeks. Four replicates of each condition were analyzed as previously described on Illumina (San Diego, CA) human version 3 microarray chips (Coppola et al., 2009); see Supplemental Methods for details). Postmortem expression data from FTD patients and controls was obtained from (Chen-Plotkin et al., 2008) (GSE13162:GSM329660-GSM329715). Network analysis was performed using previously published methods (Oldham et al., 2006; Oldham et al., 2008; Winden et al., 2009). Gene ontology (GO) analysis was performed using the DAVID functional annotation tool (David (http://david.abcc.ncifcrf.gov/) (Huang da et al., 2009a, b), see Supplemental Methods for more details.

Plasmids

Initially, the pLCIR plasmid was used containing CAG promoter, chosen because of its robust expression in neurons, driving shRNA against GRN. The control was a GFP hairpin used previously (Matsuda and Cepko, 2007). To knock down GRN in an inducible system, pTRIPZ vector was purchased from Open Biosystems (Huntsville, AL). Their scrambled pTRIPZ vector was used as a control, and their TRIPZ GRN hairpin was used to knock down GRN. The sequence of the other GRN hairpin was obtained from (Zhang et al., 2007), and was cloned into TRIPZ using the PCR shagging protocol (http://katahdin.cshl.org:9331/RNAi/html/rnai.html). FZD2 knockdown was accomplished by purchasing FZD2 hairpins in vector pLKO (Open Biosystems), and pLUIP was used to overexpress FZD2, containing a U6 promoter driving expression of FZD2 with PuroR driven by an IRES. More detailed material on plasmids and sequences can be found in Supplemental Materials and Methods.

Cell lines and cell culture

Human neural progenitor cells (NHNPs) were generated from a 17-week gestation aborted female fetus. Tissue was homogenized, plated, and cultured as previously described (Svendsen et al., 1998; Wexler et al., 2008), and further elaborated in the Supplemental Methods.

Antibodies, Reagents, and Assays

The following antibodies were used for either immunoblotting (IB) or immunofluorescence (IF): anti-GRN (rabbit polyclonal, Invitrogen; 1:500 IB), anti-β-actin (mouse monoclonal, Sigma;1:50,000 IB), anti-HA (mouse monoclonal, Covance; 1:2,000 IB), anti-polyubiquitinated conjugates (mouse monoclonal, Biomol, 1:2000 IB), anti-PCNA (rabbit polyclonal, Santa Cruz Biotechnology, 1:200), anti-cleaved-caspase-3 (rabbit polyclonal, Cell Signaling; 1:1,000 IF), anti-GFAP (goat polyclonal, Abcam; 1:1,000 IF), anti-TUJ1 (rabbit polyclonal, Millipore, 1:1,000 IF), anti-BrdU (rat monoclonal, Abcam, 1:1,000 IF), goat anti-rabbit horseradish peroxidase (Cell Signaling; 1:2,000), goat anti-mouse horseradish peroxidase (Sigma; 1:5,000), goat anti-rat Alexa Fluor 488 (1:1,1000), donkey

anti-rabbit Alexa Fluor 488 (1:1,1000), donkey anti-mouse Alexa Fluor 647 (1:1,000), donkey anti-rabbit Alexa Fluor 633 (1:1,000).

Immunocytochemistry(ICC): Cells were fixed using 4% paraformaldehyde (Sigma). Cells were permeabilized by incubating with 0.5% Triton-X for 10 minutes. Antigen retrieval for brdU staining was performed using 100 units/mL of DNase I (Worthington, Lakewood, NJ) supplemented with 1 mM $MgCl_2$. Cells were blocked for 30 minutes in 3% donkey serum, 0.2% Triton-X, diluted in TBS. Primary antibodies were diluted in blocking buffer and were incubated overnight at 4°C. Secondary antibodies were incubated for 1 hour at room temperature in the dark. All cells were counterstained with DAPI (1:5000 diluted in TBS) and coverslips were mounted using ProLong Antifade (Invitrogen). 3 biological replicates were performed per experiment. 6 high-power fields were counted per coverslip, except in relation to FZD2 manipulation experiments, in which 4 high-power fields were counted per coverslip.

Wnt Activity Assays: Dual GFP/luciferease reporters were purchased from System Biosciences (Mountain View, CA) for TCF/LEF, AP1, cJun, and NFAT. Lentivirus was produced to infect proliferating NHNP cells already harboring targeting and non-targeting GRN hairpins, producing eight different transduced lines. 6 biological replicates of each line were induced for 1 week with doxycycline and compared to its uninduced counterpart. GFP fluorescence was assessed using a Plate Reader (BioTek) to determine relative activity for each reporter in each cell type.

Mice

Progranulin knockout mice were created as reported previously (Kao et al., 2011). The mice used for the microarray study were backcrossed into the C57BL/6 strain twice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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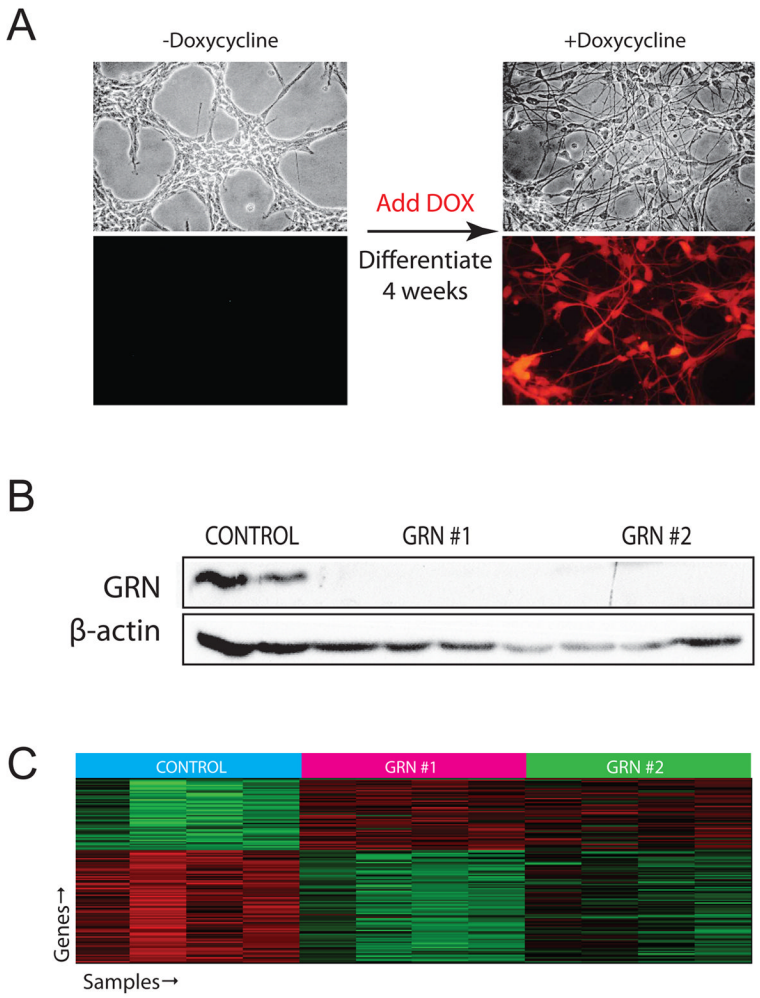

Figure 1.

An in vitro model of GRN deficiency using shRNA in NHNP cells. A. Bright-field and fluorescent images of the NHNP cells before and after differentiation in the presence of doxycycline. hNPCs were infected, selected with puromycin for 2 weeks, then differentiated for 4 weeks. Expression of hairpin was verified by visualization of robust RFP expression. No leakage of the inducible promoter is visible. B. Confirmation of GRN knockdown by Western blot. Protein was extracted from the same samples on which the arrays were run, only one band was observed for GRN and for β-actin. C. Differential expression analysis was carried out and we observed hundreds of genes changing. Genes were determined to be changing only if they were differentially expressed in the overlap of both GRN hairpins versus control. Using the strict criterion of $p<0.005$ and computing this for both hairpins individually against control, the overlap contains 153 genes (hypergeometric probability = 10E-67). Depicted here is the heatmap of all samples for these 153 genes. Most genes (152/153) common to both hairpins are differentially expressed in the same direction relative to controls, further confirming that this list of genes is robustly related to GRN knockdown. See also figures S1 and S2 and table S1.

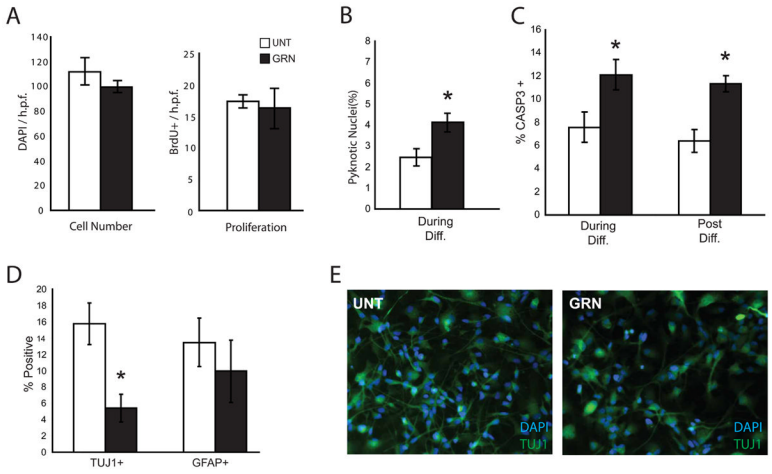

Figure 2.

Neuronal cell death occurs in differentiating NHNP cells with GRN knockdown. A. GRNi cells demonstrate no difference in cell number or proliferation. B. GRNi cells demonstrate significantly increased pyknotic nuclei. C. There is a statistically significant increase in activated CASP3 staining in GRNi cells whether GRN is lost during differentiation (left) or post-differentiation (right).

D–E. There are significantly ($P < 0.05$) fewer TUJ1+ cells but similar number of GFAP+ cells ($P = 0.50$) in cells lacking GRN. Cells were only considered to be TUJ1+ if they had a neuronal morphology. Asterisks indicate $P \leq 0.05$ and error bars are ± s.e.m. (unpaired two-tailed Student's *t*-test, n=3). See also figure S3.

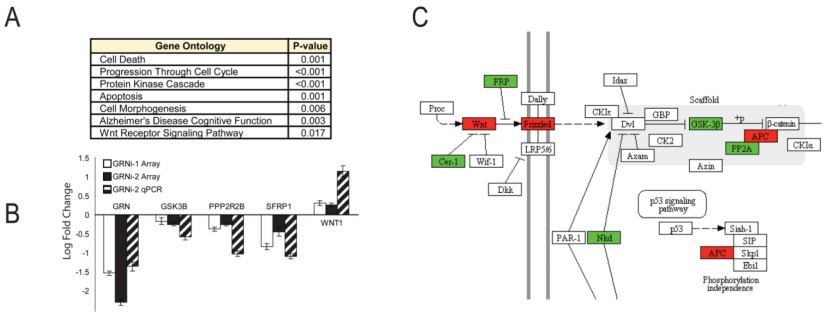

Figure 3.

A. Gene ontology analysis was carried out using DAVID on the set of differentially expressed genes. B. qRT-PCR was performed on some of the important players in the Wnt pathway to verify these gene expression changes. The left two bars show expression changes on array, and the right bar shows expression changes as measured by qPCR. qPCR was performed by comparing the second GRN hairpin versus the scrambled hairpin. C. KEGG Pathway for Wnt Signaling, with differentially expressed genes during GRNi ($p<0.05$) circled. Upregulated genes are colored red and downregulated genes are colored green. See also figures S4 and S5 and table S2.

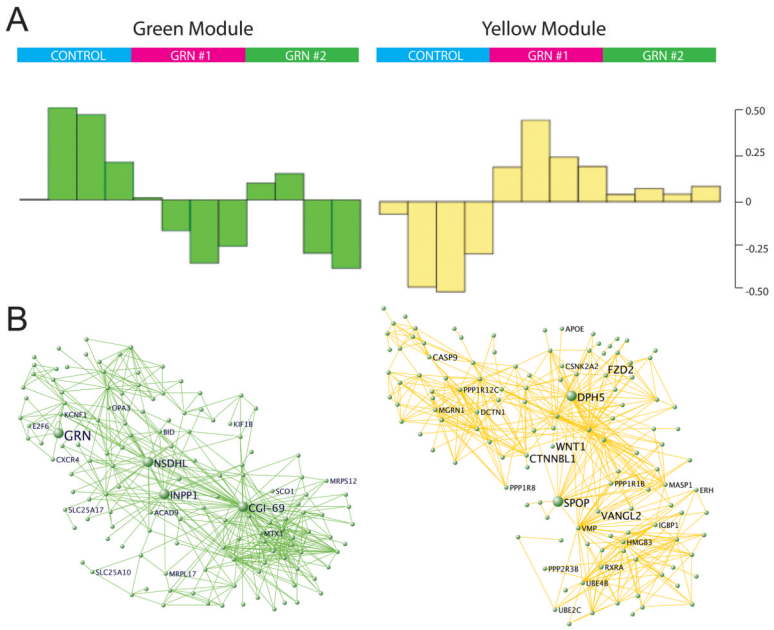

Figure 4.

WGCNA Analysis of GRN deficiency in cell culture. WGCNA analysis (see Methods) was carried out on the cell-culture data, and we chose to focus on two modules of importance: one containing GRN, and on another that is most correlated with GRN knockdown samples. A. Module eigengenes (ME) are computed from the first principle component of the gene expression data to summarize the major trend in gene expression within each module. Samples are plotted along the x-axis where each bar represents one sample, and the value of the ME for each sample is displayed on the y-axis. These plots show that these two modules are specific to the samples containing GRN knockdown. Genes in the green module are generally upregulated with GRN loss, whereas genes in the yellow module are downregulated. B. The strongest connections based on topological overlap are plotted for ease of analysis. This allows for easy visualization of highly connected genes within each module. See also figure S6 and tables S3 and S4.

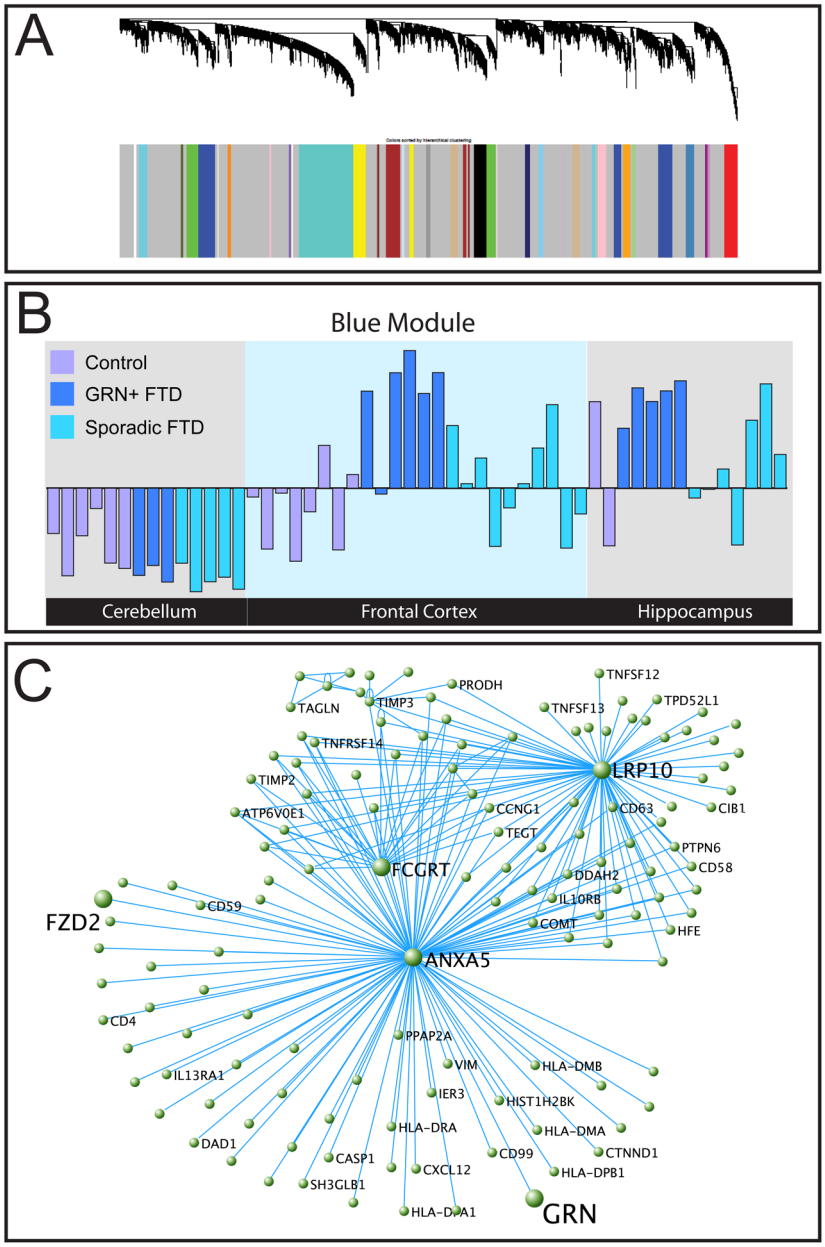

Figure 5.

WGCNA analysis on the postmortem brain dataset. A. Dendrogram showing relationship of topological overlap of genes and their relation to modules. B. First module eigengene is plotted of the blue module, a measure of the general expression of the genes in a given module. Green samples are controls, red samples are FTD with GRN mutations, and blue samples are sporadic FTD. The module containing GRN, the blue module, is positively correlated with diseased GRN+ samples. This provides further evidence that GRN+ FTD has a distinct molecular phenotype even in late-stage FTD as compared to sporadic FTD. C. A graphic depiction of the blue module using VisANT (http://visant.bu.edu). For ease of viewing, pairs of genes with the highest intramodular topological overlap are depicted, with each link corresponding to a TO value between the connected nodes. This module is notable

because it contains GRN. Its hub genes are *ANXA5* and *LRP10*, indicating that this module is likely related to apoptosis and Wnt signaling. See also figure S7 and tables S5 and S6.

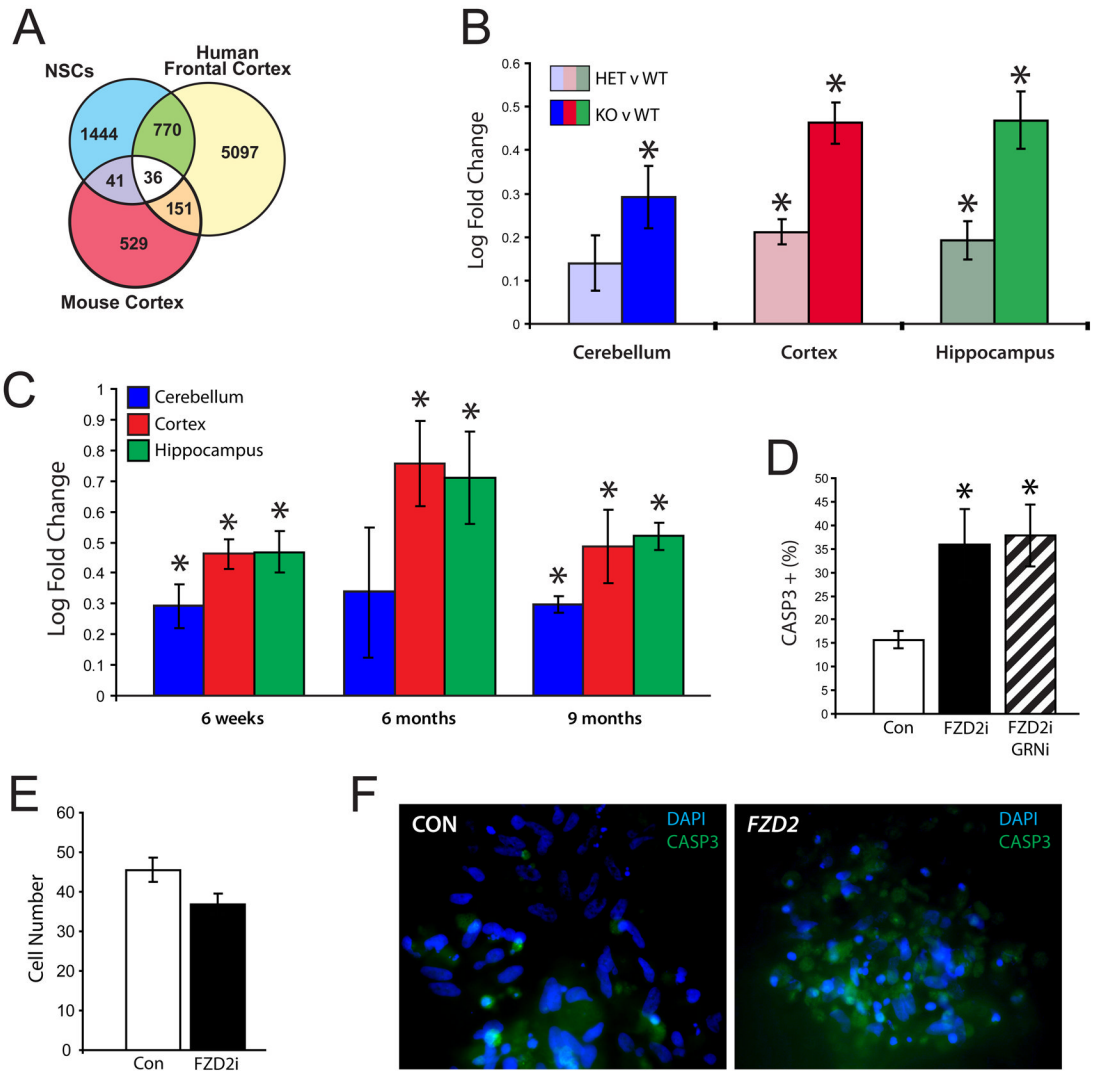

Figure 6.

A. Overlap of differential expression between NHNP cells, mouse, and FTD GRN+ patients, showing that there is greater overlap between NHNP cells and affected patient brain ($p < 0.05$, Bayesian *t*-test). B. *Fzd2* is upregulated in proportion to Grn gene dosage in mouse brain. (n=5, unpaired Student's *t*-test, $p<0.05$). Plots depict log fold change of Fzd2. C. Differential expression of FZD2 in GRN knockout mice at 6 weeks, 6 months, and 9 months (n=3–5, unpaired Student's *t*-test, $p<0.05$). Plots depict fold change of *Fzd2* in *Grn* −/− versus *Grn* +/+ mice. D, E. Differentiated NHNP cells in culture with FZD2 knockdown demonstrate a significantly larger percentage (n=3, error bars ± s.e.m., unpaired Student's *t*-test, $p<0.05$) of dying cells (D) and lower total cell number (E). However, dual FZD2/GRN knockdown demonstrate similar numbers of apoptotic cells relative to FZD2 alone (D). F. Staining showing increased activated CASP3 staining (green) in FZD2 knockdown (right) as opposed to control (left). Numerous pyknotic nuclei are evident in FZD2 knockdown cells as well. See also figure S8 and tables S7 and S8.

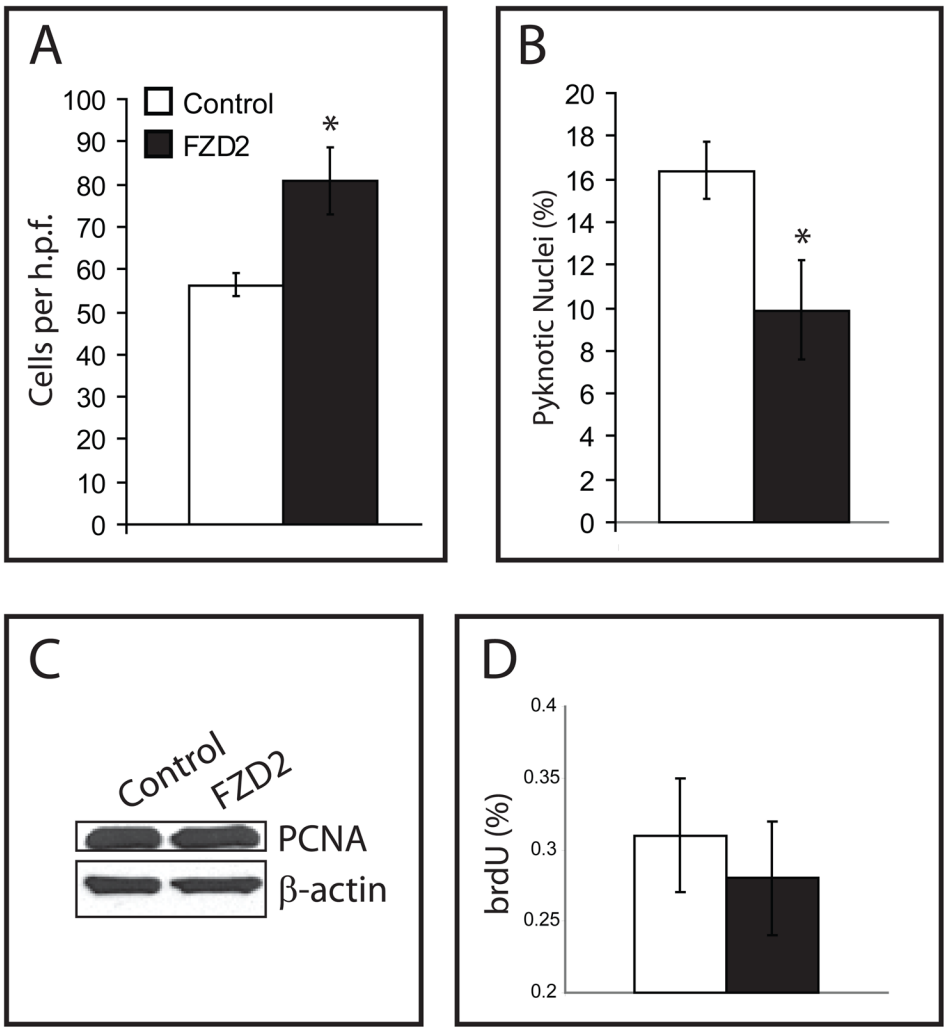

Figure 7.

A–B. GRNi cells were differentiated and after 4 weeks, and subsequently FZD2 was overexpressed for 4 weeks concurrently with GRN loss. The cells overexpressing demonstrate greater cell number (A), and fewer dying cells (B), suggesting overexpression of FZD2 is neuroprotective in the context of GRN loss (n=3, error bars ± s.e.m., unpaired two-tailed Student's *t*-test, $p<0.05$). C–D. In normal NHNP cells, FZD2 overexpression does not cause a change in proliferation as measured by PCNA immunoblotting (C) or by brdU immunostaining (D).

Table 1
Table of modules specific to GRN+ FTD disease state

Correlation of the first module eigengene with diseased state is listed here. This table characterizes the modules by gene ontology, listing the gene ontology category that is most characteristic of the module in question, and by top 3 connected genes by Kme. See also table S6.

Module	Correlation	P-Value	Top three hub genes by Kme	Notable Gene ontology (P-value)
Blue	0.66	7.95E-08	PARP4, FOXO1, RIN2	Wnt Signaling Pathway (<0.02), Apoptosis (<0.02), UbI Conjugation (<0.005)
Cyan	−0.53	4.66E-05	DNM1L, SYNJ1, NBEA	Protein Kinase (<10e-4), Nucleotide Binding (<0.002)
darkolivegreen	−0.65	1.60E-07	NAP1L3, AAK1, CAMTA1	Synaptic Transmission (<10e-4), Calcium Ion Binding (<0.01)
Green	−0.56	1.51E-05	BSCL2, ACOT7, GNG3	Oxidative Phosphorylation (<10e-18), Mitochondria (<10e-15)
lightgreen	0.58	7.86E-06	PARP12, XAF1, SP100	Antiviral Defense (<10e-17), Immun Response (<10e-9)
orange	0.52	6.88E-05	C1QA, HCLS1, VSIG4	Response to Wounding (<10e-21), Immune Response (<10e-20)
steelblue	0.67	7.31E-08	FRMD4B, PIP4K2A, RASGRP3	Myelination (<10e-7), Gliogenesis (<0.003)
tan	−0.60	2.60E-06	NSF, GLRB, AAK1	Nucleus (<3e-7), RNA Splicing (<3e-5)