Supplementary Figure and Table Legends

Figure S1. Log ratios of the probes for *GRN* on the array. For both hairpins against *GRN*, when compared to control, probes have a log ratio between –1.15 and –2.31, and p-values as computed with a Fisher's Exact Test between 1.99E-7 and 6.42E-13.

Figure S2. Immunostaining verifies differentiation of NHNP cells. A-B. Nestin staining is positive in cells differentiated for one week (A), but absent in cells differentiated for one month (B), verifying differentiation of these cells. C-D. Differentiated NHNP cells express mature markers such as MAP2 (6 weeks differentiation, panel C) and TUJ1 and GFAP (4 weeks differentiation, panel D).

Figure S3. Difference in numbers of MAP2 positive cells in GRNi cultures. NHNP cells were differentiated under two separate conditions in the absence of doxycycline, subsequently hairpins were induced in the two conditions. Condition 1: differentiate for 2 weeks, then induction for 4 weeks (left), Condition 2: differentiate for 3 weeks, then induction for 3 weeks (right). Both conditions yield fewer MAP2+ cells showing GRNi induces neuronal death. Error bars are ± s.e.m. (unpaired two-tailed Student's *t*-test, n=3).

Figure S4. An Ingenuity Network Implicates Cell Death. A custom-built network of the set of differentially expressed genes with GRN loss was designed using Ingenuity software (www.ingenuity.com). This network was notable in that the top module was significant for cell death, and a graphical depiction of this module is shown here. Red genes are upregulated in this dataset, green genes are downregulated, and gray genes have been shown to interact with genes in this dataset in the literature.

Figure S5. TCF/LEF activity in GRNi cells is decreased. Activity assay using a GFP reporter to show that canonical, but not non-canonical Wnt signaling, is increased in GRNi cells. GRN was knocked down for 1 week, and GFP was read directly in the 96 well plates by using a Plate Reader (BioTek). This GFP reporter is a specific type of GFP with a short half-life to give accurate readings for an activity assay.

Figure S6. GRN deficient cells demonstrate increased polyubiquitination. A. Immunoblotting showing an increase in polyubiquitination in GRN deficient cells. This was performed in 293T cells, but the result holds true in NHNP cells as well. B. The increase in ubiquitinated species was quantified over multiple experiments, and ImageJ software was used to quantify band intensity.

Figure S7A-G. Graphical Depiction of Modules Related to GRN+ FTD. WGCNA analysis of the postmortem brain dataset of the modules not shown in figure 5. Top: First module eigengene is plotted of each 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. Bottom: A graphic depiction of each 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.

Figure S8. Manipulation of FZD2 in vitro. Immunoblot for HA showing functionality of lentivectors used to manipulate FZD2. Lane 1: untransfected 293T cells. Lane 2:

293T cells transfected with pLUGIP-FZD2-HA, overexpressing FZD2 containing an HA tag. Lane 3: 293T cells cotransfected with the overexpression vector used in lane 2 as well as a hairpin against FZD2. Absence of a band indicates that the hairpin successfully knocks down FZD2.

Table S1. A. The first sheet contains a table consisting of the significant gene ontology categories using the original constituitive GRN knockdown system in proliferating NHNP cells lacking GRN. B. Second, a table is presented of the differentially expressed genes in proliferating cells using the inducible system expressing GRNi(96 hour timepoint, Fisher's Exact Test $p<0.05$, LogRatio>0.2). C. Summary of statistically significant gene ontology categories differentially expressed in differentiated (versus proliferating) neural progenitor cells, and the differential expression values of mature markers such as *DCX*, *TUJ1*, and *GFAP* in these differentiated cells.

Table S2. Table of 153 genes differentially expressed under differentiation of NHNP cells. The three statistical conditions are GRN hairpin #1 versus Control, GRN hairpin #2 versus Control, and both hairpins versus control. Also depicted here are gene ontology categories identified. 153 genes that met the statistical criteria were used (Bayesian *t*-test, $p<0.005$ across all conditions). David (http://david.abcc.ncifcrf.gov/) was used for gene ontology analysis. We included the following categories: molecular function and biological process levels 4 and 5, and genetic association with disease database and bind database. Categories were included if they met the statistical threshold (EASE score, $p < 0.05$) and if they had at least 3 genes in the given category.

Table S3. WGCNA Analysis of NHNP GRNi data yields 24 modules.

Table S4. Gene ontology was performed on the set of genes within these two modules.

Representative gene categories having p-values (EASE Score) less than 0.05 are shown. The green module is clearly mitochondrial as is evinced by its significant gene ontology categories. The yellow module is multi-faceted, but clearly contains cell-cycle genes, *Wnt* signaling, and Ubiquitin mediated proteolysis, all of which possibly implicate *Wnt* signaling in this module. A gene list and expression values for the submodule highly correlated with GRN knockdown is provided as well.

Table S5. Categorization of modules by brain region and disease state. A given module was deemed to be a member of a given category if the correlation of its first module eigengene with a state vector for a given category, for example cerebellum or sporadic FTD, was higher than 0.5. Correlations are depicted in the first sheet, and module membership by given category is depicted in the second. Highlighted red cells indicate module membership based on high correlation.

Table S6. Gene ontology of the blue module.

Table S7. Table of differentially expressed gene in cortex of GRN knockout mice at 6 weeks (Bayesian *t*-test, $p<0.005$, Abs(Log Ratio)>0.2, 6 months and 9 months of age (Bayesian *t*-test, $p<0.05$, Abs(Log Ratio)>0.2).

Table S8. Summary of statistically significant gene ontology categories in GRN knockout mice at both 6 months and 9 months of age (EASE score $p < 0.05$).

Supplemental Methods

Constituitive GRN Knock Down

Using the initial system to knock down GRN constituitively with a pLCIR vector proved difficult, and we initially thought that was because GRN levels can change under inflammatory conditions. This was confirmed by showing that GRN is differentially

expressed in transduced, relative to uninfected cells (data not shown). Thus, we assumed that GRN may be rising in response to lentiviral infection with GRN hairpins. To extract meaningful data from our experiment, we thus removed all genes from the list that changed normally with viral infection, easily available because we also obtained gene expression data from uninfected NHNP cells under similar conditions as the transduced NHNPs. The resultant gene list was then analyzed for gene ontology (Methods).

Plasmids

Initially, the pLCIR plasmid was used containing CAG promoter, used because of its robust expression in neurons, driving shRNA against GRN. The control was 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). In this inducible system, the transactivator protein rtTA3 and PuroR are constituitively expressed under the UBC promoter, while RDP and shRNA are inducibly regulated by an inducible tet-On CMV promoter. Their scrambled pTRIPZ vector was used as a control, their TRIPZ GRN hairpin was used to knock down GRN, sequence TGCTGTTGACAGTGAGCGACGTGTGCGTTTCAATAAAGTTTAGTGAAGCCAC AGATGTAAACTTTATTGAAACGCACACGCTGCCTACTGCCTCGGA (GRN#1). The other sequence of the other GRN hairpin (GRN#2) 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). Briefly, oligonucleotide TGCTGTTGACAGTGAGCGGGCCACTCCTGCATCTTTATAGTGAAGCCACAGAT GTATAAAGATGCAGGAGTGGCCTGCCTACTGCCTCGGA was ordered from

Invitrogen (Carlsbad, CA). This was amplified using PCR primers CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG (forward) and CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA (reverse), cut with EcoRI and XhoI, and ligated into TRIPZ. Both hairpins against GRN were used in the microarray studies, whereas all immunohistochemistry studies were conducted using GRN#2 versus scrambled control.

FZD2 hairpin sequence CCGGGCCCGACTTCACGGTCTACATCTCGAGATGTAGACCGTGAAGTCGGGCTTTTTT and the control GFP hairpin used previously (Matsuda and Cepko, 2007) was then subcloned into pLKO. HA tagged FZD2 was a generous gift of Guido Zaman and Folkert Verkaar (Schering-Plough Research Institute, The Netherlands), and it was subcloned into the pLUIP (U6 promoter driving FZD2-HA, with and IRES driving the PuroR gene) lentivector. GFP was cloned in the place of FZD2-HA as a control.

For viral production, psPAX2 and VSVG (both http://www.addgene.org) were used for viral packaging and envelope, respectively. Viruses were prepared by transfecting early-passage 293T cells (ATCC, Manassas, VA) using Fugene 6 (Roche). 293T cells were grown in neurobasal media. Viral media containing 5mg/mL polybrene was centrifuged at 3000 rpm for 5 minutes to clear cell debris, and supernatant was filtered through a 0.45 uM filter. Viral titers of at least 10^5 particles/mL were verified using Lenti-Go-Stix (Clontech). The resultant unconcentrated virus was added directly to NHNP cells.

Microarrays and WGCNA

RNA was then extracted using a microRNA kit (Qiagen, Valencia, CA). RNA concentration was determined by using a Nanodrop Spectrophotometer (Thermo, Wilmington, DE) and RNA integrity was insured by running samples on a Bioanalyzer (Agilent, Santa Clara, CA). For verifying knockdown at the protein level, protein was extracted from the organic phase of the RNA extraction, and GRN levels in the nine samples in which there was enough protein was assessed via immunoblotting. 200 ng of RNA was sent to the Southern California Genotyping Consortium at UCLA (http://scgc.genetics.ucla.edu/) and the sample concentrations were verified using a Ribogreen Assay. Samples were then hybridized to Illumina (San Diego, CA) human version 3 microarray chips. Detailed code for the microarray analysis can be found here (Coppola et al., 2009).

Network analysis was performed using previously published methods (Oldham et al., 2006; Oldham et al., 2008; Winden et al., 2009), and module merging was accomplished by using the WGCNA package in R. Briefly, expression data was imported into R (http://www.r-project.org/), and data were preprocessed by extracting hybridization dates to control for batch effects. A mask file for the Affymetrix U133A2 platform (http://masker.nci.nih.gov/ev/) was then used to control for probable bad probes. Taking each brain region separately, samples having an interarray correlation greater than 2 were removed. Expression values were scaled uniformly and quantile normalization was applied using the bioconductor package (http://www.bioconductor.org/) (Choe et al., 2005). Ingenuity Analysis (http://www.ingenuity.com) was performed using the list of 153 differentially expressed genes with GRN loss.

Differentially expressed genes or genes within a given module were compared against the *Homo Sapiens* background for enrichment within the GO categories biological process, cellular compartment, and molecular function. Only level four or five categories with more than three genes were selected. Additional categories used for GO included Protein Binding and Association with Human Disease. EASE score was used to calculate p-values, a modified Fisher Exact Test that is more conservative in examining p-values of gene lists. Only categories that had an EASE score p-values of less than 0.05 were included in these analyses. Module membership was determined using ME values. The correlation of ME values with a given state vector were then computed; for example a cerebellar vector contains ones for all cerebellar samples and zeros everywhere else. These correlation values are then used to assign membership of each module to a given state.

Cell Culture

Proliferating cells were grown in neurobasal A media (Invitrogen) supplemented with 10% BIT (Stem Cell Technologies, Vancouver Canada), 1% Anti-anti (Invitrogen), 0.5% glutamax (Invitrogen), and 2.5uM heparin (Sigma-Aldrich, St. Louis, MO). LIF, FGF2, and EGF (all Peprotech, Rocky Hill, NJ) were all added at 10 ng/mL. Cells were fed every second day. Differentiation of cells was carried out using neurobasal A media (Invitrogen), 2 ng/mL retinoic acid (Sigma-Aldrich), 10ng/mL BDNF (Peprotech), 10ng/mL NT3 (Peprotech), 10mM KCl (Fisher), 10uM forskolin (Sigma-Aldrich) and 1 ug/mL doxycycline (Sigma-Aldrich). All cells were grown on surfaces coated overnight with 50 ug/mL polyornithine (Sigma-Aldrich) followed by 5 ug/mL laminin (Invitrogen) overnight. These cells were selected using 0.5ug/mL puromycin (Sigma) 72 hours post-

transduction. Hairpins were induced when cells were seeded using 1 ug/mL doxycycline (Sigma-Aldrich).

Western Blotting

Whole protein lysates of cultured cells were prepated using 0.5% Nonidet P-40 and 250mM NaCl containing the following phosphatase and protease inhibitors: 50mM NaF (Sigma), 100μM $NaVO_4$ (Sigma), 1mM phenylmethylsulfonyl fluoride (Sigma), 1% proteinase inhibitor cocktail (Sigma P8370), and 1mM dithiothreitol (Sigma). This was added directly to cells which were immediately scraped on ice, vortexed, incubated on a rotator, centrifuged, and the supernatant was transferred to a new tube. 35ug of protein was then added to loading buffer containing 50mM dithiothreitol, boiled, and loaded onto SDS-polyacrylamide gel, and proteins were separated using electrophoresis. Gels were transferred onto PVDF membranes using wet-transfer in 20% methanol. Membranes were blocked in TBST + 5% milk for 30 minutes and were probed with primary antibody diluted in blocking buffer at 4^oC overnight. These were then probed with secondary antibody diluted in blocking buffer for one hour at room temperature. Detection was performed using Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Polyubiquitination Assays

293T cells were split, plated into 10cm plates, and transfected with 5ug of pTRIPZ plasmids containing targeting and non-targeting hairpins against GRN. Fugene-6 was used for transfection (Roche). 48 hours post-transfection, cells were harvested and protein lysate was loaded onto a polyacrylamide gel and protein was separated using

electrophoresis. Protein was transferred onto a PVDF membrane, and immunoblotted using mouse anti-polyubiquitinated conjugates (1:2000).

Proliferation Assays for FZD2 Overexpression

NHNP cells were infected with FZD2-HA and were incubated for one week. They were then reseeded into T25 flasks and onto coverslips at a density of 20,000 cells per well in a 24-well plate. These cells were then incubated for one more week and were harvested; protein lysate was produced from cells in T25 flasks and cells on coverslips were fixed with 4% PFA. Immunoblotting was performed and a prominent band was observed at the expected size of 29kD. Immunostaining was performed using the previously discussed IHC protocol staining for brdU, and percentage of brdU+ cells was quantified by cell counting (n=3 coverslips per condition).

qRT-PCR

qRT-PCR was performed using an Applied Biosystems 7900 machine according to the manufacturer's instructions. Briefly, RNA was harvested from independent samples of GRN deficient NHNP cells and cDNA was produced using Superscript III Reverse Transcriptase (Invitrogen) with random hexamers (Invitrogen). SYBR-green was used to quantify amplification of cDNA. Each probe-set was verified to be intron-spanning by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and was verified to amplify one product by verifying one peak present on the dissociation curves, and standard curves were performed to show that this assay is sensitive to changes in each gene. Three biological replicates were used for each condition, and 3 technical replicates were performed for each sample. Log fold changes are displayed here.

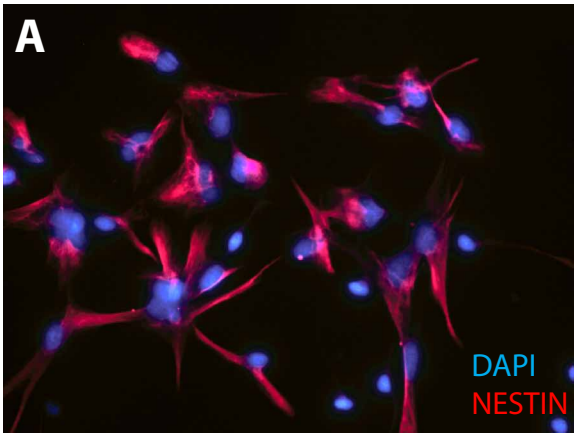
Supplementary Figure 1

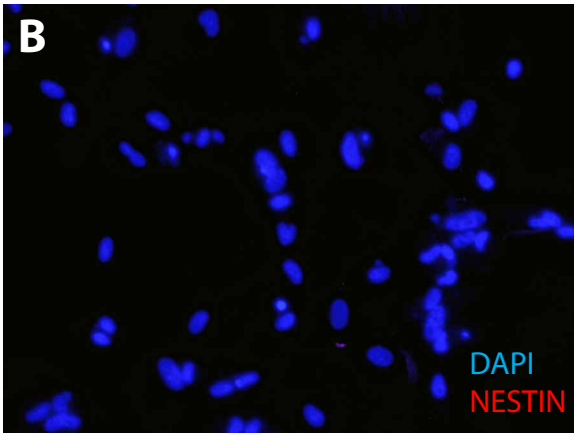
Supplemental Figure 1

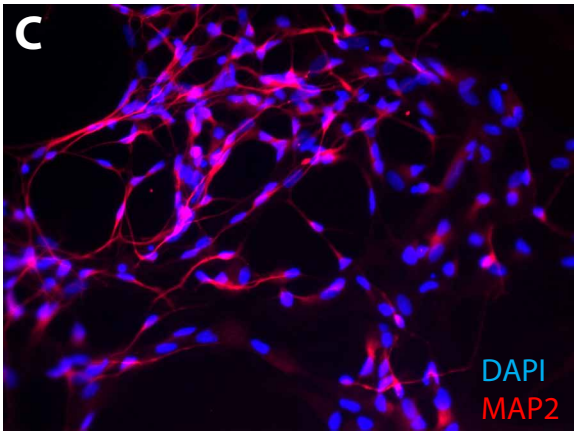
Comparison	Probe	Log Ratio	Pvalue
GRN1vsControl	ILMN_1811702	-1.53	1.12E-10
GRN2vsControl	ILMN_1811702	-2.31	6.42E-13
GRN1vsControl	ILMN_1724250	-1.15	1.99E-07
GRN2vsControl	ILMN_1724250	-1.55	6.01E-09

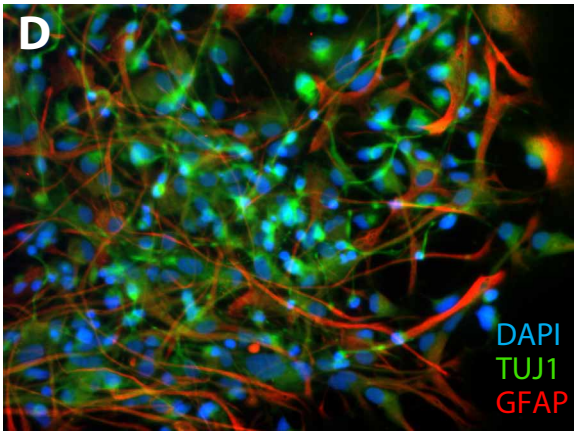
Supplementary Figure 2

Supplemental Figure 2

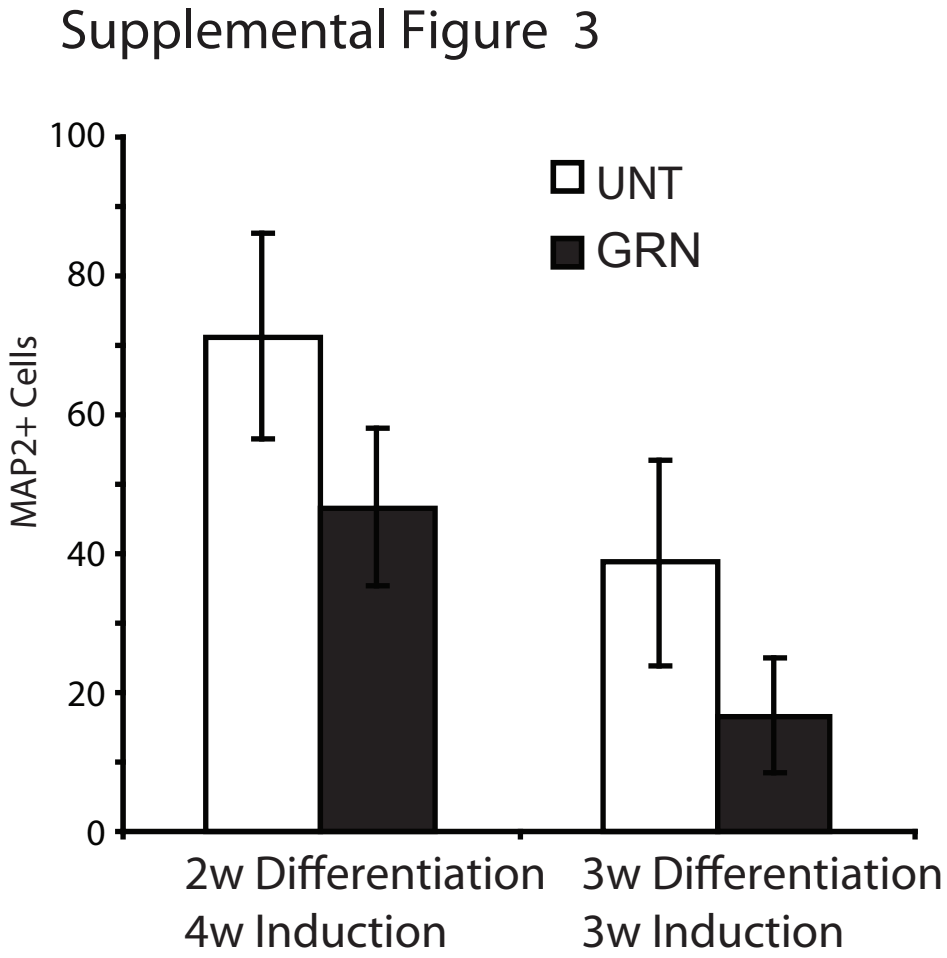






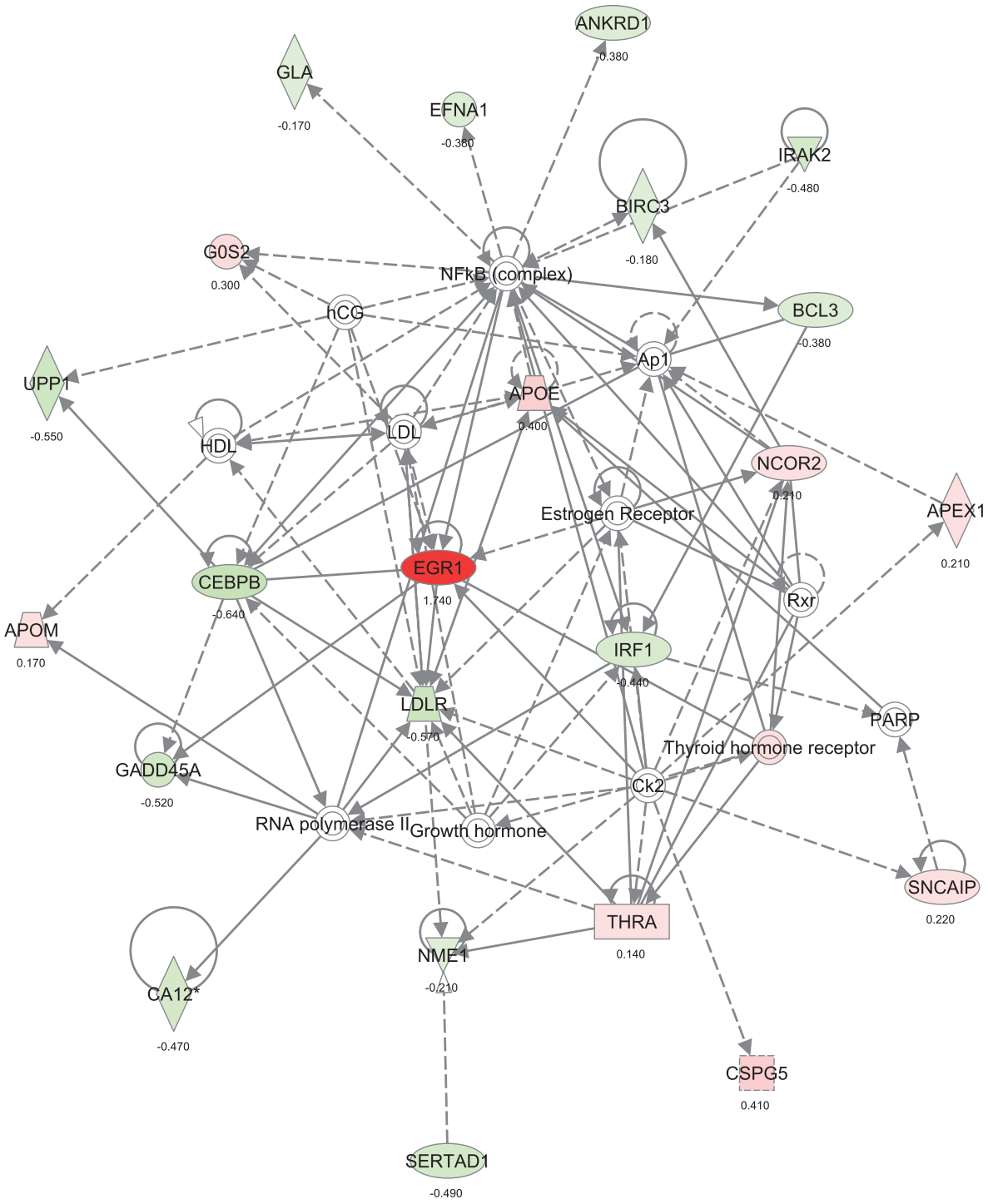


Supplementary Figure 3

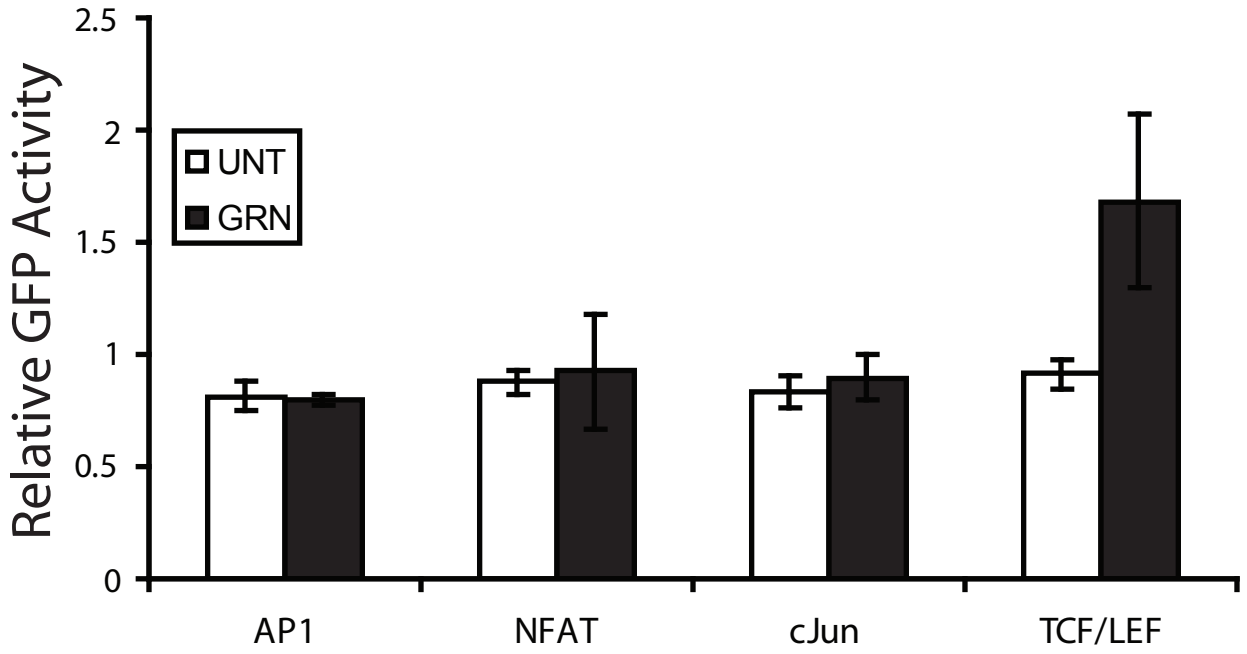


Supplementary Figure 4

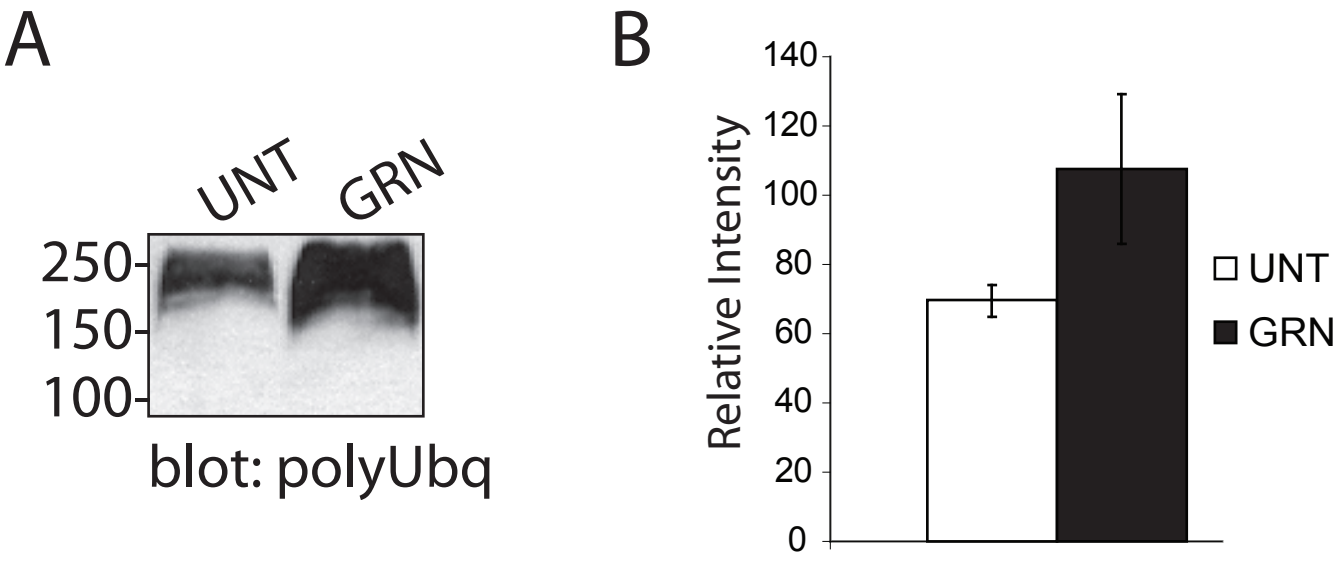
Supplemental Figure 4



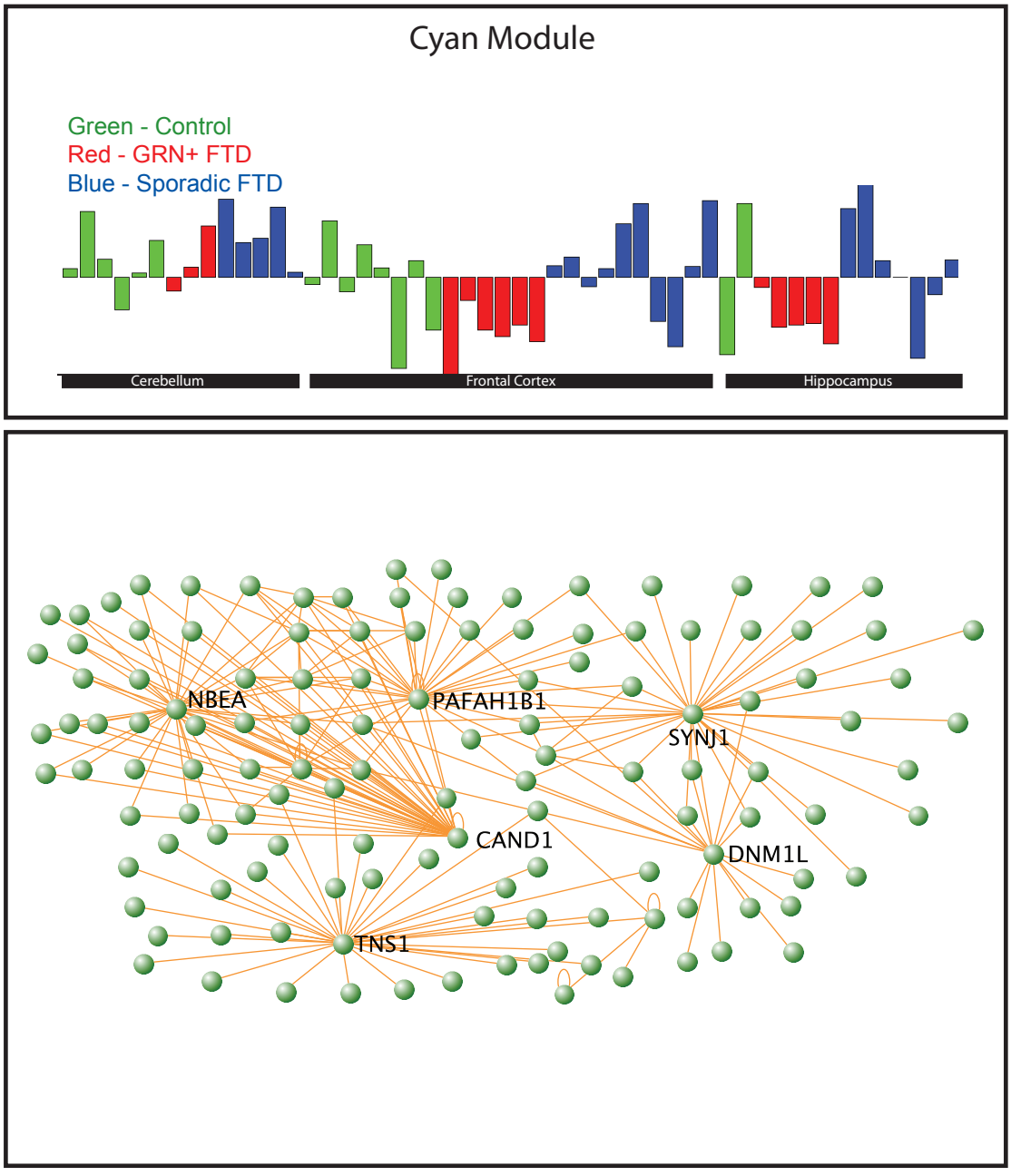
Supplemental Figure 5



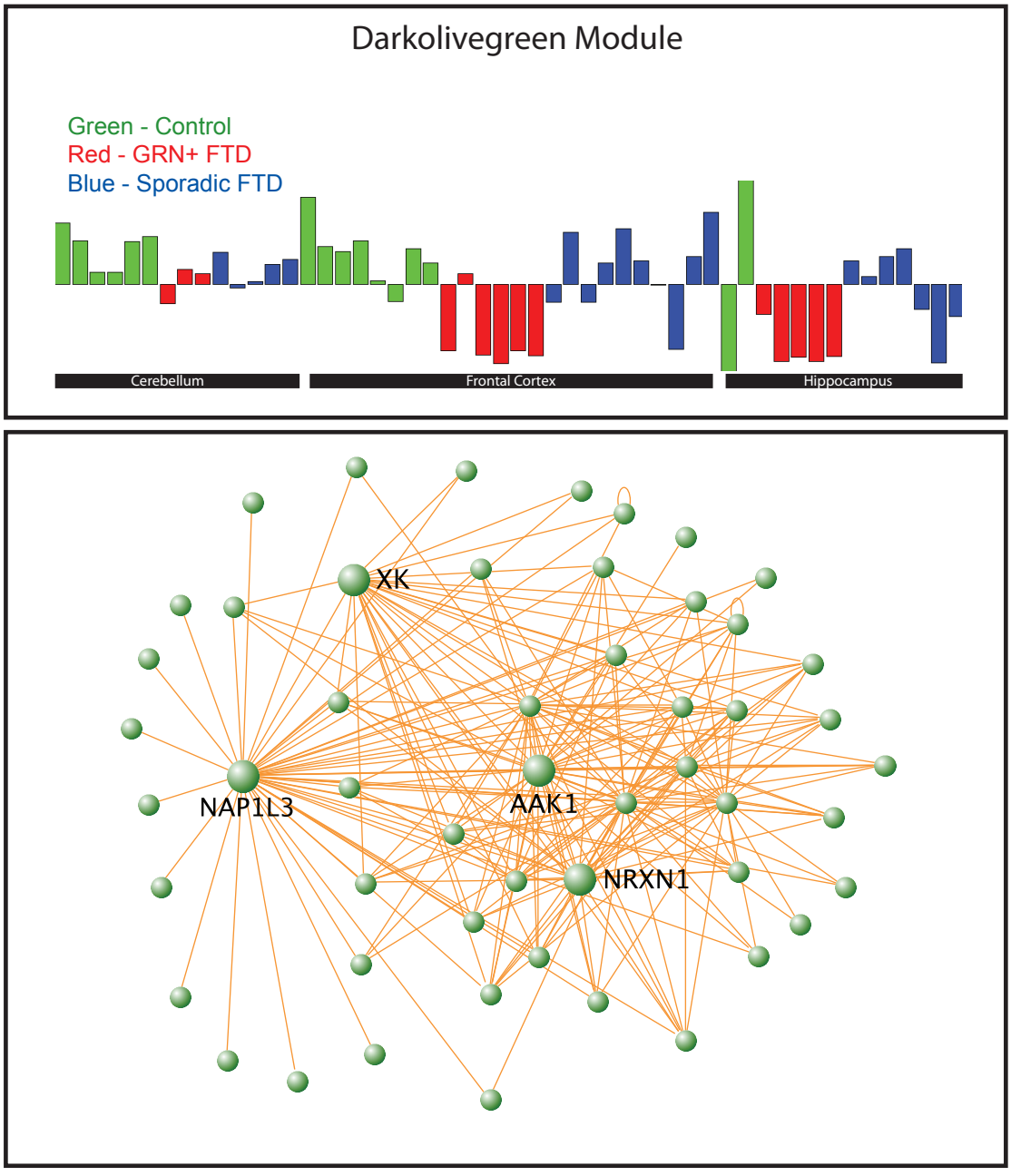
Supplemental Figure 6



Supplemental Figure 7A



Supplemental Figure 7B



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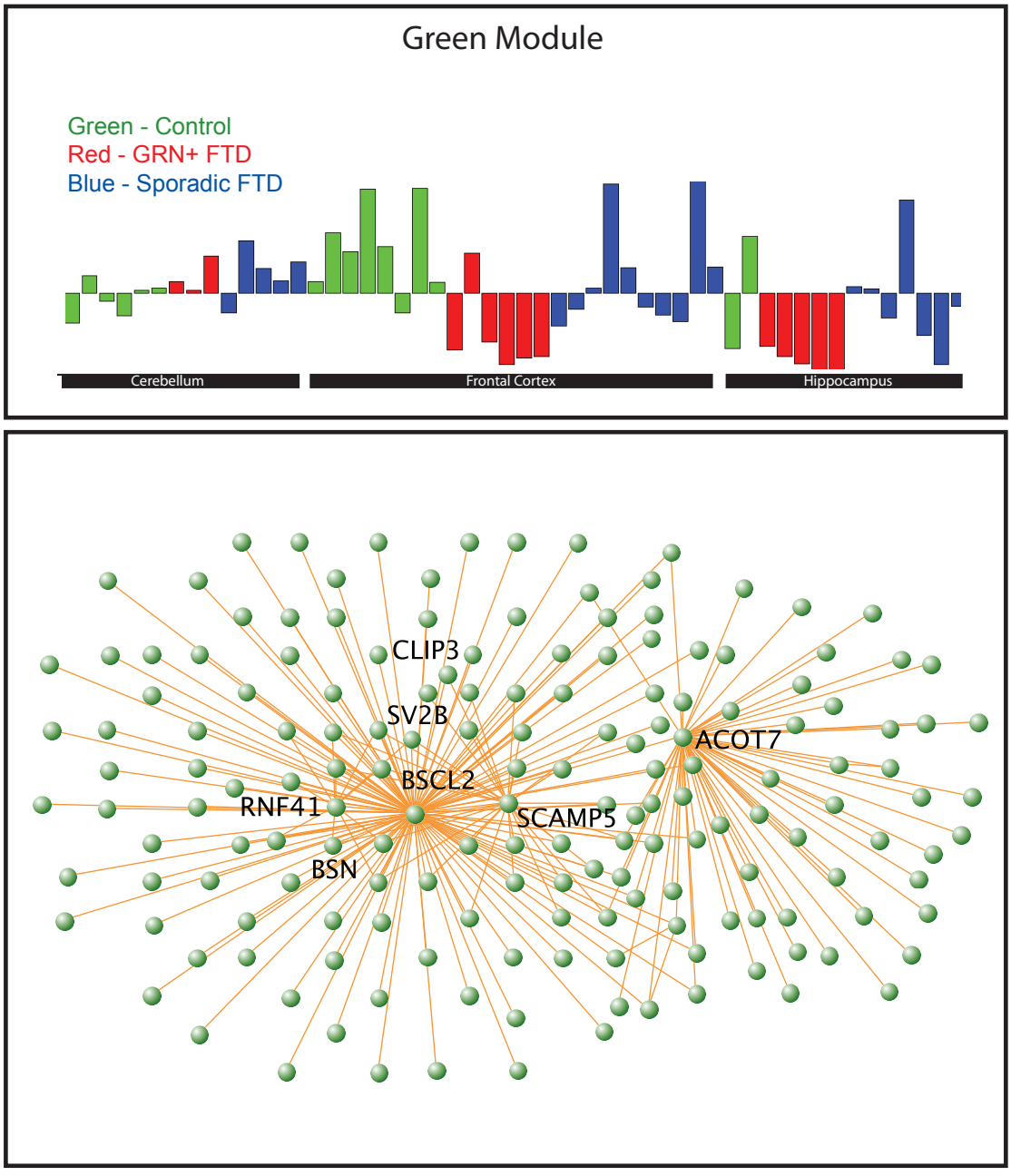

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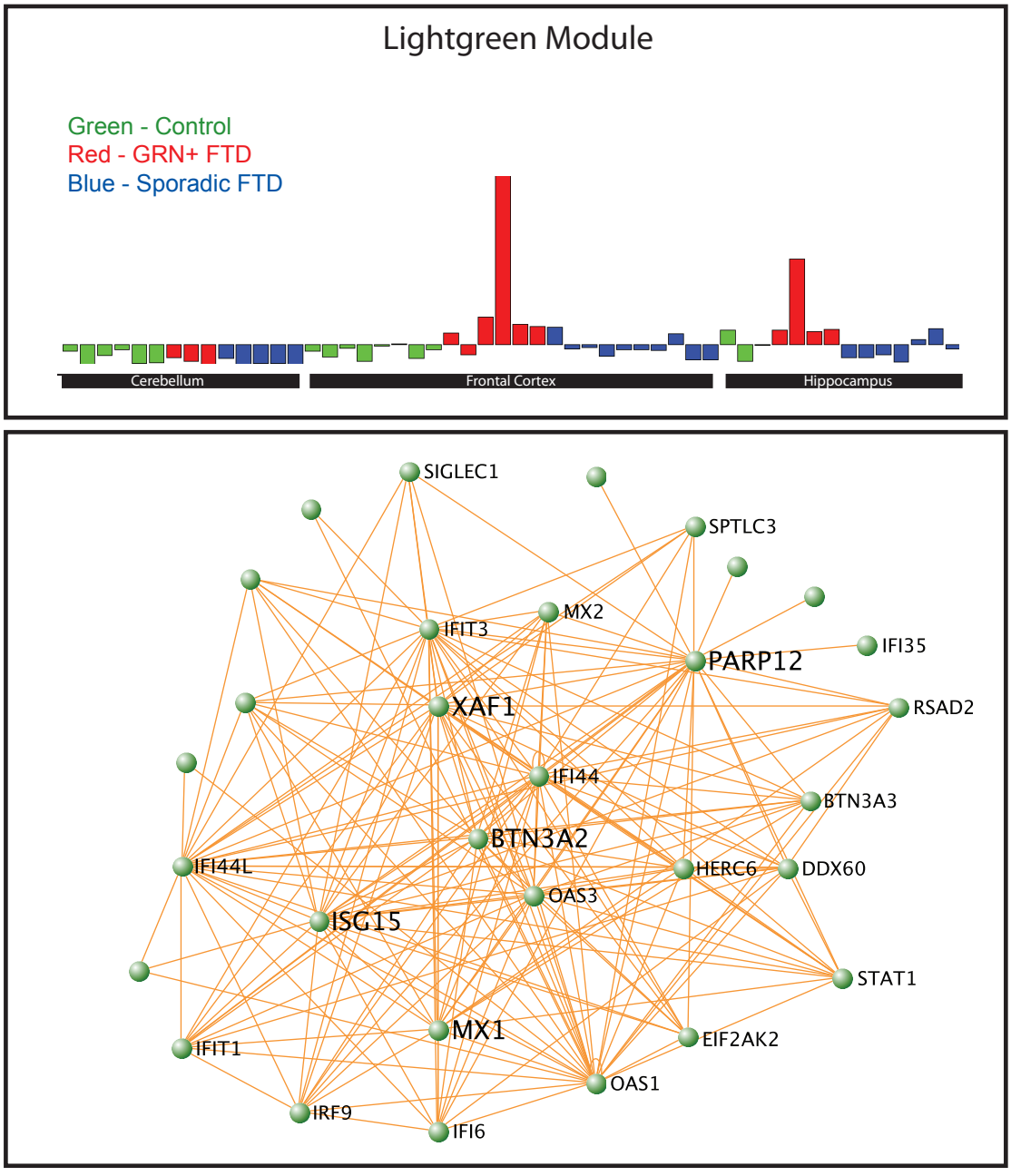

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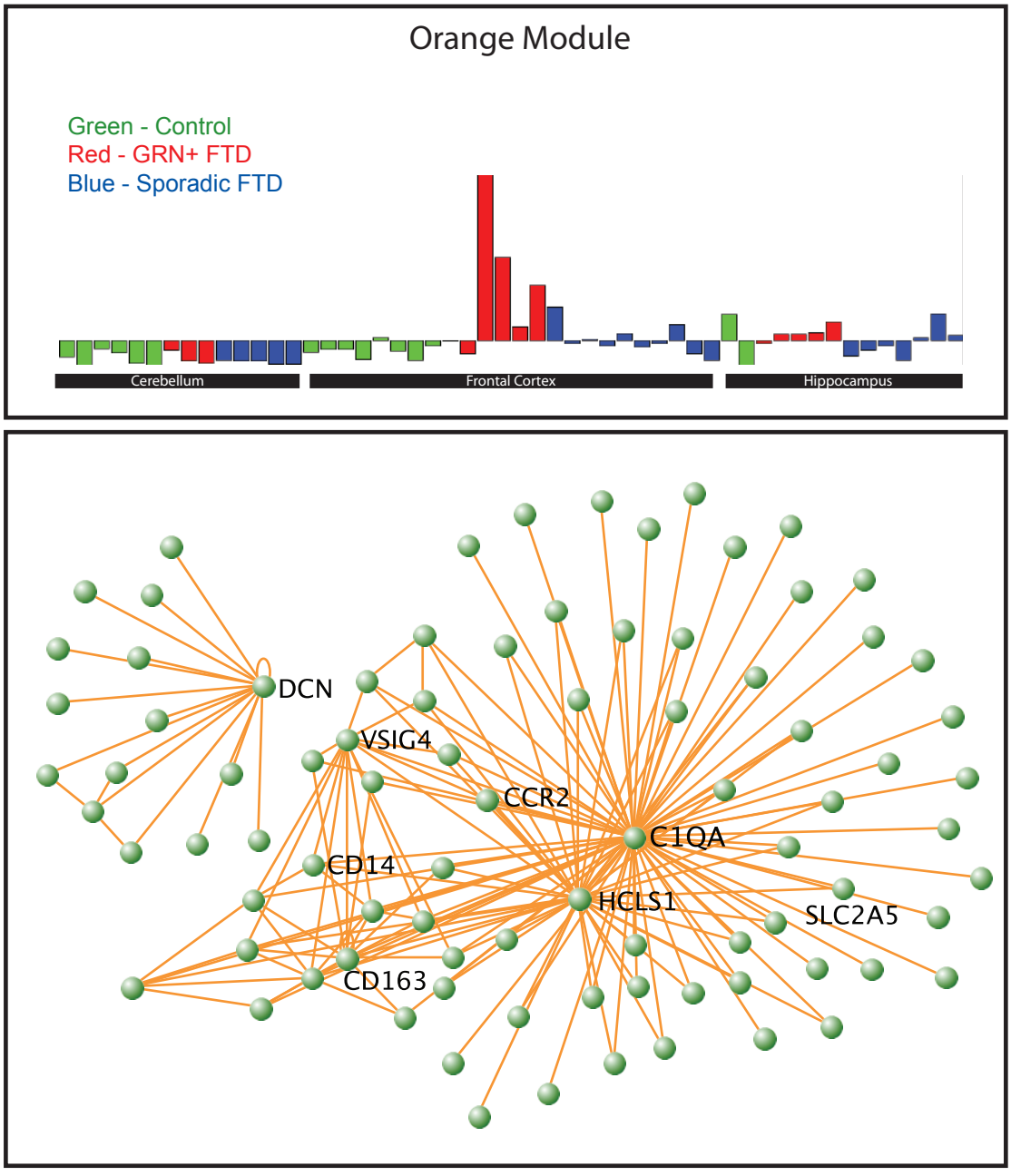

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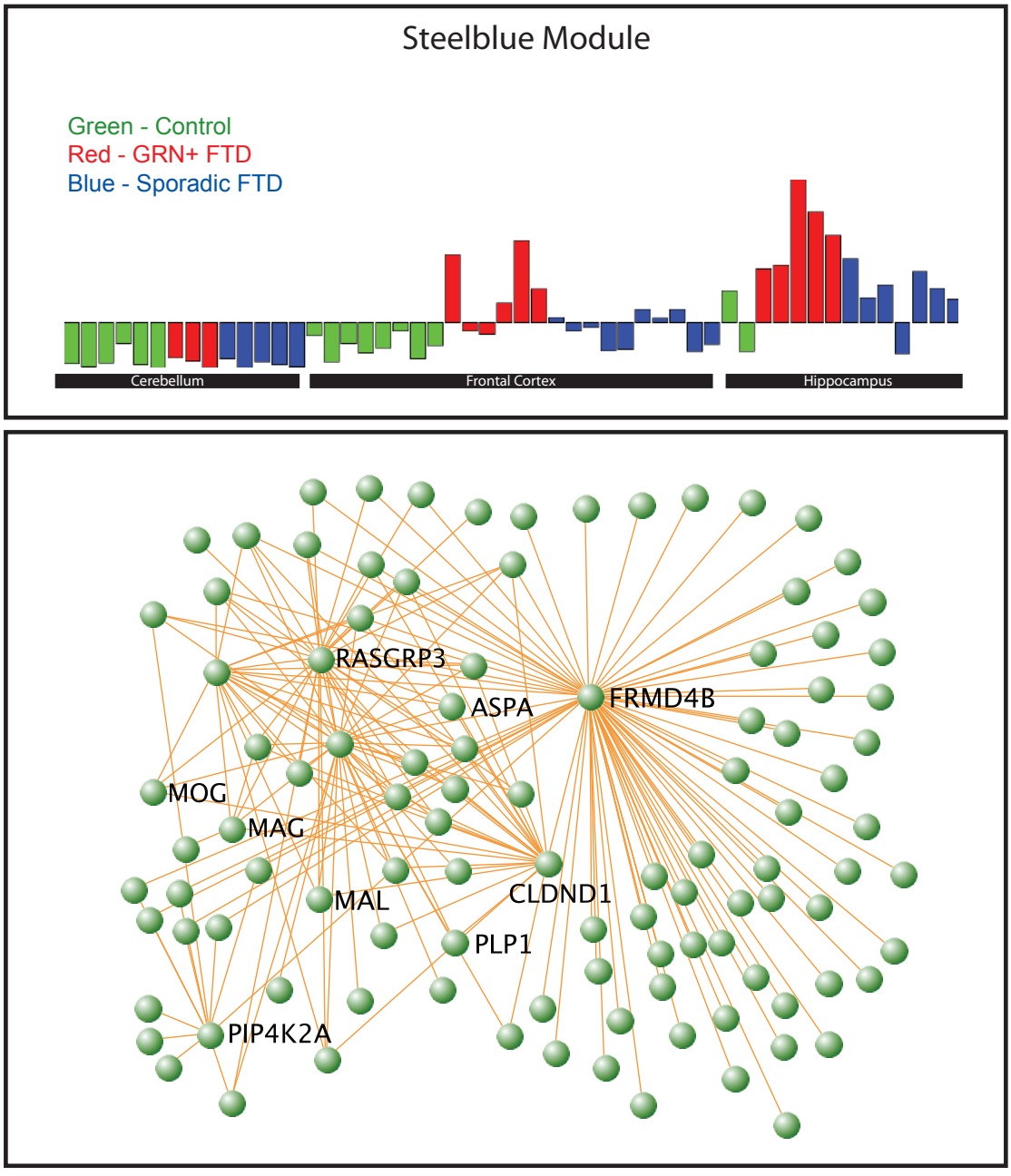
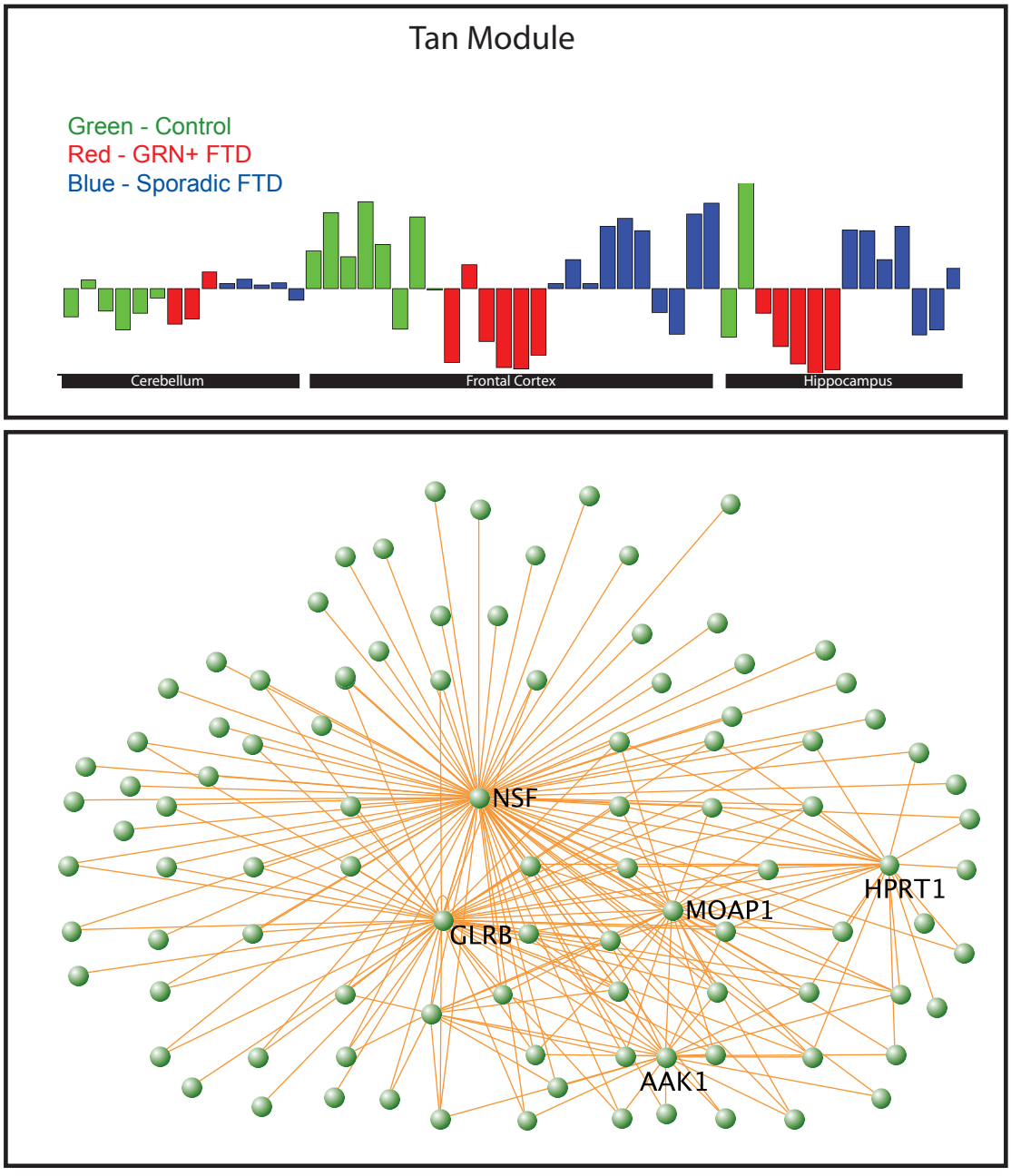


Supplemental Figure 7G



Supplemental Figure 8