Research Paper

Progranulin deficiency induces overactivation of *WNT5A* expression via TNF-α/NF-κB pathway in peripheral cells from frontotemporal dementia-linked granulin mutation carriers

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Background: Loss-of-function progranulin gene (*GRN*) mutations have been identified as the major cause of frontotemporal lobar degeneration with transactive response (TAR) DNA-binding protein 43 (TDP-43) pathology (frontotemporal lobar degeneration [FTLD]-TDP); however, little is known about the association between progranulin (PGRN) deficiency and neuronal loss in individuals with FTLD-TDP. Previously we reported enhanced proliferative activity associated with the activation of WNT5A/CDK6/pRb signalling in PGRN-deficient cells. The objective of this work was to elucidate the association between PGRN deficiency, WNT5A signalling and cell proliferation in immortalized lymphoblasts from carriers of the c.709–1G > A *GRN* mutation (asymptomatic and FTLD-TDP). **Methods:** We assessed cell proliferation in carriers of the c.709–1G > A *GRN* gene mutation and controls without *GRN* mutation and without sign of neurologic degeneration by cell counting or using an MTT assay. We used a luciferase assay to measure the nuclear factor-κ (NF-κ) activity. We evaluated messenger RNA levels using quantitative real-time polymerase chain reaction and protein levels by immunoblotting. Co-immunoprecipitation was used to analyze the interaction between PGRN and its receptors. **Results:** We enrolled 19 carriers of the *GRN* gene mutation and 10 controls in this study. The PGRN-deficient cells showed increased expression of *WNT5A* due to NF-κB signalling overactivation. We observed a competition between PGRN and tumour necrosis factor-α (TNF-α) for binding both TNF receptors (TNFR) I and II. Blocking NF-κB signalling using wedelolactone or specific antibodies against TNFRs inhibited *WNT5A* overexpression and proliferation of PGRN-deficient cells. Conversely, the activation of NF-κB signalling by TNF-α increased WNT5A-dependent proliferation of control cells. **Limitations:** All cell lines were derived from individuals harboring the same splicing *GRN* mutation. Nevertheless, most of the known *GRN* mutations lead to haploinsufficiency of the protein. **Conclusion:** Our results revealed an important role of NF-κB signalling in PGRN-associated FTLD-TDP and confirm that PGRN can bind to TNF-α receptors regulating the expression of *WNT5A*, suggesting novel targets for treatment of FTLD-TDP linked to *GRN* mutations.

Introduction

Frontotemporal lobar degeneration (FTLD) is a clinically, pathologically and genetically heterogeneous disorder resulting from the progressive deterioration of the frontal and temporal lobes of the brain.[1,2] It is the second most common form of presenile dementia (after Alzheimer disease), with a prevalence estimated to be 10–30 per 100 000 individuals between the ages of 45 and 65 years.[3,4] Depending on the affected regions, patients with FTLD can have dementia, behavioural abnormalities, language impairment or personality changes, among other conditions.[5,6] The molecular pathology is heterogeneous and is based on the type of neuronal lesions and abnormal protein aggregates present in neuroectodermic cells. In most cases, the inclusions contain either the microtubule-associated protein tau (FTLD-tau; approximately 40%) or the transactive response DNA-binding protein TDP-43 (FTLD-TDP; approximately 50%), although in a small number of cases pathological inclusions containing the fused in sarcoma (FUS) protein (FTLD-FUS) or ubiquitinated proteins (FTLD-UPS) have been described.[7–9]

A positive family history is found in 40%–50% of patients with FTLD,[10] and 10% of them present an autosomal dominant inheritance.[11] Familial forms of FTLD are caused by mutations in 3 common genes: microtubule-associated protein tau (*MAPT*), granulin (*GRN*) and chromosome 9 open reading

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frame 72 (*C9ORF72*).[12–18] Mutations in other genes, such as transactive response (TAR) DNA-binding protein-43 (*TARDBP*), valosin-containing protein (*VCP*), chromatin-modifying 2B protein (*CHMP2B*), sequestome1/p62 (*SQSTM1*) and ubiquilin-2 (*UBQLN2*) are rare causes of disease.[14,19–23]

Mutations in *GRN* have been identified as a major cause of autosomal-dominant FTLD-TDP, leading to TDP-43 inclusions by a haploinsufficiency mechanism,[12,13] but little is known about how progranulin (PGRN) deficiency induces neuronal loss in individuals with FTLD-TDP.[24]

Previous work from our laboratory showed an inverse association between WNT5A and PGRN abundance. In addition, we found an overactivation of cell proliferation associated with enhanced noncanonical WNT5A/CDK6/pRb signalling in both lymphoblasts from patients with FTLD-TDP carrying a loss-of-function *GRN* mutation (c.709–1G > A) or *GRN* knockdown SH-SY5Y neuroblastoma cells.[25] It has been suggested that an aberrant activation of the WNT5A cascade could have pathogenic significance in PGRN deficiency–linked FTLD, as it is believed that unscheduled cell cycle entry underlies neuronal loss in patients with neurodegenerative disorders.[26] The present work was undertaken to elucidate possible mechanisms involved in the PGRN deficiency–induced increases in WNT5A levels and signalling.

Little is known about how *WNT5A* is regulated; however, there is evidence that nuclear factor (NF)-κB can influence *WNT5A* expression.[27] In this sense, 2 promoter regions have been identified in the human *WNT5A* gene (promoter A and B), which contains putative NF-κB binding sites.[28] NF-κB mediates the expression of many genes involved in inflammation and it is known to be upregulated in some neurodegenerative disorders such as Alzheimer disease,[29,30] Parkinson disease[31] or amyotrophic lateral sclerosis (ALS).[32]

Tumour necrosis factor (TNF)-α is a well-known NF-κB inducer.[33] There are 2 membrane receptors of TNF-α: TNFRI and TNFRII. TNFRI is widely expressed in most cell lines and primary tissues, although TNFRII is preferentially expressed on cells of the hematopoietic lineage.[34] The binding of TNF-α to both TNFRs results in the recruitment of adaptor and interacting proteins that activate the IκB kinase (IKK) complex leading to phosphorylation and degradation of IkBα and activation of NF-κB.[35,36] Work from different laboratories supports the hypothesis that PGRN directly binds to TNFRs, blocking their interaction with TNF-α.[37–39] Therefore, we hypothesized that PGRN deficiency would eventually facilitate TNF-α/NF-κB signalling, which in turn may control WNT5A expression and signalling.

Methods

Materials

All components for cell culture were obtained from Invitrogen. We obtained recombinant human progranulin from Enzo and recombinant human TNF-α from R&D Systems. We purchased polyvinylidene fluoride (PVDF) membranes from Merk-Millipore, and the Luciferase Reporter Assay System was obtained from Promega. Other reagents were of molecular biology grade. We obtained antibodies against human NF-κB p50 (H-119) (sc-7178), NF-κB p65 (H-286) (sc-7151), β-actin (sc-81178) and α-tubulin (sc-23948) from Santa Cruz Biotechnologies; antibodies against phospho-IκBα (Ser32) (14D4) (#2859), IκBα (#9242) and NF-κB phospho-p65 (Ser536) (#3031) from Cell Signalling; and antibodies against TNFRI and II from Hycult Biotech. Anti-WNT5A and anti-PGRN (EPR3781) were obtained from Abcam, and mouse anti-human Lamin B1 was from Calbiochem. The kinase inhibitor PD332991 was kindly provided by Pfizer, and we obtained the NF-κB inhibitor wedelolactone (WDL) from Merk-Millipore.

Cell lines

We recruited carriers of the c.709–1G > A *GRN* gene mutation (presymptomatic and symptomatic) and control individuals without *GRN* mutation and without sign of neurologic degeneration from 7 different families of Basque descent with a homogeneous genetic background to participate in this study. In all patients, FTD was diagnosed in the Hospital Universitario Donostia using consensus criteria, as published elsewhere.[40]

All study protocols were approved by the Hospital Universitario Donostia and the Spanish Council of Higher Research Institutional Review Board and are in accordance with National and European Union Guidelines. In all cases, peripheral blood samples to determine the presence of the c.709–1G > A *GRN* mutation and to establish the lymphoblastoid cell lines were obtained after the patients or their relatives provided written informed consent.

Establishment of lymphoblastic cell lines was performed in our laboratory as previously described[41] by infecting peripheral blood lymphocytes with the Epstein–Barr virus (EBV). Cells were grown in suspension in T flasks in an upright position, in approximately 8 mL of RPMI-1640 medium that contained 2 mM of L-glutamine, 100 mg/mL of streptomycin/penicillin and 10% (v/v) fetal bovine serum (FBS). The flasks were maintained in a humidified 5% CO_2 incubator at 37°C. Fluid was routinely changed every 3 days by removing the medium above the settled cells and replacing it with an equal volume of fresh medium.

GRN *knockdown neuroblastoma SH-SY5Y cell lines*

Stable *GRN* knockdown neuroblastoma SH-SY5Y cells (Clone # 207) was provided by Drs. Joselin and Wu from the Center for Genetic Medicine (Northwestern University, Chicago, Il USA). *GRN* knockdown was achieved using pSUPERIOR RNAi construct as previously described.[42] We designed a sequence of 19 nucleotides targeted against nucleotides 207–226 (#207) of the human *GRN* messenger RNA (mRNA). The 64 nt short hairpin RNA sense and antisense primer sequences were 5′-GATCCCCGGCCACTCCTGCATCTTTATTCAAGAGATAAAGATGCAGGAGTGGCCTTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAGGCCACTCCTGCATCTTTATCTCTTGAATAAAGATGCAGGAGTGGCCGGG-3′.

The sense and antisense primer pairs were annealed and ligated into the pSUPERIOR vector (OligoEngine) according to

the manufacturer's instructions. The vector control was also stably introduced into SH-SY5Y cells to generate the control cell line.

Determination of cell proliferation

Cell proliferation was determined by total cell counting using a TC10TM Automated Cell Counter from Bio-Rad Laboratories. The EBV-immortalized lymphocytes from controls and *GRN* mutation carriers were seeded at an initial cell concentration of 1×10^6 cells/mL^{-1}. Cells were enumerated every day thereafter. In some experiments, cell proliferation was assessed using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay.[43]

Immunological analysis

Cell extract

To prepare whole cell extracts the lymphoblasts were seeded at an initial density of 1×10^6 cells $\times$ mL^{-1}, and 24 hours later cells were harvested, washed in phosphate-buffered saline (PBS) and then lysed in ice-cold lysis buffer (50 mM Tris pH 7.4, 150 mM of NaCl, 50 mM of NaF, 1% Nonidet P-40), containing 1 mM of sodium orthovanadate, 1 mM of phenylmethylsulfonylfluride (PMSF), 1 mM of sodium pyrophosphate and protease inhibitor complete mini mixture (Roche). The protein content of the extracts was determined using a de Pierce BCA Protein Assay kit (Thermo Scientific).

Western blot analysis

We fractionated 50–100 µg of protein from cell extracts on an SDS polyacrylamide gel and transferred it to PVDF membrane. The membranes were then blocked with 5% BSA (Sigma) and incubated overnight at 4ºC, with primary antibodies in the following concentrations: β-actin (1:1000), α-tubulin (1:1000), anti-human Lamin B (1:500), anti-WNT5A (1:500), anti-PGRN (1:500), NF-κB p50 (1:1000), NF-κB p65 (1:200), NF-κB phospho-p65 (Ser536) (1:1000), phospho-IkBα (1:500), IBa (1:1000), anti-TNF-RI (1:500) and TNFR-II (1:500). The specificity of the antibodies used in this work was checked by omitting the primary antibody in the incubation medium. Signals from the primary antibodies were amplified using species-specific antisera conjugated with horseradish peroxidase (Bio-Rad) and detected with a chemiluminiscent substrate detection system. Protein band densities were quantified using Image J software (National Institutes of Health) after scanning the images with a GS-800 densitometer from Bio-Rad.

Co-immunoprecipitation assay

After cell extracts preparation, 1 mg of protein was subjected to immunoprecipitation overnight at 4ºC with anti-PGRN antibody or an irrelevant Ig. Samples were incubated with protein A Sepharose (GE Healthcare Bio-Sciences), and the resultant immunoprecipitates were washed 3 times in ice-cold lysis buffer. The samples were then treated with protein sample buffer and boiled before immunoblotting. Western blotting was performed using the TNFRI and TNFRII antibodies. Membranes were incubated with anti-PGRN antibody as positive control of the experiment.

Reporter transfection and luciferase activity assay

Lymphoblasts from controls and carriers of the *GRN* mutation (2×10^6 cells) were transiently transfected with 2 µg of NF-κB-luciferase reporter plasmid (3EnhConA) using the Amaxa nucleofector II device (Lonza) and the P-16 program. To evaluate transfection efficiency and cell viability, cells were co-transfected with the GFP-expressing construct pmaxGFP (Amaxa Biosystems). Twenty-four hours after nucleofection, 3×10^5 cells were staining with propidium iodide and analyzed by flow cytometry. The transfection efficiency was approximately 17%. Luciferase activity measurement took place 24 hours after nucleofection using the Luciferase Assay System (Promega) according to the manufacturer's instructions.

Quantitative real-time PCR

Total RNA was extracted from cell cultures using Trizol reagent (Invitrogen). We quantified RNA yields spectrophotometrically, and RNA quality was checked using the A260:A280 ratio and on a 1.2% agarose gel to determine the integrity of 18S and 28S rRNA. RNA was then treated with DNase I Amplification grade (Invitrogen). One microgram was reverse transcribed with the Superscript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate using TaqMan Universal PCR MasterMix No Amperase UNG (Applied Biosystems) reagent according to the manufacturer's protocol. Primers were designed using the Universal Probe Library Set, Human (Roche Applied Science) and used at a final concentration of 20 mM. The following sequences of forward and reverse primers were used: 5′- ATTGTACTGCAGGTGTACCTTAAAAC-3′ and 5′-ACCCCCTTATAAATGCAACTGTTC-3′ for *WNT5A* and 5′-CCAACCGCGAGAAGATGA-3′ and 5′-CCAGAGGCGTACAGGGATAG-3′ for β-actin. Quantitative real-time PCR was performed in the Bio-Rad iQ5 system using a thermal profile of an initial 5-min melting step at 95 ºC followed by 40 cycles at 95 ºC for 10 s and 60 ºC for 60 s. Relative mRNA levels of the genes of interest were normalized to β-actin expression using the simplified comparative threshold cycle delta-delta Ct method ($2^{-[DCT\ WNT5A\text{-}DCT\ Actin]}$).

Statistical analysis

Statistical analyses were performed using Graph Pad Prism software version 6. Data are presented as means ± standard errors of the mean (SEM). Normality was checked using the Shapiro–Wilk test. Parametric tests were therefore used in the statistical analysis. Based on the expertise achieved in previous works[25,44,45] we expected that with the sample size that we used and the significance level that we established the variability within groups would be low enough and the power to detect differences between groups would be high enough to

ensure a statistical power above 0.9. Statistical significance was estimated using the Student *t* test or, when appropriate, using 1-way and 2-way analyses of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. We considered results to be significant at $p < 0.05$.

Results

Study sample

A total of 29 individuals from 7 families were enrolled in this study: 19 carriers of the *GRN* gene mutation (12 presymptomatic and 7 symptomatic) and 10 controls. Table 1 summarizes the demographic characteristics and the plasma levels of PGRN of all participants enrolled in this study.

WNT5A *expression and signalling is enhanced in PGRN-deficient lymphoblasts*

Data in Fig. 1 confirm and extend previous results from our laboratory by showing that lower cellular and secreted levels of PGRN found in lymphoblasts harboring the c.709–1G > A *GRN* mutation are associated with a significant increase in both cellular content and secreted WNT5A protein (Fig. 1A and B). In consonance, PGRN-deficient lymphoblasts, either from asymptomatic individuals or patients with FTLD-TDP, showed higher *WNT5A* mRNA expression levels (Fig. 1C). The increased *WNT5A* mRNA levels were blunted by the addition of 100 ng/mL of rhPGRN (Fig. 1C), resulting in the normalization of the enhanced proliferative activity of lymphoblasts harboring the *GRN* mutation (Fig. 1D). This dose of rhPGRN falls within the plasma concentration of PGRN found in control individuals[25,46] (Table 1).

NF-κB is overactivated in PGRN-deficient lymphoblasts

Because it has been reported that *WNT5A* expression could be regulated by NF-κB,[27] we were interested in assessing whether PGRN deficiency influences NF-κB activity. Fig. 2A showed a clear increase of NF-κB activation in PGRN-deficient lymphoblasts, as examined using a NF-κB luciferase reporter assay. As NF-κB activation occurs via phosphorylation of IkBα followed by proteasome-mediated degradation of this protein, resulting in the release and nuclear translocation of active p50 and p65 NF-κB subunits,[47,48] we determined that the intracellular levels of total and phosphorylated IκBα in controls and carriers of *GRN*-mutated lymphoblasts. We also performed a fractionation of cell extracts to look for nuclear p50 and p65 NF-κB subunit enrichment in PGRN-deficient cells. Fig. 2B shows increased levels of phosphorylated IkBα protein along with a diminution of total IκBα protein in cellular extracts from PGRN-deficient lymphoblasts, thereby facilitating the translocation of NF-κB subunits to the nucleus. As shown in Fig. 2C and 2D, the levels of p50 and p65 NF-κB subunits are increased in the nucleus of cells carrying the *GRN* mutation. In addition, we observed a significant decrease of these proteins in the cytosolic compartment of PGRN-deficient cells. Taken together these results indicate that PGRN deficiency is associated with activation of NF-κB signalling.

Table 1: Characteristics of individuals enrolled in this study

		c.709–1G > A *GRN* mutation carriers	
Characteristic	Control (*n* = 10)	Asymptomatic (*n* = 12)	FTLD (*n* = 7)
Age range, yr	31–69	34–72	54–70
Mean age, yr	51.8 ± 4.3	56.8 ± 3.2	65.3 ± 2.3
Sex, female:male	5:5	6:6	7:0
Age at onset, yr	—	—	60.7 ± 1.7
Phenotype	Asymptomatic	Asymptomatic	FTD-bv; CBS
Serum PGRN levels (ng/mL)	126.7 ± 13	47.5 ± 7.3	44 ± 5.6

CBS = corticobasal syndrome; FTD-bv = frontotemporal dementia (behaviour); PGRN = progranulin.

NF-κB inhibition blunts the serum-mediated enhanced proliferation of PGRN-deficient lymphoblasts

We evaluated the impact of experimentally modulating NF-κB activity using WDL, a selective inhibitor of IκB kinase,[49] on the proliferative activity and cellular content of WNT5A in controls and PGRN-deficient lymphoblasts.

Treatment with escalating concentrations of WDL (0–5 μM) suppressed the cell proliferation, which we assessed using the MTT reduction assay, in a dose-dependent manner (Fig. 3A). Maximal effects were found at the concentration of 2.5 μM. The treatment with this concentration of WDL for 72 hours was effective in decreasing the total number of PGRN-deficient lymphoblasts with no effect in control cells (Fig. 3B). As shown in Fig. 3C, WDL was able to decrease the levels of both *WNT5A* mRNA and WNT5A protein in PGRN-deficient lymphoblasts without affecting control cells. The effectiveness of WDL blocking NF-κB activation in PGRN-deficient lymphoblasts is shown in Fig. 3D. The addition of WDL (2.5 μM) was sufficient to inhibit the enhanced phosphorylation of IκBα protein and thus to increase the levels of total IκBα in the lymphoblasts of the *GRN*-mutation carriers. Taken together these results demonstrated that the PGRN haploinsufficiency–induced increase in levels of WNT5A and the enhanced proliferation of lymphoblasts harboring the *GRN* mutation depends on the activation of NF-κB.

PGRN interacts with TNF-α receptors in EBV-immortalized lymphoblasts

It has been reported that PGRN interacts with the TNF-α receptors, TNFRI and II,[37] raising the possibility that disruption of PGRN–TNFRI/II interactions may underlie FTLD pathogenesis. On these grounds, we considered the possibility that PGRN haploinsufficiency might induce overactivation of TNF-α/NF-κB signalling, which results in increased levels of WNT5A and cell proliferation. First, we checked if PGRN effectively binds to both TNFRs by immunoprecipitating PGRN with an anti-PGRN antibody, and then we probed for TNFRs with antibodies against TNFRI and II (Fig. 4A, left panel). A reverse experiment immunoprecipitating with

TNFRI and II antibodies is shown in the right panel of Fig. 4A. We found that PGRN binds to both TNFRI and II. Then, we treated lymphoblasts from controls and carriers of the *GRN* mutation with anti-TNFR antibodies or with soluble TNF-α and determined the proliferative activity, NF-κB activation and levels of WNT5A (Fig. 4 and Fig. 5).

Treatment of controls and PGRN-deficient lymphoblasts with anti-TNFRI or anti-TNFRII antibodies normalized the

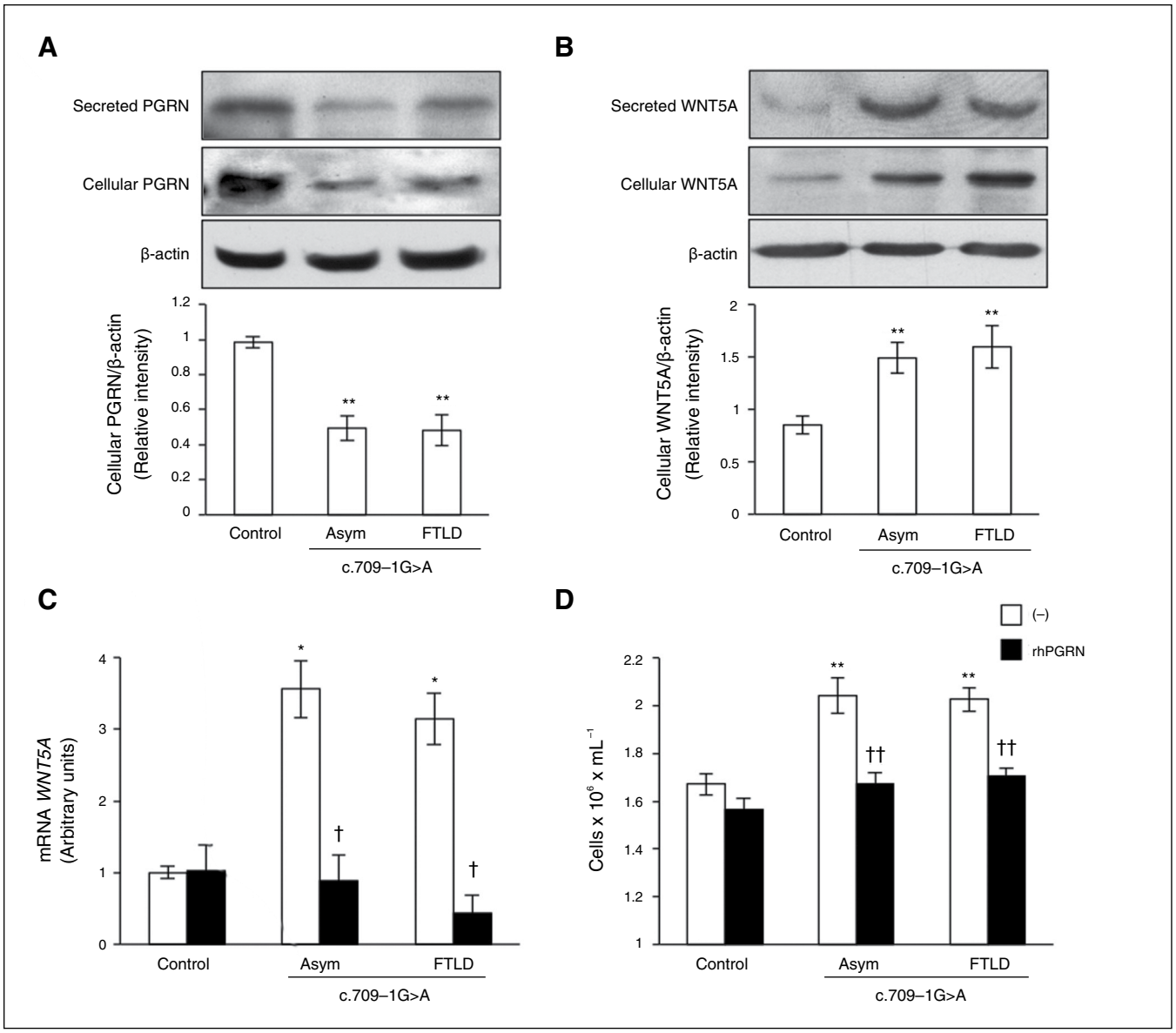

Fig. 1: WNT5A expression and cell proliferation in progranulin (PGRN)-deficient lymphoblasts and control cells. (**A**) Immortalized lymphocytes from controls and carriers of the c.709–1G > A *GRN* mutation, either asymptomatic or patients with frontotemporal lobar degeneration (FTLD)-TDP, were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$. Twenty-four hours later, cells and conditioned medium were collected and processed to detect PGRN and WNT5A protein levels using Western blotting. Representative immunoblots show the cellular content and secreted levels of WNT5A protein in control and PGRN-deficient cells. Data represent the means ± standard errors of the mean (SEM) of the levels of PGRN and WNT5A observed in 11 experiments carried out with all the individuals enrolled in this study. (**B**, **C**) Statistical analysis was performed using 1-way analysis of variance (ANOVA; PGRN: $F_{2,25} = 19.13$, WNT5A: $F_{2,23} = 15.89$, $^{**}p < 0.01$ compared with control cells). Cells were grown in the absence (white bars) or presence (black bars) of 100 ng/mL of recombinant human PGRN (rhPGRN). (**B**) Twenty-four hours after treatment, cells were harvested to analyze *WNT5A* mRNA expression by quantitative polymerase chain reaction. Values shown are the means ± SEM for 5 experiments carried out with 5 different cell lines from controls and *GRN* mutation carriers (asymptomatic and FTLD-TDP). Statistical analysis was performed using 2-way ANOVA ($F_{2,27} = 3.12$, $^{*}p < 0.05$ compared with control cells, †$p < 0.05$ compared with untreated cells). (**C**) To asses cell proliferation, samples were taken 72 hours after rhPGRN treatment and counted using a TC10 Automated Cell Counter from BioRad. Values shown are the means ± SEM for 10 different observations carried out with all the cell lines enrolled in this study. We performed 2-way ANOVA ($F_{2,51} = 3.39$, $^{**}p < 0.01$ compared with control cells, ††$p < 0.01$ compared with untreated cells).

proliferative activity of cells harboring the *GRN* mutation without significantly affecting the proliferation of control cells (Fig. 4B). Blocking just 1 of the TNFRs was enough to blunt the enhanced proliferative response of PGRN-deficient lymphoblasts, as we didn't observe additive effects in cell proliferation after treatment with both anti-TNFR antibodies (Fig. 4B). By contrast, as shown in Fig. 4C, the addition of soluble TNF-α increased proliferation of control cells, but not that of PGRN-deficient lymphoblasts. This effect of TNF-α on control cells was prevented in the presence of recombinant human PGRN (rhPGRN). In addition, TNF-α partially blunted the effect of exogenous rhPGRN, decreasing the total cell number in PGRN-deficient lymphoblasts (Fig. 4C).

We then evaluated the effect of anti-TNFRs and exogenous TNF-α treatments on NF-κB activity and WNT5A levels. As expected, both anti-TNFRs decreased NF-κB overactivation in asymptomatic patients and patients with FTLD, which was indicated by the inhibition of the phosphorylation of IκBα (Fig. 5A). In agreement with the idea that NF-κB is involved in the increased levels of WNT5A in PGRN-deficient cells, Fig. 5B shows that the blockade of TNFRs decreased the cellular content of WNT5A in lymphoblast-carriers of the *GRN* mutation to values close to those of control cells (Fig. 5B). Conversely, the addition of TNF-α–activated NF-κB signalling (Fig. 5C) increased WNT5A protein levels in control cells to values close to those observed in lymphoblasts harboring the *GRN* mutation (Fig. 5D).

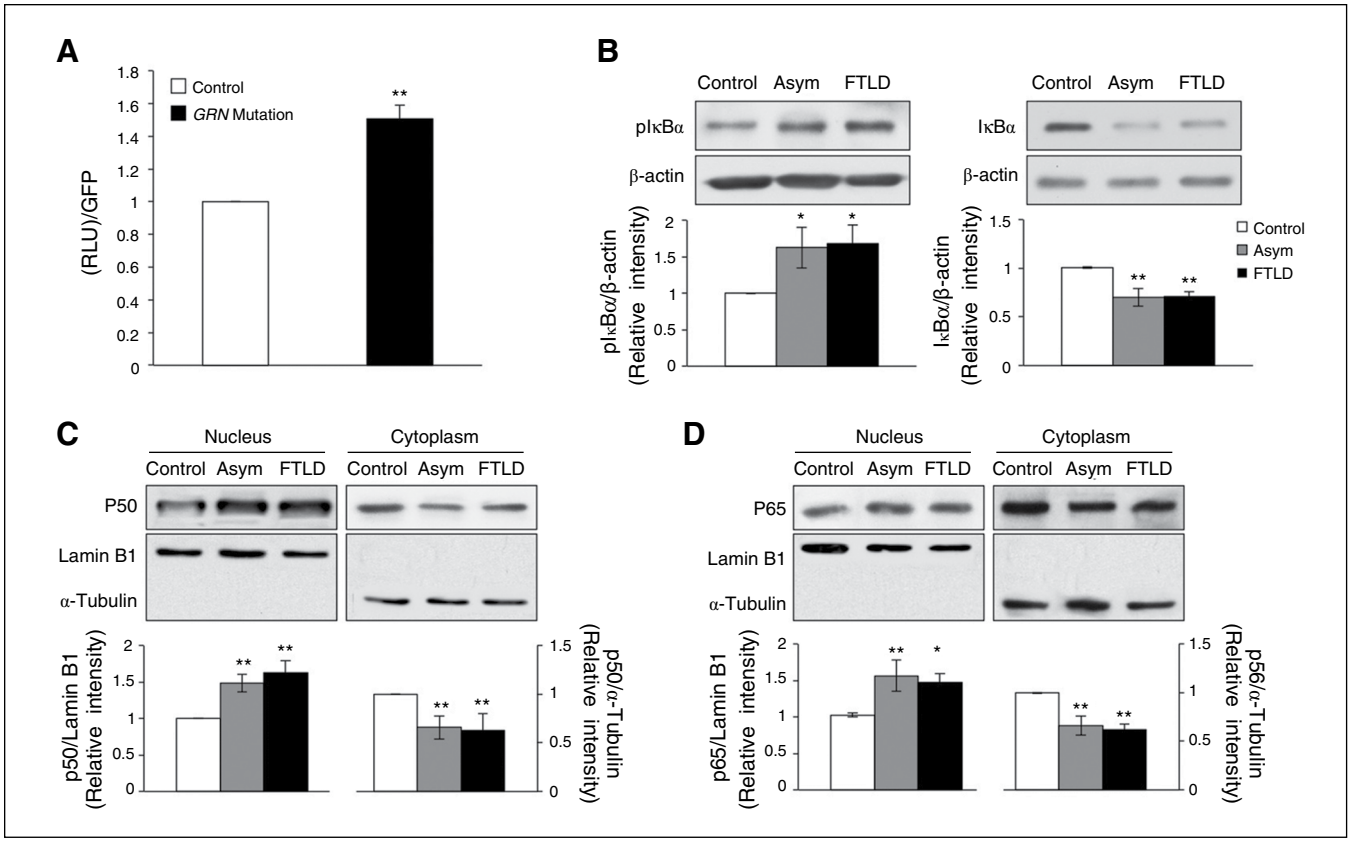

Fig. 2: Nuclear factor (NF)-κB signalling pathway in control and progranulin (PGRN)-deficient lymphoblasts. (**A**) Immortalized lymphoblasts from controls and carriers of the c.709–1G > A *GRN* mutation were transiently transfected with a NF-κB-luciferase reporter plasmid (3EnhancerConA). To evaluate the effectiveness of transfection, cells were co-transfected with a green fluorescent protein (GFP)-reporter plasmid. Twenty-four hours after nucleofection, cells were harvested and lysed to determine luciferase activity using the Luciferase Assay System (Promega). The graph corresponds to the mean ± standard error of the mean (SEM) of 3 independent experiments relativized by GFP. Statistical analysis was performed using the Student *t* test ($t_4 = 6.203$, $^{**}p < 0.01$ compared with control cells). (**B**, **C** and **D**) Immortalized lymphoblasts from controls and carriers of the c.709–1G > A *GRN* mutation, asymptomatic or patients with frontotemporal lobar degeneration (FTLD)-TDP, were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$, and 24 hours later cells were collected and lysed to prepare whole cell extracts or to separate nuclear and cytosolic fragments. Levels of proteins were analyzed using Western blotting. (**B**) Representative immunoblots show the levels of pIkBα (left panel) and IkBα (right panel). Data represent means ± SEM of different experiments carried out with 8 controls and 12 *GRN* mutation carriers (6 asymptomatic and 6 FTLD). β-actin was used as loading control. Statistical analysis was performed using 1-way analysis of variance (ANOVA; IkBα: $F_{2,26} = 9.7$, pIkBα: $F_{2,19} = 4.69$, $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with control cells). (**C** and **D**) Representative immunoblots showing the nuclear (left) and cytosolic (right) content of (**C**) p50 or (**D**) p65 NF-kB subunits. Lamin B1 and α-tubulin antibodies were used as loading and purity control of the nuclear and cytosolic fractions, respectively. Data represent means ± SEM of different experiments using 7 controls, 8 asymptomatic *GRN* mutation carriers and 6 patients with FTLD. Statistical analysis was performed using 1-way ANOVA (Nuclear p65: $F_{2,13} = 5.676$, $p < 0.05$; cytosolic p65: $F_{2,13} = 13.32$, $p < 0.01$; Nuclear p50: $F_{2,37} = 8.715$, $p < 0.01$; cytosolic p50: $F_{2,37} = 18.00$, $p < 0.01$ compared with control cells).

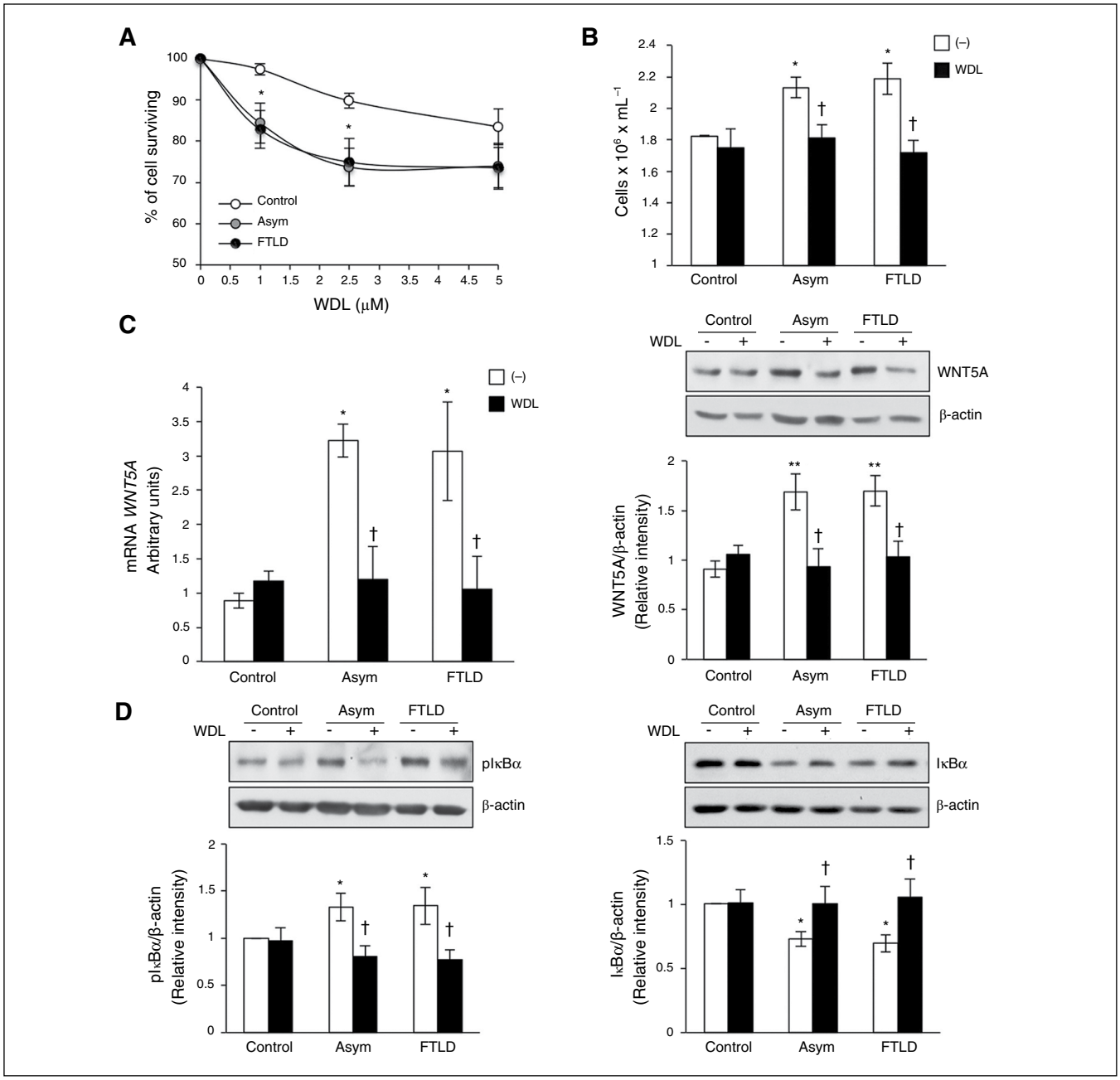

Fig. 3: Effect of wedelolactone (WDL) on nuclear factor (NF)-κB activation, cell proliferation and WNT5A expression of control and progranulin (PGRN)-deficient lymphoblasts. (**A**) A total of 100 000 cells per well were seeded in a 96-well plaque in absence or presence of increasing doses of WDL (0–5 mM), and the cell survival was measured using the MTT assay. Results are the mean of 3 replicates in 4 different experiments, and are expressed as the percentage of cell survival of treated cells compared with untreated ones. Statistical analysis was performed using 2-way analysis of variance (ANOVA; $F_{2,32} = 12.03$, $^*p < 0.05$ compared with control cells). (**B**) Effect of the treatment with WDL (2.5 mM) on proliferation of control and PGRN-deficient lymphoblasts. Immortalized lymphocytes were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$, and aliquots were taken for cell counting 72 hours after the drug administration. Data shown are the means ± standard errors of the mean (SEM) of different experiments carried out with 4 individuals of each type. We used 2-way ANOVA to carry out the statistical analysis ($F_{2,14} = 3.73$, $^*p < 0.05$ compared with control cells, †$p < 0.05$ compared with untreated cells). (**C**) Effect of the WDL (2.5 mM) treatment on *WNT5A* mRNA (left panel) and WNT5A protein levels (right panel). A representative immunoblot is shown. Data represent the means ± SEM of 5 experiments for qualitative polymerase chain reaction (PCR) and 9 for Western blotting carried out with 5 individuals of each type. Statistical analysis was performed using 2-way ANOVA (*WNT5A* mRNA: $F_{2,17} = 2.692$, WNT5A protein: $F_{2,37} = 4.58$, $^*p < 0.05$ and $^{**}p < 0.01$ compared with control cells, †$p < 0.05$ compared with untreated cells). (**D**) Representative immunoblots show the effectiveness of WDL treatment in preventing the activation of the NF-κB pathway through the inhibition of IkBα phosphorylation (left panel) and IkBα protein degradation (right panel). Densitometric data represent the means ± SEM of 6 independent experiments carried out with 5 individuals of each type. Statistical analysis was performed using 2-way ANOVA (pIkBα: $F_{2,21} = 3.77$, IkBα: $F_{2,24} = 3.44$, $^*p < 0.05$ and $^{**}p < 0.01$ compared with control cells, †$p < 0.05$ compared with untreated cells).

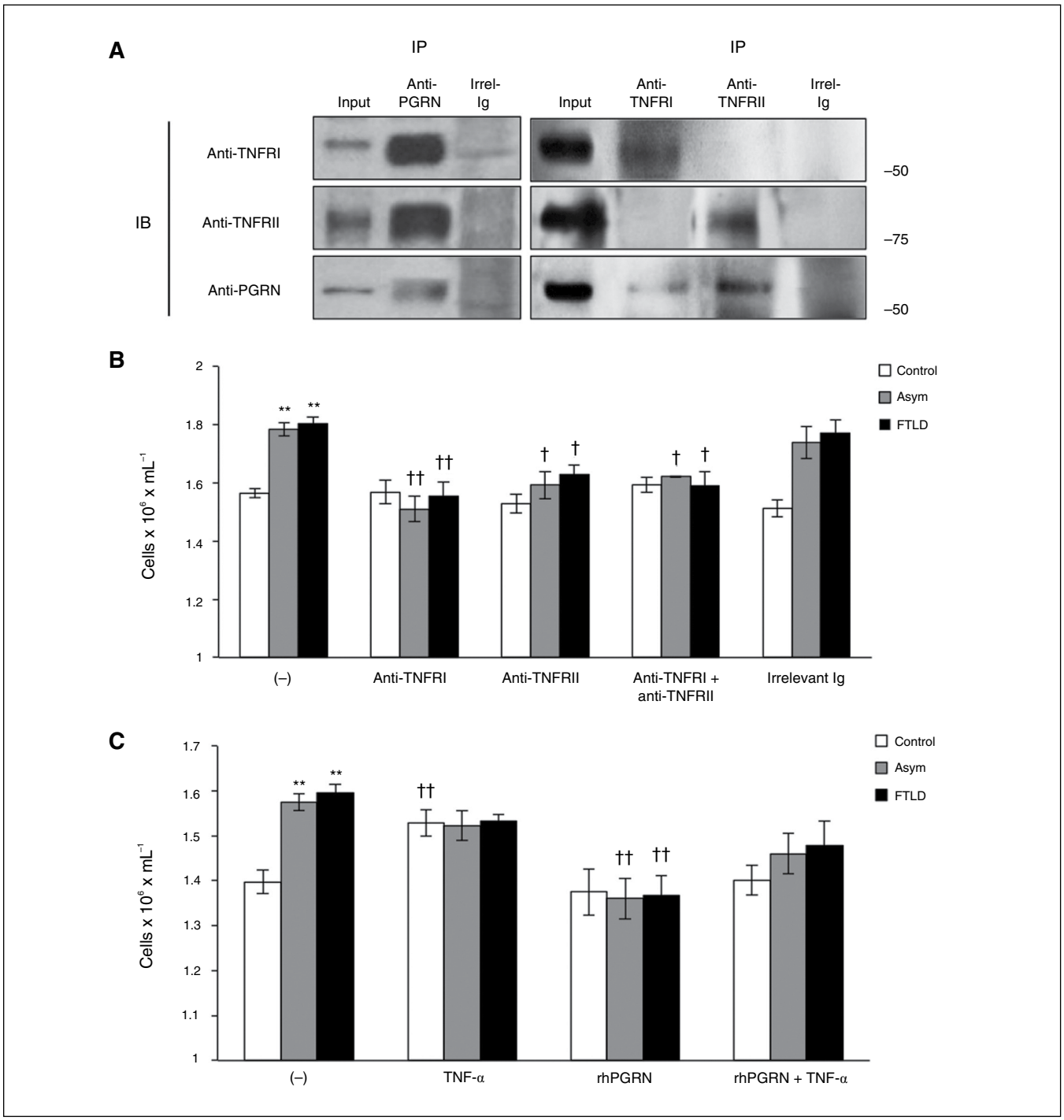

Fig. 4: Cross-talk between progranulin (PGRN) and tumour necrosis factor (TNF)-α receptors in Epstein–Barr virus (EBV)–immortalized lymphoblasts. (**A**) Lysates from EBV-immortalized lymphocyte cultures were co-immunoprecipitated with anti-TNF receptor (TNFR)I, TNFRII and PGRN antibodies or an irrelevant Ig and probed for TNFRs or PGRN using specific antibodies. We took 100 mg of total cell lysates as input for the co-immunoprecipitated proteins. The experiment was repeated 3 times, yielding similar results. (**B** and **C**) Immortalized lymphocytes from controls and carriers of the c.709–1G > A *GRN* mutation, asymptomatic or patients with frontotemporal lobar degeneration (FTLD)-TDP, were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$ in presence or absence of antibodies against (**B**) TNFRs (I and II) or an irrelevant Ig or (**C**) exogenous TNF-α and PGRN. Seventy-two hours after treatment, samples were taken and cells were counted using a TC10 Automated Cell Counter from BioRad. Values shown are the means ± standard errors of the mean (SEM) for 7 independent experiments carried out with all the cell lines derived from the participants enrolled in this study. We used 2-way analysis of variance (ANOVA) to carry out the statistical analysis (TNFRI and II: $F_{8,113} = 4.09$, TNF-α–PGRN: $F_{6,143} = 4.07$).

The enhanced activity of CDK6 in PGRN-deficient lymphoblast contributes to the NF-κB activation-mediated increased WNT5A expression

Previous work from our laboratory demonstrated that the PGRN haploinsufficiency–induced stimulation of proliferation was associated with the activation of CDK6/pRb cascade.[45] Since it has been reported that CDK6 may affect the transcriptional activity of NF-κB by its ability to phosphorylate the p65 subunit at serine 536,[50,51] we considered it interesting to evaluate whether this kinase could influence total levels of WNT5A. To address this issue we treated the cells with a known CDK6 activity inhibitor (PD332991). As expected, carriers of the *GRN* mutation presented higher levels of pSer536p65 NF-κB subunit

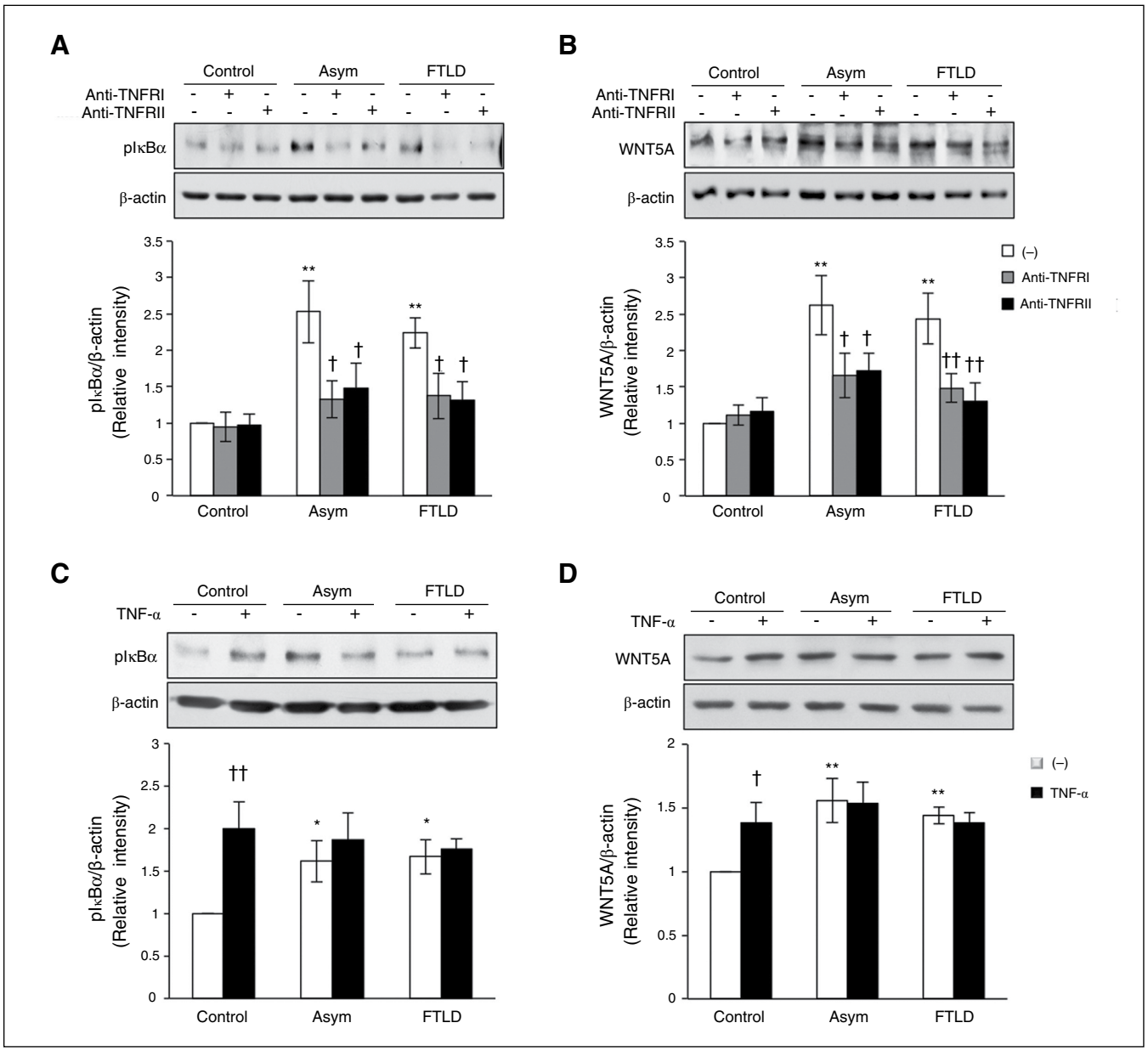

Fig. 5: Effect of modulating nuclear factor (NF)-κB activity on WNT5A levels. Immortalized lymphocytes from controls and carriers of the c.709–1G > A *GRN* mutation were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$ in the presence or absence of antibodies against (**A** and **B**) tumour necrosis factor receptors (TNFRs) I and II or (**C** and **D**) exogenous TNF-α. Twenty-four hours after treatment, cells were harvested, lysed to obtain the whole cell extract and analyzed using Western blotting. Representative immunoblots show pIkBα and WNT5A levels after the addition of (**A** and **B**) anti-TNFRs or (**C** and **D**) TNF-α treatment. Data shown are the means ± standard errors of the mean (SEM) for different observations for each protein carried out with 5 controls, 5 asymptomatic individuals and 5 patients with frontotemporal lobar degeneration (FTLD). Statistical analysis was performed using 2-way analysis of variance (pIkBα–TNFRs: $F_{4,36} = 1.546$, WNT5A–TNFRs: $F_{4,38} = 3.11$, pIkBα–TNF-α: $F_{2,18} = 6.21$, WNT5A-TNF-α: $F_{2,26} = 2.55$, $^*p < 0.05$ and $^{**}p < 0.01$ compared with control cells, $\dagger p < 0.05$ and $\dagger\dagger p < 0.01$ compared with untreated cells).

than controls. The inhibition of CDK6 activity by PD332991 selectively decreased the overphosphorylation of p65 NF-κB subunit in PGRN-deficient cells without affecting the pSer536p65 levels of control cells (Fig. 6A). Under these experimental conditions, blocking CDK6-associated kinase activity leads to the decrease of cell proliferation (Fig. 6B) of PGRN-deficient lymphoblasts to values close to those of control cells. Moreover, the addition of PD332991 blocked the enhanced expression of WNT5A observed in lymphoblasts from both presymptomatic and symptomatic carriers of the c.709–1G > A *GRN* mutation.

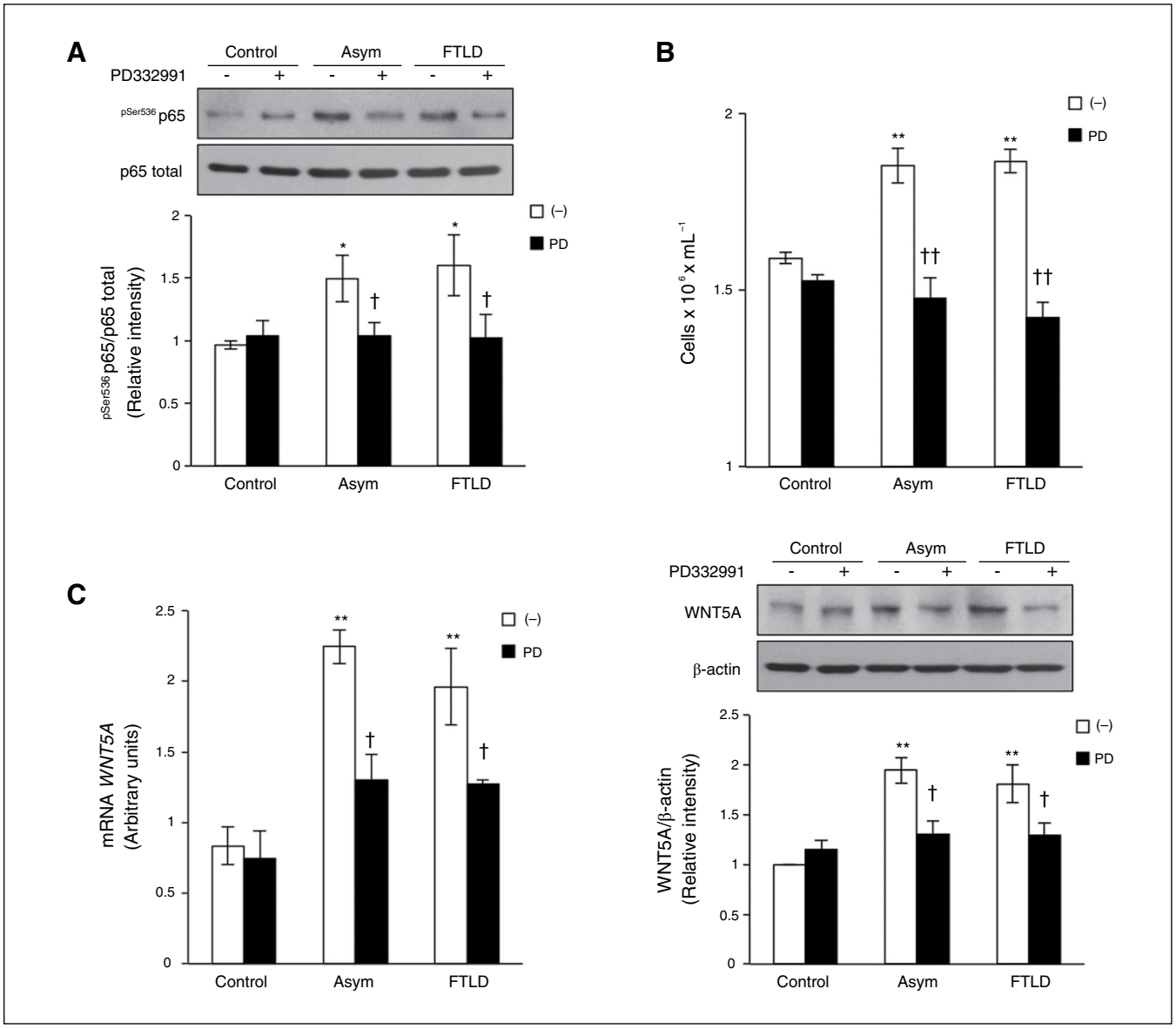

Fig. 6: Effect of CDK6 kinase activity inhibition on nuclear factor (NF)-κB activation and WNT5A expression. Immortalized lymphocytes from controls and carriers of the c.709–1G > A *GRN* mutation were seeded at an initial density of $1 \times 10^6 \times mL^{-1}$ and treated during 24 hours with the CDK6 kinase inhibitor PD332991 (1 mM). (**A**) Representative immunoblot shows the levels of phosphorylated Ser536p65 after PD332991 treatment. We used total p65 as loading control. Values represent the means ± standard errors of the mean (SEM) for 10 different observations carried out with 5 controls, 5 asymptomatic individuals and 5 patients with frontotemporal lobar degeneration (FTLD). Statistical analysis was performed using 2-way analysis of variance (ANOVA; $F_{2,33} = 3.50$, $^*p < 0.05$ compared with control cells, †$p < 0.05$ compared with untreated cells). (**B**) Cell counting 72 hours after PD treatment. Values shown are the means ± SEM for counts carried out with all the cell lines enrolled in this study. We used 2-way ANOVA to perform the statistical analysis ($F_{2,30} = 13.40$, $^{**}p < 0.01$ compared with control cells, ††$p <$ 0.01 compared with untreated cells). (**C**) *WNT5A* mRNA levels assessed using quantitative polymerase chain reaction (PCR; left panel) and WNT5A protein levels assessed using Western blotting (right panel) after PD332991 treatment. Representative immunoblot is shown, with values representing the means ± SEM for 10 independent experiments for Western blotting and quantitative real-time–PCR carried out with 7 controls, 7 asymptomatic individuals and 7 patients with FTLD. Statistical analysis was performed using 2-way ANOVA (WNT5A protein levels: $F_{2,44} = 6.719$, *WNT5A* mRNA levels: $F_{2,24} = 2.943$, $^{**}p < 0.01$ compared with control cells, †$p < 0.05$ compared with untreated cells).

PGRN deficiency increases NF-kB activity and WNT5A expression of SH-SY5Y neuroblastoma cells

Previous work form our laboratory has shown that PGRN-deficient neuroblastoma SH-SY5Y cells (*GRN* KD) presented enhanced WNT5A protein levels and signalling.[25] Here we addressed the issue as to whether these changes were accompanied by enhanced NF-κB activity. First, in agreement with our previous observations, we detected higher *WNT5A* mRNA expression levels in GRN KD cells than in control cells (Fig. 7A). Second, as shown in Fig. 7B, *GRN* KD cells displayed higher levels of pIkBα (left panel) together with a decrease in total IkBα content (right panel). Moreover a significant increase in the nuclear content of p50 and p65 NF-κB subunits was found in these PGRN-deficient neuronal cells (Fig. 7C and D). Taken together these results indicate that PGRN deficiency stimulates NF-κB activity in this neuronal model of FTLD-TDP, as it does in peripheral cells from patients with FTLD-TDP.

Discussion

Mounting evidence has suggested that some biochemical dysfunctions affecting the brains of patients with neurodegenerative diseases, including FTD, may also be traced outside the central nervous system (CNS).[46,52–54] For this reason, peripheral cells, such as fibroblasts or blood lymphocytes, have been used extensively in the search for useful biomarkers that may correlate with expression and/or progression of disease.[55–58] In this regard, it has been shown that peripheral cells from patients with neurodegenerative disorders display cell cycle disturbances,[45,59–62] reflecting the unscheduled cell cycle re-entry of postmitotic neurons and leading to apoptotic cell death.[63–65] The present work was undertaken to elucidate

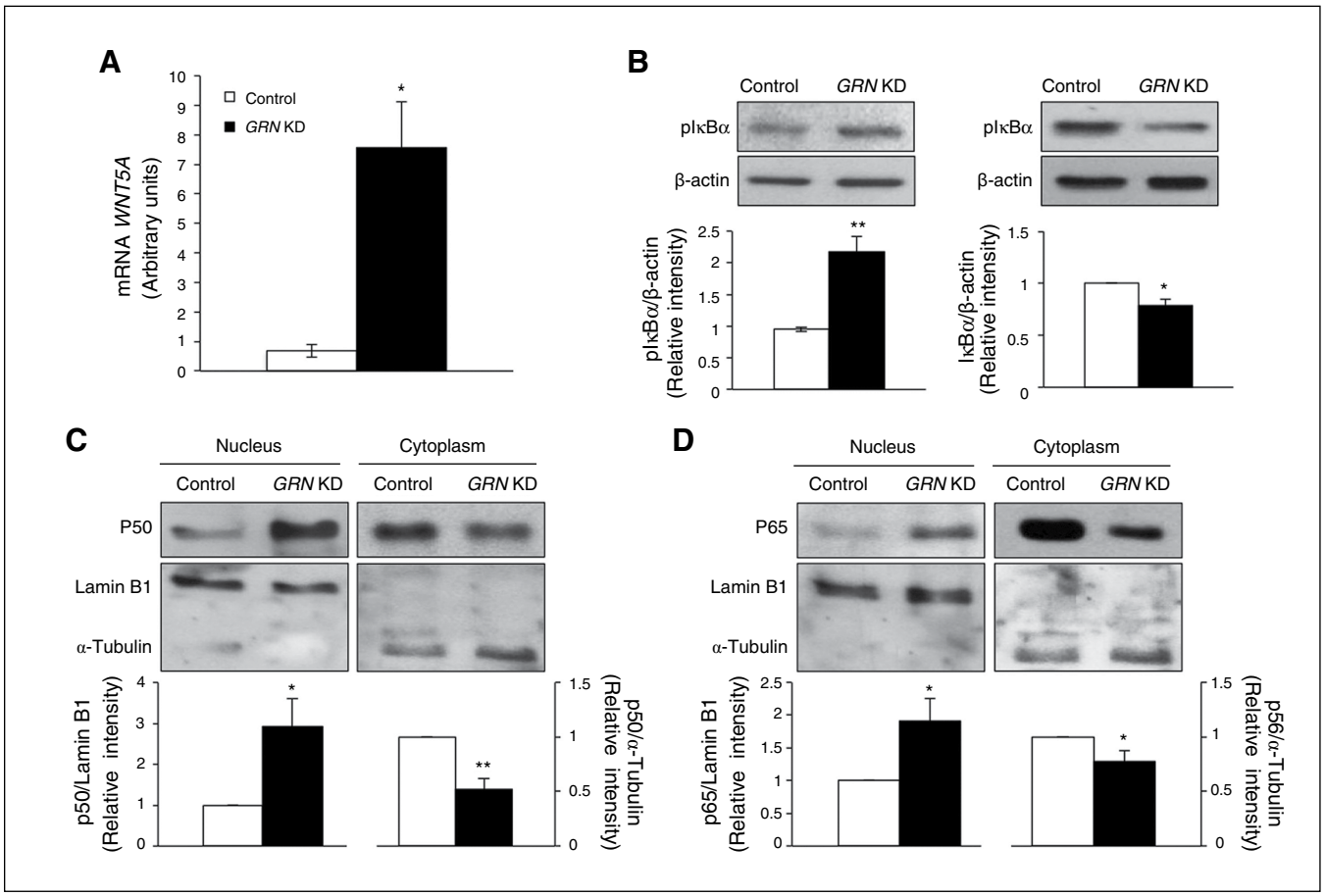

Fig. 7: Nuclear factor (NF)-κB activation and WNT5A expression in progranulin (PGRN)-deficient SH-SY5Y neuroblastoma cells. (**A**) *WNT5A* mRNA levels of control and *GRN* knockdown cells. Data represent the means ± standard errors of the mean (SEM) of 3 independent experiments. We used the Student *t* test to carry out the statistical analysis (t_4 = 2,88, *$p < 0.05$ compared with control cells). (**B**) Representative immunoblots show the levels of pIkBα (left panel) and IkBα (right panel) of control and *GRN* knockdown neuroblastoma cells. Data represent means ± SEM of 6 different experiments for pIkBα (t_{10} = 4.273) and 4 for IkBα (t_6 = 3.21). Statistical analysis was performed using the Student *t* test. β-actin was used as loading control (*$p < 0.05$ and **$p < 0.01$ compared with control cells). (**C** and **D**) Representative immunoblots show the nuclear and cytosolic content of (**C**) p50 or (**D**) p65 NF-κB subunits in control and *GRN* knockdown cells. Lamin B1 and α-tubulin antibodies were used as loading and purity control of the nuclear and cytosolic fractions, respectively. Data represent the means ± SEM of 5 different experiments. Statistical analysis was performed using the Student *t* test (nuclear p50: t_8 = 3.15, cytosolic p50: t_8 = 4.94, nuclear p65: t_8 = 2.9, cytosolic p65: t_8 = 2.4, *$p < 0.05$ and **$p < 0.01$ compared with control cells).

possible mechanisms involved in the PGRN deficiency–induced WNT5A expression and signalling in PGRN-deficient lymphoblasts from patients with FTLD-TDP that were considered responsible for the increased activation of the CDK6/pRb-mediated cell proliferation.[25] The main finding of our study is that PGRN deficiency facilitates TNF-α/NF-κB signalling to regulate WNT5A expression.

We investigated whether WNT5A expression and signalling could be regulated by NF-κB activity based on 2 pieces of information: first, NF-κB putative binding sites have been identified in the 2 alternative promoters A and B of the human *WNT5A* gene,[28,66] and second, we considered that PGRN deficiency could favour TNF-α-induced NF-κB activation as TNFRs have been proposed to serve as the binding receptor for PGRN.[37]

Several lines of evidence support the idea that *WNT5A* expression is regulated through NF-κB signalling in carriers of the c.709–1G > A *GRN* mutation. First, PGRN-deficient lymphoblasts showed higher NF-κB activity than control cells. Second, inhibition of NF-κB activation by WDL resulted in the decrease of *WNT5A* expression together with the normalization of the proliferative activity of mutant lymphoblasts. Third, the blockade of the NF-κB activation by using antibodies to both TNFRs (I and II) was also effective in decreasing WNT5A levels and proliferation of PGRN-deficient cells. Finally, the addition of soluble TNF-α induced the activation of NF-κB and increased *WNT5A* expression and proliferation of control cells. Although all the cell lines used were derived from individuals harboring the same splicing *GRN* mutation, the demonstration that rhPGRN rescued the phenotype of diseased lymphoblasts as well as the finding that NF-κB is also overactivated in *GRN* KD SH-SY5Y neuroblastoma cells allows the conclusion that PGRN deficiency induces the expression of *WNT5A* through the NF-κB pathway.

It has previously been reported that PGRN binds to TNFRs with high affinity.[37] Other authors did not find evidence for a direct interaction between PGRN and TNFRs;[67] however, their inability to demonstrate the direct binding of PGRN to the TNFRs could have been due to improper folding of the rhPGRN used or to inappropriate chip selection for the surface plasmon resonance. Since then, several groups independently

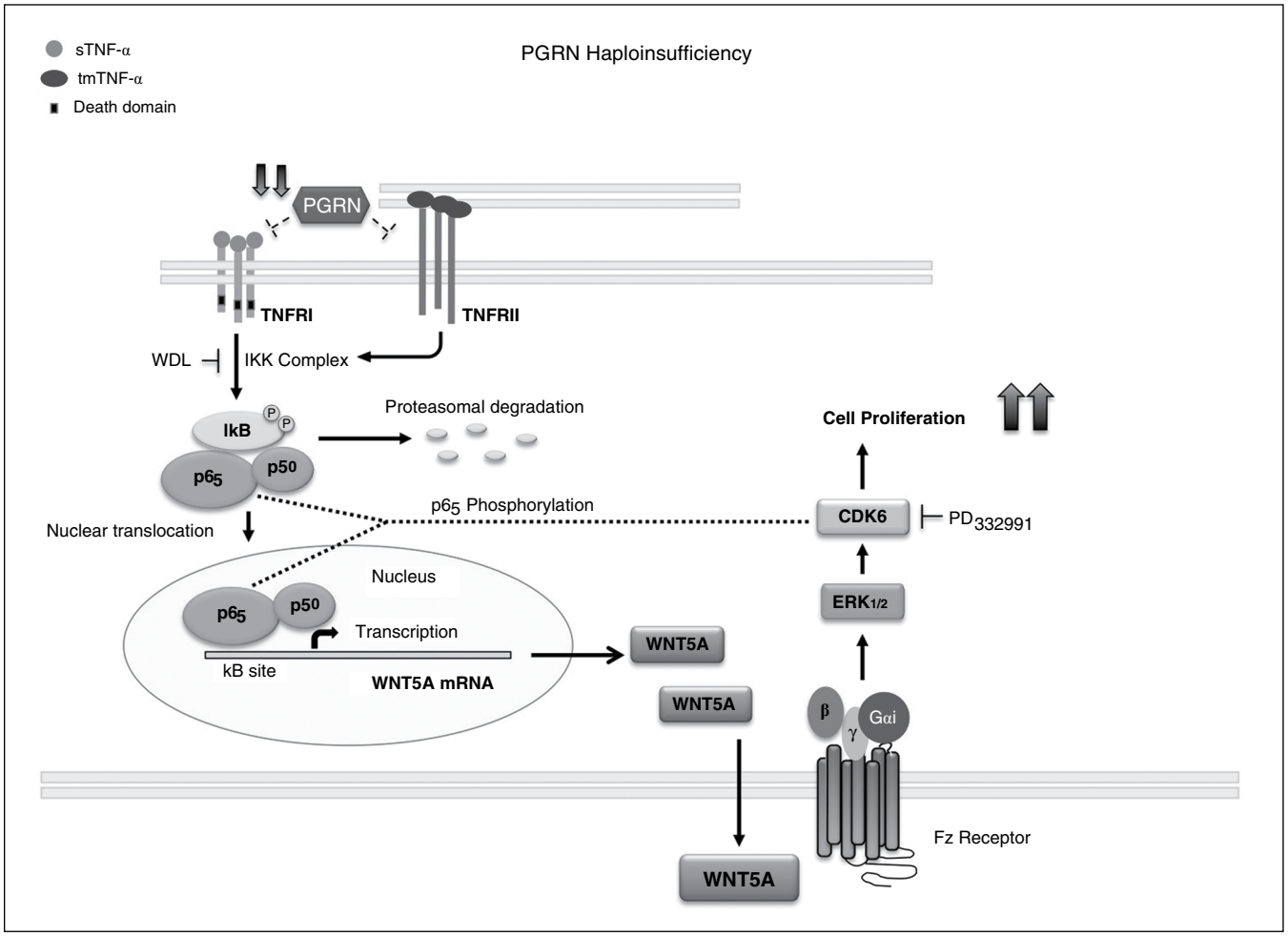

Fig. 8: Diagram summarizing the progranulin (PGRN) deficiency-related nuclear factor (NF)-κB activation and WNT5A expression; PGRN deficiency favours tumour necrosis factor (TNF)-α/NF-κB signalling that results in the increase of WNT5A expression and signalling. The WNT5A-induced stimulation of CDK6 activity is responsible for the enhanced proliferation of PGRN-deficient lymphoblasts and amplifies the NF-κB-mediated activation of WNT5A transcription through the p65 NF-κB subunit phosphorylation.

confirmed the PGRN–TNFR interaction and the inhibitory effect of this binding on TNF-α-induced effects.[39,68,69] Our results suggest the existence of a physical interaction between PGRN and both TNFRs (I and II), as we found that PGRN co-immunoprecipitates with either TNFRI or II. Moreover a functional interaction between PGRN and TNFRs is likely, as we found opposite effects after treatment with anti-TNFR antibodies or exogenous TNF-α on NF-κB signalling, *WNT5A* expression and proliferative activity of human lymphoblasts. Together these results suggest the cross-talk of TNF-α and PGRN signalling pathways to regulate the NF-κB-mediated expression of *WNT5A*, which in turn determines the proliferative activity of human lymphoblasts. It has been reported that $Grn^{-/-}$ mice developed a more severe collagen-induced inflammatory arthritis than their control littermates as the result of the competition between TNF-α and PGRN for signalling through TNFR.[37] Moreover, previous work showed that PGRN is a therapeutic target for rheumatoid arthritis.[70] On these grounds it would be interesting to evaluate if individuals harboring loss-of-function *GRN* mutations are more susceptible to inflammatory pathologies. A deficiency in PGRN could eventually disinhibit TNF-α-mediated signalling in neurons and/or microglia, leading to a persistent proinflammatory state over the lifetime that ultimately could contribute to neurodegeneration.

Generally, NF-κB activation depends on the phosphorylation and subsequent degradation of IkBα, an inhibitory NF-κB protein that forms a complex with p50 and p65 NF-κB subunits. Once released, NF-κB heterodimer is translocated into the nucleus, where it activates the transcription of its target genes.[71] However, it has been shown that NF-κB activity can also be regulated by phosphorylation of the p50 and p65 NF-κB subunits.[72,73] Of particular interest for our work is a report indicating that the p65 subunit of NF-κB is phosphorylated at Ser536 by CDK6.[50] As already mentioned, PGRN-deficient lymphoblasts display increased levels and kinase activity of CDK6 protein.[44,45] On these grounds we considered the possibility that the enhanced CDK6 levels could contribute to increased NF-κB activity in these cell lines. Our results show that the treatment of PGRN-deficient cells with PD332991, a specific CDK6 kinase activity inhibitor, inhibited p65 NF-κB subunit phosphorylation, decreased WNT5A levels and blunted the enhanced proliferative response of the PGRN-deficient lymphoblasts.

Taken together, these findings suggest the existence of a positive feedback loop where the overactivation of WNT5A/CDK6 signalling induced by PGRN deficiency could contribute to maintaining higher levels of WNT5A protein by favouring the phosphorylation of the p65 subunit and the nuclear transcriptional activity of NF-κB. On the other hand, this connection between NF-κB and CDK6 suggests a role of this kinase in inflammation and indicates that CDK6 could contribute to increase the proliferative activity by means other than pRb activation.

Limitations

A limitation of the present work is that all cell lines used were derived from individuals harboring the same splicing *GRN* mutation. Nevertheless, since most of the known *GRN* mutations lead to haploinsufficiency of the protein, one can assume that PGRN deficiency mediates the stimulation of WNT5A signalling.

Conclusion

We provide evidence that PGRN deficiency favours TNF-α/NF-κB signalling, which then results in the increase of WNT5A expression and signalling. Moreover, the WNT5A-induced stimulation of CDK6 activity, in addition to being responsible for the enhanced proliferation of PGRN-deficient lymphoblasts, amplifies the NF-κB-mediated activation of *WNT5A* transcription. The proposed scenario is represented schematically in Fig. 8.

In agreement with previous work, our results show no differences in the proliferative activity, NF-κB signalling and WNT5A levels among lymphoblasts derived from carriers of the c.709–1G > A *GRN* mutation who are asymptomatic or who have a clinical diagnosis of FTD.[25,45] Because most of the asymptomatic carriers are younger than the patients with FTD, it is suggested that these features are probably early etiologically relevant events during the development of this disorder. These presymptomatic *GRN* mutation carriers showed no symptoms of disease, but previous studies have described poorer neuropsychological performance and distinctive age-related cortical thinning in asymptomatic individuals compared with controls[74,75] that may reflect a presymptomatic phase of the disease.

Changes detected in peripheral cells from patients with FTLD-TDP might not fully reflect those in the FTLD brain; however, mounting evidence suggest that, in addition to neuronal damage, there are also peripheral aspects of the disease.[44,46,54,76–79] Altered activation of both TNF-α and WNT5A signalling has been found in the brains of patients with several types of neurodegenerative disorders.[80–82] Assuming that the TNF-α and WNT5A-mediated cell cycle dysfunction reported here could be peripheral sign of FTLD associated with PGRN deficiency, we think that targeting TNF-α and/or WNT5A signalling could have potential implications for designing novel therapeutic strategies.

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