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Beyond known mutations: Functional evidence for LRRK2 pathway activation in a subset of Parkinson's disease patients

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Article

Keywords:

Posted Date: December 9th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-8294746/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at npj Parkinson's Disease on September 26th, 2026. See the published version at https://doi.org/10.1038/s41531-026-01575-6.

Abstract

Background

Pathogenic *LRRK2* variants cause Parkinson's disease (PD) through increased kinase activity, with *VPS35* p.D620N and other rare *LRRK2* variants showing similar effects. Findings in idiopathic PD suggest broader LRRK2 pathway involvement. As LRRK2-targeted therapies advance, identifying patients with elevated pathway activity beyond known mutations is essential.

Methods

LRRK2-dependent pRab10^{Thr73} phosphorylation was quantified in blood neutrophils from 181 genetically enriched PD patients and 71 controls. Whole-exome sequencing identified pathogenic and rare variants. For a subset of PD patients with LRRK2 hyperactivation, an siRNA knock-down screen targeting genes carrying rare variants was performed in A549 cells.

Results

VPS35 p.D620N carriers showed marked hyperactivation, defining a reference threshold. Fourteen percent of PD patients without known pathogenic variants showed similar or higher activation. Knock-down studies implicated genes including *NECAP2*, *EIF5A2*, and *TECPR1* in modulating LRRK2 signaling.

Conclusions

A subset of PD patients without known mutations exhibits LRRK2 hyperactivation, supporting a broader "LRRK2-driven" mechanism and the need for scalable biomarkers.

Introduction

Parkinson's disease (PD) is a common neurodegenerative movement disorder affecting millions of individuals worldwide, but there is no cure or disease modifying treatment[1]. While most cases are idiopathic with no clearly defined cause, approximately 5–15% can now be attributed to known genetic determinants, including high penetrance monogenic variants (e.g., *LRRK2, PRKN, VPS35*) and strong risk alleles (e.g., *GBA1*)[2, 3]. These, however, don't explain all PD cases where a monogenetic cause is suspected but not confirmed; for example in individuals who report a positive family history of PD (27%)[3] and especially when accompanied with early PD onset. Moreover, recent genome-wide association studies (GWAS) indicate that the 90 established PD risk loci account for only 16–36% of the heritable risk in idiopathic PD, altogether implying that many other genetic contributors to PD have yet to be identified[4].

Among known PD genes, *LRRK2* - which encodes the large multidomain leucine-rich repeat kinase 2 (LRRK2) - represents the most common monogenic cause of PD, with pathogenic variants found in 1–2% of sporadic and 4% of familial PD cases[3, 5]. While p.G2019S is the most frequent pathogenic variant in LRRK2, a systematic overview of published data on clinical and genetic spectrum of LRRK2 variants in PD has identified over 200 additional, predominantly missense variants, most of which remain classified as variants of uncertain clinical significance[6]. Functional data are available for fewer than half of these variants, with 23 reported to increase LRRK2 kinase activity[7]. Current evidence indicates that disease causing pathogenic and likely pathogenic *LRRK2* variants (e.g., p.R1067Q, p.R1441G/C, p.F1700L and p.V1447L) enhance LRRK2 kinase activity and lead to hyperphosphorylation of downstream Rab GTPase substrates, particularly Rab10, both in cellular models and in clinical samples from variant carriers[7–10]. Aberrant Rab phosphorylation disrupts membrane trafficking and endolysosomal homeostasis and contributes to the underlying pathogenic mechanism driving *LRRK2*-associated PD[11–14]. Consequently, LRRK2 has emerged as a high value therapeutic target, with LRRK2 small molecule kinase inhibitors and LRRK2 lowering

therapies, including degraders and anti-sense oligonucleotides, currently being trialled in both idiopathic and genetically enriched LRRK2 PD.

To support these therapeutic efforts, reliable biomarkers are required to assess LRRK2 kinase activity and target engagement in human biospecimens[15]. A robust quantitative multiplexed immunoblotting assay has been developed to measure LRRK2-dependent Rab10 phosphorylation at Threonine 73 (pRab10^{Thr73}) in peripheral blood neutrophils and monocytes, providing a direct readout of LRRK2 kinase activity *in vivo*[16]. This assay enables systematic evaluation of LRRK2 pathway activation across patient cohorts and has previously shown that pathogenic variants such as p.R1441G and p.G2385R significantly increase LRRK2-dependent Rab10 phosphorylation[7, 17, 18]. Additionally, the same approach has enabled reclassification of rare *LRRK2* variants, (e.g., p.R1067Q, p.V1447L and p.F1700L) from variants of uncertain significance to pathogenic or likely pathogenic based on elevated kinase activity in patient samples[8–10].

Emerging evidence suggests that LRRK2 dysfunction extends beyond rare *LRRK2* variant carriers. For example, the pathogenic *VPS35* p.D620N increases LRRK2 kinase activity - as measured by elevated Rab10^{Thr73} phosphorylation - in peripheral blood neutrophils and monocytes from mutation carriers and model systems[19, 20]. These findings support the view that VPS35 and LRRK2 converge on the same endolysosomal signalling pathway[21]. Similarly, the recently identified PD-associated *Rab32* p.S71R variant increases LRRK2-dependent Rab10^{Thr73} phosphorylation when overexpressed together with LRRK2 in HEK293 cells[22, 23]. Conversely, knock-down of the LRRK2-counteracting phosphatase, PPM1H, increases Rab10^{Thr73} phosphorylation[24] and results in significant ciliary loss in a group of striatal interneurons in mice, mirroring the effects of activating LRRK2 variants (p.R1441G, p.G2019S)[25]. Notably, ciliary impairments have also been observed not only in postmortem brains of *LRRK2* p.G2019S carriers but also in idiopathic PD patients[26], suggesting that LRRK2-linked cellular pathologies extend to sporadic disease. Clinically, LRRK2-associated PD is often indistinguishable from idiopathic PD[27], and common LRRK2 polymorphisms have been linked to increased disease risk[4]. Together, these findings underscore a broader role for LRRK2 within a convergent PD-relevant signalling network.

In this study, we investigated LRRK2 kinase pathway activation in a large PD cohort enriched for genetic predisposition (i.e., early onset PD and / or family history), all of whom underwent whole-exome sequencing to identify known-PD causing mutations, rare variants in established PD genes, and rare variants across the entire exome. Fresh peripheral blood neutrophils were collected from all participants, including healthy controls, to assess LRRK2 kinase activation as described previously[8–10, 17, 18]. Patients were then stratified by LRRK2-dependent Rab10^{Thr73} phosphorylation to determine whether aberrant kinase activation occurred beyond known rare *LRRK2* and *VPS35* variant carriers. Elevated LRRK2 kinase activity, reaching levels comparable to those observed in *VPS35* p.D620N mutation carriers, was detected in approximately 14% of PD cases. For the genes harbouring rare variants identified in a subset of patients with increased LRRK2 activity (n = 4), we performed siRNA-mediated knockdown screening in A549 cells to identify potential genetic factors contributing to abnormal LRRK2 kinase activation. This led to the identification of several potentially interesting hits whose knockdown up- or down-regulated Rab10 phosphorylation.

Together, these findings reveal a distinct subset of PD patients with functional LRRK2 kinase pathway activation independent of known pathogenic variants in LRRK2 or VPS35. These individuals may represent an underlying "LRRK2-driven" PD subgroup who may benefit from LRRK2 targeted therapies, underscoring the need for biomarkers to guide precision stratification in PD.

Methods

Patient recruitment and blood sample collection

The majority of participant recruitment was conducted through Parkinson's disease outpatient clinics at the departments of neurology of the Klinik Ottakring and the Medical University of Vienna. Patients and healthy controls were invited to participate under existing IRB approval (EK Nr: 1844/2019, Medical University of Vienna, Austria) and all participants provided informed written consent prior to enrolment. Additionally, several patients were included from an existing Dundee study under ethical

approval issue by North East - Newcastle & North Tyneside 2 Research Ethics Committee, United Kingdom (REC reference:19/ES/0031). Clinical data were collected for all participants with an established diagnosis of PD according to MDS clinical diagnostic criteria[28]. This included but was not limited to age, biological sex, ethnicity, age at symptom onset, age at diagnosis, and self-reported family history of PD or tremor. For healthy control participants, data collected included age and biological sex. 20-40mL of blood were collected per participant into EDTA-coated BD Vacutainer tubes by venepuncture for genetic testing and neutrophil isolations. Samples were typically processed within 2–4 hours of collection to ensure best sample quality.

Genetic sequencing

Whole exome sequencing was performed using SureSelect Human All Exon v5 and v6 kits (Agilent Technologies) and Twist Human Exome 2.0 Plus Comprehensive Exome Spike-in and Mitochondrial Panel (Twist Bioscience) for enrichment. Sequencing was conducted as 100-bp paired-end runs on an Illumina HiSeq4000 or NovaSeq6000 systems. Bioinformatic analysis was performed using the exome variant analysis pipeline of the Institute of Human Genetics (Technical University of Munich, Germany). In brief, raw data processing involved read alignment against the human assembly GRCh37/hg19 using a BurrowsWheeler-Aligner, variant calling using Samtools and variant annotation using Custom-Perl-scripts. At least 95% of targeted exome bases were covered to a depth of 20x or greater in all samples. Copy number variants (CNVs) were called using the Exome Depth algorithm (version 1.1.10). Identity by state analysis was used to determine relatedness between individuals and conducted with in-house pearl scripts using all variants with a read depth > 10x, a genotype quality > 30 and a mapping quality > 50.

Human peripheral blood neutrophil isolations

Neutrophil isolations were performed via immunomagnetic negative selection following previously described method[16]. Briefly, fresh blood was transferred into 50mL falcon tubes and EasySep™ direct Human Neutrophil Isolation Cocktail (50 µL/mL of sample) and EasySep™ Direct RapidSpheres magnetic beads (1:1 ratio) were added to the tubes. Blood was subsequently mixed and incubated (5min, room temperature (RT)), following which, the tubes were topped up to 50 mL with PBS containing 1 mM EDTA, mixed again by inversion and placed into the Easy 50 EasySep™ Magnet for 10min. The enriched cell suspensions were transferred to fresh tubes, magnetic beads were added again with 5min incubation, placed into the magnet for 5min and transferred to fresh tubes once more. To remove residual magnetic beads, tubes were placed in the magnet for an additional 10min. Once transferred to a fresh tube, suspensions were then diluted to 40 mL with PBS and centrifuged at 450 x g, 5min. Supernatants were discarded, leaving neutrophil pellets. Pellets were then directly washed with PBS and lysed in 1% (v/v) Triton lysis buffer (50 mM Tris/HCl (pH 7.4), 1% (v/v) Triton X-100, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM 2-glycerophosphate, 5 mM sodium pyrophosphate, 270 mM sucrose, 0.1% (v/v) 2-mercapthoethanol, complete EDTA-free protease inhibitor cocktail and freshly supplemented with 1µg/mL microcystin-LR and 0.5mM diisopropylfluorophosphate (DIFP)). Lysates were cleared by centrifugation (17,000 x g, 15min, 4°C).

siRNA knockdown in A549 cells

Gene knockdown was achieved using small interfering RNAs (siRNA) to transiently reduce target gene expression in A549 cells. The siRNAs utilized in the experiments can be found summarized in Supplementary Table 1. siRNAs were resuspended in siRNA buffer diluted in RNAse-free water to a final concentration of 10 µM. For each experiment A549 cells were seeded in 6-well plates and once the cells have reached 30–50% confluency they were transfected with relevant siRNAs. 12.5 nM (2.5 µL) of each siRNA was mixed with 5 µL of transfection reagent Lipofectamine RNAiMax all in 200 µL of Opti-MEM Reduced Serum Medium and incubated for 5 minutes at RT before being added to the cells. Following transfections the cells were incubated for 72hrs at 37C°, 5% CO_2. The cells were subsequently lysed with ice cold 1% Triton X-100 lysis buffer and cleared by centrifugation (17,000 x g, 15min, 4°C). The siRNA screen was performed in two batches with 80 proteins knockdown in batch

1 and 79 in batch 2 including negative and positive controls in each batch. Two cell dishes were transfected per biological replicate, with two independent biological replicates in total.

Quantitative immunoblotting

Total protein concentration of all cell lysates was determined using Pierce™ BCA Protein Assay Kit. Cell lysates were diluted to a final concentration of 1–2 µg/µL in 4x NuPage LDS Sample Buffer supplemented with 5% β-mercaptoethanol and boiled at 96°C for 10min. Equal amounts of protein were loaded on NuPAGE 4–12% Bis-Tris gradient gels alongside a molecular weight protein ladder. For clinical sample analysis, on each gel a healthy control derived neutrophil "intergel control" (IGC) was included for normalization purposes and to enable comparison of results between the gels. Electrophoresis was carried out in 1x MOPS SDS running buffer (90V 30min, then 120V for ~ 60-90min). Proteins were transferred to a 0.45 µm nitrocellulose membrane at 90V for 90 minutes in 1x transfer buffer (48 mM Tris-HCl, 39 mM glycine). Once transferred, the membranes were briefly stained with Ponceau S dye and cut accordingly to molecular weight of the target proteins of interest to visualize. Membranes were washed in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween20) and blocked (30min, RT) in 5% non-fat dry milk diluted in TBS-T. The membranes were washed with TBS-T and left to incubate overnight at 4°C in primary antibodies (prepared in TBS-T containing 5% BSA and 0.02% NaN_3) (Supplementary Table 2). The next day, after 30min wash in TBS-T, the membranes were incubated (1hr, RT) with multiplexed fluorescent secondary antibodies (Supplementary Table 2) diluted in 5% milk in TBS-T. Finally, membranes were washed three times for 10 minutes each in TBS-T and the signals were visualized using a LI-COR Odyssey CLx imaging system.

Data analysis

Statistical tests carried out using GraphPad Prism 10. For statistical analyses involving multiple groups, the Kruskal-Wallis test was carried out followed by Dunn's multiple comparisons test with Bonferroni correction. Pairwise comparisons were performed using Mann-Whitney two-tailed test.

Results

Cohort description and identification of PD-associated variants

We enrolled 181 patients with PD, the majority with a high likelihood of genetic predisposition, defined by early disease onset (≤50 years old)[29] and / or family history of PD in first- or second-degree relatives, along with 71 healthy controls, between 2020–2024. Based on these criteria, genetically predisposed PD patients were classified into four distinct subgroups according to family history and age at PD onset (Table 1). The first group consisted of 35 individuals with EOPD and a positive family history, with a mean age at onset of ~ 44 (± 6.1) years, the second comprised 62 patients with EOPD but no family history (42 ± 6.4 years), the third consisted of 55 patients with a positive family history but later disease onset (61 ± 6.1 years), and the fourth included 28 patients with an age at onset ≥50 years and no reported family history (57 ± 5.8 years) (Table 1). Healthy control participants (n = 71) were recruited as closely age matched as possible to the PD patients with an average age at assessment of approximately 48 (± 17.2) years. Healthy volunteers were self-reported fit and well with no history of neurodegenerative diseases or family history of PD.

DNA from the majority of PD patients underwent whole exome sequencing, enabling identification of both known and likely pathogenic variants in PD associated genes as well as rare variants across the entire exome. Copy number variations in relevant genes (e.g. *PRKN*) could also captured by this method. Three PD patients and one non-manifesting carrier harboured the pathogenic *VPS35* p.D620N (Table 2). All four individuals belonged to previously reported multigenerational families showing segregation of the *VPS35* p.D620N variant with the disease[20]. The *LRRK2* p.G2019S variant was identified in five participants - four with early onset PD and one with later disease onset at age 70. Three PD patients harboured biallelic *PRKN* variants consistent with autosomal recessive early-onset PD[30]. Of these, patient W120 carried homozygous deletions of *PRKN* exon 2 and had developed juvenile-onset PD at age 14, while patients W142 (AAO: 18) and W81 (AAO: 38), both with early-

onset disease, had compound heterozygous deletions of *PRKN* exon 2 in combination with previously reported pathogenic variants p.R275W and p.C253Y, respectively[31, 32] (Table 2).

In addition, five PD patients carried the *LRRK2* p.N2081D variant, which is predominantly associated with inflammatory bowel disease and has been shown to modestly increase LRRK2 kinase activity in a cellular overexpression model (Table 3)[7, 33]. Moreover, 14 patients carried variants in the PD risk gene *GBA1* (Table 2). Rare variants were also identified in genes associated with atypical or syndromic forms of parkinsonism, including *PLA2G6* (n = 1), *GCH1* (n = 1), *Rab39b* (n = 1) (Table 3). Patient W213 carried both the *LRRK2* p.N2081D variant and 2 biallelic *PLA2G6 variants* p.P223L and p.T319M. Variants in *PLA2G6* have previously been associated with more complex forms of parkinsonism such as dystonia parkinsonism and autosomal recessive EOPD[34, 35]. Patient W199 was found to carry a small deletion in Rab39b and small loss-of-function deletions in Rab39b have been associated with X-linked intellectual disability and early onset parkinsonism[36]. Interestingly, a pathogenic p.K224R variant in *GCH1*, identified in patient W257, is linked to dopa-responsive dystonia (DRD) and was reported in several studies to appear more commonly in PD patients compared to healthy controls[37, 38].

Overall, approximately, 14% of the cohort carried known or likley pathogenic variants in PD-associated genes. The remaining patients without identified pathogenic variants constituted the genetically undefined group, which was subsequently investigated to explore potential mechanisms of aberrant LRRK2 kinase activation and to identify “LRRK2-like” individuals with functional pathway dysregulation despite the absence of known mutations.

Functional assessment of LRRK2 kinase activity identifies a subset of PD patients with elevated Rab10 phosphorylation

To determine whether LRRK2 kinase activity is altered in our cohort, peripheral blood neutrophils were isolated from fresh blood from all participants, including healthy controls, and analysed by immunoblotting for LRRK2-dependent Rab10 phosphorylation at threonine 73 site as described before[8–10, 16]. Neutrophils were treated with and without a selective LRRK2 kinase inhibitor to confirm that any observed Rab10 phosphorylation was LRRK2 kinase dependent.

We found that *VPS35* p.D620N carriers exhibited a robust (≥ 2-fold) increase in LRRK2-dependent Rab10 phosphorylation, whereas none of the five *LRRK2* p.G2019S carriers showed any elevation (Fig. 1a,c). These results are consistent with previous reports[17, 19], and the degree of kinase activation observed in *VPS35* p.D620N carriers was therefore used to define a cutoff threshold for hyperactivation across the PD cohort.

This threshold enabled the identification of additional PD patients with comparable or greater degree of LRRK2 kinase activation to that observed in carriers of the pathogenic *VPS35* p.D620N variant. Based on this criterion, 26 PD patients (14%) from the genetically enriched cohort were found to exhibit LRRK2 kinase activation of similar or higher magnitude than *VPS35* p.D620N carriers (Fig. 1a). Interestingly, there was also a slight but statistically significant increase in Rab10 phosphorylation levels in the PD patient group overall compared with the healthy control group (Fig. 1b).

Although no significant differences in Rab10 phosphorylation were observed between the predefined clinical groups, patients with increased Rab10 phosphorylation were represented across all four groups (Fig. 1c). Notably, the largest proportion of PD patients with increased LRRK2 kinase activity were those with early onset PD (n = 11), none of whom were pathogenic biallelic *PRKN* variant carriers. *GBA1* variant carriers did not show elevated Rab10 phosphorylation compared with controls, except for one (W34, p.T408M) (Supplementary Fig. 1). In addition, several other patients carrying variants in PD-relevant genes were among the 26 PD patients with the highest Rab10 phosphorylation levels, reaching effect sizes comparable or higher to those observed in *VPS35* p.D620N carriers (Supplementary Fig. 1). This subgroup included two of the five carriers of the *LRRK2* p.N2081D variant (W300 and W191) and W257, who harboured *GCH1* p.K224R.

siRNA screen to identify candidate modifiers of LRRK2 kinase pathway activation

To explore potential genetic factors underlying LRRK2 kinase hyperactivation, we prioritised four PD patients (W34, W110, W130 and W144) who showed between 2 6-fold increases in LRRK2-dependent Rab10 phosphorylation. These individuals were selected because they were available for an independent second blood collection for LRRK2 kinase activation studies in neutrophils. Increased LRRK2 kinase activation was confirmed in three of the four subjects, whereas Rab10 phosphorylation levels had normalised in participant W34 (Fig. 2a). Clinically, W34, W130 and W144 presented with early-onset PD, while W110 developed PD at age 56. All four were male, and only W34 had reported first-degree family history of PD. Whole-exome sequencing identified approximately 180–240 rare missense variants per individual defined as a minor allele frequency (MAF) < 1000 (Fig. 2b). In W144, extensive consanguinity was detected (> 100 Mb runs of homozygosity), prompting for additional filtering for homozygous variants (MAF < 0.03), which yielded 21 additional candidate genes.

To identify genetic modifiers of LRRK2 signalling, we performed an siRNA-mediated knockdown screen in A549 cells targeting prioritized genes harbouring rare variants in these 4 individuals. Candidate genes were systematically prioritised amongst the genes harbouring rare variants based on three criteria: (1) Rarity in the population, selecting heterozygous missense variants reported no more than five times in the gnomAD v2.1 database[39] (all homozygous variants in W144 were retained); (2) Cellular relevance, confirming protein expression in neutrophils, the cell type in which the LRRK2 activation phenotype was observed; and (3) Model suitability, confirming expression in A549 cells, the model used for siRNA screening (Fig. 2b). This multistep filtering reduced the candidate list from 182–244 to 35–45 genes per patient, plus six genes carrying homozygous variants in W144. siRNA-mediated knockdown of LRRK2, PPM1H, and VPS35 were included as positive controls to validate assay sensitivity given their well-documented impact on LRRK2 kinase signalling, yielding the expected effects: complete loss of pRab10^{Thr73} upon LRRK2 knockdown, a ~ 1.5-fold increase with PPM1H silencing, and a ~ 0.4-fold reduction following VPS35 depletion (Fig. 2b,c, Supplementary Fig. 2). Using these benchmarks, several genes with comparable or stronger effects were identified. NECAP2 depletion produced the strongest increase in Rab 10 phosphorylation (~ 1.6-fold), followed by EIF5A2, XYLT1, CD22, ALG9, and TECPR1 (1.3–1.5-fold). In contrast, knockdown of CDK12, EXOC8, FAM105A, NUP205, and ATRIP reduced Rab10 phosphorylation to VPS35-like levels (< 0.4-fold). It is worth noting that genes whose knockdown resulted in reduced Rab10 phosphorylation also displayed reduced total LRRK2 levels (Supplementary Fig. 3). Nevertheless, together these findings identify novel genetic candidates for LRRK2 kinase pathway modulation. While patient W34 was identified to carry a p.T408M variant in *GBA1* (Table 2), the gene was not targeted with siRNA due to the common nature of the variant and the availability of GBA1 knock-out A549 cells. Our results previously demonstrated that lack of GBA1 in these cells does not influence LRRK2-dependent Rab10 phosphorylation when compared to wild-type cells (Supplementary Fig. 4).

Discussion

The aetiology of PD is highly heterogenous, reflecting contributions from monogenic causes, common genetic risk factors, and non-genetic influences such as ageing, environmental exposures, and inflammatory processes. Pathogenic variants in *LRRK2*, *SNCA* and *PRKN* account for a subset of early-onset and familial PD[2, 3], yet a large proportion of such cases remain genetically unresolved[40]. Despite advances in sequencing technologies, progress in understanding the genetic architecture of PD remains limited by the underuse of genetic testing in routine clinical care[41], the difficulty of interpreting variants of uncertain significance, the absence of clear genotype-directed treatments, and the lack of integrated functional data to support variant interpretation.

Pathogenic variants in *LRRK2* are among the most common monogenic causes for PD, most recently reported in 3% of individuals among 12,580 PD patients analysed[3]. These variants exert their pathogenic effects through increased LRRK2 kinase activity, leading to dysregulation of endolysosomal trafficking and downstream lysosomal stress. The pathogenic *VPS35* p.D620N variant, affecting a key component of the retromer complex, similarly enhances LRRK2 kinase activity[19]. Importantly, LRRK2 targeting therapeutics are already being tested in clinical trials, underscoring the translational relevance of understanding how LRRK2 activation arises across both genetic and non-genetic forms of PD. We hypothesized that LRRK2 operates within a broader signalling network relevant to PD and that additional, yet unidentified genetic and other factors may influence its activation, thereby contributing to disease pathogenesis.

In this study, we examined a genetically enriched PD cohort (n = 181) and stratified participants by LRRK2-dependent Rab10^{Thr73} phosphorylation in peripheral blood neutrophils. Consistent with previous reports, *VPS35* p.D620N carriers exhibited marked Rab10 hyperphosphorylation[19] and were used to establish a threshold for LRRK2 kinase activation. By contrast, *LRRK2* p.G2019S carriers showed no elevation in Rab10 phosphorylation in neutrophils, supporting previous results[17, 42], despite the variants modest, but established kinase-activating effect in cellular overexpression and knock-in animal models [7, 43]. This discrepancy likely reflects the limited sensitivity and dynamic range of current pRab10^{Thr73} assays in neutrophils rather than a true absence of LRRK2 activation. It may also be that other substrates or assays (e.g., Rab12 phosphorylation) are better suited to capture LRRK2 pathway activity, although robust assays for these readouts are not yet established. Notably, *LRRK2* p.G2019S carriers have been reported to exhibit elevated urinary bis(monoacylglycero)phosphate (BMP) levels - an independent marker of lysosomal dysfunction downstream of LRRK2 activation - supporting increased LRRK2 kinase activity *in vivo*[44, 45]. In contrast, *GBA1* variant carriers, while representing one of the most common genetic risk groups for PD[2, 3, 46], showed neither increased Rab10^{Thr73} phosphorylation levels in this study nor elevated urinary BMPs in prior reports[45], suggesting that GBA1-related pathology affects lysosomal biology through mechanisms largely independent of LRRK2 kinase activation. Additionally, none of the 3 *PRKN*-associated PD cases displayed evidence of LRRK2 pathway activation nor the carriers of the compound heterozygous variants in *PLA2G6*, p.P223L and p.T319M (W213) and carrier of a small deletion in *Rab39B* gene (W199). Interestingly, patient W257 with a heterozygous *GCH1* p.K224R variant, exhibited a five-fold increase in Rab10 phosphorylation. Although there are currently no reports of a direct interaction between LRRK2 and GCH1, our proposed approach would be to first validate the increased Rab10 phosphorylation in an independent blood collection and, if reproducible, investigate additional patients before pursuing mechanistic studies.

The most striking finding was that 14% (n = 26) of all PD patients, excluding those carrying *VPS35* p.D620N, showed LRRK2 kinase activation comparable or greater than that observed in VPS35 p.D620N carriers, suggesting the presence of yet-unidentified modifiers influencing LRRK2 function. Individuals with elevated Rab10 phosphorylation were detected across all clinical groups but were most frequent in early-onset PD, supporting the notion that LRRK2 pathway activation may extend beyond known LRRK2 and VPS35 mutation carriers, which are typically associated with late-onset disease. We also observed a small but statistically significant increase in LRRK2-dependent Rab10 phosphorylation in PD patients compared with controls, even after excluding *VPS35* p.D620N carriers from the analysis (Supplementary Figure). Together, these findings provide evidence for a subset of PD patients with elevated LRRK2 kinase activity and a likely genetic contribution, consistent with the emerging concept of a “LRRK2-like” molecular phenotype within idiopathic PD.

To investigate the underlying genetic basis of this phenotype, we combined whole-exome sequencing with an siRNA-mediated functional screen targeting genes harbouring rare variants in a subset of four PD patients with LRRK2 kinase hyperactivation in neutrophils. The screen identified several genes whose knockdown either increased *(NECAP2, EIF5A2, XYLT1, CD22, ALG9, TECPR1)* or decreased *(EXOC8, FAM105A, NUP205, ATRIP)* LRRK2-dependent Rab10 phosphorylation. Notably, NECAP2 depletion resulted in the strongest increase in Rab10 phosphorylation, exceeding that of PPM1H knockdown. NECAP2 encodes an endosomal adaptor protein involved in clathrin coat formation and receptor recycling - pathways closely linked to LRRK2’s established role in endolysosomal trafficking. These results further suggest a mechanistic intersection between endosomal homeostasis and LRRK2 activation that merits further investigation. Although siRNA-based methods have limitations e.g. transient knock-down, potential off-target effects, and variable silencing efficiency, and inability to capture gain of function effects, they enable scalable, pathway-anchored screening of functionally relevant candidates. We independently confirmed LRRK2 hyperactivation in three of the four patients, underscoring the biological robustness of the phenotype. We also note that variability in neutrophil Rab10 phosphorylation likely reflects a combination of biological immune related influences and technical factors inherent to the assay and biomatrix, such as pre-analytical handling or possibly time-to-processing. While we minimised these variables through standardised Standard Operating Procedures and appropriate controls, residual technical variability cannot be fully excluded.

Definitive mechanistic validation through CRISPR/Cas9 knock-out or knock-in of candidate genes, rescue experiments and functional studies will be essential next steps, but these were outside the scope of the present study, which was designed to identify and prioritise candidate modifiers through integrated genetics-plus-function screening focused on LRRK2. Looking

ahead, integrating functional assays with large-scale population datasets from gnomAD, UK Biobank, and the Global Parkinson's Genetics Program (GP2) will refine variant interpretation and help delineate LRRK2-driven disease subtypes. Combining such functional biomarkers with complementary readouts - such as urinary BMP levels - may enable precise patient stratification and broaden the population who could benefit from LRRK2-targeted therapies.

In summary, we provide evidence that LRRK2 kinase hyperactivation occurs in a subset of PD patients without known pathogenic variants, plausibly driven by additional genetic modifiers or convergent cellular pathways. Functional LRRK2 biomarkers in peripheral blood neutrophils provide proof of concept for guiding precision interventions as LRRK2-targeted therapies advance through clinical development. However, neutrophils are a challenging biomatrix for large-scale implementation due to time and labour-intensive isolation process. An important next step toward broader clinical application will be the development of alternative or complementary assay formats that can be more easily standardised across clinical trials.

Declarations

Data availability

Data that support the findings of this study can be found in Supplementary materials with raw data deposited in Zenodo (10.5281/zenodo.17661460).

Acknowledgments

We sincerely want to thank the patients and healthy volunteers for their willingness to participate in research and the generous donation of the blood samples for this study. We want to thank Karolina Zeneviciute for providing data regarding the impact of GBA1 knock-out on LRRK2 signalling pathway in A549 cells.

Author Contributions

NP: Investigation, data curation, formal analysis, writing of original draft, visualization, WP: Resources, Review and editing of the manuscript, JH: Resources, investigation,CB: Resources, AZ: Conceptualization, review and editing of the manuscript, ES: Conceptualization, supervision, formal analysis, writing of original draft and editing of the manuscript. All authors read and approved the final manuscript.

Funding Sources and Conflict of Interest:

ES was supported by a CSO Senior Clinical Academic Fellowship (SCAF/18/01) and NP by a Carnegie Trust PhD studentship (PHD010656).

The authors declare that there are no conflicts of interest relevant to this work.

References

1. Luo Y, Qiao L, Li M, Wen X, Zhang W, Li X. Global, regional, national epidemiology and trends of Parkinson's disease from 1990 to 2021: findings from the Global Burden of Disease Study 2021. Frontiers in Aging Neuroscience. 2025;16:1498756.
2. Cook L, Verbrugge J, Schwantes-An T-H, Schulze J, Foroud T, et al. Parkinson's disease variant detection and disclosure: PD GENEration, a North American study. Brain. 2024;147(8):2668–79.
3. Westenberger A, Skrahina V, Usnich T, Beetz C, Vollstedt E-J, et al. Relevance of genetic testing in the gene-targeted trial era: the Rostock Parkinson's disease study. Brain. 2024;147(8):2652–67.

Tables

Table 1. Demographic characteristics of the study's cohort. Patients were grouped based on the presence of early-onset Parkinson's disease (EOPD) and / or presence of reported family history of PD. The table includes the number of individuals per group, mean age at assessment, mean age at onset (AAO) and sex distribution. Mean values for age and AAO are presented with standard deviation (±SD). NMC: non-manifesting carrier.

	PD Patients n=181				**Healthy controls n=71**	**VPS35 D620N NMC n=1**
	AAO <50 years old		**AAO >50 years old**			
Family History	*Positive n=35*	*Negative n=62*	*Positive n=56*	*Negative n=28*		
Age at Onset (mean ± SD)	44.3 (6.1)	41.7 (6.4)	61.2 (6.2)	56.8 (5.8)	N/A	N/A
Age at Assessment (mean ± SD)	55.7 (7.0)	52.1 (9.2)	69.0 (8.0)	66.5 (8.1)	47.9 (17.0)	42
Sex (f/m)	12/23	20/42	28/28	13/15	40/31	1/0

Table 2. Demographic characteristics of a subset of patients carrying variants in PD-associated genes. This includes variants that are either disease causing or strong risk variants. For each individual, sex, age as reported at assessment, age at disease onset (AAO) and family history of PD are summarized. The gene and the associated variant at nucleotide and protein level are also reported. For compound heterozygous variant the second variant is also specified. Asterisk marked families are from the Zimprich, Alexander, et al. *(2011)* study[21]. NMC: Non-manifesting carrier, Het: Heterozygous, Hom: Homozygous.

ID	Sex	Age	AAO	Gene	Variant		Inheritance pattern	Causality	Family History
					Nucleotide	Amino Acid			
w67	m	64	63	*VPS35*	c.1858G>A	p.Asp620Asn	Het	Pathogenic	Multigenerational family: Family A*
w129	f	48	46	*VPS35*	c.1858G>A	p.Asp620Asn	Het	Pathogenic	Multigenerational family: Family B*
W05	f	68	47	*VPS35*	c.1858G>A	p.Asp620Asn	Het	Pathogenic	Multigenerational family: Family B*
W06	f	42	NMC	*VPS35*	c.1858G>A	p.Asp620Asn	Het	Pathogenic	Multigenerational family: Family B*
w120	f	27	14	*PRKN*		Deletion of Exon 2	Hom	Pathogenic	
w142	m	69	18	*PRKN*	- c.823C>T	Deletion of Exon 2, p.Arg275Trp	Compound Het	Pathogenic	Mother PD
w81	f	53	38	*PRKN*	- c.758G>A	Deletion of Exon 2 Cys253Tyr	Compound Het	Pathogenic	
w108	m	52	48	*LRRK2*	c.6055G>A	p.Gly2019Ser	Het	Pathogenic	Father PD, aunt PD
w172	f	50	43	*LRRK2*	c.6055G>A	p.Gly2019Ser	Het	Pathogenic	Grandmother PD
w223	f	60	54	*LRRK2*	c.6055G>A	p.Gly2019Ser	Het	Pathogenic	
HP90	f	74	70	*LRRK2*	c.6055G>A	p.Gly2019Ser	Het	Pathogenic	Paternal uncle PD
HP92	f	55	37	*LRRK2*	c.6055G>A	p.Gly2019Ser	Het	Pathogenic	
w105	m	63	50	*GBA1*		splice site	Het	Risk	Two brothers PD, cousin PD
w98	m	62	40	*GBA1*	c.1223C>T	p.Thr408Met	Het	Risk	
w146	f	78	57	*GBA1*	c.1223C>T	p.Thr408Met	Het	Risk	Two sisters PD, daughter PD
w193	m	42	41	*GBA1*	c.1223C>T	p.Thr408Met	Het	Risk	
w34	m	53	35	*GBA1*	c.1223C>T	p.Thr408Met	Het	Risk	Father PD, maternal uncle PD
w145	m	52	40	*GBA1*	c.1448T>C	p.Leu483Pro	Het	Risk	
w249	m	65	38	*GBA1*	c.1448T>C	p.Leu483Pro	Het	Risk	
w93	m	62	45	*GBA1*	c.1093G>A	p.Glu365Lys	Het	Risk	
w103	m	54	41	*GBA1*	c.1093G>A	p.Glu365Lys	Het	Risk	
w118	m	54	42	*GBA1*	c.1093G>A	p.Glu365Lys	Het	Risk	Father PD
w177	m	57	57	*GBA1*	c.1093G>A	p.Glu365Lys	Het	Risk	Sister PD (W234), father PD
w234	f	60	55	*GBA1*	c.1093G>A	p.Glu365Lys	Het	Risk	Sister PD (W177), father PD
w101	m	49	42	*GBA1*	c.1226A>G	p.Asn409Ser	Het	Risk	

w141	m	70	49	*GBA1*		Deletions of Ex 6 and 7	Het	Risk

Table 3. Demographic characteristics of a subset of patients carrying variants in PD-relevant genes. This includes variants that have yet to distinguished as causative for PD but have been previously associated with atypical parkinsonism, increased PD risk or reported at higher frequency in PD cases compared to healthy controls. For each individual, sex, age as reported at assessment, age at disease onset (AAO) and family history of PD are summarized. The PD-relevant gene and associated variant are also reported. For compound heterozygous variant the second variant is also specified. DRD: Dopa-responsive dystonia, Het: Heterozygous, Hom: Homozygous.

ID	Sex	Age	AAO	Gene	Variant		Inheritance pattern	Variant Causality	Family History
					Nucleotide	Amino Acid			
W300	m	50	45	*LRRK2*	c.6241A>G	p.Asn2081Asp	Het	Possibly benign	Father PD
W191	f	51	42	*LRRK2*	c.6241A>G	p.Asn2081Asp	Het	Possibly benign	Grandfather PD
W208	m	53	39	*LRRK2*	c.6241A>G	p.Asn2081Asp	Het	Possibly benign	
W83	f	53	38	*LRRK2*	c.6241A>G	p.Asn2081Asp	Het	Possibly benign	
w213	m	54	49	*LRRK2*	c.6241A>G	p.Asn2081Asp	Het	Possibly benign	
				PLA2G6	c.668C>T, c.956C>T	p.Pro223Leu, p.Thr319Met	Compound het	Associated with Parkinsonism, causality likely pathogenic	
w257	m	60	51	*GCH1*	c.671A>G	p.Lys224Arg	Het	Pathogenic for DRD, Unclear for PD	
w199	m	56	53	*Rab39b*		p.Gly20_Cys23del	Het	Associated with X-linked intellectual disability and early onset parkinsonism, variant causality unclear	

Figures

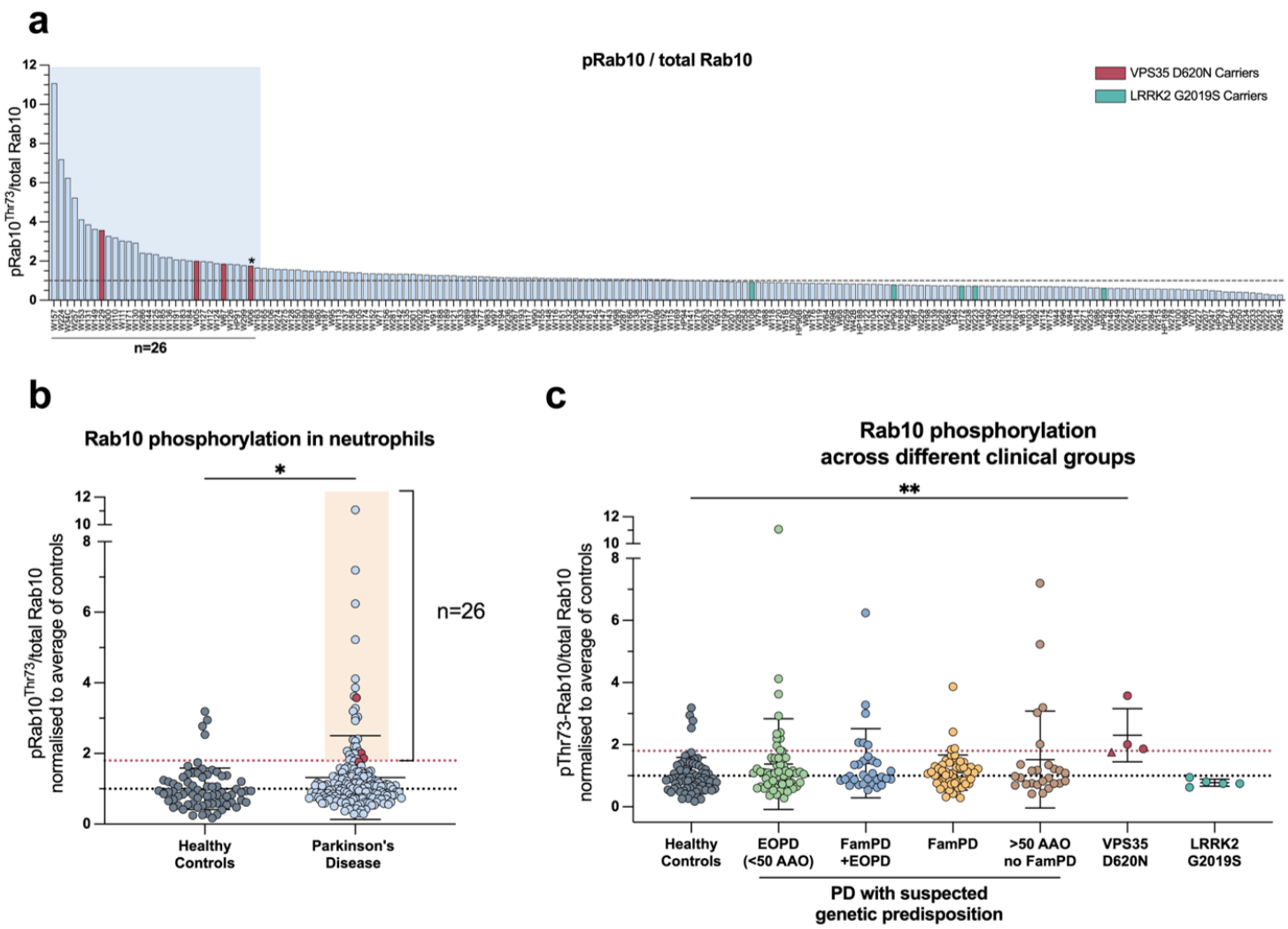


Figure 1

A subset of PD patients exhibits increased LRRK2 dependent Rab10 phosphorylation in peripheral human blood neutrophils. (**a**) Rab10 phosphorylation was measured by multiplexed quantitative immunoblotting in neutrophils from all PD patients and presented as pRab10/total Rab10 in a descending order. Three carriers of the VPS35 p.D620N variant are highlighted in red, with the non-manifesting carrier indicated with an asterisk. Five LRRK2 p.G2019S carriers are shown in teal. (**b**) LRRK2-dependent Rab10 phosphorylation levels in neutrophils of the PD patient group (n=181) including the non-manifesting VPS35 p.D620N carrier compared to healthy control group (n=71). VPS35 p.D620N carriers are marked in red. Statistical analysis: Mann Whitney's two-tailed test, *$p < 0.05$ (**c**) pRab10 / total Rab10 levels across all clinical groups. Statistical analysis: Kruskal-Wallis test followed by Dunn's post host test with Bonferroni correction, **$p < 0.01$. All values were normalized to the mean of healthy controls (y=1) represented as mean ± SD. Red dotted line at y=1.8 represents the lowest pRab10 value from VPS35 p.D620N participant.

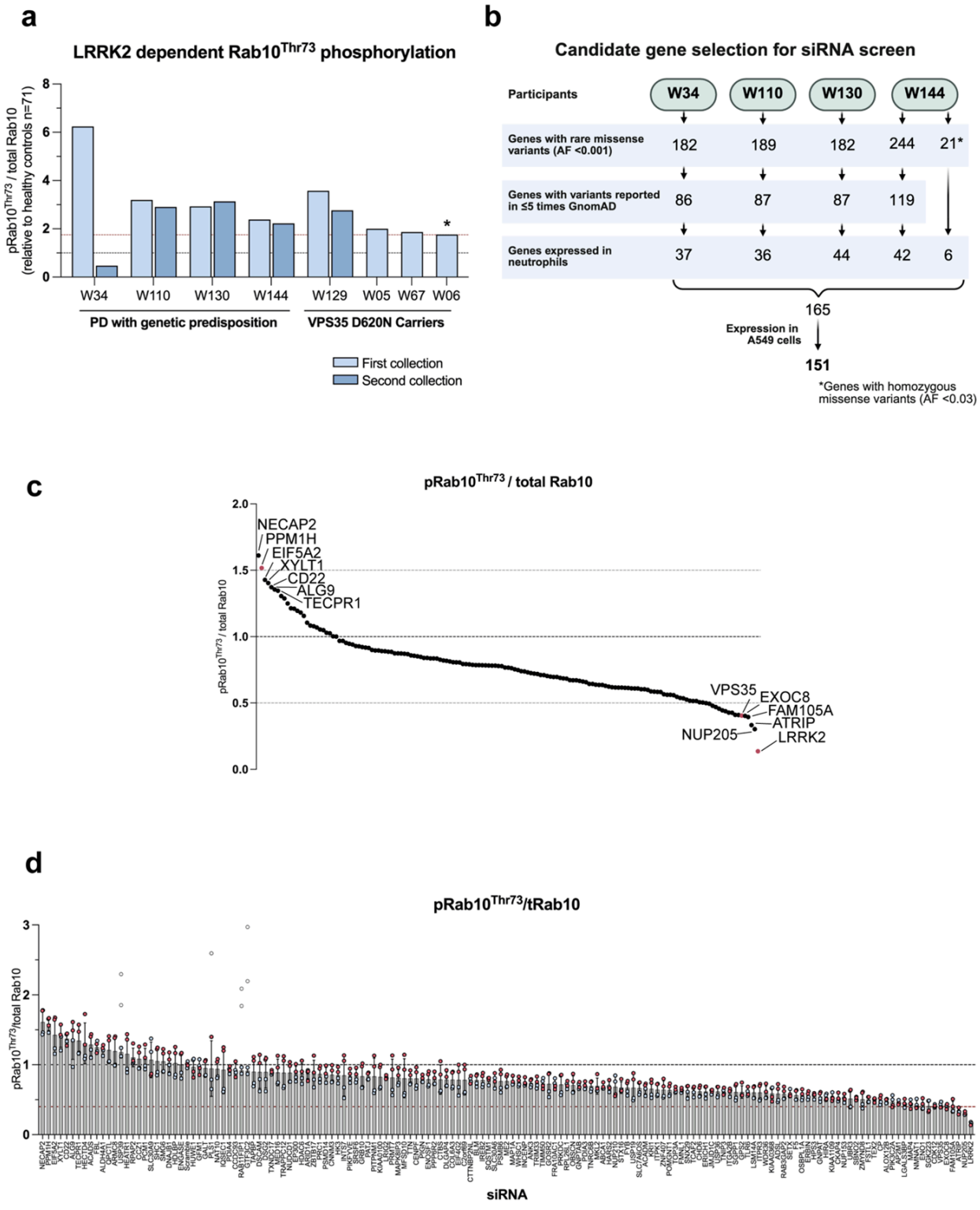


Figure 2

siRNA screen identifies potential novel regulators of LRRK2 signalling pathway. (**a**) Summary of Rab10 phosphorylation levels in neutrophils as analysed by immunoblotting from first and second independent blood collections in patients W34, W144, W110 and W130. Four VPS35 p.D620N carriers were included for comparison purposes with a second blood collection available for patient W129. The non-manifesting carrier is marked with an asterisk. All values were normalized to 71 healthy controls (y=1). Red dotted line at y=1.8 represents the lowest pRab10 value from VPS35 p.D620N patient. (**b**)Schematic outline detailing screening strategy for gene inclusion in the siRNA screen. Gene and protein expression in A549 cells was determined using proteomic database Copica (https://copica.proteo.info/#/home) and transcriptomic database Human Protein Atlas

(https://www.proteinatlas.org/). Created in https://BioRender.com (**c**) Summary of Rab10 phosphorylation levels from siRNA screen as analysed by immunoblotting and presented as pRab10 / total Rab10 levels in a descending order. Values represent the mean of two biological replicates. (**d**) Bar chart illustrating mean pRab10 / total Rab10 levels for individual siRNAs with values for each replicate included. Biological replicates are distinguished by red and blue colours. In white are marked outliers excluded from the overall ranking of pRab10 values due to impaired sample quality. Red line at y=0.4 represents the value from VPS35 knockdown. Each data point represents cells coming from a different dish of cells. Error bars represent ±SD. Each value was normalized to the non-targeting control (Scramble), y=1.

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