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BLOOD RNA BIOMARKERS IN PRODROMAL PARK4 AND REM SLEEP BEHAVIOR DISORDER SHOW ROLE OF COMPLEXIN-1 LOSS FOR RISK OF PARKINSON'S DISEASE

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Summary Statement:

We identified pathway effects and molecular biomarkers of synucleinopathy in a large PD family with alpha-synuclein-duplication, thus establishing lab procedures for risk diagnostics. Furthermore, we defined complexin-1 as a potential biomarker for both prodromal (REM sleep behavior disorder) and manifest PD.

Abstract

Parkinson's disease (PD) is a frequent neurodegenerative process at old age. Accumulation and aggregation of the lipid-binding SNARE complex component alpha-synuclein (SNCA) underlies this vulnerability and defines stages of disease progression. Determinants of SNCA levels and mechanisms of SNCA neurotoxicity are intensely investigated. In view of physiological SNCA roles in blood to modulate vesicle release, we studied blood samples from a new large pedigree with *SNCA* gene duplication (PARK4 mutation), to identify effects of SNCA gain-of-function as potential disease biomarkers. The expression of other Parkinson's disease gene was not, but complexin-1 (*CPLX1)* mRNA downregulation was correlated with genotype. In global RNAseq profiling of blood from presymptomatic PARK4, bioinformatics detected significant upregulations for platelet activation, hemostasis, lipoproteins, endocytosis, lysosome, cytokine, toll like receptor signalling and extracellular pathways. In PARK4 platelets, stimulus-triggered degranulation was impaired. Strong *SPP1*, *GZMH*, and *PLTP* mRNA upregulations were validated in PARK4. When analysing cases with REM sleep behaviour disorder (RBD), the most specific known prodromal stage of general PD, only blood *CPLX1* levels were altered. Validation experiments confirmed an inverse mutual regulation of *SNCA* and *CPLX1* mRNA levels. In the 3'-UTR of the *CPLX1* gene we identified a SNP that is significantly associated with PD risk. In summary, our data define *CPLX1* as PD risk factor and provide functional insights into the role and regulation of blood alpha-synuclein levels. The novel blood biomarkers of PARK4 in this Turkish family may become useful for PD prediction.

Introduction:

Parkinson's disease (PD) is the second most frequent age-associated brain degeneration disorder, affecting about 1% of the population over 65 years. The PD-specific progressive movement deficit is mostly due to the severe affliction and cell death of midbrain nigrostriatal dopaminergic neurons (Braak et al., 2003). Surviving neurons in vulnerable regions exhibit aggregates predominantly consisting of the protein alpha-synuclein (SNCA), which are visualized as Lewy neurites and Lewy bodies, both in sporadic late-onset PD and in most familial early-onset PD variants (Goedert et al., 2013).

Autosomal dominant PD with early clinical manifestation was observed in rare families, leading to the identification of SNCA protein missense mutations (PARK1 variant) such as A53T and of *SNCA* gene duplication/triplication events (PARK4 variant) as the strongest causes of this pathology (Polymeropoulos et al., 1997; Singleton et al., 2003). Further recruitment of PD families led to the identification of several disease genes responsible for monogenic PD (Corti et al., 2011). In addition, genome-wide association studies (GWAS) of very large collectives of late manifesting sporadic PD cases identified two regions on chromosome 4 (*SNCA* locus and *CPLX1/GAK/TMEM175/DGKQ* locus) that contain genetic variants predisposing to multifactorial PD (Lill et al., 2012; Nalls et al., 2014). Variations in the *SNCA* gene 3'-untranslated region (3'-UTR) and its promoter correlated strongly with PD risk (Rhinn et al., 2012).

SNCA is physiologically concentrated in axon terminals. It is associated with the lipid membranes of synaptic vesicles and interacts with synaptobrevin, a component of the SNARE complex, modulating vesicle exocytosis and neurotransmission (Diao et al., 2013). Its toxic gain-of-function leads over time to impaired synaptic vesicle release and synaptic failure (Garcia-Reitbock et al., 2010; Nemani et al., 2010; Platt et al., 2012; Janezic et al., 2013). Current investigations aim to elucidate SNCA-triggered pathology, concentrating on disease stages before the occurrence of irreversible cell loss, when neuroprotective therapies might still be efficacious. In the prodromal stage of PD, non-motor symptoms such as hyposmia, constipation, depression or rapid eye movement (REM) sleep behavior disorder (RBD) were documented, out of which RBD is now recognised as the most specific and predictive prodromal phenotype. Individuals suffering from RBD carry a risk of >85% to manifest PD after 15-20 years and the associated neurodegenerative process is a synucleinopathy in 95% of cases (Stiasny-Kolster et al., 2005; Albers et al., 2012; Boeve et al., 2013; Iranzo et al., 2013; Mahowald and Schenck, 2013; Iranzo et al., 2014).

SNCA is abundantly expressed in blood (Shin et al., 2000; Barbour et al., 2008). The accumulation of SNCA in short-lived blood cells was found to result in diverse subtle phenotypes. Enhanced apoptotic vulnerability of human PARK1 lymphocytes and SNCA-transfected myeloma and leukemia cell lines to oxidative stress (Kim et al., 2004; Battisti et al., 2008), impaired innate immune functions of mouse leukocytes with SNCA overexpression (Gardai et al., 2013), and dose-dependent inhibition of alpha-granule release in human platelets exposed to exogenous SNCA (Park et al., 2002) provide evidence that biomarkers of elevated SNCA abundance and of the risk of synucleinopathy may be identified in peripheral tissues.

Our identification of a novel large pedigree of autosomal dominant PD due to *SNCA* gene duplication with 12 presymptomatic PARK4 heterozygotes now provided a unique opportunity to explore blood biomarkers and permitted the definition of a molecular signature at RNA level that predicts PARK4. For validation, the results were assessed in individuals with a risk to develop PD due to manifestation of RBD as a highly specific prodromal sign. Our data on blood biomarkers as a diagnostic tool may contribute to assess the risk of multifactorial PD in individuals without positive family history. The most relevant biomarker is the SNARE component complexin-1, which acts as risk factor for PD by itself.

Results:

RNA levels in blood from presymptomatic PARK4 heterozygotes are reduced for *CPLX1*

An exceptionally large pedigree with autosomal dominant PD (Fig. 1A) was identified in Turkey. Blood DNA genotyping demonstrated a genomic tandem duplication at the *SNCA* locus as a known PD cause (Singleton et al., 2003) (Fig. 1B) and detected presymptomatic PARK4 heterozygotes, 12 of which were available and included into this study. The two clinically affected family members plus the twelve presymptomatic heterozygotes (mean age 45.5, range from 29 to 56 years, 6 males) versus twelve age-matched control relatives (mean age 44.6, from 31 to 57 years, 8 males) underwent overnight fasting and had whole peripheral blood protein and RNA samples collected and processed in parallel, to assess the SNCA-dependent expression profiles of blood. As a consequence of the tandem duplication, the blood mRNA levels of the neighbouring genes *GPRIN3, SNCA,* and *MMRN1* were increased to about 1.5 fold in blood of PARK4 cases versus control relatives (11 versus 9). Given that the *MMRN1* gene dosage also has been implicated with cognitive decline (Nishioka et al.,

2006; Fuchs et al., 2007; Mutez et al., 2011), it will be interesting to perform a neuropsychological characterization of the PARK4 heterozygotes in this family in the future, but currently the cognitive score in one patient is still unaffected (MMSE = 30). In an initial candidate-gene study we studied the blood expression of all PARK genes, of the previously reported PD blood biomarker *ST13* and of promising transcripts that encode putative SNCA-interactor proteins (Fig. 2A,B) and showed altered levels in our previous global transcriptome profiling of midbrain tissue in a carefully characterized synucleinopathy mouse model with dopaminergic deficits, impaired synaptic plasticity and mitochondrial dysfunction (Kurz et al., 2010; Platt et al., 2012; Tozzi et al., 2012; Gispert et al., 2014; Subramaniam et al., 2014; Brehm et al., 2015b; Gispert et al., 2015). *SNCA* gene duplication in the Turkish pedigree showed no correlation with the expression of other genes with known association to PD risk or of the previously reported PD blood biomarker *ST13* (Scherzer et al., 2007). A nominally significant inverse correlation was observed for *CPLX1* and a trend towards inverse correlation for *YWHAE* (Fig. 2B), two phenomena that we had previously identified as synucleinopathy markers in mouse mutant midbrain (Gispert et al., 2014; Brehm et al., 2015a). *CPLX1* levels were reduced to 0.67-fold, so the effect size was moderate; in spite of the nominal significance ($p = 0.04$), a demonstration of actual significance after Bonferroni or Benjamini-Hochberg correction would be possible only in much larger sample collections. Quantitative immunoblots of protein extracts from corresponding whole blood PARK4 samples showed a ~1.5-fold accumulation of SNCA monomer, but no SNCA aggregates were detectable - even after delipidation for epitope-unmasking – probably due to the short lifespan of blood cells (Sharon et al., 2003) (Fig. 1C). The protein levels of complexin-1 could not be detected reliably in blood. Together with previous mouse findings (Chandra et al., 2004; Gispert et al., 2014), these data suggest that *CPLX1* mRNA levels in blood mirror the gain-of-physiological-function of SNCA rather than aggregation pathology. This provides proof-of-principle that blood RNA profiling may aid the development of risk prediction biomarkers for synucleinopathies.

Blood platelets from presymptomatic PARK4 heterozygotes suggest reduced stimulus-triggered degranulation in absence of SNCA aggregates.

Because exogenous recombinant human *SNCA* and deletion of murine *Scna* are known to modulate the stimulus-triggered vesicular release from blood platelets (Park et al., 2002; Reheman et al., 2010), we investigated platelet function in two presymptomatic PARK4 heterozygotes (male aged 48 years and female aged 45 years) and two age- and sex-

matched first degree control relatives (male aged 43 years, female aged 45 years) who could travel from Turkey to Germany. As revealed by flow cytometry, stimulation-induced degranulation increased the cell surface presence of the alpha-granule antigen CD62P (P-selectin) and the lysosomal CD63 antigen. In comparison with their control relatives and with normal German population, these degranulation-dependent changes were diminished in presymptomatic PARK4 heterozygotes (Table 1). Electron microscopic analysis did not detect protein aggregates in the PARK4 platelets (Fig. 3). The presymptomatic PARK4 heterozygotes did not report any manifest blood coagulation disorder. These functional data may explain the expression dysregulation of the SNARE complex component *CPLX1* as a compensatory effort within the platelet activation pathway.

Global RNA profiling of blood from presymptomatic PARK4 heterozygotes detects several strongly upregulated pathways

Global expression profiling by RNAseq in blood samples from five presymptomatic PARK4 heterozygotes (male aged 53 years, female aged 59 y, male aged 50 y, female aged 47 y, male aged 50 y) versus five age- and sex-matched controls (male aged 45 y, female aged 54 y, male aged 57 y, female aged 42 y, male aged 42 y) was employed to identify additional molecular effects of *SNCA* gene duplication. All data were deposited at the ENA public database (accession PRJEB8960) and a table containing the ranked gene list of expression changes will be provided to readers upon request. In order to identify pathway dysregulations, the data were assessed bioinformatically with Gene Set Enrichment Analysis (GSEA). Downregulated pathways were not apparent, but several strong upregulations (with p-values = 0.0 and q-values = 0.0) were documented and concerned the cytokine signalling in immune system (Normalized Enrichment Score, NES = 4.8), adaptive immune system (NES 4.5), hemostasis (NES 4.4), lysosome (NES 4.3), platelet activation signalling and aggregation (NES 4.1), innate immune system (NES 4.0), endocytosis (NES 3.9), toll like receptor signalling (NES 3.3), metabolism of lipids and lipoproteins (NES 3.3), and extracellular region (NES 2.9) These unbiased findings are in excellent agreement with the known features and functions of SNCA in blood such as altered coagulation and altered immune competence, and may reflect a compensatory increase in the well-established lysosomal degradation of SNCA (Westbroek et al., 2011).

GZMH, _SPP1_, _PLTP_ blood qPCR shows increase in presymptomatic PARK4, but cannot distinguish prodromal multifactorial PD (RBD), while _CPLX1_ is useful as biomarker in PARK4 and RBD

Studies on presymptomatic PARK4 heterozygotes: Particularly strong effects within the lysosome, immune, and lipid pathways were studied as promising biomarker candidates for PARK4. We decided to validate the strong *GZMH* transcript upregulation (to 204%, $p = 0.04$ in pairwise *t*-test) by the independent qPCR technique in the same individuals plus additional members of the PARK4 pedigree, because the lysosomal enzymes cathepsin D and B, but not cathepsin G-like 2 (or granzyme H, GZMH) were previously implicated in degradation versus aggregation of SNCA (Crabtree et al., 2014; Tsujimura et al., 2014). The similarly strong upregulation (to 244%, $p = 0.01$) of the immunity regulator osteopontin (*SPP1*) transcript was assessed, because osteopontin levels were previously identified as a biomarker of PD in blood serum, cerebrospinal fluid, microglia, and affected neurons (Iczkiewicz et al., 2006; Maetzler et al., 2007; Shi et al., 2015). The similarly strong upregulation (to 189%, $p = 0.08$) of the phospholipid transfer protein (*PLTP*) transcript was assessed, because PLTP modifies ataxia, Alzheimer's disease, tau phosphorylation, and serves as lipopolysaccharide interactor (Dong et al., 2009; Gautier and Lagrost, 2011; Albers et al., 2012). The qPCR analysis confirmed significant increases for both transcripts (to 179% with $p = 0.009$ for *GZMH*, to 201% with $p = 0.016$ for *SPP1*) (Fig. 4A). In comparison, the recently published longitudinally dynamic biomarkers of PD in blood, *HNF4A* and *PTBP1*, did not show significantly altered levels at this prodromal stage ($n = 9$ control versus 12 PARK4 individuals).

Studies on patients with RBD, but asymptomatic for Parkinson motor symptoms: To investigate whether these candidate biomarkers might also be useful for risk prediction in individuals without PD family history who manifest symptoms that might represent incipient PD (Postuma et al., 2012), we collected blood RNA from overnight fasted individuals with REM sleep behaviour disorder (RBD). The qPCR analysis of 46 RBD cases (mean age 65 years, range from 34 to 83, with 36 males; since sampling, five cases converted to PD, one to Lewy Body Dementia, without DATscan confirmation) versus 19 matched controls (mean age 61 years from 30 to 74, with 12 males) failed to detect a significant increase for *GZMH* ($p = 0.29$), for *SPP1* ($p = 0.35$; but upon exclusion of one exceptionally high value among controls, an upregulation to 174% with $p = 0.04$ was observed), and for *PLTP* ($p = 0.66$). In contrast, a significant downregulation was confirmed for the *CPLX1* transcript (to 74%,

p = 0.015; upon exclusion of one exceptionally high value among controls, a statistical trend towards downregulation with p = 0.06 remained) (Fig. 4B).

To define the stability of *CPLX1* mRNA levels in blood as a biomarker further, their dependence on gender and age was investigated. Combining the younger control individuals from Turkey with the older control individuals from Central Europe, the expression did not show a significant correlation with age in linear regression analysis (p = 0.33). No deviation from linearity was detectable in the runs test (p = 0.53). There was no dependence on gender (p = 0.50). The PARK4 cohort showed a trend towards reduced *CPLX1* values at older age (p = 0.08) and a trend towards lower values in females (p = 0.08).

Overall, the blood qPCR observations validate the RNAseq findings and suggest that *GZMH*, *SPP1* and *PLTP* upregulations are indeed strong effects of PD pathogenesis. However, they may not be specific enough for the risk diagnostics of sporadic multifactorial PD, whereas the comparatively subtle *CPLX1* downregulation is useful in large populations with parallel sample processing, even if its fold-change is too small for individual diagnostics.

Reduced *CPLX1* mRNA in human *SNCA*-transfected neuroblastoma

To further evaluate the alpha-synuclein-triggered expression changes, we transiently overexpressed wild-type alpha-synuclein in human SH-SY5Y neuroblastoma cells, a widely used PD cell model with catecholaminergic properties. A downregulation of *CPLX1* mRNA in parallel to accumulation of complexin-1 protein occurred within two days after transfection (Fig. 5). This demonstrates that these effects are initiated early in neural cells. The observed upregulation of complexin-1 protein in RIPA fractions of these cells after 2 days contrasts with the downregulation of complexin-1 protein in 8 M urea fractions that was recently observed in the prefrontal cortex of autopsied PD patients at old age (Dumitriu et al., 2016) and may be explained by altered CPLX1 solubility, by progression from acute to chronic SNCA gain-of-function, or by other differences between neuroblastoma cells versus cortical postmitotic neurons.

Does complexin-1 protein aggregate in PD midbrain?

Protein levels of alpha-synuclein and of complexin-1 are increased in midbrain tissue from patients with idiopathic PD and from mouse models of synucleinopathy (Basso et al., 2004; Gispert et al., 2014). Such accumulations may be due to elevated gene dosage with increased transcript levels or alternatively be due to impaired degradation during an

aggregation process. The latter mechanism may be responsible for the decreased *SNCA* transcript levels that were described in some PD cases (Dächsel et al., 2007). Several other components of the SNARE complex that mediate membrane fusion and vesicle exocytosis, namely SNAP-25, syntaxin-1, and synaptobrevin-2, are contained in Lewy bodies and are depleted from surrounding neural tissue in PD (Garcia-Reitbock et al., 2010; Mukaetova-Ladinska et al., 2013). To test whether complexin-1 is aggregating within Lewy bodies, as was previously observed for alpha-synuclein and 14-3-3, we performed immunohistochemical analyses of PD patient midbrains. A low-titer antibody (Acris) detecting complexin-1 with high specificity stained aggregates in neurites and neuronal perikarya of fixed tissue after antigen retrieval (Fig. 6A-D). Double immunofluorescence revealed co-localization of alpha-synuclein and complexin-1 in fixed tissue only after maximal exposure and contrast adjustment of the channel depicting complexin-1 (Fig. 6E-H). These observations might support the notion that complexin-1 is sequestered into Lewy bodies, but of course the evidence must be considered with caution given that numerous proteins may be entrapped in aggregates without a specific role in pathogenesis.

The recent report of a 20% reduction of CPLX1 protein in 8 M urea fractions of prefrontal cortex from autopsied PD patients (Dumitriu et al., 2016) is also compatible with a sequestration of complexin-1 into Lewy bodies. Given that we documented a 33% reduction of *CPLX1* expression in presymptomatic PARK4 blood, the dysregulation appears to be relatively stable over decades and may not be useful as biomarker of disease-progression from prodromal to final stages of PD. Therefore, extensive investigations of *CPLX1* expression in PD patients of varying severity were not attempted.

CPLX1 gene variant enhances PD risk

It remains unclear whether *CPLX1* modulation is neuroprotective or rather contributes to pathogenesis. Interestingly, the *CPLX1* gene is encoded within a locus of confirmed association with PD risk according to GWAS meta-analyses (Lill et al., 2012). The corresponding PD risk haplotype, with an odds ratio of 1.45 at chromosome 4p16.3, spanned the genes *PCGF3 – LOC100129917 – CPLX1 – GAK - TMEM175 – DGKQ*. The GWAS approach cannot reliably dissect such loci further, although it can detect relative disease risks as low as 1.1-fold. In the latest meta-analysis of GWAS studies in PD (Nalls et al., 2014), two SNPs in the intron 1 of *CPLX1* directly next to the *GAK* gene reached genome-wide significance (rs76444973 and rs34006598), but it is difficult to predict how these variants would influence the function of CPLX1 protein. We therefore employed a complementary

approach and searched for a low-frequency *CPLX1* gene variant, which shows its risk association already in small case-control collectives and explains a substantial part of the disease association contained in the locus. For this purpose 360 random patients with idiopathic general (non-monogenic) PD and 358 controls were studied regarding several candidate SNPs outside the linkage disequilibrium block around *GAK*, within the *CPLX1* gene 3'-UTR. The selected SNPs show minor allele frequencies between 0.1 and 0.5 in Caucasian populations and were available as Taqman genotyping assays. Significant association with PD risk was observed for the G allele of the SNP rs10794536 (Table 2), with an odds ratio of 1.33. This 3'-UTR variant might influence the stability of *CPLX1* mRNA. These data suggest that complexin-1 not only serves as a downstream marker of the physiological alpha-synuclein function, but also modulates PD risk.

***Cplx1* gene ablation upregulates alpha-synuclein levels**

In order to determine whether complexin-1 loss-of-function enhances or alleviates the susceptibility to PD, the relevant brain region was studied in a *Cplx1* null mouse. This mouse line was previously shown to have an early and strong cerebellar phenotype (Reim et al., 2001; Glynn et al., 2005). In addition these mice display dystonia, shuffled walking and reduced novelty seeking, several signs that are characteristic effects of nigrostriatal dysfunction; intriguingly, these mice also display resting tremor, a diagnostic hallmark in humans for the onset of intermediate stage PD (Glynn et al., 2005). However, only cerebellar but not cerebral tissues were investigated to date. Brains of the *Cplx1* null mice revealed significantly increased levels (1.3-1.5-fold) of *Snca* mRNA and alpha-synuclein protein already by 3 months of age (Fig. 7). The data provide further evidence that complexin-1 levels are not only a downstream marker of alpha-synuclein function, but are also involved in alpha-synuclein abundance, apparently by reciprocal feedback expression regulation.

Discussion:

In summary, we report a new exceptionally large PARK4 pedigree, provide proof-of-principle that the risk for a future manifestation of PD is reflected in the global transcriptome of blood, identify diverse pathways and in particular the levels of *SPP1-GZMH-PLTP-CPLX1* as biomarkers of pathogenesis, show subtle *CPLX1* downregulations to distinguish

prodromal PD (presymptomatic PARK4 heterozygote and RBD individuals) cohorts from controls, and suggest complexin-1 loss-of-function to act as PD risk factor.

25 years ago, the characterization of the large Italian Contursi PARK1 kindred triggered research into PD genetics (Golbe et al., 1990; Polymeropoulos et al., 1996; Polymeropoulos et al., 1997). Now, the cooperativity of the Turkish PARK4 pedigree is a unique opportunity for further studies into human synucleinopathy such as brain imaging, and the existence of 12 presymptomatic heterozygotes of the *SNCA* gene duplication in this family may be crucial for the identification of a molecular signature of disease risk. Our deposition of PARK4 blood RNAseq data at the European Nucleotide Archive database aims to drive this effort, ensuring that PD risk, which is triggered by the genetically defined synucleinopathy of PARK4 in the Turkish pedigree and mirrored by specific pathways and molecular biomarkers, can now be compared with diverse ongoing data collections in manifest PD patients, usually without family history, and with multifactorial pathogenesis (Scherzer et al., 2007; Marek et al., 2011; Santiago and Potashkin, 2015).

It is unlikely that diagnostics of PD risk can be based on a single technique or a single molecular biomarker, so we evaluated the global transcriptome and the pathway dysregulations by GSEA bioinformatics. These approaches recently permitted us to identify three gene expression changes in dependence of alpha-synuclein function in brain tissue, concerning the chemotaxis factor *Lect1* in one digenic and two monogenic PD mouse models (Gispert et al., 2015) and the midbrain-selective transcription factor *Foxp1* as well as the SNARE complex component *Cplx1* in a synucleinopathy model and SNCA-deficient mouse mutants (Gispert et al., 2014). While we were unable to detect substantial *FOXP1* and *LECT1* expression in human fresh whole blood PAXgene samples - in spite of strong signals having been reported in microarray studies of purified peripheral mononuclear blood cells -, we found mRNA complexin-1 levels downregulated in whole blood in the Turkish PARK4 pedigree. The observation that increased abundance of SNCA - a known modulator of the SNARE complex and of vesicle dynamics - modifies the expression of another SNARE interactor, CPLX1, led us to hypothesize that altered physiological functions of SNCA at earliest stages of the accumulation and aggregation process will trigger immediate molecular and cellular responses in a subtle but cumulative manner, which may be exploited as biomarkers. This notion was supported by GSEA pathway dysregulations for the metabolism of lipids and lipoproteins and for endocytosis in response to the PARK4 mutation in the lipid-binding endocytosis modulator SNCA (Diao et al., 2013). Furthermore, this concept was also substantiated by dysregulated hemostasis and platelet activation pathways, with

corresponding preliminary documentation of impaired platelet degranulation in PARK4 blood, in the absence of detectable protein aggregates, in excellent agreement with previous publications *in vitro* and in mouse on a role of SNCA for platelet activation (Park et al., 2002; Reheman et al., 2010).

Our efforts to identify additional molecular biomarkers with stronger effect sizes focused on *GZMH* levels as representative of the lysosome pathway upregulation, on *SPP1* levels as representative of the immunity pathway upregulation, and on *PLTP* levels as representative of the lipid metabolism pathway. The data clearly confirmed their role as biomarkers of PARK4 at least in this Turkish pedigree, so it may now become interesting to test if progressive stages of synucleinopathy can be correlated with expression levels of these factors or of other pathway components, whether these factors represent molecular targets of neuroprotective therapies, or are risk factors themselves. Interestingly, a moderate mRNA upregulation for the lysosomal enzyme glucocerebrosidase (acid beta-glucosidase, GBA, log2 fold change = 0.31) was notable in the PARK4 blood RNAseq GSEA; GBA is known to contribute to SNCA degradation and its mutations act as modifiers of PD risk (Yap et al., 2011; Sidransky and Lopez, 2012; Fishbein et al., 2014). But there are many triggers of lysosomal induction and of immunity responses beyond alpha-synuclein accumulation and PD, and this probably explains why *GZMH* and *SPP1* upregulations are not specific for RBD cases, but detectable also among some control individuals.

In spite of the small effect size of *CPLX1* mRNA downregulation, this observation reproducibly distinguished cohorts of presymptomatic PARK4 heterozygotes in blood, of RBD cases in blood, and neuroblastoma cells after SNCA transfection. The corresponding protein levels are difficult to quantify in blood due to the abundance of hemoglobin, albumin and fibrinogen, and because small fold-changes are difficult to detect with antibody-dependent techniques and non-linear enzyme kinetics. CPLX1 protein accumulated in neuroblastoma cells (proteins extracted with RIPA buffer) in our experiments and is known to accumulate together with SNCA in midbrain tissue (proteins extracted with 7 M urea, 2 M thiourea and 4% CHAPS), while it is decreased in the prefrontal cortex of patients with sporadic PD (proteins extracted with 8 M urea) (Basso et al., 2004; Dumitriu et al., 2016). Thus the abundance of CPLX1 protein in fractions of different solubility appears to vary between different brain areas or in dependence on the progression of alpha-synuclein aggregation. We speculate that the accumulation of insoluble alpha-synuclein through protein interaction sequestrates CPLX1 protein, leading to its accumulation in solubility, later in insolubility, while a homeostatic negative autoregulation reduces the *CPLX1* mRNA levels.

The decrease of *CPLX1* mRNA has clear advantages as risk biomarker in peripheral tissue, while complexin-1 protein redistribution might become a biomarker of disease progression in nervous tissue. The formation of Lewy bodies has been shown to deplete SNARE complex components from neurons, although it is technically difficult to demonstrate their presence within the protein inclusion bodies (Garcia-Reitbock et al., 2010; Mukaetova-Ladinska et al., 2013). The unequivocal co-localization of SNCA and CPLX1 in aggregates is challenging. Co-immunoprecipitation studies failed to detect a direct association, but the high detergent conditions needed to enrich SNCA oligomers / fibrils would not leave protein associations intact.

Thus, instead of pursuing the physical interaction between SNCA and CPLX1 proteins we decided to assess their genetic interaction further. Two lines of evidence, (1) the identification of the *CPLX1* gene variant rs10794536 that is associated with PD risk already in a relatively small case-control study, and (2) the demonstration that genetic ablation of *Cplx1* in mouse brain leads to an elevation of *SNCA* levels that is comparable to PARK4, both argue that complexin-1 also has a role as risk factor in PD, not only a role as biomarker. The role of complexin-1 appears to extend beyond monogenic synucleinopathy cases into sporadic PD, in view of (i) its accumulation in mass-spectrometry proteomics of PD midbrain autopsies, (ii) the GWAS meta-analyses of risk loci for sporadic PD, and (iii) our findings of *CPLX1* blood level downregulation in RBD cases.

It is noteworthy in this context that a recent report showed a knock-in mutation in another SNARE protein, unphosphorylatable S187A-substituted SNAP-25, to trigger increased abundance and mislocalization of SNCA, together with SNARE assembly problems and presynaptic dilatation, but without neurodegeneration until the mouse age of 1 year. These authors concluded that dysfunctions at the SNARE complex in presynapses may induce elevated SNCA abundance and thus lead to an insidious neurodegenerative process (Nakata et al., 2012; Yasuda et al., 2013). This scenario is in good agreement with the recently identified genetic risk factors for idiopathic PD in cases without monogenic traits. The reproducible GWAS loci of PD risk (Lill et al., 2012; Nalls et al., 2014) are surprisingly enriched in genes relevant for vesicle dynamics, such as the *SYT11* (encoding synaptotagmin-11) and *RAB25* genes at the chromosome 1q21.1-22 PD risk locus, the *RAB7L1* gene at the chromosome 1q32 PARK16 locus, the *NSF* gene (encoding N-ethylmaleimide-sensitive factor) within the risk haplotype at the chromosome 17q21 PD risk locus named after *MAPT*, and the *SYT4* gene (encoding synaptotagmin-4) at the chromosome 18q12.3 PD risk locus named after *RIT2*. A remaining major GWAS PD risk locus contains

the *LRRK2* gene, which is responsible for autosomal dominant PARK8 and encodes protein modulating synaptic vesicles cycling and interacting with syntaxin-1B / NSF / clathrin / AP2 (Piccoli et al., 2011). Thus, the *SNCA* and *CPLX1* 3'-UTR variants might be representative for various SNARE / RAB vesicle cycle alterations that modulate PD risk.

Overall, our initial characterization of an exceptionally large PARK4 pedigree from Turkey provided insights into the prodromal stage of monogenic synucleinopathy cases, defining a molecular signature in blood transcriptome that may be relevant for predictive diagnostics in groups of at-risk individuals. Although several expression differences between groups with presymptomatic PD versus controls were identified with statistical significance, individual differences at present would be insufficient for predictive diagnostics. Most importantly, the data identify CPLX1 as biomarker and modifier gene of PD risk.

Materials and Methods:

Neurological examination of members of the PARK4 family, which originated in the North Eastern region of Turkey, to document clinical manifestation or presymptomatic PARK4 heterozygote status adhered to the UK PD Society Brain Bank criteria (Hughes et al., 1992).

Genotyping. Genomic DNA from blood samples in EDTA tubes stored at +4 °C was extracted with MagNA Pure Systems (Roche), after informed written consent and genetic counselling, with approval from the Ethics Commission of Boğaziçi University Istanbul. The molecular genetic diagnosis of PARK4 in an index PD patient from this family (female aged 51 with UPDRS score 7 after seven years disease duration, showing rigidity, resting tremor and bradykinesia, having 60 months of Levodopa-treatment with some benefit, but dyskinesias) was originally made by semi-quantitative multiplex PCR and multiplex ligation-dependent probe amplification (P051 kit) of *SNCA* exons 3 and 4 in comparison to *Parkin* and *TTR* exons 4, then extended to examine 22 pedigree members. Demonstration of *SNCA* gene duplication was complemented by the detection of the associated 4q21-3-4q22.3 haplotype of 17 microsatellite markers (D4S2691, D4S2409, D4S2462, D4S2622, D4S1542, D4S2929, D4S2371, D4S2461, D4S2304, D4S3459, D4S3457, D4S1544, D4S410, D4S1089, D4S414, D4S2404, D4S2364) across 11 Mb around the *SNCA* locus in 12 pedigree members to demonstrate co-segregation. Further genotyping experiments were performed for the PARK4 family members using DNA probes, specific to the *SNCA* gene, and quantitative real-time PCR method. Expression levels of the beta-Actin, *GAPDH* and

TBP genes were separately used as endogenous controls for normalization. The gene expression fold changes were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Molecular karyotyping. Two PARK4 blood samples were assessed for copy number variation using a SurePrint G3 Human CGH (comparative genomic hybridisation) microarray 2x400K design 021850 (Agilent Technologies). Agilent's labelling kit was utilized and all procedures were carried out according to the manufacturer's instructions. Scanning was performed on an Agilent microarray scanner and raw data were processed by Feature Extraction 9.5. Deleted and amplified regions were determined on Agilent's Genomic Workbench Standard Edition 5.0.14. A minimum of four consecutive probes had to be affected to make a decision. The aberration detection threshold of the ADM-2 algorithm was set to 5.9.

Whole blood transcriptomics and protein analysis. After overnight fasting of the individuals, whole blood samples from the PARK4 family were taken (after informed written consent and with approval from the Ethics Commission of Boğaziçi University Istanbul) into PAXgene (Qiagen) and EDTA tubes. Also after overnight fasting, whole blood samples were collected into PAXgene tubes from a RBD cohort comprising 46 cases and 19 healthy relatives (cases with narcolepsy were excluded), after written informed consent with approval from the Ethics Commission at Marburg University. Always processing cases and controls in parallel, after 2 hours incubation of the PAXgene tubes at room temperature, storage at -80 °C occurred, until the RNA was extracted after a further overnight room temperature incubation, using the corresponding PAXgene kit. The isolated RNA was cleaned up by DNAse-I amplification grade (Invitrogen) and converted to cDNA by SuperScript III Reverse Transcriptase (Invitrogen). Quality and quantity of the cDNA was determined by spectrophotometry and RNA integrity numbers (Agilent Bioanalyzer). Quantitative RT-PCR was performed employing TaqMan assays (Applied Biosystems) Hs01018439_s1 (*GPRIN3*), Hs00201182_m1 (*MMRN1*), Hs00411197_m1 (*LRRK2*), Hs01084510_m1 (*GIGYF2*), Hs00223032_m1 (*ATP13A*), Hs01026447_m1 (*ATXN3*), Hs00697109_m1 (*DJ-1*), Hs00260868_m1 (*PINK1*), Hs00832556_sH (*ST13*), Hs01038318_m1 (*PARK2*), Hs00201825_m1 (*FBX07*), Hs00188233_m1 (*UCHL1*), Hs00164683_m1 (*GBA*), Hs00234883_m1 (*HTRA2*), Hs00185926_m1 (*PLA2G6*), Hs00608185_m1 (*SNCB*), Hs00705917_s1 (*YWHAG*), Hs01103386_m1 (*SNCA*), HS00793604_m1 (*YWHAB*), HS00356749_g1 (*YWHAE*), Hs00362510_m1 (*CPLX1*), Hs00277212_m1 (*GZMH*), Hs00959010_m1 (*SPP1*), Hs00272126_m1 (*PLTP*), Hs00230853_m1 (*HNF4A*) and

Hs00914687_g1 (*PTBP1*). After studies of various loading controls (e.g. *GAPDH, ACTN, RPL13A, TBP, SDH and YWHA2*) versus *SNCA* expression in whole blood, Hs99999910_m1 (*TBP*) was observed to show the smallest variance and so it was included as housekeeping control for each expression test. A total of 25 ng cDNA was used in each reaction. The PCR conditions were 2 min at 50 ºC, 10 min at 95 °C, 40 cycles of 15 sec at 95 °C and 1 min at 60 °C. The gene expression changes were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Whole blood protein analysis. For protein extraction from the EDTA tubes, 300 µl blood were lysed with equal amount of 1% SDS-RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Igepal CA-630 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF and one tablet Complete Protease Inhibitor Cocktail (Roche)] and sonicated for 10 sec. The blood lysates were rotated at 4 °C for 30 min and centrifuged at 4 °C for 30 min. The supernatants were depleted in hemoglobin content using a commercial kit (HemogloBind, Biotech) following the manufacturer's instructions. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific). For immunoblotting, proteins were diluted to 4 µg/µl and mixed with equal amount of loading buffer [250 mM Tris/HCl pH 6.9, 20% glycerol, 4% SDS, 10% mercaptoethanol, 0.005% bromophenolblue]. Samples were incubated at 65 °C for 16 hours prior to loading to the 12% polyacrylamide gels and transferred to PVDF membranes. The membrane was blocked in 5% milk powder and incubated with primary antibodies against alpha-synuclein (1:1,000, BD 610786), beta-synuclein (1:1,000 from Abcam ab6165 and from Upstate 36-009), complexin-1 (1:500, Acris AP51050PU-N) and beta-actin (1:10,000 from Sigma), visualizing the signals by the ECL method. Densitometric analysis was carried out using the ImageJ software.

Blood platelet flow cytometry. After applying a light tourniquet, which was immediately released after venipuncture, blood was drawn using a 21-gauge butterfly needle. Blood was collected in 4.5 ml coagulation tubes containing 0.106 mol/L sodium citrate (Sarstedt AG) for flow cytometry evaluations. The following directly conjugated monoclonal mouse anti-human antibodies were obtained from BD Biosciences: phycoerythrin (PE)-conjugated anti-CD62P, PE-conjugated anti-CD63 and (Perc-CP)-conjugated anti-CD61. Platelet immunostaining was performed as follows: 20 µL diluted citrated whole blood (1:10, Dulbecco´s modified phosphate Saline without Ca^{++} and Mg^{++} (DBPS), Gibco BRL) were incubated with saturating concentrations of monoclonal antibodies for 15 minutes at RT. Thereafter samples were diluted with 1mL DPBS and analyzed immediately by FACS

Calibur flow cytometer (BD Biosciences). Platelets were identified through CD61 positivity and their characteristic light scatter. Each analysis was performed on 5000 platelets. Quantification of surface expression of given antigens was obtained using CellquestPro software (BD Biosciences). The mean fluorescence intensity was used to define molecule expressions on platelet surface. Results were compared to 20 healthy Central European volunteers (control group). To characterize α-granule and lysosome release response upon platelet activation, 20 μL 1:10 diluted citrated whole blood was stimulated with thrombin receptor agonist peptide (TRAP-6, final concentration: 10 μmol/L, Roche) and DPBS (control) for 10 minutes at room temperature. Platelets were immunostained as described above.

Platelet electron microscopy. Approximately 16 ml blood was collected in 8.2 ml coagulation tubes containing 0.106 mol/L sodium citrate (Sarstedt AG). Fresh platelet-rich plasma (PRP) was prepared by centrifugation at 140 × *g* for 10 min at room temperature. After being stored for 30 min, PRP samples were stimulated with TRAP-6: final concentration 30 μmol/L for 30 seconds. Fixation of suspended platelets was accomplished by combining the sample with an equal volume of 0.1% glutaraldehyde in White's saline (a 10% solution of a 1:1 mixture of: (1) 2.4 mmol/L NaCl, 0.1 mmol/L KCH, 46 mmol/L $MgSO_4$ and 64 mmol/L $Ca(NO_3)_2$. $4H_20$; and (2) 0.136 mol/L $NaHCO_3$, 8.4 mmol/L NaH_2PO and 0.1g/L of phenol red, pH 7.4). After 15 min the samples were centrifuged to pellets, washed and resuspended in 3% glutaraldehyde in the same buffer, resuspended and maintained at 4 ºC for 30 min, then sedimented to pellets. The supernatant was removed and replaced with 1% osmic acid in distilled water containing 1.5% potassium ferrocyanide for 1 h at 4 ºC. All samples were dehydrated in a graded series of alcohols and embedded in Epon. Thin sections of 100 nm were cut from the plastic blocks on a Leica ultramicrotome, and were examined after staining with uranyl acetate and lead citrate to enhance contrast. Samples were examined on a Technai F30 electron microscope at 300 kV equipped with US 4000 camera.

RNAseq. Global blood RNA sequencing for the five PARK4 versus five age and sex matched healthy individuals was outsourced to Alacris Theranostics GmbH, Berlin, Germany. Strand-specific RNA sequencing libraries were prepared from 500 ng of total RNA according to the Illumina TruSeq stranded mRNA protocol. mRNA was selected using oligo(dT) Dynabeads. Sequencing was performed on Illumina HiSeq2500 platform, using paired-end 2x50 bp sequencing mode. For each library, 40-50 mln of passed filter reads were

obtained. Raw data was processed according to the manufacturer's instructions. Illumina's bcl2fastq software v1.8.2 was used for base calling and demultiplexing. The sequencing reads were mapped to the human genome reference GRCh37/hg19 using bwa (version 0.5.9, http://bio-bwa.sourceforge.net/). Exonic reads in correct genomic orientation, defined by Ensembl Release 73 (http://www.ensembl.org/index.html), were counted using custom python scripts. Expression values were calculated using RPKM normalization with custom R scripts (reads per kilobase per million mapped reads (Mortazavi et al., 2008)). Since we observed huge amounts of hemoglobin in the samples, reads on hemoglobin genes were removed prior to the expression analysis. Differential expression between disease and control samples was calculated with the R bioconductor package edgeR (Robinson et al., 2010) (http://www.bioconductor.org/packages/release/bioc/html/edgeR.html) applying an exact test for overdispersed data. As an additional criterion to judge variance and restrict the number of biomarker candidates, a custom R script was used to calculate pairwise *t*-tests between disease and control expression values for each gene. All data were publically deposited at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the accession number PRJEB8960 / ERP010003.

Bioinformatic systems biology. RNAseq data from 5 presymptomatic PARK4 heterozygotes and 5 matched controls were analyzed to compute average fold changes for each gene and rank them according the *t*-test statistics. For duplicate entries, the maximum value was used. The ranked list of gene symbols thus generated was subjected to nonspecific filtering and assessed by Gene Set Enrichment Analysis (GSEA) using the Java-based version GSEA-P (Subramanian et al., 2005; 2007). Permutations were performed on gene sets owing to the low number of biological replicates. We used the c2 (online pathway databases, Pubmed publications, expert of domain knowledge), c5 (Gene Ontology categories) and cc (Gene Ontology cellular component) gene sets from the MSigDB database (v4.0, May 2013, http://www.broadinstitute.org/gsea/msigdb/index.jsp) to analyze the data sets.

Transient transfection of wild-type alpha-synuclein in SH-SY5Y cells. The open reading frames corresponding to wild-type alpha-synuclein were cloned into the plasmid pcDNA3.1(+) (Invitrogen), between the restriction sites KpnI and NotI. Transient overexpression was performed by nucleofection using 1.5 million SH-SY5Y cells (recently authenticated and tested for contamination) and 2 micrograms of plasmid together with Amaxa nucleofector Kit V (Lonza). Fourty eight hours post transfection, total RNA was isolated using the RNeasy kit (QIAGEN), cDNA was synthesized using 1 microgram of total RNA and relative mRNA levels were determined by qPCR using the TaqMan probes *SNCA*

(Hs00240906_m1), *CPLX1* (Hs00362510_m1) and *TBP* (Hs99999910_m1) (Applied Biosystems). Relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ formula. Total protein was isolated with lysis buffer [Tris-HCl 137 mM pH 6.8, sodium dodecyl sulfate 4%, glycerol 20% and protease inhibitors 1X (Roche)]. Thirty micrograms of protein were resolved in a 4-12% gradient gel (Novex NP0322BOX), transferred to PVDF membrane, and blocked with 5% skim-milk in PBS-Tween 0.1%. The following antibodies were used to detect alpha-synuclein (Covance #SIG-39730-200), complexin 1+2 (Synaptic systems #122002), beta-Actin (Sigma-Aldrich #A5441) and GAPDH (Calbiochem #CB1001).

Brain autopsy processing and immunohistochemistry were performed as described previously (2010a; Seidel et al., 2010b), employing primary antibodies for complexin-1 (1:25 Acris AP51050PU-N and 1:100 SySy 122002) and alpha-synuclein (1:2000 BD Biosciences 610786). For antigen-retrieval, sections were treated with 99% formic acid, 3 min at room temperature. Single immunostainings of complexin-1 were performed with a peroxidase-conjugated secondary antibody and the ABC kit, and visualized with the diaminobenzidine (DAB) method. Double immunostainings with either of the complexin-1 antibodies combined with the alpha-synuclein antibody were performed with secondary Cy3 anti-rabbit and alexa 488-anti-mouse antibodies. Autofluorescence was quenched with application of 0.06% Sudan Black in 70% ethanol for 10 min at room temperature. Stainings were conducted on 4 clinically confirmed idiopathic PD patients and 2 control cases without history of neuropsychiatric disease (PD cases 08-27, 09-12, 09-87 and 09-237; controls 12-10084 and 12-10079). The high-titer antibody detecting both complexin-1 and complexin-2 (SySy) did not stain any aggregates. Informed consent was obtained, both the University Medical Centre Groningen and the Goethe University Hospital Frankfurt ethics commissions approved the study.

Gene variants of CPLX1 were analysed in PD and control samples that were previously described (Lavedan et al., 1998; Leroy et al., 1998; Rissling et al., 2004; Gispert et al., 2005; Moller et al., 2008) and were collected at the Düsseldorf and Marburg University Hospitals in Germany. The SNPs rs1052595, rs2242236, rs11248041, rs7340883, rs6816868, rs10794536 were studied, employing 2-5ng genomic DNA, 1x TaqMan Genotyping Mastermix (AppliedBiosystems 4371355), 1x TaqMan SNP genotyping assay in 20 µl (Invitrogen) and running the reaction under standard conditions in a StepOnePlus apparatus (AppliedBiosystems).

Mouse breeding and characterization with brain dissection was carried out as described in the literature (Glynn et al., 2005; Gispert et al., 2009). The diet of ataxic *Cplx1*-/-

mice was supplemented with DietGel Recovery 72-06-5022 (ClearH_2O). Extraction of protein and RNA was carried out as previously described (Gispert et al., 2013). The transcript expression studies employed TaqMan assays (AppliedBiosystems) Mm00447333_m1 (*Snca*) and Mm00446973_m1 (*Tbp*). Quantitative immunoblotting used the following primary antibodies for mouse alpha-synuclein (1:1,000 BD Biosciences 610786), complexin-1 (1:500 Acris AP51050PU-N and 1:1,000 SySy 122002), beta-actin (1:10,000 Sigma A5441) and their corresponding secondary antibodies (GE Healthcare UK Limited LNA931V/AG for ECL-anti-mouse-HP from sheep and LNA934V/AG for ECL-anti-rabbit-HP from donkey).

Statistical analyses presented in bar graphs were performed by unpaired Student's t-tests, those of *CPLX1* level association with age or gender were performed with linear regression, runs test and 2-way ANOVA, all being conducted and plotted with the Prism 5.04 software (GraphPad, La Jolla, CA, USA). The allele association studies were carried out after excluding deviations from Hardy-Weinberg equilibrium by χ^2 statistics for genotype and allele distribution.

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Competing Interests:

No competing interests declared.

Author Contributions:

Lahut S: Organization and execution of research and statistical analysis. Writing of the first draft.

Gispert S: Design of research and statistical analysis. Review and Critique.

Omur O: Organization and execution of research and statistical analysis.

Tireli H: Clinical investigation of the PARK4 family. Review and Critique.

Basak AN: Organization and design of research. Review and Critique.

Oertel W: Organization and design of research. Review and Critique.

Auburger G: Organization and design of research. Review and Critique.

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References:

Albers JJ, Vuletic S, Cheung MC (2012) Role of plasma phospholipid transfer protein in lipid and lipoprotein metabolism. Biochim Biophys Acta 1821:345-357.

Barbour R, Kling K, Anderson JP, Banducci K, Cole T, Diep L, Fox M, Goldstein JM, Soriano F, Seubert P, Chilcote TJ (2008) Red blood cells are the major source of alpha-synuclein in blood. Neurodegener Dis 5:55-59.

Basso M, Giraudo S, Corpillo D, Bergamasco B, Lopiano L, Fasano M (2004) Proteome analysis of human substantia nigra in Parkinson's disease. Proteomics 4:3943-3952.

Battisti C, Formichi P, Radi E, Federico A (2008) Oxidative-stress-induced apoptosis in PBLs of two patients with Parkinson disease secondary to alpha-synuclein mutation. J Neurol Sci 267:120-124.

Boeve BF et al. (2013) Clinicopathologic correlations in 172 cases of rapid eye movement sleep behavior disorder with or without a coexisting neurologic disorder. Sleep Med 14:754-762.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, Braak E (2003) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197-211.

Brehm N, Rau K, Kurz A, Gispert S, Auburger G (2015a) Age-Related Changes of 14-3-3 Isoforms in Midbrain of A53T-SNCA Overexpressing Mice. J Parkinsons Dis 5:595-604.

Brehm N, Bez F, Carlsson T, Kern B, Gispert S, Auburger G, Cenci MA (2015b) A Genetic Mouse Model of Parkinson's Disease Shows Involuntary Movements and Increased Postsynaptic Sensitivity to Apomorphine. Mol Neurobiol 52:1152-1164.

Chandra S, Fornai F, Kwon HB, Yazdani U, Atasoy D, Liu X, Hammer RE, Battaglia G, German DC, Castillo PE, Sudhof TC (2004) Double-knockout mice for alpha- and beta-synucleins: effect on synaptic functions. Proc Natl Acad Sci U S A 101:14966-14971.

Corti O, Lesage S, Brice A (2011) What genetics tells us about the causes and mechanisms of Parkinson's disease. Physiol Rev 91:1161-1218.

Crabtree D, Dodson M, Ouyang X, Boyer-Guittaut M, Liang Q, Ballestas ME, Fineberg N, Zhang J (2014) Over-expression of an inactive mutant cathepsin D increases

endogenous alpha-synuclein and cathepsin B activity in SH-SY5Y cells. J Neurochem 128:950-961.

Dächsel JC, Lincoln SJ, Gonzalez J, Ross OA, Dickson DW, Farrer MJ (2007) The ups and downs of alpha-synuclein mRNA expression. Mov Disord 22:293-295.

Diao J, Burré J, Vivona S, Cipriano DJ, Sharma M, Kyoung M, Südhof TC, Brunger AT (2013) Native alpha-synuclein induces clustering of synaptic-vesicle mimics via binding to phospholipids and synaptobrevin-2/VAMP2. Elife 2:e00592.

Dong W, Albers JJ, Vuletic S (2009) Phospholipid transfer protein reduces phosphorylation of tau in human neuronal cells. J Neurosci Res 87:3176-3185.

Dumitriu A, Golji J, Labadorf AT, Gao B, Beach TG, Myers RH, Longo KA, Latourelle JC (2016) Integrative analyses of proteomics and RNA transcriptomics implicate mitochondrial processes, protein folding pathways and GWAS loci in Parkinson disease. BMC Med Genomics 9:5.

Fishbein I, Kuo YM, Giasson BI, Nussbaum RL (2014) Augmentation of phenotype in a transgenic Parkinson mouse heterozygous for a Gaucher mutation. Brain 137:3235-3247.

Fuchs J, Nilsson C, Kachergus J, Munz M, Larsson EM, Schule B, Langston JW, Middleton FA, Ross OA, Hulihan M, Gasser T, Farrer MJ (2007) Phenotypic variation in a large Swedish pedigree due to SNCA duplication and triplication. Neurology 68:916-922.

Garcia-Reitbock P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, Ghetti B, Della Corte L, Spano P, Tofaris GK, Goedert M, Spillantini MG (2010) SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. Brain 133:2032-2044.

Gardai SJ, Mao W, Schule B, Babcock M, Schoebel S, Lorenzana C, Alexander J, Kim S, Glick H, Hilton K, Fitzgerald JK, Buttini M, Chiou SS, McConlogue L, Anderson JP, Schenk DB, Bard F, Langston JW, Yednock T, Johnston JA (2013) Elevated alpha-synuclein impairs innate immune cell function and provides a potential peripheral biomarker for Parkinson's disease. PLoS One 8:e71634.

Gautier T, Lagrost L (2011) Plasma PLTP (phospholipid-transfer protein): an emerging role in 'reverse lipopolysaccharide transport' and innate immunity. Biochem Soc Trans 39:984-988.

Gispert S, Trenkwalder C, Mota-Vieira L, Kostic V, Auburger G (2005) Failure to find alpha-synuclein gene dosage changes in 190 patients with familial Parkinson disease. Arch Neurol 62:96-98.

Gispert S, Kurz A, Brehm N, Rau K, Walter M, Riess O, Auburger G (2014) Complexin-1 and Foxp1 Expression Changes Are Novel Brain Effects of Alpha-Synuclein Pathology. Mol Neurobiol Aug 12. [Epub ahead of print].

Gispert S, Brehm N, Weil J, Seidel K, Rub U, Kern B, Walter M, Roeper J, Auburger G (2015) Potentiation of neurotoxicity in double-mutant mice with Pink1 ablation and A53T-SNCA overexpression. Hum Mol Genet 24:1061-1076.

Gispert S, Parganlija D, Klinkenberg M, Drose S, Wittig I, Mittelbronn M, Grzmil P, Koob S, Hamann A, Walter M, Buchel F, Adler T, Hrabe de Angelis M, Busch DH, Zell A, Reichert AS, Brandt U, Osiewacz HD, Jendrach M, Auburger G (2013) Loss of mitochondrial peptidase Clpp leads to infertility, hearing loss plus growth retardation via accumulation of CLPX, mtDNA and inflammatory factors. Hum Mol Genet 22:4871-4887.

Gispert S et al. (2009) Parkinson phenotype in aged PINK1-deficient mice is accompanied by progressive mitochondrial dysfunction in absence of neurodegeneration. PLoS One 4:e5777.

Glynn D, Drew CJ, Reim K, Brose N, Morton AJ (2005) Profound ataxia in complexin I knockout mice masks a complex phenotype that includes exploratory and habituation deficits. Hum Mol Genet 14:2369-2385.

Goedert M, Spillantini MG, Del Tredici K, Braak H (2013) 100 years of Lewy pathology. Nat Rev Neurol 9:13-24.

Golbe LI, Di Iorio G, Bonavita V, Miller DC, Duvoisin RC (1990) A large kindred with autosomal dominant Parkinson's disease. Ann Neurol 27:276-282.

Hughes AJ, Daniel SE, Kilford L, Lees AJ (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. J Neurol Neurosurg Psychiatry 55:181-184.

Iczkiewicz J, Jackson MJ, Smith LA, Rose S, Jenner P (2006) Osteopontin expression in substantia nigra in MPTP-treated primates and in Parkinson's disease. Brain Res 1118:239-250.

Iranzo A, Fernandez-Arcos A, Tolosa E, Serradell M, Molinuevo JL, Valldeoriola F, Gelpi E, Vilaseca I, Sanchez-Valle R, Llado A, Gaig C, Santamaria J (2014) Neurodegenerative disorder risk in idiopathic REM sleep behavior disorder: study in 174 patients. PLoS One 9:e89741.

Iranzo A, Tolosa E, Gelpi E, Molinuevo JL, Valldeoriola F, Serradell M, Sanchez-Valle R, Vilaseca I, Lomena F, Vilas D, Llado A, Gaig C, Santamaria J (2013)

Neurodegenerative disease status and post-mortem pathology in idiopathic rapid-eye-movement sleep behaviour disorder: an observational cohort study. Lancet Neurol 12:443-453.

Janezic S, Threlfell S, Dodson PD, Dowie MJ, Taylor TN, Potgieter D, Parkkinen L, Senior SL, Anwar S, Ryan B, Deltheil T, Kosillo P, Cioroch M, Wagner K, Ansorge O, Bannerman DM, Bolam JP, Magill PJ, Cragg SJ, Wade-Martins R (2013) Deficits in dopaminergic transmission precede neuron loss and dysfunction in a new Parkinson model. Proc Natl Acad Sci U S A 110:E4016-4025.

Kim S, Jeon BS, Heo C, Im PS, Ahn TB, Seo JH, Kim HS, Park CH, Choi SH, Cho SH, Lee WJ, Suh YH (2004) Alpha-synuclein induces apoptosis by altered expression in human peripheral lymphocyte in Parkinson's disease. Faseb J 18:1615-1617.

Kurz A, Double KL, Lastres-Becker I, Tozzi A, Tantucci M, Bockhart V, Bonin M, Garcia-Arencibia M, Nuber S, Schlaudraff F, Liss B, Fernandez-Ruiz J, Gerlach M, Wullner U, Luddens H, Calabresi P, Auburger G, Gispert S (2010) A53T-alpha-synuclein overexpression impairs dopamine signaling and striatal synaptic plasticity in old mice. PLoS One 5:e11464.

Lavedan C et al. (1998) Absence of mutation in the beta- and gamma-synuclein genes in familial autosomal dominant Parkinson's disease. DNA Res 5:401-402.

Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, Harta G, Brownstein MJ, Jonnalagada S, Chernova T, Dehejia A, Lavedan C, Gasser T, Steinbach PJ, Wilkinson KD, Polymeropoulos MH (1998) The ubiquitin pathway in Parkinson's disease. Nature 395:451-452.

Lill CM et al. (2012) Comprehensive research synopsis and systematic meta-analyses in Parkinson's disease genetics: The PDGene database. PLoS Genet 8:e1002548.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

Maetzler W, Berg D, Schalamberidze N, Melms A, Schott K, Mueller JC, Liaw L, Gasser T, Nitsch C (2007) Osteopontin is elevated in Parkinson's disease and its absence leads to reduced neurodegeneration in the MPTP model. Neurobiol Dis 25:473-482.

Mahowald MW, Schenck CH (2013) REM sleep behaviour disorder: a marker of synucleinopathy. Lancet Neurol 12:417-419.

Marek K et al. (2011) The Parkinson Progression Marker Initiative (PPMI). Prog Neurobiol 95:629-635.

Moller JC, Rissling I, Mylius V, Hoft C, Eggert KM, Oertel WH (2008) The prevalence of the G2019S and R1441C/G/H mutations in LRRK2 in German patients with Parkinson's disease. Eur J Neurol 15:743-745.

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 5:621-628.

Mukaetova-Ladinska EB, Andras A, Milne J, Abdel-All Z, Borr I, Jaros E, Perry RH, Honer WG, Cleghorn A, Doherty J, McIntosh G, Perry EK, Kalaria RN, McKeith IG (2013) Synaptic proteins and choline acetyltransferase loss in visual cortex in dementia with Lewy bodies. J Neuropathol Exp Neurol 72:53-60.

Mutez E, Lepretre F, Le Rhun E, Larvor L, Duflot A, Mouroux V, Kerckaert JP, Figeac M, Dujardin K, Destee A, Chartier-Harlin MC (2011) SNCA locus duplication carriers: from genetics to Parkinson disease phenotypes. Hum Mutat 32:E2079-2090.

Nakata Y, Yasuda T, Fukaya M, Yamamori S, Itakura M, Nihira T, Hayakawa H, Kawanami A, Kataoka M, Nagai M, Sakagami H, Takahashi M, Mizuno Y, Mochizuki H (2012) Accumulation of alpha-synuclein triggered by presynaptic dysfunction. J Neurosci 32:17186-17196.

Nalls MA et al. (2014) Large-scale meta-analysis of genome-wide association data identifies six new risk loci for Parkinson's disease. Nat Genet 46:989-993.

Nemani VM, Lu W, Berge V, Nakamura K, Onoa B, Lee MK, Chaudhry FA, Nicoll RA, Edwards RH (2010) Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. Neuron 65:66-79.

Nishioka K, Hayashi S, Farrer MJ, Singleton AB, Yoshino H, Imai H, Kitami T, Sato K, Kuroda R, Tomiyama H, Mizoguchi K, Murata M, Toda T, Imoto I, Inazawa J, Mizuno Y, Hattori N (2006) Clinical heterogeneity of alpha-synuclein gene duplication in Parkinson's disease. Ann Neurol 59:298-309.

Park SM, Jung HY, Kim HO, Rhim H, Paik SR, Chung KC, Park JH, Kim J (2002) Evidence that alpha-synuclein functions as a negative regulator of Ca(++)-dependent alpha-granule release from human platelets. Blood 100:2506-2514.

Piccoli G, Condliffe SB, Bauer M, Giesert F, Boldt K, De Astis S, Meixner A, Sarioglu H, Vogt-Weisenhorn DM, Wurst W, Gloeckner CJ, Matteoli M, Sala C, Ueffing M (2011) LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. J Neurosci 31:2225-2237.

Platt NJ, Gispert S, Auburger G, Cragg SJ (2012) Striatal dopamine transmission is subtly modified in human A53Talpha-synuclein overexpressing mice. PLoS One 7:e36397.

Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, Sanges G, Stenroos ES, Pho LT, Schaffer AA, Lazzarini AM, Nussbaum RL, Duvoisin RC (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science 274:1197-1199.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, Nussbaum RL (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045-2047.

Postuma RB, Aarsland D, Barone P, Burn DJ, Hawkes CH, Oertel W, Ziemssen T (2012) Identifying prodromal Parkinson's disease: pre-motor disorders in Parkinson's disease. Mov Disord 27:617-626.

Reheman A, Tasneem S, Ni H, Hayward CP (2010) Mice with deleted multimerin 1 and alpha-synuclein genes have impaired platelet adhesion and impaired thrombus formation that is corrected by multimerin 1. Thromb Res 125:e177-183.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell 104:71-81.

Rhinn H, Qiang L, Yamashita T, Rhee D, Zolin A, Vanti W, Abeliovich A (2012) Alternative alpha-synuclein transcript usage as a convergent mechanism in Parkinson's disease pathology. Nat Commun 3:1084.

Rissling I, Geller F, Bandmann O, Stiasny-Kolster K, Korner Y, Meindorfner C, Kruger HP, Oertel WH, Moller JC (2004) Dopamine receptor gene polymorphisms in Parkinson's disease patients reporting "sleep attacks". Mov Disord 19:1279-1284.

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139-140.

Santiago JA, Potashkin JA (2015) Network-based metaanalysis identifies HNF4A and PTBP1 as longitudinally dynamic biomarkers for Parkinson's disease. Proc Natl Acad Sci U S A 112:2257-2262.

Scherzer CR, Eklund AC, Morse LJ, Liao Z, Locascio JJ, Fefer D, Schwarzschild MA, Schlossmacher MG, Hauser MA, Vance JM, Sudarsky LR, Standaert DG, Growdon JH, Jensen RV, Gullans SR (2007) Molecular markers of early Parkinson's disease based on gene expression in blood. Proc Natl Acad Sci U S A 104:955-960.

Seidel K, den Dunnen WF, Schultz C, Paulson H, Frank S, de Vos RA, Brunt ER, Deller T, Kampinga HH, Rub U (2010a) Axonal inclusions in spinocerebellar ataxia type 3. Acta Neuropathol 120:449-460.

Seidel K, Schols L, Nuber S, Petrasch-Parwez E, Gierga K, Wszolek Z, Dickson D, Gai WP, Bornemann A, Riess O, Rami A, Den Dunnen WF, Deller T, Rub U, Kruger R (2010b) First appraisal of brain pathology owing to A30P mutant alpha-synuclein. Ann Neurol 67:684-689.

Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA, Selkoe DJ (2003) The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. Neuron 37:583-595.

Shi M, Movius J, Dator R, Aro P, Zhao Y, Pan C, Lin X, Bammler TK, Stewart T, Zabetian CP, Peskind ER, Hu SC, Quinn JF, Galasko DR, Zhang J (2015) Cerebrospinal fluid peptides as potential Parkinson disease biomarkers: a staged pipeline for discovery and validation. Mol Cell Proteomics.

Shin EC, Cho SE, Lee DK, Hur MW, Paik SR, Park JH, Kim J (2000) Expression patterns of alpha-synuclein in human hematopoietic cells and in Drosophila at different developmental stages. Mol Cells 10:65-70.

Sidransky E, Lopez G (2012) The link between the GBA gene and parkinsonism. Lancet Neurol 11:986-998.

Singleton AB et al. (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science 302:841.

Stiasny-Kolster K, Doerr Y, Moller JC, Hoffken H, Behr TM, Oertel WH, Mayer G (2005) Combination of 'idiopathic' REM sleep behaviour disorder and olfactory dysfunction as possible indicator for alpha-synucleinopathy demonstrated by dopamine transporter FP-CIT-SPECT. Brain 128:126-137.

Subramaniam M, Althof D, Gispert S, Schwenk J, Auburger G, Kulik A, Fakler B, Roeper J (2014) Mutant alpha-synuclein enhances firing frequencies in dopamine substantia nigra neurons by oxidative impairment of A-type potassium channels. J Neurosci 34:13586-13599.

Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP (2007) GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics 23:3251-3253.

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005) Gene set enrichment analysis:

a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102:15545-15550.

Tozzi A, Costa C, Siliquini S, Tantucci M, Picconi B, Kurz A, Gispert S, Auburger G, Calabresi P (2012) Mechanisms underlying altered striatal synaptic plasticity in old A53T-alpha synuclein overexpressing mice. Neurobiol Aging 33:1792-1799.

Tsujimura A, Taguchi K, Watanabe Y, Tatebe H, Tokuda T, Mizuno T, Tanaka M (2014) Lysosomal enzyme cathepsin B enhances the aggregate forming activity of exogenous alpha-synuclein fibrils. Neurobiol Dis 73C:244-253.

Westbroek W, Gustafson AM, Sidransky E (2011) Exploring the link between glucocerebrosidase mutations and parkinsonism. Trends Mol Med 17:485-493.

Yap TL, Gruschus JM, Velayati A, Westbroek W, Goldin E, Moaven N, Sidransky E, Lee JC (2011) Alpha-synuclein interacts with Glucocerebrosidase providing a molecular link between Parkinson and Gaucher diseases. J Biol Chem 286:28080-28088.

Yasuda T, Nakata Y, Choong CJ, Mochizuki H (2013) Neurodegenerative changes initiated by presynaptic dysfunction. Transl Neurodegener 2:16.

Figures

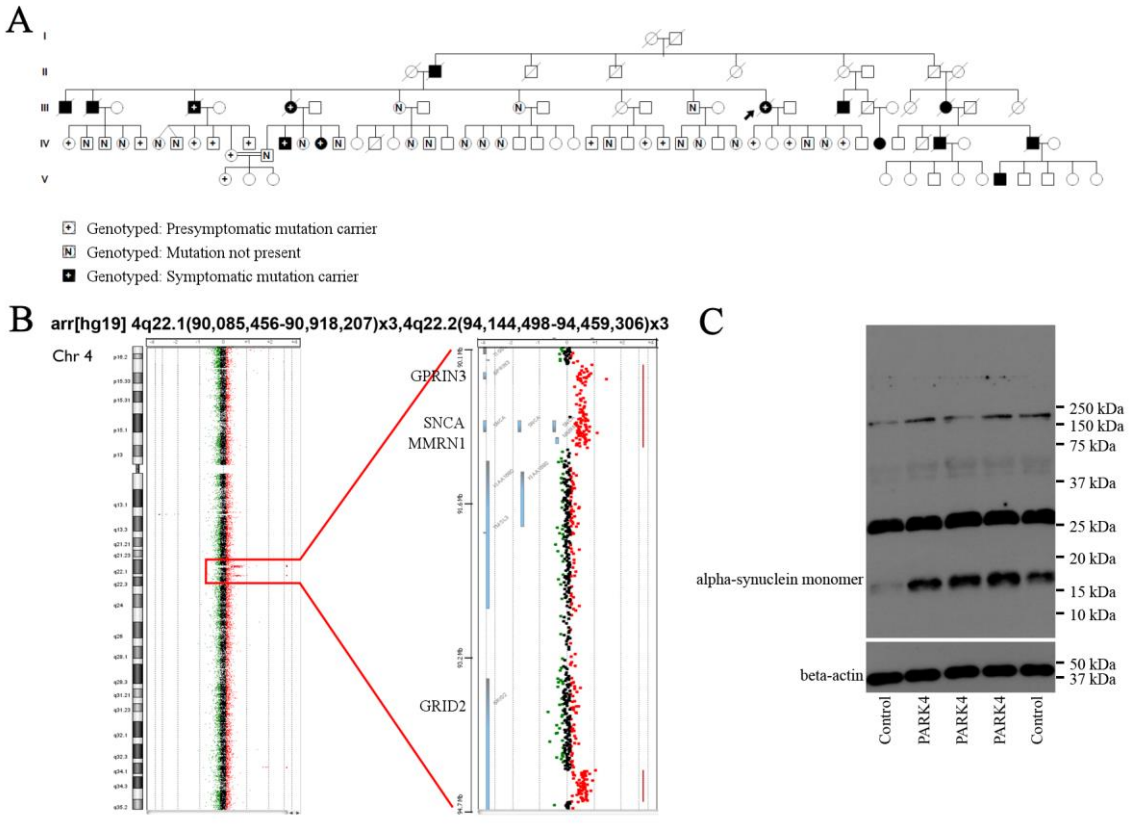

Figure 1: Large kindred with autosomal dominant PD inheritance due to PARK4 mutation with 12 presymptomatic PARK4 heterozygotes (A) Pedigree structure with genotype of *SNCA* gene duplication. Squares and circles denote male and female individuals, respectively. Black filling versus the black symbol '+' inside, illustrates clinically manifested PD versus presymptomatic PARK4 heterozygote status, respectively. The letter 'N' indicates the individuals genotyped and found not to carry the PARK4 mutation. Samples from the rest of the individuals were unavailable to us, thus, their status was indicated according to the information gained from the family. **(B)** DNA from the Turkish PARK4 family exhibits duplications at the *SNCA* locus. The analysis of the chromosome 4q22.1-22.2 region by molecular karyotyping using Agilent array CGH revealed two adjacent copy number gains on chromosome 4 (left chromosome view, right gene view). The first duplication, 833-862 kb in size, carries *GPRIN3*, *SNCA*, and *MMRN1* in their entirety. The second duplication in close

proximity affects exons 7-13 of the *GRID2* gene. The genomic position coordinates as annotated by hg19 nomenclature are indicated above for both chromosome 4 fragments that exist in 3 copies. **(C)** PARK4 blood shows upregulation of alpha-synuclein monomer, but no high molecular weight aggregates in limited mobility zone of gels. Whole blood protein extracts were depleted in hemoglobin, analyzed on polyacrylamide blots subjected to delipidation, then studied for alpha-synuclein (above) and beta-actin (below, as a loading control) immunoreactivity.

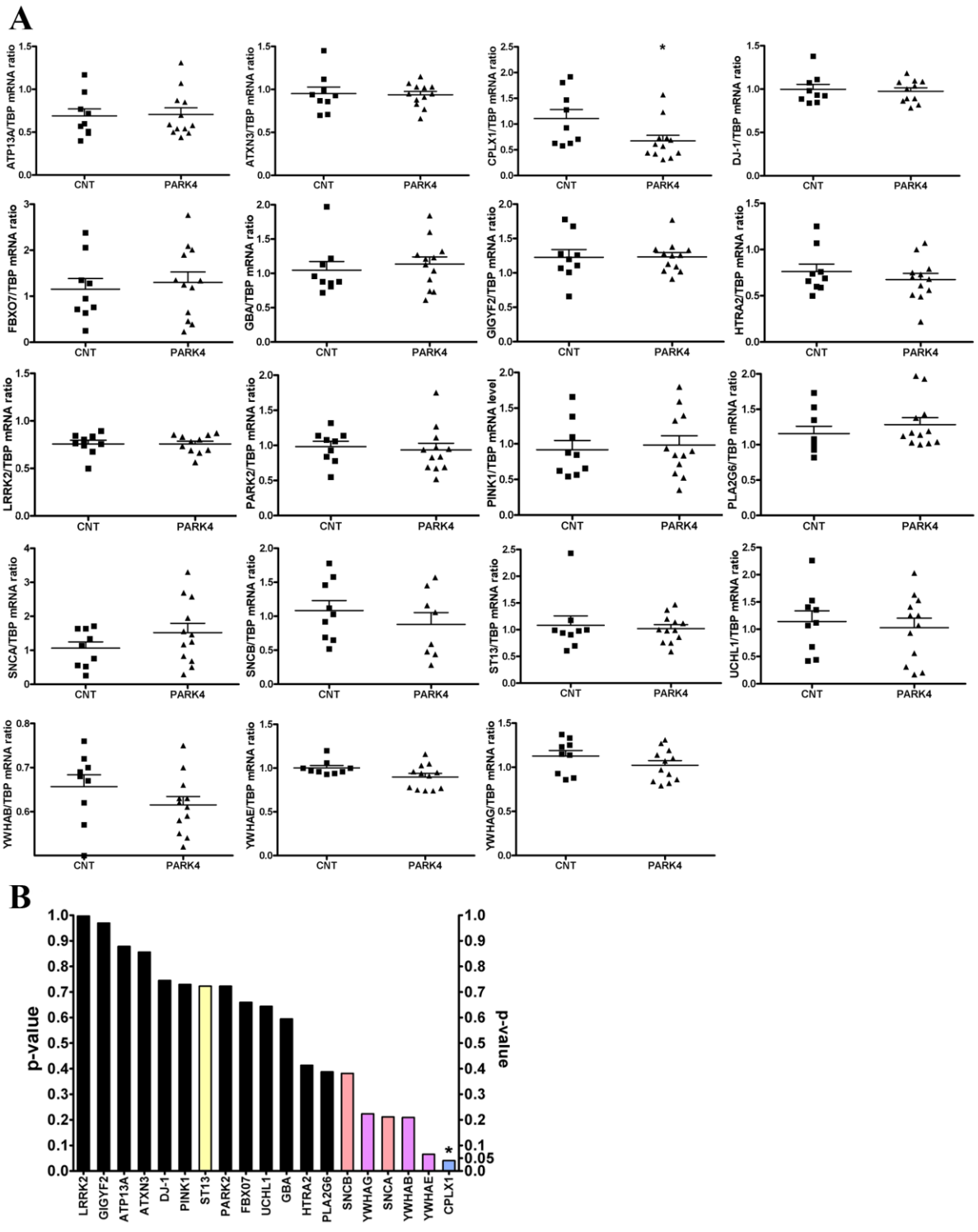

Figure 2: Correlation between whole blood mRNA levels (qPCR) of candidate genes and PARK4 genotype, comparing 14 adult PARK4 heterozygotes versus 9 age-matched control relatives. (A) Scattergrams represent the expression levels in whole blood of individual transcripts determined by qPCR and normalized versus TBP loading control, with mean and SEM. **(B)** The bar graph summarizes these scattergrams by illustrating nominal unpaired Student t-test p-values. The selected candidate genes are either known to be mutated

in monogenic PD (black) or were previously claimed to constitute a blood expression biomarker of PD (yellow) or represent components of the interactome of alpha-synuclein (SNCA), including beta-synuclein (SNCB) (orange), 14-3-3 isoforms (YWHAG, YWHAB, YWHAE) (purple) and complexin-1 (CPLX1) (blue) within the presynaptic SNARE complex.

972 * illustrates p-value < 0.05, ** p < 0.01.

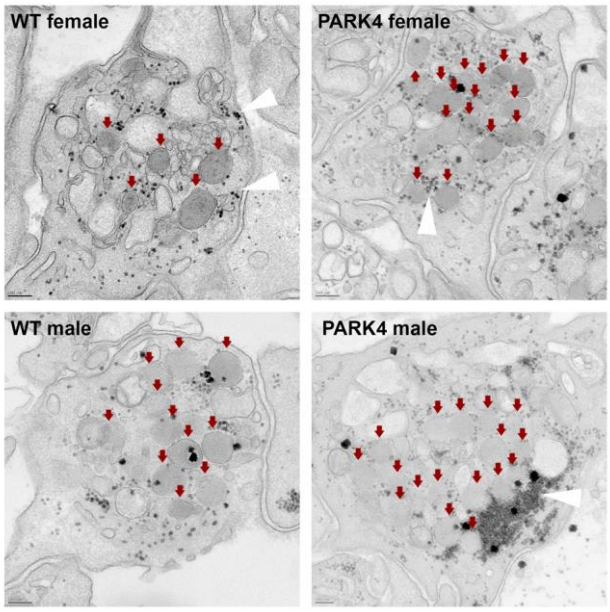

Figure 3: Electron microscopy of blood platelets after stimulus-triggered degranulation are depicted, illustrating centrally clustered alpha granules (red arrows) and glycogen granula (white arrowheads), but no detectable protein aggregates in PARK4 cases versus matched WT relatives. The bar length represents 200 nm (n = 2 control versus 2 PARK4 individuals).

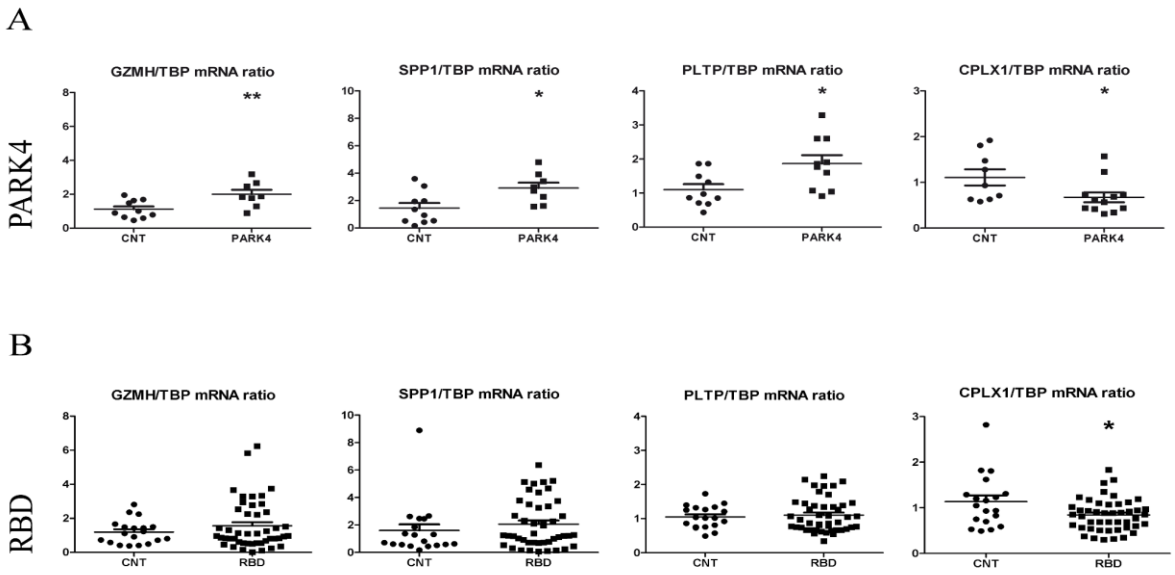

Figure 4: Blood RNAseq validation. mRNA levels assessed by qPCR **(A)** in PARK4 show downregulation for *CPLX1*, upregulations for *GZMH*, *SPP1*, and *PLTP* (n = 9 control versus 12 PARK4 individuals), **(B)** in RBD show significant downregulation only for CPLX1, only an upregulation for SPP1 after removal of one outlier value among controls (n = 19 controls versus 46 RBD cases), and no relevant changes for *GZMH* and *PLTP*. The individual value plots show mean and SEM. * illustrates p-value < 0.05, ** p < 0.01.

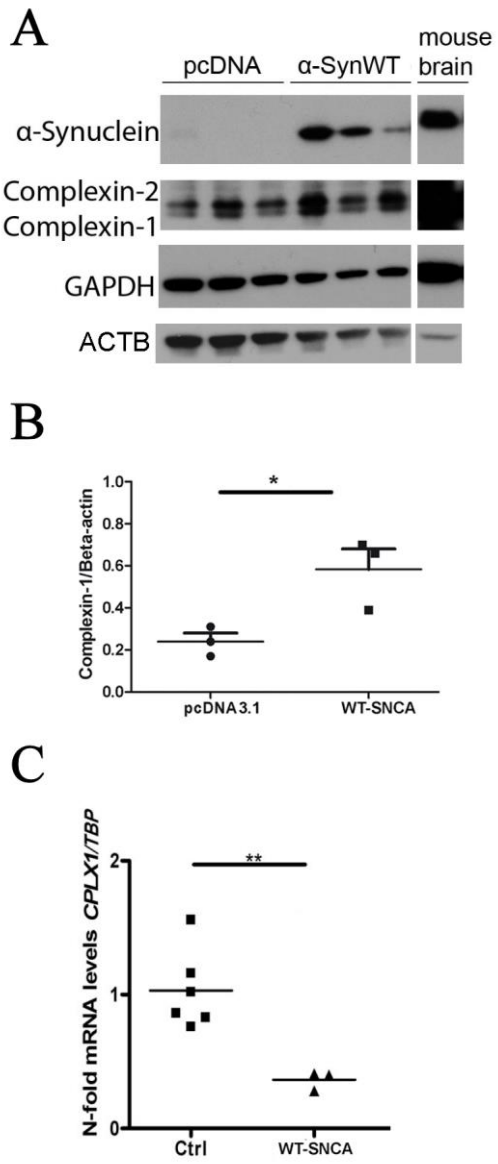

Figure 5: Accumulation of complexin-1 protein and downregulation of *CPLX1* mRNA levels after overexpression of wild type alpha-synuclein in SH-SY5Y cells. Human SH-SY5Y neuroblastoma cells were transiently transfected with plasmid pcDNA3.1(+) as empty vector control or wild-type alpha-synuclein (α-syn). **(A)** Immunoblot confirmation of successful alpha-synuclein overexpression and of the effect on complexin-1 protein levels on the second day after transfection, using GAPDH and beta-Actin (ACTB) as loading control (n = 3 versus 3 versus 3 in independent experiments). **(B)** Densitometric quantification of complexin-1 (antibody from Synaptic systems) versus ACTB ratios, normalized against control, in a scattergram analysis with t-test. **(C)** Analysis of corresponding *CPLX1* mRNA levels at 2 days after transfection. Asterisks indicate statistical significance versus control group (n = 6 versus 3 versus 12). ** illustrates p-value < 0.01 and *** p < 0.001.

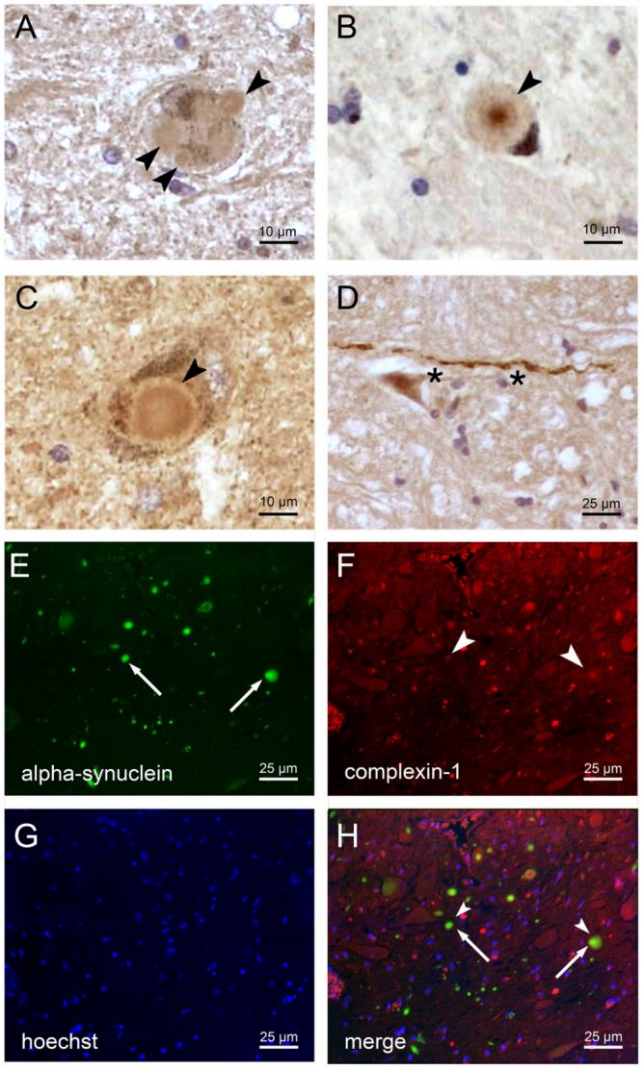

Figure 6: PD midbrain autopsies contain cytoplasmic and neuritic aggregates. Tissue sections (5 µm thick) from PD patients were stained with anti-complexin-1 (Acris) or double stained with anti-complexin-1 and anti-alpha-synuclein antibodies. **(a-c)** Neurons in the substantia nigra exhibiting Lewy body-like structures mildly stained with anti-complexin-1 (arrowheads). **(d)** Complexin-1 immunopositive Lewy neurite-like structure (asterisks) in the medulla at the level of the motor vagus nucleus. **(e-h)** Double immunostaining depicting possible co-localization of alpha-synuclein (green) and complexin-1 (red), only after maximal exposure and contrast adjustment in the red channel. Sections were counterstained with Hoechst dye.

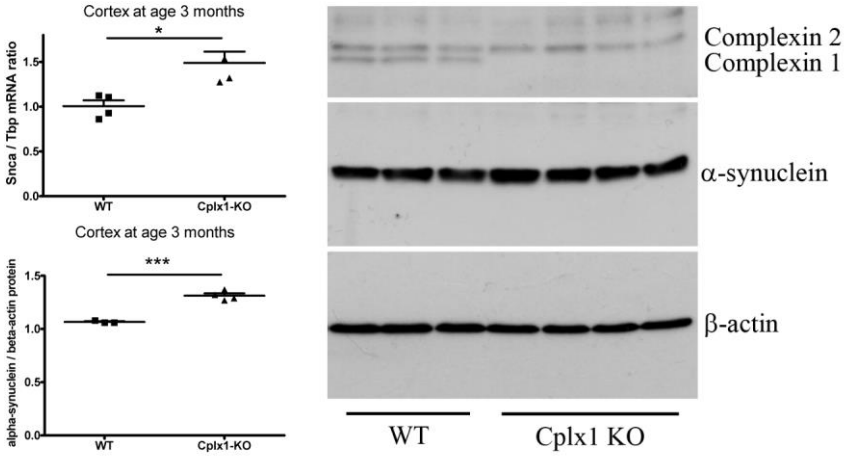

Figure 7: Elevated alpha-synuclein mRNA and protein levels in adult mouse *Cplx1*-/- cortex. Normalized fold changes are shown for qPCR (n = 4 versus 4, left above) and immunoblot quantitation (n = 3 versus 4, left below) in scattergrams, together with representative scans of Western blots for complexin 1/2, alpha-synuclein and beta-actin (right). * illustrates p-value < 0.05 and *** p < 0.001.

Tables

Table 1: Stimulation-induced blood platelet degranulation:

	Controls (n = 20) Median (range)	PARK4 family			
		WT 43y	WT 44y	MUTANT 49y	MUTANT 52y
CD62P before stimulation	5.80 (14.01 - 2.17)	15.69	13.18	6.97	10.71
CD62P after stimulation	599.86 (797.37 - 379.10)	458.67	466.2	315.76	101.22
CD63 before stimulation	12.8 (17.2 - 6.2)	10.03	14.57	11.31	15.82
CD63 after stimulation	166.3 (212.6 - 123.9)	190.75	164.18	105.08	32.33

Individual mean fluorescence values (for the PARK4 family members) or median values and range (for controls) are given for the cell surface localization of alpha-granule-derived CD62P (P-selectin) and lysosome-derived CD63 antigen, as quantified by flow cytometry.

Table 2: Significant association of the *CPLX1*-3'UTR-SNP rs10794536 G-allele with PD risk.

Group	Genotypic frequencies (%)			Allelic frequencies (%)	
	GG	GT	TT	G	T
PD Patients (n = 360)	12 (3.33%)	116 (32.22%)	232 (64.44%)	140 (19.44%)	580 (80.56%)
Controls (n = 358)	5 (1.40%)	100 (27.93%)	253 (70.67%)	110 (15.36%)	606 (84.64%)
		$\chi 2 = 4.97$ P = 0.083		$\chi 2 = 4.16$ **P = 0.041**	

Approximately half of the patient and control samples were recruited at Düsseldorf and Marburg Universities, with both case-control collectives showing similar trends in the chi-square statistics for allele and genotype associations.