The GBAP1 pseudogene acts as a ceRNA for the glucocerebrosidase gene GBA by sponging miR-22-3p

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Mutations in the GBA gene, encoding lysosomal glucocerebrosidase, represent the major predisposing factor for Parkinson's disease (PD), and modulation of the glucocerebrosidase activity is an emerging PD therapy. However, little is known about mechanisms regulating GBA expression. We explored the existence of a regulatory network involving GBA, its expressed pseudogene GBAP1, and microRNAs. The high level of sequence identity between GBA and GBAP1 makes the pseudogene a promising competing-endogenous RNA (ceRNA), functioning as a microRNA sponge. After selecting microRNAs potentially targeting both transcripts, we demonstrated that miR-22-3p binds to and down-regulates GBA and GBAP1, and decreases their endogenous mRNA levels up to 70%. Moreover, over-expression of GBAP1 3′-untranslated region was able to sequester miR-22-3p, thus increasing GBA mRNA and glucocerebrosidase levels. The characterization of GBAP1 splicing identified multiple out-of-frame isoforms down-regulated by the nonsense-mediated mRNA decay, suggesting that GBAP1 levels and, accordingly, its ceRNA effect, are significantly modulated by this degradation process. Using skin-derived induced pluripotent stem cells of PD patients with GBA mutations and controls, we observed a significant GBA up-regulation during dopaminergic differentiation, paralleled by down-regulation of miR-22-3p. Our results describe the first microRNA controlling GBA and suggest that the GBAP1 non-coding RNA functions as a GBA ceRNA.

The glucocerebrosidase gene (GBA) encodes for the enzyme glucocerebrosidase (GCase), which catalyzes the hydrolysis of the membrane glucosylceramide (GlcCer) to ceramide and glucose. GCase is mainly a lysosomal enzyme and only partly associated with the outer surface of the cell membrane. GCase deficiency leads to the accumulation of the substrate, responsible for the multi-organ clinical manifestations of Gaucher's disease (MIM #606463), one of the most common lysosomal storage disorders. While biallelic mutations in GBA are responsible for Gaucher's disease, heterozygous GBA variants have been repeatedly associated with susceptibility to Parkinson's Disease (PD). Importantly, Gaucher's and PDs have been connected due to the clinical observation of parkinsonism and Lewy Bodies (LB) pathology in a fraction of patients with Gaucher's disease. Compared with the general population, patients with the milder form of Gaucher's disease (type 1) have a 20-fold increased lifetime risk of developing parkinsonism, whereas the odds ratio for any GBA mutation in PD patients compared to controls was greater than 5 in a multi-center analysis including more than 5000 cases and 4000 controls. Several studies confirmed that GBA mutations, in particular the two most common ones (p.N370S and p.L444P), are more frequent in PD patients than in healthy controls, demonstrating that genetic lesions in this gene are a

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common risk factor for the disease\(^8\).\(^9\). Recently, we proved the strong relationship between GBA mutations and PD progression and survival\(^11\).

Despite many efforts, the mechanism underlying the relation between GBA mutations and the development of PD remains unclear. There are studies supporting a gain-of-function effect of the mutated protein (promoting \(\alpha\)-synuclein aggregation), as well as others supporting a loss-of-function mechanism (leading to substrate accumulation, and hence affecting \(\alpha\)-synuclein processing and clearance)\(^12\).\(^13\).\(^14\). Widespread deficiency of GCase activity has been demonstrated in the brains of PD patients carrying GBA mutations, but it is also significant that PD patients without GBA mutations were shown to exhibit deficiency of GCase in the substantia nigra (SN) as well as in blood\(^13\).\(^14\). Moreover, neurons and brains of PD patients showed accumulation of GlcCer that directly influences the abnormal lysosomal storage of \(\alpha\)-synuclein oligomers, thus resulting in a further inhibition of the GCase activity. These findings suggested that the bi-directional effect of GlcCer and \(\alpha\)-synuclein accumulation forms a positive feedback loop that may lead to a self-propagating disease\(^15\).\(^16\). Recent data also linked GCase impairment to the cell-to-cell propagation of \(\alpha\)-synuclein aggregates\(^16\). Based on the above-mentioned evidence, it is plausible that dysregulated GBA levels could represent a common feature in PD, whereas loss-of-function GBA mutations could constitute the specific trigger responsible for PD development in the GBA-associated disease.

Dysregulation of GBA expression may, in theory, be due to altered epigenetic, transcriptional, and/or post-transcriptional regulatory mechanisms. In particular, RNA-based networks, characterized by interactions between a specific mRNA, microRNAs (miRNAs), and competitive endogenous RNAs (ceRNAs), are emerging as post-transcriptional regulators of gene expression\(^17\). Moreover, accumulating evidence points to deregulation of noncoding RNAs as an important and largely unexplored regulatory layer in human neurodegenerative disorders, such as PD\(^18\).\(^19\).

MiRNAs are ~20-nucleotide-long regulatory RNAs that act as post-transcriptional regulators of gene expression by repressing target mRNAs translation and/or by inducing mRNA degradation. About 2000 miRNAs have been experimentally validated in humans and many more have been predicted bioinformatically, making them a major class of regulators\(^20\). Each miRNA might inhibit the expression of multiple target mRNAs, whose recognition is based on imperfect complementary binding between miRNAs and their target sites, usually located within the 3’ untranslated region (3’UTR)\(^21\). Recently ceRNAs were described as a novel category of regulatory RNAs: these transcripts compete with mRNAs for miRNAs, acting as molecular “sponges” and thus influencing mRNA levels\(^22\).\(^23\).\(^24\).\(^25\).\(^26\).\(^27\).\(^28\).\(^29\).\(^30\).\(^31\).\(^32\).\(^33\).\(^34\).\(^35\).\(^36\).\(^37\).\(^38\).\(^39\).\(^40\).\(^41\).\(^42\).\(^43\).\(^44\).\(^45\).\(^46\).\(^47\).\(^48\).\(^49\).\(^50\).\(^51\).\(^52\).\(^53\).\(^54\).\(^55\).\(^56\).\(^57\).\(^58\).\(^59\).\(^60\).\(^61\).\(^62\).\(^63\).\(^64\).\(^65\).\(^66\).\(^67\).\(^68\).\(^69\).\(^70\).\(^71\).\(^72\).\(^73\).\(^74\).\(^75\).\(^76\).\(^77\).\(^78\).\(^79\).\(^80\).\(^81\).\(^82\).\(^83\).\(^84\).\(^85\).\(^86\).\(^87\).\(^88\).\(^89\).\(^90\).\(^91\).\(^92\).\(^93\).\(^94\).\(^95\).\(^96\).\(^97\).\(^98\).\(^99\).\(^100\).\(^101\).\(^102\).\(^103\).\(^104\).\(^105\).\(^106\).\(^107\).\(^108\).\(^109\).\(^110\).\(^111\).\(^112\).\(^113\).\(^114\).\(^115\).\(^116\).\(^117\).\(^118\).\(^119\).\(^120\).\(^121\).\(^122\).\(^123\).\(^124\).\(^125\).\(^126\).\(^127\).\(^128\).\(^129\).\(^130\).\(^131\).\(^132\).\(^133\).\(^134\).\(^135\).\(^136\).\(^137\).\(^138\).\(^139\).\(^140\).\(^141\).\(^142\).\(^143\).\(^144\).\(^145\).\(^146\).\(^147\).\(^148\).\(^149\).\(^150\).\(^151\).\(^152\).\(^153\).\(^154\).\(^155\).\(^156\).\(^157\).\(^158\).\(^159\).\(^160\).\(^161\).\(^162\).\(^163\).\(^164\).\(^165\).\(^166\).\(^167\).\(^168\).\(^169\).\(^170\).\(^171\).\(^172\).\(^173\).\(^174\).\(^175\).\(^176\).\(^177\).\(^178\).\(^179\).\(^180\).\(^181\).\(^182\).\(^183\).\(^184\).\(^185\).\(^186\).\(^187\).\(^188\).\(^189\).\(^190\).\(^191\).\(^192\).\(^193\).\(^194\).\(^195\).\(^196\).\(^197\).\(^198\).\(^199\).\(^200\).

Results

MiR-22-3p targets GBA and GBAP1. Since there is no information on miRNAs modulating GBA expression, we searched bioinformatically for miRNAs potentially targeting both GBA and its pseudogene. Predictions were performed using eight sources of software; candidate miRNA selection was performed by prioritizing miRNAs: i) predicted by at least five algorithms; ii) containing at least 7-nucleotide perfect seed match with GBA and GBAP1 3’UTRs; iii) known to be expressed in the brain and previously implicated in neurodegenerative diseases. These filtering steps allowed the selection of three candidate miRNAs: miR-22-3p, miR-132, and miR-212. For functional validation, we prioritized miR-22-3p and miR-132, since they were expressed at a higher level in both the cerebellum and frontal cortex (Supplementary Table 1).

To verify that GBA/GBAP1 can be targets of miR-22-3p and/or miR-132, we cloned both miRNA precursors in a suitable expression vector, and over-expressed them in HeLa cells for 24 hours. The results of these experiments showed that miR-22-3p over-expression can significantly reduce GBA and GBAP1 endogenous mRNA levels (up to 72%; \(P < 0.0003\)). Conversely, no GBA modulation was detected after miR-132 over-expression (Fig. 1A).

To confirm these results, the 3’UTRs of GBA and GBAP1 were cloned downstream of the luciferase gene in the psiCHECK2 vector. These UTRs differ for only 6 nucleotides, none of them mapping in the predicted binding sites for miR-22-3p and miR-132. We cotransfected in HeLa cells each of these reporter plasmids together with the vector expressing either the miR-22-3p or miR-132 precursor. The results of transfection experiments substantially confirmed previous observations, i.e. miR-22-3p was able to target both GBA and GBAP1 UTRs (37% and 34% reduction, respectively; \(P < 0.0001\)). Conversely, miR-132 did not affect the expression of the reporter gene (Fig. 1B), and was hence not further investigated.

To better unravel the functional impact of miR-22-3p on the expression of GBA/GBAP1, we decided to study miR-22-3p/GBA/GBAP1 expression profiles in 11 cell lines. Real-time reverse-transcription (RT)-PCR evidence a ubiquitous expression of GBA in the analyzed lines, with highest levels present in HeLa and glioblastoma cells, and lowest levels in HepG2 cells. GBAP1 was present in all cell lines, though at lower levels than GBA (from 186 to 1.8 times less) (Supplementary Figure 1A). MiR-22-3p showed a nearly ubiquitous expression profile, with highest levels in HepG2 and glioblastoma cells, and lowest levels in HEK293 (Supplementary Figure 1B).

Based on these expression profiles, we decided to repeat miR-22-3p over-expression experiments in HEK293 cells. Results were comparable to those observed in the HeLa cell line, with GBA and GBAP1 endogenous mRNA levels significantly decreased, after 24 hours, up to 44% (\(P < 0.05\); Fig. 1C). We then confirmed the effects of
Figure 1. MiR-22-3p targets GBA and GBAP1. (A) Endogenous expression levels of GBA and GBAP1 after pre-miR-22-3p or pre-miR-132 over-expression in HeLa cells. Cells were collected 24 hours after transfection and total RNA extracted. Expression levels, measured by real-time RT-PCRs, are shown as normalized rescaled values, setting as 1 the value measured in cell transfected with an empty vector (psiUX, mock). (B) Luciferase reporter assays of GBA or the GBAP1 3′UTR after pre-miR-22-3p or pre-miR-132 over-expression in HeLa cells. 48 hours after transfection, cells were collected and protein lysates prepared for reporter assays. Renilla luciferase activity was normalized against the firefly luciferase activity, setting as 1 the value measured in cells cotransfected with an empty vector (psiCHECK2, no miRNA overexpression). (C–E) Effect of pre-miR-22-3p over-expression in HEK293 cells. Panel C shows the effect on the endogenous GBA and GBAP1 transcripts, measured by real-time RT-PCR 24 hours after transfection. Panel D shows the reduction of GBA protein, as assessed by Western blot analysis, 48 or 96 hours after transfection. A representative blot (right) and the densitometric analysis of three independent experiments (left) are shown. Panel E reports the effect on the
endogenous GBA-specific GCcase activity. In all cases, the value measured in cells cotransfected with an empty vector (psUX, no miRNA over-expression) was set as 1. (F) Luciferase reporter assays of GBA or GBA1 3’UTRs, with or without the putative miRNA recognition element (ΔMRE), after miR-22-3p over-expression in HEK293 cells. Cells were collected 48 hours after transfection and lysates prepared for reporter assays. Renilla luciferase activity was normalized against the firefly luciferase activity, setting as 1 the value measured in cells cotransfected with an empty vector (psICHECK2, no miRNA over-expression). The mir-22-3p sensor served as positive control. (G) MiR-22-3p/miR-132 fold increase reached in each over-expression experiment (detailed below histograms). Error bars represent means ±SEM of 3 independent biological replicates, each performed at least in triplicate. In all panels, the reference value, set as 1, is indicated by a dotted line. Significance levels of t-tests are shown. *P < 0.05; **P < 0.01; ***P < 0.005.

miR-22-3p over-expression also at the protein level. Western blot analysis showed a ~20% reduction in the GBA protein level at both 48 hours and 96 hours after transfection (Fig. 1D). Measurements of endogenous GCcase activity demonstrated a 10% and 18% down-regulation after 48 hours and 96 hours of transfection, respectively (P = 0.012 and P < 0.003; Fig. 1E).

Finally, the specific binding of miR-22-3p to GBA and GBA1 3’UTRs was demonstrated by deleting the miR-22-3p putative miRNA responsive element (ΔMRE) in the reporter constructs containing the relevant UTR, and subsequently cotransfecting each mutagenized plasmid together with the miR-22-3p expressing one. In these experiments, a luciferase construct containing miR-22-3p antisense sequences (miR-22-3p sensor) was used as a positive control. Our data showed that miR-22-3p responsiveness strictly depends on the presence of the predicted responsive element in the 3’UTR, since its deletion completely abolishes the miRNA-mediated regulation (Fig. 1E). As expected, the level of luciferase activity in the miR-22-3p sensor control dramatically dropped (95% reduction).

**GBAP1 acts as a ceRNA titrating miR-22-3p and up-regulating GBA.** We first verified the coexpression of GBA/GBAP1/miR-22-3p in a broad range of samples (20 human tissues as well as 24 different cerebral regions). The three transcripts were all ubiquitously expressed (Supplementary Figure 2A,B). In particular, GBA showed minimal expression in the skeletal muscle and the highest level in the medial temporal cortex (17 fold the skeletal muscle). GBA1 expression levels weakly correlated with those of GBA (Pearson’s correlation coefficient of 0.53, P < 0.0013, Supplementary Figure 2C), in agreement with the possible ceRNA role of GBA1. Interestingly, GBA1 highest expression levels were registered in the brain, where the disproportion between the gene and pseudogene levels is one of the lowest (ratio 1:1.5). Concerning miR-22-3p, although no consistent anti-correlation was found across the analyzed tissues, the highest miR-22-3p levels were detected in the tissues with lowest GBA/GBAP1 expression (Supplementary Figure 2).

These results prompted us to verify if altered levels of GBAP1 could indeed modify the expression of GBA. First, as a proof of concept, we over-expressed both the 3’UTR of GBAP1 and the miR-22-3p hairpin in HEK293 cells. Concurrently, the over-expression experiment was conducted using as sponge the 3’UTR of GBAP1 without the miR-22-3p responsive element. We showed that GBAP1 3’UTR over-expression causes a significant increase in the levels of endogenous GBA mRNA only in the presence of the miR-22-3p binding site (1.72 fold; P = 0.019; Supplementary Figure 3A). We also evaluated the ceRNA effect at the protein level, by measuring the GCcase activity upon miR-22-3p and GBAP1 3’UTR over-expression. Our data confirmed that the GBAP1 3’UTR, containing the miR-22-3p binding site, causes a significant increase of GCcase activity (1.11 fold; P = 0.013) (Supplementary Figure 3B).

Second, considering the high levels of miR-22-3p measured in HepG2 cells (16-fold the levels measured in HEK293; Supplementary Figure 1), we over-expressed in this cell line the 3’UTR of GBAP1 alone (with or without the miR-22-3p responsive element) and measured its effect on endogenous GBA. We observed a significant increase in the levels of endogenous GBA mRNA, once again only in the presence of the miR-22-3p binding site (1.68 fold; P = 0.0016; Fig. 2A). The GBAP1 ceRNA effect through miR-22-3p sponging was confirmed by measuring the expression levels of known miR-22-3p targets, i.e., the SP1 and SIRT1 genes, which both resulted in up-regulation of ~1.7 fold (P < 0.015). Conversely, no up-regulation was observed for the CELFI1 transcript (Fig. 2A), which does not contain any miR-22-3p responsive element. These results were corroborated by the measurements of GCcase activity and GBA protein levels in HepG2 cells under the same experimental conditions (GCcase activity: 1.13 fold increase, P = 0.049; GBA protein: 1.40 fold increase, P = 0.020) (Fig. 2B). Finally, a similar overexpression experiment was repeated using as ceRNA the miR-22-3p sensor, which, in principle, represents the “perfect” miRNA sponge. As expected, we observed an up-regulation of all miR-22-3p targets (Supplementary Figure 4).

**The GBAP1 ceRNA effect could be modulated by the nonsense-mediated mRNA decay (NMD) pathway.** To better unravel the reciprocal regulation of the couple GBA/GBAP1, we decided to comprehensively study GBA and GBAP1 alternative splicing patterns and the possible regulation of expression of these two genes operated by NMD. To capture the vast majority of all possible splicing events, long-range RT-PCR assays were designed to completely cover both genes (Fig. 3A). The specific amplification of either GBA or GBAP1 in each assay was assured by anchoring one primer to exon 9, in correspondence of the pseudogene-specific 55-bp deletion. RT-PCR assays were performed on RNA extracted from HepG2 cells treated or not with the NMD inhibitor cycloheximide. This analysis allowed the identification of multiple alternatively-spliced isoforms for GBAP1; conversely, GBA did not show any detectable alternative isoform (Fig. 3A). Notably, the heterogeneity of the splicing pattern of
GBAP1 increased after cycloheximide treatment, suggesting that multiple pseudogene splicing isoforms may be modulated by NMD. A tentative reconstruction of the main splicing variants of GBAP1 was performed by a combination of isoform-specific semi-nested RT-PCRs and DNA sequencing, highlighting the presence of multiple transcripts containing a premature termination codon (Supplementary Figure 5).

The global effect of NMD degradation on GBA and GBAP1 levels was also investigated by semi-quantitative real-time RT-PCR. This analysis showed a significant increase in the expression level of GBAP1 in treated cells (4.18 and 3.92 fold in HEK293 and HepG2 cells, P = 0.045 and P = 0.0034, respectively), confirming that this pseudogene is down-regulated by NMD (Fig. 3B). Also GBA transcripts were up-regulated upon NMD inhibition (2.28 and 2.35 fold in HEK293 and HepG2 cells), a rather unexpected result given the lack of out-of-frame GBA isoforms in our preliminary analysis. However, these results suit the hypothesis that GBAP1 levels may influence GBA expression through a ceRNA effect. As control, in-frame and out-of-frame PRKCA isoforms, known to be insensitive/sensitive to the NMD blockage32, were also analyzed and yielded the expected results (Fig. 3B).

GBA, GBAP1, and miR-22-3p are expressed in induced pluripotent stem cells (iPSCs)-derived neuronal cells. To be relevant for the molecular pathogenesis of PD, the GBA/GBAP1/miR-22-3p network should work in tissues affected by the disease process, e.g. dopaminergic (DA) neurons. We thus verified the expression of GBA, GBAP1, and miR-22-3p in iPSCs and iPSC-derived neuronal cells (after 35 days of differentiation). Semi-quantitative real-time RT-PCR assays were performed on total RNA extracted from iPSCs/neurons derived from fibroblasts of six healthy controls and four PD patients (all carrying GBA mutations).

Figure 2. GBAP1 acts as a ceRNA titrating miR-22-3p and up-regulating GBA. (A) Effect of GBAP1 3’UTR (with or without the miR-22-3p recognition element, ΔMRE) over-expression on the endogenous transcript levels of indicated miR-22-3p targets in HepG2 cells. 24 hours after transfections, cells were collected for extracting total RNA for measurements by semi-quantitative real-time RT-PCRs of: i) GBA; ii) SP1 (Sp1 Transcription Factor; known miR-22-3p target, positive control)28; iii) SIRT1 (Sirtuin 1; known miR-22-3p target, positive control)29; and iv) CELF1 (CUGBP, Elav-Like Family Member 1; negative control). The value measured in cells transfected with an empty vector (psiCHECK2, mock) was set as 1. (B) Effect of GBAP1 3′UTR (wild type or ΔMRE) over-expression on GCase activity, measured 96 hours after transfections. (C) Effect of GBAP1 3′UTR (wild type or ΔMRE) over-expression on GBA protein level, measured by Western blot 96 hours after transfections. A representative blot (right) and the densitometric analysis (left) are shown. Error bars represent: means ± SEM of 3 (A) or 4 (B) independent biological replicates, each performed at least in triplicate; means ± SD of 3 independent biological replicates (C, GBAP1 3′UTR). In all panels, the reference value, set as 1, is indicated by a dotted line. Significance levels of t-tests are shown. *P < 0.05; **P < 0.01.
**Figure 3.** GBAP1 codes for multiple alternatively-spliced isoforms and is modulated by NMD. (A) Analysis of GBA and GBAP1 splicing patterns. In the upper part of the panel, a schematic representation of GBA (reference sequence: NM_001005741.2) and GBAP1 (reference sequence: NR_002188.2) genes is reported. Exons are indicated by boxes, introns by lines. The 55-bp-long sequence characterizing GBA exon 9 is specified by a grey rectangle. The scheme is approximately to scale. The overlapping fragments amplified by RT-PCRs to analyze the GBA and GBAP1 splicing patterns are indicated by dashed lines and a letter. In the lower part of the panel, the electrophoretic analysis (agarose gels 2%) of RT-PCR amplicons is shown. RT-PCRs were performed on RNA extracted from HepG2 cells treated (+) or untreated (−) with the NMD inhibitor cycloheximide. On the top of each gel, letters indicate the relevant RT-PCR amplicons. (B) Demonstration of the NMD-mediated degradation of GBAP1 transcripts. The two panel shows expression levels of GBAP1 and GBA isoforms in HEK293 and HepG2 cells, untreated or treated for 8 hours with cycloheximide. Expression levels of endogenous GBAP1/GBA isoforms were measured by semi-quantitative real-time RT-PCRs. Results are presented as normalized rescaled values, setting as 1 the value of the untreated samples (dotted line). The expression level of the Connexin 43 or 32 transcripts, known to be insensitive to NMD, were used in the normalization step.
RT-PCRs performed on out-of-frame and in-frame PRKCA isoforms, known to be respectively sensitive and insensitive to the NMD blockage, represent the positive and negative control. Error bars represent means + SEM of 3 independent biological replicates, each performed at least in triplicate. Significance levels of t-tests are shown. *$P < 0.05$; **$P < 0.01$.

All three players of the regulatory circuit were expressed both in iPSCs and iPSC-derived neurons, respectively. The process of differentiation towards neurons is accompanied by a significant up-regulation of GBA (8 fold in controls, $P = 0.024$; 3 fold in patients, $P = 0.029$) and by a parallel increase in expression levels of GBAP1 (Fig. 4A and B). In addition, we detected a trend for down-regulation of the GBA transcript in PD patients with respect to controls in DA neurons (0.54 fold, $P = 0.057$). Finally, consistently with the observed up-regulation of GBA/GBAP1 during neuronal differentiation, we detected lower expression levels of miR-22-3p in DA neurons with respect to their precursors (0.39 fold in controls; 0.18 fold in patients) (Fig. 4C).

### Discussion

Despite substantial efforts over the past few years to understand the role of long non-coding RNAs (lncRNAs) in health and disease, only some of them have been investigated for their biological function. One promising, although debated, idea assigning to lncRNAs a generalized function is the “ceRNA hypothesis”, based on the fact that specific RNAs can limit miRNA activity through sequestration, thus up-regulating the expression of miRNA target genes. In particular, two classes of lncRNAs are increasingly recognized as main ceRNA contributors, i.e. circular RNAs and pseudogene-derived transcripts. Indeed, transcribed pseudogenes, mostly deriving from duplication events, are considered optimal ceRNA candidates, as they share miRNA-binding sites with the ancestral genes. To date, a number of pseudogenes have been experimentally demonstrated to act as ceRNAs, including: PTENP1 and KRAS1P, OCT4-pg4, BRAF P1, and CYP4Z2P. In this study, we describe a novel ceRNA-based network involving GBA, its pseudogene GBAP1, and miR-22-3p (Fig. 5).

The molecular evolution, expression pattern, and mechanisms of transcriptional regulation of GBA have been previously investigated, mainly because of its direct link with Gaucher’s disease. On the other hand, the few data available on GBA post-transcriptional regulation principally stem from a screening aimed to identify miRNAs regulating the GCase activity in p.N370S homozygous Gaucher fibroblasts. This screening involved 875 miRNAs and evidenced at least three candidates (miR-127-5p, miR-16-5p, and miR-195-5p), exhibiting a Z-score of at least +3, with substantial consequences on the GCase activity. However, in all cases, the miRNA effect did not seem to be mediated by a direct binding of the miRNA to GBA transcripts; rather, miRNAs acted either on the LIMP-2 receptor, which is involved in the trafficking of GCase from the endoplasmic reticulum to the lysosome, or on the expression levels of known modifiers of the GCase activity. Hence, our work identifies miR-22-3p as the first miRNA directly targeting GBA. Interestingly, in the publicly-available dataset of Siebert and coworkers, miR-22-3p mimic resulted to down-regulate GCase activity ($Z$-score = $-1.5$; suggestive $P = 0.066$), according to our results.

Concerning GBAP1, sparse information is available to date, and it is primarily focused on the evolution of the GBAP1 locus as an example of a very recently acquired pseudogene. We hence extensively studied GBAP1 splicing pattern and expression profile, showing that it is subjected to multiple physiologic in-frame and out-of-frame splicing events and that it is broadly expressed, though often at low levels (Supplementary Figure 2). More interestingly, we showed that GBAP1 is targeted by NMD, which seems to be the main mechanism regulating its expression level: blocking NMD, the ratio between GBA and GBAP1 substantially increased (on average from 1/100 to 1/68 in HepG2 cells, and from 1/9 to 1/4 in HEK293 cells). Of course, the GBAP1 expression control exerted through NMD raises the question about the pseudogene translation, since RNAs should undergo its expression level: blocking NMD, the ratio between GBA and GBAP1 substantially increased (on average from 1/100 to 1/68 in HepG2 cells, and from 1/9 to 1/4 in HEK293 cells). Of course, the GBAP1 expression control exerted through NMD raises the question about the pseudogene translation, since RNAs should undergo degradation of bound miRNAs, possibly enhancing GBAP1 efficiency as miRNA sponge, as suggested for other pseudogenes.

The relevance of post-transcriptional regulation in determining the low GBAP1 expression is also suggested by the observation that GBAP1 proximal and distal promoters show high level of sequence identity with those of GBA, and are hence predicted to have similar transcriptional strength. For instance, the presence of two TATA boxes and two CAAT boxes in the proximal promoter of GBAP1 exactly recapitulates the architecture of in-cis regulatory elements characterizing the GBA proximal promoter. Moreover, epigenetic marks are not substantially different when comparing the gene and the pseudogene promoters, as inferred from the UCSC Genome Browser ENCODE tracks ([http://genome.ucsc.edu](http://genome.ucsc.edu/); release Feb. 2009, GRCh37/hg19). Indeed, our in-house preliminary data, obtained with reporter constructs, show that the activity of GBAP1 promoters does reach the transcriptional levels of the corresponding GBA promoters (data not shown).

Our overexpression experiments in different cell lines clearly demonstrated that GBAP1 3′UTR, at supraphysiologic concentrations, can modulate GBA mRNA levels through a miR-22-3p-mediated regulatory circuit. However, these results do not necessarily imply that this ceRNA-based regulation may also work in more physiologic conditions. To confirm that GBAP1 can act as a GBA ceRNA without overexpression, we exploited the predicted differential sensitivity to NMD of the gene and pseudogene transcripts (see Fig. 3A). Cycloheximide treatment allowed us to increase the relative abundance of the endogenous GBAP1 mRNA of around 4 times the basal level and was accompanied by a 2-fold increase in GBA transcripts, not directly attributable to NMD, and compatible with a ceRNA effect (Fig. 3B). Hence, in specific cells or developmental stages, up-regulation of...
GBAP1, resulting from post-transcriptional or epigenetic regulatory mechanisms, might titrate miRNAs away from the GBA protein-coding transcripts, thus providing a physiologic ceRNA effect.

The existence of an RNA-based network controlling GBA expression suggests the intriguing possibility that miR-22-3p or GBAP1 dysregulation could also be associated with PD. In this frame, we investigated GBA/GBAP1/miR-22-3p expression pattern in disease-relevant tissues using in-silico analyses of microarray datasets publicly available through the Gene Expression Omnibus repository (see Supplementary Materials and Methods), as well as in-vivo measurements performed on RNA extracted from iPSCs and iPSC-derived neuronal cells of PD patients and controls. GBA (A), GBAP1 (B), and miR-22-3p (C) expression levels were measured by semi-quantitative real-time RT-PCRs in up to six iPS and iPSC-derived neuronal cells of cases and controls. Boxplots show expression levels according to the disease status; boxes define the interquartile range; the thick line refers to the median. Results are presented as normalized rescaled values. Significance level for differences between groups was calculated by a Wilcoxon-Mann-Whitney test, and showed only if significant. *P < 0.05.
PD cases and controls. In particular, we retrieved three microarray datasets evaluating differential gene expression in the SN of post-mortem brains, for a total of 51 cases and 42 controls (Supplementary Table 2). In the meta-analysis, we measured a significant down-regulation of both GBA and GBAP1 transcripts in PD patients ($P < 0.05$; Supplementary Figure 6). Notably, we observed the same significant down-regulation for GBA transcripts in iPS-derived DA neurons of PD patients; accordingly, miR-22-3p was slightly, although not significantly, up-regulated in cases vs. controls (on average 1.96 fold, $P = 0.13$; Fig. 4).

A few studies have reported a potential neuroprotective effect of miR-22-3p in rat models of cerebral ischemia-reperfusion injury, as well as in Huntington's and Alzheimer's disease, through a reduction in inflammation and apoptosis43,44. However, other studies suggested a pro-senescence role of miR-22 in endothelial progenitor cells, in cancer, and in the aging heart and brain28,45–47. While the neuroprotective effects of miR-22 have suggested enhancing its expression as a potential therapeutic strategy for the treatment of neurodegenerative conditions, it may well be that miR-22 overexpression represents a pathophysiologic response to protect the cell from injury and stress also triggering other non-beneficial effects, like increased aging and reduced GCase activity.

In conclusion, we are aware of the fact that the connection between the RNA-based network and PD pathogenesis presented here has not been formally proven. However, one can easily imagine a link between the down-regulation of the sister transcripts GBA/GBAP1 - or, conversely, the up-regulation of miR-22-3p - and an aberrant α-synuclein metabolism, as already theorized12. A confirmed dysregulation of the GBA/GBAP1/miR-22-3p circuit in PD patients would suggest possible novel therapeutic strategies, based either on the direct control of the expression of the miRNA/pseudogene, or on the modulation of the NMD pathway aimed at up-regulating GBAP1 levels48.

**Methods**

**Plasmid constructs.** MiR-22-3p and miR-132 precursors were inserted into the psiUX expression vector (kindly provided by Prof. I. Bozzoni, Università di Roma La Sapienza, Rome, Italy). GBA and GBAP1 3’UTRs were directionally cloned downstream of the renilla luciferase gene in the psiCHECK2 reporter plasmid (Promega, Madison, USA). All constructs were produced by PCR amplifying the relevant genomic region from the DNA of a healthy subject using an appropriate PCR primer couple (Supplementary Table 3), and subsequently by cutting the amplified products with the proper restriction enzyme. Restricted products were ligated into the relevant plasmid.
The constructs carrying the GBA and GBAP1 3′UTR deleted of the miR-22-3p binding site (ΔMRE) were obtained by site-directed mutagenesis, by means of the QuikChange kit (Agilent Technologies, Santa Clara, USA), following the manufacturer protocol.

A pGL3-control luciferase construct containing a single perfectly-complementary miR-22-3p antisense sequence (miR-22-3p sensor), kindly provided by Dr. Da-Zhi Wang (Children’s Hospital Boston and Harvard Medical School), was used as a positive control27.

All plasmids were purified using the PureYield™ Plasmid Miniprep System kit (Promega) according to the manufacturer’s instructions. All recombinant and mutagenized vectors were verified by conventional Sanger sequencing, as described50.

Prediction of GBA/GBAP1-targeting miRNAs. Predictions were performed using publicly-available algorithms: microRNA.org49, MicroCosm Targets50, PITA51, as well as the miRWalk2 suite52.

Cell cultures and transfection experiments. HEK293, HepG2, and HeLa cells (kind gift of Prof. D. Fornasari and Prof. A. Rollier, University of Milan, Milan, Italy) were cultured according to the standard procedures. All cell lines were routinely tested for mycoplasma contamination.

For miRNA over-expression experiments, cells were cotransfected using 3.5 µg (HeLa) or 875 ng (HEK293) of the psiUX plasmid expressing either miR-22-3p or miR-132 precursors.

For the miRNA-target interaction analysis, HEK293 cells were cotransfected using 300 ng of the psiUX plasmid expressing miR-22-3p together with 720 ng of the psiCHECK2 plasmid containing the relevant 3′UTR.

For the ceRNA-effect analysis, HEK293 cells were cotransfected using 300 ng of the psiUX plasmid expressing miR-22-3p together with 300 ng of the psiCHECK2 plasmid containing the GBA 3′UTR. HepG2 cells were transfected with 300 ng of the GBAP1 3′UTR only or with 300 ng of the miR-22-3p sensor (as positive “spoon” control).

In each experiment, an equal number of cells (2.5 * 10^5 for HeLa, 3 * 10^5 for HEK293, 4 * 10^5 for HepG2) were transfected with the Polyplus jetPRIME (EuroClone, Wetherby, UK) in 6-well plates, as described by the manufacturer. Depending on the measurement to be performed at the end of experiment, cells were collected 24, 48, 72, or 96 hours after transfection (detailed in the relevant figure legend), to obtain either total RNA, or cell lysates (see below).

RNA samples. Expression profiles of GBA, GBAP1, and miR-22-3p were determined using RNA from: a panel of 20 human tissues (First Choice total RNA; Ambion, Austin, USA), a panel of 24 human cerebral regions (Clontech Laboratories, Palo Alto, USA), 11 cell lines, iPSCs, and DA neurons differentiated from iPSCs (see below).

RNA from cell lines, iPSCs, DA neurons, as well as transfected cells was isolated using the Eurozol kit (Euroclone), according to the manufacturer’s protocol. RNA concentration/quality was assessed using the Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, USA).

Semi-quantitative real-time RT-PCR. For the evaluation of expression levels of specific genes, random hexamers and the Superscript-III Reverse Transcriptase (Invitrogen, Carlsbad, USA) were used to perform first-strand cDNA synthesis starting from 1 µg of RNA extracted from cells, or RNA derived from a panel of human tissues. From a total of 20 µL of the RT reaction, 1 µL was used as template for amplifications using the FastStart SYBR Green Master Mix (Roche, Basel, Switzerland) on a LightCycler 480 (Roche), following a touchdown thermal protocol. Expression levels were normalized using HMBS (hydroxymethylbilane synthase gene) and ACTB (β-actin) as housekeeping genes. To discriminate between the quasi-identical GBA and GBAP1 genes, we took advantage of the 55-bp deletion in exon 9 characterizing GBA/GBAP1 spread along the two genes.

MiR-22-3p and miR-132 levels were measured by real-time RT-PCR by a poly(A) tailing and a universal reverse transcription approach, using the miRNA First Strand Synthesis kit (Agilent Technologies) and starting from 300 ng of total RNA, according to the manufacturer’s instructions. RT-PCR reactions were performed using the universal reverse primer (Agilent Technologies) and miRNA-specific forward primers, as described53. U6 snRNA was used as housekeeping gene. Real-time reactions were performed as described above.

In all cases, real-time RT-PCR assays were performed at least in triplicate on a LightCycler 480, and expression levels were analyzed by the GeNorm software54. Correlation between GBA/GBAP1/miR-22-3p expression profiles was calculated using the Pearson’s correlation. Pearson’s coefficients <−0.5 and >0.5 are considered as anti-correlation and positive correlation, respectively. P values <0.05 were considered as statistically significant.

Primer couples used in RT-PCR assays are listed in Supplementary Table 3.

Luciferase assays. For miRNA-target interaction assays, the activities of firefly/renilla luciferase were measured in lysates from transfected cells by using the Dual-Luciferase Reporter Assay System (Promega) and the Wallac 1420 VICTOR® V reader (PerkinElmer, Waltham, USA). The values of renilla luciferase were normalized against the corresponding values of firefly luciferase.

Western blot analysis. Cells were lysed in water containing protease and phosphatase inhibitors (Sigma-Aldrich, Saint Louis, USA) on ice using an ultrasonic homogenizer. Total cell protein content was measured using the DC Protein Assay (Bio-Rad, Hercules, USA). In total, 40–50 µg of the protein lysate was loaded on a 10% polyacrylamide gel and transferred on a PVDF membrane (GE Healthcare, Freiburg, Germany). Blots were incubated with primary antibodies overnight at 4°C on a shaker platform (Anti-GBA ab128879 1:2,500,
Abscam, Cambridge, USA; anti-GAPDH G9545 1:7,000, Sigma) and were then probed with anti-rabbit IgG-HRP secondary antibody (1:2,000, Cell Signaling, Leiden, The Netherlands) for 1 h at room temperature. Visualization was done by using Westar ETA C.2.0 ECL Substrate for Western Blotting (Cyanagen, Bologna, Italy). For quantitative measurements, membranes were acquired using the Uvitec Cambridge technology (Eppendorf, Hamburg, Germany). Image analysis was performed with the Uvitec software.

**GCase enzymatic activity assays.** Cells to be assayed were washed twice with phosphate buffered saline (PBS), harvested, and then lysed in water containing complete protease inhibitor cocktails (Roche). Total cell protein content was measured using the Micro BCA assay reagent (Pierce, Rockford, USA). Cells lysates were transferred to a 96-well microplate and assays were performed in triplicate. Cell-lysate associated GCase activity was analyzed using 4-methylumbelliferyl-β-D-glucopyranoside (MUB-Glc; Glycosynth, Warrington, UK), solubilized at a final concentration of 6 mM in Mcllvaine Buffer (0.1 M Citrate/0.2 M Phosphate, pH 5.2) containing 0.1% Triton X-100. As Triton is a selective inhibitor of β-glucosidase (GBA2) activity, these conditions allowed the specific measurement of GBA-related GCase activity 55,56. The reaction mixtures were incubated at 37°C under gentle shaking. The fluorescence was recorded after transferring 10 μL of the mixture in the microplate and adding 190 μL of 0.25 M glycine, pH 10.7. The fluorescence was detected by a Wallac 1420 VICTOR® II V reader. Data were expressed as pmoles of converted substrate/mg cell proteins × hour.

**GBA and GBAP1 splicing pattern and sensitivity to the NMD pathway.** Analysis of GBA/GBAP1 splicing patterns and susceptibility to NMD was undertaken in HepG2 and HEK293 cell lines. Cells were plated at a density of 4 × 10^4 per 6-well dish and, after 72 hours, treated for 8 hours with cycloheximide (100 μg/mL; dissolved in dimethyl sulfoxide) or with the vehicle alone. After the treatment, cells were washed with PBS and total RNA extracted.

For the analysis of the splicing pattern, a set of gene-specific or pseudogene-specific RT-PCR assays (Supplementary Table 3) was designed to catch the vast majority of possible alternative splicing events. RT-PCRs were performed as described above. The main amplified products, recovered from the agarose gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega), were directly sequenced to confirm their identity.

Variations in the expression levels of GBA/GBAP1 upon treatment were quantified by real-time RT-PCR assays using as reference an NMD-resistant transcript (i.e., Connexin 43 or Connexin 32 mRNAs, whose coding sequences are all contained in a single exon, for HEK293 and HepG2, respectively). The NMD-sensitive and insensitive PRKCA transcripts were used as controls 32.

**Fibroblast-derived iPSCs.** iPSC lines derived from skin fibroblasts of six controls and four PD patients carrying heterozygous GBA mutations (p.I444P, n = 2; p.N370S, n = 2) were previously described 37 and were obtained following the protocol of Takahashi and colleagues 38. These iPSCs were subjected to neuronal differentiation for 35 days in vitro, according to Kriks and collaborators’ protocol 39.

This study has the approval of the local Ethics Committees (Parkinson Institute, ASST “Gaetano Pini-CTO”, Milan, Italy; IRCCS Foundation Ca’ Granda Ospedale Maggiore Policlinico, Milan, Italy; Medical Faculty and the University Hospital Tübingen, Tübingen, Germany) and was performed according to the Declaration of Helsinki. Signed informed consent was obtained from all participants.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the article and its Supplementary material file or from the corresponding authors on request.

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Author Contributions
L.S. and V.R. performed the experiments, analyzed the data, and participated in writing the manuscript; M.S. and M.A. performed the GCase activity experiments; R.K., A.D.F., and M.D. produced iPSCs and differentiated neuronal cell lines from patients and controls; S.G. oversaw patient sample collection and selection for the study; G.S., S.D. and R.A. designed and coordinated the study, analyzed the data and wrote the manuscript.

Additional Information
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