Parkinson's disease protein PARK7 prevents metabolite and protein damage caused by a glycolytic metabolite

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Cells are continuously exposed to potentially dangerous compounds. Progressive accumulation of damage is suspected to contribute to neurodegenerative diseases and aging, but the molecular identity of the damage remains largely unknown. Here we report that PARK7, an enzyme mutated in hereditary Parkinson's disease, prevents damage of proteins and metabolites caused by a metabolite of glycolysis. We found that the glycolytic metabolite 1,3-bisphosphoglycerate (1,3-BPG) spontaneously forms a novel reactive intermediate that avidly reacts with amino groups. PARK7 acts by destroying this intermediate, thereby preventing the formation of proteins and metabolites with glycerate and phosphoglycerate modifications on amino groups. As a consequence, inactivation of PARK7 (or its orthologs) in human cell lines, mouse brain, and Drosophila melanogaster leads to the accumulation of these damaged compounds, most of which have not been described before. Our work demonstrates that PARK7 function represents a highly conserved strategy to prevent damage in cells that metabolize carbohydrates. This represents a fundamental link between metabolism and a type of cellular damage that might contribute to the development of Parkinson's disease.

Parkinson's disease is the second most common neurodegenerative disease (1). It is characterized by progressive debilitating motor symptoms (i.e., spontaneous shaking, stiffness, slow movements, and postural instability) (1). Currently, there is no causal therapy, and this is in part due to our insufficient knowledge about the pathogenesis of this disease. Loss of a vulnerable neuron population (i.e., dopaminergic neurons in the substantia nigra) plays a key role in this disease, and the formation of insoluble aggregates of the protein α-synuclein (Lewy bodies) seems to contribute to disease progression. Most cases of Parkinson's disease occur at old age, a major risk factor for neurodegenerative diseases in general (2). Cells are continuously exposed to reactive compounds that can damage cellular components. The progressive accumulation of damage likely contributes to aging and age-associated diseases (3, 4). Why some individuals develop Parkinson's disease and most others don't, is unknown. Evidence from rare hereditary cases indicates that the failure to eliminate damaged mitochondria can play an important role in the development of Parkinson's disease (5–7). It is also increasingly clear that the neurons affected in Parkinson's disease are particularly sensitive to oxidative stress and toxins (8–10).

Reactive oxygen species are clearly a major cause of cellular damage (11). Yet, physiological metabolic activity produces many other dangerous compounds (4) that are often overlooked. We are only starting to understand the pathways that preempt or repair this type of damage (12, 13). In the present paper we reveal that the enzyme PARK7 prevents damage caused by a metabolite from glycolysis, the key metabolic pathway for carbohydrates in most cells and organisms. Inactivating mutations in PARK7 (also called DJ1) lead to autosomal recessive Parkinson's disease (14). Patients present with early-onset disease that slowly progresses and is accompanied by the formation of Lewy bodies (15, 16). PARK7 knockout mice develop hypokinesia and defects in the same neuronal population affected by nonhereditary Parkinson's disease (17–19). Surprisingly, PARK7 also plays a role in cancer (20, 21) and a wide range of cellular functions (reviewed in refs. 22 and 23) that include the protection from reactive oxygen species (24–30), the maintenance of mitochondrial function (31, 32), and chaperone activity (33, 34). Yet, it remained enigmatic which enzymatic activity of PARK7 was required for these functions.

PARK7 is part of an evolutionary conserved enzyme family that relies on a key cysteine residue for its activity (35, 36). In PARK7, this cysteine residue is easily oxidized and has been

Significance

Reactive compounds cause cellular damage that is suspected to contribute to aging and neurodegenerative diseases. Oxidative stress and environmental factors likely contribute to this. Here we report that an enzyme mutated in Parkinson's disease can prevent damage of metabolites and proteins caused by a metabolite from the central pathway of sugar metabolism. Inactivation of this enzyme in model systems, ranging from flies to human cells, leads to the accumulation of a wide range of damaged metabolites and proteins. Thus, this enzyme represents a highly conserved strategy to prevent damage in cells that metabolize sugars. Overall, we discovered a fundamental link between carbohydrate metabolism and a type of cellular damage that might contribute to the development of Parkinson's disease.

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The authors declare no competing interest.

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reported to serve as a scavenger for reactive oxygen species (24–30). Yet, it remained unclear how the oxidized protein would be reduced again to maintain its protective function. Recently, evidence has been presented that PARK7 (and bacterial homologs) can remove covalent adducts produced by a reaction between the metabolite methylglyoxal and amino groups on proteins, metabolites, and nucleic acids (37–40). These studies seemed to represent a potential breakthrough, since they suggested that Parkinson’s disease might be caused by the failure to maintain proteins and DNA “clean” and functional. More recently, these findings have been disputed (41, 42), and convincing evidence has been presented that this apparent deglycating activity is not due to the active removal of these adducts, but rather due to the degradation of free methylglyoxal, which is in rapid equilibrium with these modifications (43, 44). Consistent with this, we did not observe an increase of methylglyoxal-modified proteins or nucleotides in several PARK7 knockout cell lines (SI Appendix, Fig. S1).

We therefore hypothesized that the real enzymatic function of PARK7 that underlies its pleiotropic cellular effects might still be unknown. Using a combination of biochemical, genetic, and analytical approaches in human cell lines, mouse brain, and fruit flies, we discovered that PARK7 prevents damage of metabolites and proteins that is caused by the glycolytic intermediate 1,3-bisphosphoglycerate (1,3-BPG). Remarkably, some patients with genetic inactivation of the enzyme that utilizes 1,3-BPG also develop early-onset Parkinson’s disease (45), indicating that damage caused by 1,3-BPG may play a pathogenetic role in patients without PARK7 mutations as well.

Results

PARK7 Prevents Accumulation of a New Class of Metabolites. The molecular function of PARK7 should be detectable in a variety of experimental settings, given that PARK7 has been found to be the most stably expressed protein across a wide variety of tissues and organisms (46). Hence, we analyzed extracts from cancer cell lines, mouse brain, and Drosophila melanogaster with a highly sensitive liquid chromatography-mass spectrometry (LC-MS) approach. We observed that the concentrations of several unknown metabolites were increased 3- to 50-fold in PARK7 knockout cell lines (Fig. 1A–F and SI Appendix, Fig. S2), whereas these compounds were close to the limit of detection in wild-type samples. We identified these metabolites as glyceraldehyde-adducts of the physiological metabolites glutamate (Glu), reduced (GSH), and oxidized (GSSG) glutathione, glutamine (Gln), glycerophosphorylethanolamine (GPE), and lysine (Glu), reduced (GSH), and oxidized (GSSG) glutathione, gluta-

A Glycolytic Metabolite Is Responsible for the Formation of Glycerate-Modified Metabolites. Glycerate-modified metabolites have not been described before, but Moellering and Cravatt (47) have previously reported that proteins can be modified with phosphoglycerate when the glycolytic metabolite 1,3-BPG spontaneously reacts with amino groups in lysine side-chains. Furthermore, they observed that cells can dephosphorylate phosphoglycerate-modified peptides by unknown phosphatases. We reasoned that if amino groups in proteins can react with 1,3-BPG, then amino groups in metabolites might also undergo the same reaction. In a second step, the resulting phosphoglycerate adducts could be dephosphorylated by phosphatases to produce glycerate adducts (Fig. 2A).

We first tested this hypothesis in vitro. To this end, we produced 1,3-BPG with phosphoglycerate kinase in the presence of the six metabolites that were modified in PARK7 knockout models (Fig. 2 B–D). Under these conditions, we observed a continuous increase in phosphoglycerate-modified metabolites (Fig. 2D), consistent with 1,3-BPG being the source for these modifications.

Next, we investigated whether 1,3-BPG was required for the formation of glycerate-modified metabolites in cells. To do this, we modulated cellular 1,3-BPG levels. On the one hand, we knocked down the enzyme that produces 1,3-BPG in Drosophila, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and, on the other hand, we knocked down the enzyme that consumes it, phosphoglycerate kinase (PGK) (Fig. 2 A and E). In PARK7 knockout cells, we observed an increase in glycerate-modified metabolites when PGK was knocked down, whereas levels were decreased when GAPDH was knocked down (Fig. 2F). Similar tendencies were also observed when we knocked down these enzymes in wild-type cells, though the low abundance made quantifications difficult (Fig. 2 G and H). Overall, these observations are consistent with glycerate-modifications forming first as phosphoglycerate modifications, followed by subsequent dephosphorylation by yet unknown phosphatases. This model would also explain why DJ1β-deficient D. melanogaster show increases of both N-glyceryl-lysine and N-phosphoglyceroyl-lysine (Fig. 1 I and J).

PARK7 Deficiency Leads to the Accumulation of Proteins Damaged by Phosphoglycerate and Glycerate Modifications. Free lysine with phosphoglycerate or glycerate modifications was increased in PARK7/DJ1β-deficient samples. We therefore wondered whether proteins with these modifications on lysine residues would also accumulate. To test this, we analyzed proteolytic digests from mouse brain using nano-LC-MS. The identification of phosphoglycerate-modified peptides was greatly facilitated by using common phosphopeptide-enrichment protocols (Fig. 3A). Thus, we found 62 phosphoglycerate-modified peptides from 51 proteins (see Fig. 3B and SI Appendix, Fig. S3 and Datasets S2, S3, and S5 for comparison with standards and MS3), which were exclusively detectable or of much higher abundance in PARK7 knockout samples.

Phosphoglycerate-modified metabolites were almost completely converted into glycerate-modified metabolites in vivo. Glycerate-modified proteins have never been described before, but we reasoned that many phosphoglycerate-modified proteins might also be converted to glycerate-modified proteins, which may accumulate in PARK7 knockout samples. We therefore analyzed proteolytic digests of mouse brain samples without prior phosphopeptide enrichment. This revealed 76 glycerate-modified peptides from 46 proteins, and 12 phosphoglycerate-modified peptides from 10 proteins (Fig. 3C and SI Appendix, Fig. S3B and Datasets S2, S4, and S5 for comparison with standards and MS3). Again, almost all modified peptides were found at much higher levels in PARK7 knockout samples.

Next, we investigated whether the accumulation of modified proteins could be rescued by re-expression of PARK7 in knockout samples.
Fig. 1. PARK7 and its orthologs prevent accumulation of glycerate- and phosphoglycerate-modified metabolites. (A–F) N-glyceroyl-glutamate (A), N-glyceroyl-GSH (B), N-glyceroyl-GSSG (C), N-glyceroyl-glutamine (D), N-glyceroyl-glycerophosphoethanolamine (GPE) (E), and N-c-glyceroyl-lysine (F) were quantified by LC-MS in HCT116 wild-type and two PARK7 knockout clones, as well as wild type and Park7 knockout mouse brain. (G and H) Western blot analysis (G) and quantification of the indicated metabolites (H) in wild-type or PARK7 knockout HCT116 cells, where wild-type or catalytic mutant PARK7, yajL, or spDJ1 were re-expressed. "Control vector" denotes cells transduced with lentiviruses with an empty expression cassette. (I and J) Quantification of the indicated metabolites in DJ1 knockout and wild type flies. Values are means ± SEM (n ≥ 3) and were normalized within each metabolite. *P < 0.05 in comparison to the wild-type condition (A–F, I, and J) and the knockout control with the control vector (G and H).
cells. We found 30 phosphoglycerate- and 2 glycerate-modified peptides from 21 proteins in PARK7-deficient HCT116 cells. Most modified peptides were much more abundant than in wild-type cells, and re-expression of PARK7 in these cell lines brought their abundance down to wild-type levels (Fig. 3D and E), proving that the observed changes were caused by the loss of PARK7.

Samples from DJ1β-deficient D. melanogaster contained 51 phosphoglycerate-modified peptides from 26 different proteins, which were either absent or much less abundant in samples from wild-type flies (Fig. 4A). Curiously, we were unable to detect any glycerate-modified proteins in Drosophila DJ1β knockout samples. This indicated that the phosphates acting on phosphoglycerate modifications in mouse brain and HCT116 cells might be absent in Drosophila.

At this point, it was unclear why some lysines were modified with phosphoglycerate and others with glycerate. To better understand this, we decided to analyze a single protein in depth. We chose to focus on hemoglobin, which represents more than 95% of the protein content of red blood cells. We found glycerate-modifications on 12 lysine residues and the N terminus of hemoglobin at much higher levels in PARK7 knockout mice than in wild-type controls (Fig. 4B). All of these modifications affected surface-exposed lysine residues (Fig. 4C), consistent with the idea that not only a reactive metabolite but also a phosphatase needs to have access to the site of modification. In contrast, only a single phosphoglycerate modification could be quantified. Remarkably, it affected a residue located on the inside of the hemoglobin tetramer, suggesting that access for phosphatases might be limited, thereby preventing the conversion of phosphoglycerate into glycerate (Fig. 4D). This indicated that the accessibility determines which lysine residues retain phosphoglycerate modifications and which ones become dephosphorylated. Remarkably, we found evidence that more than half of the surface-exposed lysine residues of the hemoglobin tetramer can be modified with glycerate.

A nonenzymatic modification of amino groups would be expected to occur in an indiscriminate manner, depending on the accessibility and the protonation state of the amino group.
Fig. 3. Glyceraldehyde-3-phosphate and phosphoglycerate-modified proteins accumulate in PARK7 deficiency. (A) Schematic representation of the experimental setup. (B–E) LC-MS analysis of proteolytically digested mouse brain (B and C) or cancer cell line (D and E) samples with (phosphopeptide enriched in B and D) or without (input in C and phosphopeptide enrichment. N-phosphoglyceroyl-lysine modifications are presented in yellow and N-glyceraldehyde-lysine modifications are presented in red. PARK7 wild type or knockout HCT116 cells were transduced with a lentiviral vector driving expression of wild type PARK7 or an empty expression cassette (control). Peptides levels were normalized to the abundance of the corresponding proteins and normalized to the median of the abundances across samples. Asterisks indicate peptides that could be derived from more than one protein isoform. Raw data are available online (Dataset S2 Proteomic data, and ProteomeXchange (62) under the accession number PXD029410.)

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Consistent with this, we not only observed modified lysine residues, but also modified N termini of proteins (Figs. 3 B–D and 4B). Modifications were mainly found in highly abundant proteins (Fig. 4E), which are expected to be more easily detected. This includes the glycolytic enzymes that had previously been found by Moellering and Cravatt (47) to be phosphoglycerate-modified. Several affected proteins are known to interact with phosphorylated organic acids (e.g., enolase, phosphoglycerate mutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate phosphatase, the mitochondrial adenine nucleotide translocator SLC25A4, and hemoglobin) and might have an intrinsic affinity for 1,3-BPG. However, this is not a requirement since more than half of all accessible lysine residues in hemoglobin were found to be modified, suggesting that most proteins can be modified and potentially damaged by glycerate and phosphoglycerate modifications. Thus, many modified proteins of lower abundance undoubtedly remain to be discovered.

PARK7 Prevents Damage Caused by a Glycolytic Metabolite without Destroying It. The striking increase in glycerate- and phosphoglycerate-modified proteins and metabolites upon PARK7 loss-of-function indicated that PARK7 either removes these modifications (hypothesis 1 and 2 in Fig. 5A) or prevents their formation (hypothesis 3 in Fig. 5A). To distinguish between these models, we quantified newly synthesized glycerate-modified metabolites in cells by following the incorporation of $^{13}$C atoms after treatment with U- $^{13}$C-glucose. Given that the glycerate-modification is expected to incorporate three carbons from glucose, we assumed that newly formed glycerate-modified metabolites would be detected as m+3 forms, whereas the preexisting fraction would present as m+0 (Fig. 5A). We performed these experiments in an HCT116 PARK7 knockout clone where we acutely induced the expression of either wild-type or catalytic mutant CI005 PARK7 from a doxycycline-inducible promoter (Fig. 5B). If PARK7 prevents the formation of newly synthesized glycerate-adducts, we expected that it would only reduce the abundance of the m+3 fraction, whereas both the m+0 and the m+3 fraction would be affected if PARK7 removed the glycerate modification. Following induction of PARK7 expression, the m+3 fraction was more than twofold lower, whereas no change was observed when mutant PARK7 was expressed (Fig. 5 C–H). This demonstrates that PARK7 does not actively remove glycerate adducts. Furthermore, recombinant PARK7 did not have any activity on phosphoglycerate adducts in vitro (Fig. 5I).

Taken together, PARK7 prevents the formation of phosphoglycerate and glycerate modifications of metabolites, but does not remove them. This was puzzling since our experiments (Fig. 2) and the work of Moellering and Cravatt (47) had indicated that 1,3-BPG causes the formation of phosphoglycerate adducts. How could...
Parkinson’s disease protein PARK7 prevents metabolite and protein damage caused by a glycolytic metabolite

**Fig. 5.** PARK7 prevents formation of the glycerate and phosphoglycerate modifications but does not remove them. (A) Rationale for the 13C tracing experiment to determine whether PARK7 prevents formation of adducts or removes them. Six hours after induction of PARK7 expression in PARK7 knockout (KO) cell lines, cells were incubated with U-13C-glucose for 6 h. (B) Western blot of acute doxycycline-induced re-expression of PARK7 in PARK7 KO HCT116 cells. (C–H) De novo synthesized (black, m+3) and residual old (white, m+0) N-glyceroyl-glutamate (C and D), N-glyceroyl-GSH (E and F), and phosphoglycerate (G and H) were assessed by following 13C incorporation upon treatment with U-13C-glucose after acute re-expression of wild-type or mutant PARK7 in PARK7 knockout cell lines. Results are expressed in arbitrary units (AU) or as the fraction of each metabolite to the median of the PARK7-treated condition (I). Asterisks denote statistical significance (*P < 0.05) compared to the noninduced condition with wild-type vector (C–H) or the buffer control (I). AU, arbitrary units.

PARK7 prevent formation of these adducts without destroying this important metabolite and affecting glycolysis? To resolve this question, we recapitulated the formation of adducts in vitro (Fig. 6 A–C). We produced 1,3-BPG in the presence of metabolites with free amino groups (Fig. 6A). This led to the formation of phosphoglycerate-modified metabolites, similar to what we had previously observed (Fig. 2 B–D). In contrast, formation of phosphoglycerate-modified metabolites was almost completely prevented when this reaction was performed in the presence of PARK7 or its distant homologs spDJ-1 (S. pombe) or yajL (E. coli) (Fig. 6C). Strikingly, 1,3-BPG levels remained unchanged (Fig. 6B). Of note, PARK7 carrying a mutation in the key catalytic cysteine residue, C106S, did not affect adduct formation nor 1,3-BPG levels (Fig. 6B and C). Similar results were also observed when we incubated metabolites with enzyme-free 1,3-BPG (SI Appendix, Fig. S4). Again, addition of recombinant PARK7 did not change concentrations of 1,3-BPG (SI Appendix, Fig. S4B), but almost completely prevented the formation of phosphoglycerate-modified metabolites. These findings clearly demonstrated that PARK7 prevents 1,3-BPG-dependent modifications without affecting 1,3-BPG levels.

To test whether PARK7 could also prevent phosphoglycerate modifications on proteins, we set up reactions that produced 1,3-BPG via PGK, but where GAPDH was also present in the reaction mixture (similar to Fig. 6A). The analysis of protein modifications revealed numerous phosphoglycerate-modifications on both GAPDH and PGK, which were strongly reduced in abundance in the presence of PARK7 (Fig. 6D). Of note, comparable results were also obtained when 1,3-BPG was produced via GADPH (SI Appendix, Fig. S5).

Taken together, these observations unequivocally demonstrate that PARK7 prevents the formation of 1,3-BPG–dependent modifications without affecting 1,3-BPG levels. Thus, PARK7 must destroy a reactive intermediate that spontaneously forms from 1,3-BPG and avidly reacts with amino groups. To demonstrate this, we set up a competition experiment between glutamate and the nucleophile cysteamine (Fig. 6E). If glutamate reacted directly with 1,3-BPG, then formation of phosphoglycerate-modified glutamate should only be decreased by the addition of cysteamine if this addition depletes 1,3-BPG levels. In contrast, if cysteamine and glutamate compete in a reaction with a reactive degradation product of 1,3-BPG, then the addition of cysteamine should reduce the formation of phosphoglycerate-modified glutamate without affecting 1,3-BPG levels. As shown in Fig. 6F, the addition of 1 mM cysteamine did not affect 1,3-BPG levels. In contrast, phosphoglycerate-modified glutamate production was...
reduced by more than 90% (Fig. 6G). This demonstrates that 1,3-BPG is not directly responsible for the formation of phosphoglycerate adducts (Fig. 7A). We also noted that even weaker nucleophiles (such as ammonium ions) can prevent the formation of adducts and react with the reactive intermediate (Fig. 6G), making direct identification by MS difficult.

**Tentative Identification of the Reactive Metabolite as Cyclic-1,3-Phosphoglycerate Formed by an Intramolecular Attack from 1,3-BPG.** To glean insights into the identity of the elusive reactive metabolite, we searched for products of 1,3-BPG that were absent in vitro reactions containing PARK7. This revealed a metabolite with an m/z of 166.9751, which was absent when we produced 1,3-BPG via phosphoglycerate kinase in the presence of PARK7 or its orthologs (Fig. 7B, presenting the same samples as in Fig. 6C). Based on its coelution with a synthetic standard, its resistance to alkaline phosphatase, the loss of a carboxylic acid function in MS2, and based on its hydrolysis into both 2-phosphoglycerate and 3-phosphoglycerate, this metabolite was identified as cyclic-2,3-phosphoglycerate (SI Appendix, Fig. S6). Interestingly, while PARK7 prevented the formation of cyclic-2,3-phosphoglycerate, it was unable to degrade it (Fig. 7C). This indicated that cyclic-2,3-phosphoglycerate is not the elusive PARK7 substrate but rather formed downstream of this substrate.

To form cyclic-2,3-phosphoglycerate, either the hydroxyl group on carbon 2 or the phosphate group on carbon 3 need to be activated, for example by attaching a good leaving group. Coming from 1,3-BPG, the most parsimonious explanation is the formation of cyclic-1,3-phosphoglycerate, which can form by an intramolecular attack of 1,3-BPG (Fig. 7D). A nucleophilic attack by amino groups would explain the formation of phosphoglycerate-adducts. Likewise, we hypothesized that a nucleophilic attack on carbon 1 by the catalytic cysteine residue of PARK7 would lead to the formation of a thioester, which would then hydrolyze to form 3-P-glycerate (Fig. 7D). In such a model, PARK7 should be able to convert 3-P-glycerate to the enzyme-linked thioester, even if the equilibrium of the hydrolysis is expected to be far on the side of 3P-glycerate. To test this, we incubated 3-P-glycerate in the presence or absence of PARK7 in a reaction where 60% of the water molecules contained $^{18}$O (Fig. 7E). If PARK7 could form a thioester with 3P-glycerate, we would expect that hydrolysis of the thioester back to 3-phosphoglycerate would incorporate $^{18}$O into the molecule. When we incubated 3-P-glycerate in the presence of PARK7, we observed a twofold increase in 3-phosphoglycerate with a two-unit mass increase. After correction for natural isotope distribution of $^{13}$C, this corresponds to $^{18}$O incorporation in 1.5% of 3-P-glycerate molecules, whereas none was incorporated in presence of CI06S mutant PARK7 or without enzyme (Fig. 7F). These observations are consistent with a nucleophilic attack of PARK7 on carbon 1 of cyclic-1,3-phosphoglycerate, which is eventually converted to 3P-glycerate.

As indicated above, the reactive intermediate was very sensitive to the presence of nucleophiles, which likely precluded its detection by LC-MS. Nevertheless, we wondered whether the kinetics of adduct formation might allow us to determine its half-life. We reasoned that the concentration of the reactive intermediate should reach a steady-state in a reaction with constant 1,3-BPG levels (Fig. 7G, state 1). Under these conditions, the rate of the formation and the rate of the degradation of the reactive metabolite should be identical. While we could
PARK7 knockout

Fig. 7. Tentative identification and characterization of the reactive compound as cyclic-1,3-phosphoglycerate. (A) Schematic representation of the formation of N-glycerol- and N-phosphoglyceroyl-modifications in proteins and metabolites. (B) Quantification of an ion with an m/z of 166.9751 by LC-MS in reactions, where 1,3-BPG was continuously produced in the presence or absence of PARK7 and related proteins (same setup and samples as Fig. 6 A–C). The metabolite was identified as cyclic-2,3-phosphoglycerate (SI Appendix, Fig. S6). (C) Cyclic-2,3-phosphoglycerate was quantified after preformed cyclic-2,3-phosphoglycerate was incubated in presence of PARK7 enzyme or its storage buffer. (D) Working model of cyclic-1,3-phosphoglycerate as the reactive intermediate, its formation, its fate and its degradation by PARK7. (E) Exchange reaction with H218O expected to occur if PARK7 forms 3-phosphoglycerate via a thioester intermediate. While the equilibrium of the thioester hydrolysis is far on the side of 3-phosphoglycerate, incorporation of 18O into 3-phosphoglycerate in the presence of PARK7 would indicate that a thioester has been formed transiently. (F) Working model of the protein metabolism of a metabolite/protein

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not determine the concentrations directly, we were able to determine adduct formation when we added cysteamine at concentrations that almost completely depleted this metabolite (Figs. 6G and 7G, state 2). This led to the rapid formation of N-phosphoglyceroyl-cysteamine within <5 min, by reacting with the preexisting reactive metabolite (Fig. 7H). Subsequently, N-phosphoglyceroyl-cysteamine increased much slower but at a constant rate, which should correspond to the rate of the formation of the reactive intermediate (Fig. 7G, state 2). Based on this rate, the reactive intermediate present in steady state before addition of cysteamine would have needed 12 min to be formed (SD = 3.8 min). Given that formation rate and degradation rate of the reactive metabolite in steady state should be identical, we estimate that the half-life of the reactive intermediate is 8.4 min. Of note, addition of PARK7 at the same time as cysteamine led to the formation of N-phosphoglyceroyl-cysteamine at 4x lower rate. This indicates that 75% of the reactive metabolite is being destroyed by PARK7 even in the presence of a 50-fold excess of the nucleophile cysteamine.

Discussion

PARK7 Solves a Universal Problem of Glycolysis. Degradation of glucose in glycolysis produces two molecules of ATP. This can only be achieved via the formation of 1,3-BPG. Thus, damage caused by this metabolite has to be dealt with in all cells that perform glycolysis, explaining the functional conservation of PARK7 during evolution. Our data shows that PARK7 achieves the seemingly impossible task of preventing the damage caused by 1,3-BPG without affecting levels of this key glycolytic metabolite. This reveals a so far unknown chemical property of 1,3-BPG: it does not directly modify amino groups efficiently. Rather, this modification proceeds mostly via a reactive intermediate, which can be eliminated by PARK7 before it reacts with amino groups of metabolites and proteins. In fact, the necessity for PARK7 to compete with amino groups might explain why PARK7 is highly abundant and why its concentration is kept the same across different cell types and organisms (46). PARK7 destroys a reactive metabolite that was not previously known to exist. While we were unable to detect this metabolite by mass spectrometry, indirect evidence indicates that it is cyclic-1,3-phosphoglycerate, which can form by an intramolecular attack of 1,3-BPG.

The discovery that PARK7 destroys a short-lived reactive metabolite might stimulate the search for other enzymes that serve to inactivate other short-lived reactive metabolites that might escape detection by mass spectrometry. Like PARK7, such enzymes might play a role in preventing cellular damage and the development of degenerative diseases.

A Solution to the Long-Standing Enigma of PARK7 Function? More than 2,000 papers have been published on PARK7. It is therefore legitimate to ask whether the PARK7 function reported here is the core function or whether it is simply one more function for a legitimate to ask whether the PARK7 function reported here is the core function or whether it is simply one more function for a
mitochondrial metabolism (24, 53), and can increase glycolytic flux (54). This phenotype partially overlaps with the phenotype that is observed upon inactivation of other genes mutated in early-onset Parkinson’s disease (53). Several of these play a role in mitophagy (e.g., PINK1 and Parkin) (5–7), a mitochondrial quality-control mechanism. When they are inactivated, this can lead to mitochondrial dysfunction, increased reactive oxygen species production, and increased glycolytic flux (8, 9, 55). Oxidative stress and mitochondrial dysfunction have received most of the attention as a cause for neuronal cell death. However, an increase in neuronal glycolytic flux can also affect the viability of neurons. For example, a forced activation at the level of phosphofructokinase reduces viability of neurons, whereas an inhibition at this step protects neurons from detrimental effects of ischemia-reperfusion injury and excitotoxicity (56–58). These observations have been explained by reciprocal changes of the flux in the pentose phosphate pathway, which via the production of NADPH normally helps cells to resist against oxidative damage (56). Nevertheless, activation of phosphofructokinase may also increase 1,3-BPG levels and thereby the formation of phosphoglycerate and glycerate adducts. Thus, it is conceivable that modified proteins and metabolites may contribute to the phenotype observed in other models of hereditary Parkinson’s disease and neuronal toxicity. This might explain why PARK7 can rescue part of the phenotype of PARK1 deficiency (53).

New Roads for Preventive or Therapeutic Interventions? While patients with genetic PARK7 deficiency are rare, the catalytic cysteine 106 of PARK7 is easily inactivated by oxidative stress (59). This suggests that PARK7 might be intermittently inactivated even in individuals without mutations in this protein. As a consequence, modified metabolites might form, and specific modified proteins might accumulate over the course of the lifetime of an organism. To what extent this contributes to the pathogenesis of nonhereditary Parkinson’s disease will need to be investigated.

The formation of phosphoglycerate and glycerate adducts is expected to depend on the cellular concentration of 1,3-BPG. Consistent with this, we observed that knockdown of PGK (i.e., the enzyme that consumes 1,3-BPG) leads to increased levels of glycerate-modified metabolites even when PARK7 is fully functional (Fig. 2 G and H). Therefore, accumulation of modified metabolites or proteins might play a role in the development of early-onset Parkinsonism in a subset of patients with PGK1 deficiency (45).

In reverse, a reduction of cellular 1,3-BPG levels might slow down disease progression by reducing the formation of phosphoglycerate and glycerate adducts. This therapeutic goal may be achieved by stimulating glycolytic enzymes downstream of 1,3-BPG or by inhibiting upstream enzymes. At the same time, these interventions need to ensure that overall energy metabolism and the delicate metabolic interplay between different cell types in the brain are maintained (60). It has recently been found that PGK activators can improve the outcome of experimental PD models (51). It is tempting to speculate that these effects were partially caused by a reduction in phosphoglycerate and glycerate adducts. In any case, our work suggests that dietary or pharmacologic interventions to reduce cellular 1,3-BPG levels should be explored as future therapeutic or preventive approaches.

Methods
A detailed description of the methods is contained in SI Appendix.

Resource Availability. Further information and requests for resources and reagents should be directed to G.T.B. Proteomics datasets have been submitted to ProteomeXchange (62) under the accession no. PXD0023410.

Plasmids. Vectors for CRISPR/Cas9-mediated gene-targeting were generated based on the vector pX459 as described in Sanjana et al. (63). Plasmids for the lentiviral expression of shRNAs were produced in a system related to the miR-E system (64). Constitutive and inducible expression in mammalian cells was achieved using lentiviral constructs driven by a CMV promoter (65) or a doxycycline-inducible promoter from the pTRIPZ plasmid (OpenBiosystem), respectively.

Cell Culture. We generated knockout clones by transient transfection with CRISPR/Cas9 plasmids using Lipofectamine 2000, followed by 48-h puromycin selection. Recombinant lentiviruses were produced by transient transfection of HEK293T cells with lentivector vectors and packaging plasmids pPAX2 and pMD2.G using calcium phosphate coprecipitation (66). The virus-containing supernatant was recovered to infect target cell lines, followed by a 3-d puromycin selection.

Animals. Drosophila D1Jl(JA93) and tissues of the mouse line B6-Cg- Park7+tm1a(Liras) were snap-frozen and underwent metabolomic and proteomic analysis. Mouse experiments were performed following the European and Belgian legislation for care and use of laboratory animals, and approved by the University’s Animal Welfare Committee.

Production of Recombinant Proteins and In Vitro Assays. Recombinant proteins were produced in E. coli and purified via a hexahistidine tag. The activity of these proteins (in combination with commercially available enzymes) was assessed by following product formation with LC-MS. Concentrations of 1,3-BPG were determined using a coupled enzymatic assay.

LC-MS Analysis of Metabolites. Metabolites were analyzed using an Agilent 6550 ion funnel mass spectrometer in negative mode, in comparison to synthetic standards. Their abundance was quantified using the M51 [M-H]- peak area.

Nano-LC-MS Analysis of Peptides. After reduction, alkylation and proteolytic digestion, peptides were analyzed by an Orbitrap Fusion Lumos tribrid mass spectrometer, and (phospho)glycerate-modified peptides were identified based on the HCD MS2 spectra using Proteome Discoverer 2.4 (Thermo). Peptides were quantified using M51 precursor intensity. We confirmed the identity of several peptides by M53 and by comparison to synthetic standards.

Western Blotting. Cell lysates were prepared in RIPA buffer and Western blots were performed as previously described (67). Primary antibodies were anti-PARK7 (Santa-Cruz, clone D4, 1:1000), anti-PGK1 (Proteintech 17811–1-AP, 1:4000), anti-GAPDH (Proteintech 60004–1-lg, 1:2000), anti–p-JNK (Cell Signaling #9284; 1:1000), anti–phospho-h2AX (Millipore; 1:1000), anti-GLO1 (1:1000) and antimethylglyoxal (recognizing the MG-H1 modification of arginines, Cell Biolabs STA-011, 1:5000) diluted in Tris-buffered saline (150 mM NaCl, 20 mM Tris-Cl pH 7.4, 0.05% Tween-20) containing 2% bovine serum albumin (Sigma).

Data Availability. Mass spectrometry proteomics data have been deposited at ProteomeXchange, www.proteomeexchange.org/ (accession no. PXD029410) via the PRIDE partner repository (62). Processed mass spectrometry data are presented in Datasets S2–S5. All other study data are included in the main text and supporting information.

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