

ments. In any case, the questions remain: What kind of elements associated with these processes would have arrived relatively early in chordate evolution and then become practically frozen in birds and mammals? And what mechanisms would underlie this, allowing them to resist virtually all further change?

Note added in proof: We recently became aware of related observations made by Boffelli *et al.* (37).

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Supporting Online Material

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A Family with Severe Insulin Resistance and Diabetes Due to a Mutation in *AKT2*

Stella George,^{1*} Justin J. Rochford,^{1*} Christian Wolfrum,³ Sarah L. Gray,¹ Sven Schinner,¹ Jenny C. Wilson,¹ Maria A. Soos,¹ Peter R. Murgatroyd,¹ Rachel M. Williams,² Carlo L. Acerini,² David B. Dunger,² David Barford,⁴ A. Margot Umpleby,⁵ Nicholas J. Wareham,⁶ Huw Alban Davies,⁷ Alan J. Schafer,⁸ Markus Stoffel,³ Stephen O'Rahilly,^{1†} Inês Barroso^{8,9}

Inherited defects in signaling pathways downstream of the insulin receptor have long been suggested to contribute to human type 2 diabetes mellitus. Here we describe a mutation in the gene encoding the protein kinase *AKT2/PKBβ* in a family that shows autosomal dominant inheritance of severe insulin resistance and diabetes mellitus. Expression of the mutant kinase in cultured cells disrupted insulin signaling to metabolic end points and inhibited the function of coexpressed, wild-type *AKT*. These findings demonstrate the central importance of *AKT* signaling to insulin sensitivity in humans.

Most forms of diabetes are likely to be polygenic in origin, although a number of monogenic forms are being recognized (1, 2). Although rare, these monogenic examples offer insight into the function of the affected gene in humans as well as offering important clues to understanding more common forms.

We have been screening genomic DNA from 104 unrelated subjects with severe insulin resistance for mutations in genes that are implicated in insulin signaling. We iden-

tified a missense mutation in the serine/threonine kinase gene *AKT2* in one Caucasian proband. *AKT2* (also known as *PKBβ*) is highly expressed in insulin-sensitive tissues and is activated in response to growth factors and related stimuli (3, 4), a process that requires its phosphorylation by the phosphoinositide-3 phosphate-dependent kinase activities designated *PDK1* and *PDK2* (3). The proband (Fig. 1D, iii/1) is a nonobese 34-year-old female who developed diabetes mellitus at 30 years of age. The proband, her nonobese mother, her maternal grandmother, and a maternal uncle were all heterozygous for a G-to-A substitution predicted to result in an R-to-H substitution at amino acid 274 (Fig. 1, A and B) (5). All were markedly hyperinsulinemic (table S1), and the mother and maternal grandmother developed diabetes mellitus in their late thirties. Three other first-degree relatives available for study were all clinically normal, with normal fasting glucose and insulin, and were homozygous for the wild-type *AKT2* sequence (Fig. 1D and table S1). This mutation was not found in the genomic DNA of 1500 Caucasian control subjects from the United Kingdom.

R²⁷⁴ forms part of an RD sequence motif within the catalytic loop of the *AKT2* kinase

¹Department of Clinical Biochemistry, ²Department of Paediatrics, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ, UK. ³Laboratory of Metabolic Diseases, Rockefeller University, New York, NY 10021, USA. ⁴Section of Structural Biology, Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, UK. ⁵Department of Diabetes, Endocrinology and Internal Medicine, Guy's, King's and St. Thomas' School of Medicine, London, UK. ⁶Medical Research Council Epidemiology Unit, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 8RN, UK. ⁷Darent Valley Hospital, Darent Wood Road, Dartford, Kent DA2 8DA, UK. ⁸Incyte, 3160 Porter Drive, Palo Alto, CA 94304, USA. ⁹Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK.

*These authors contributed equally to this work.

†To whom correspondence should be addressed. E-mail: sorahill@hgmpr.mrc.ac.uk

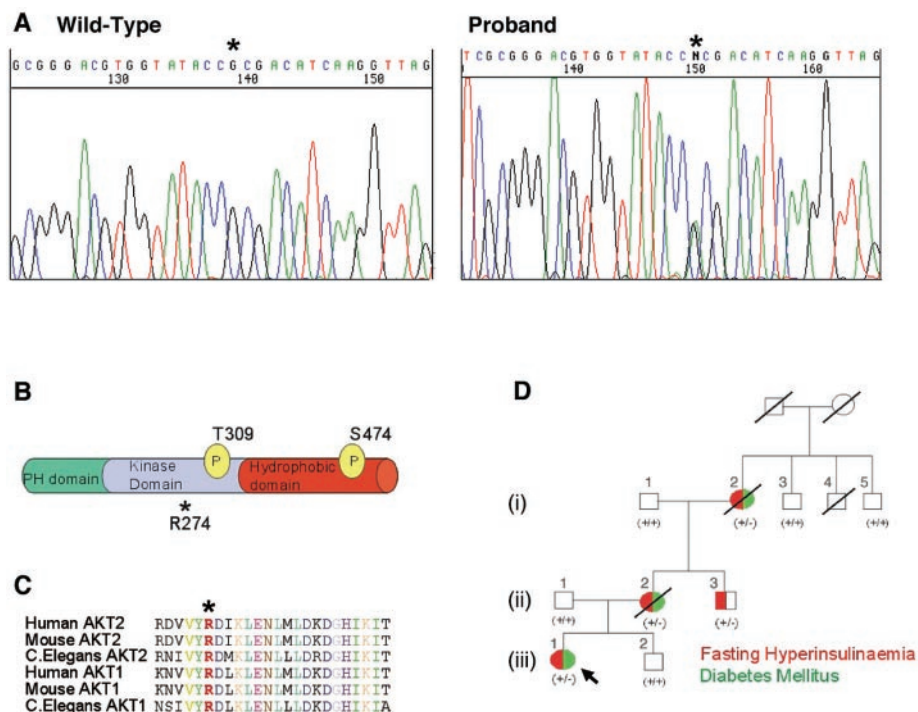


Fig. 1. Detection of a nonconservative heterozygous mutation in *AKT2* that cosegregates with severe insulin resistance. **(A)** Direct sequencing of genomic DNA from the proband (right) and a control subject (left). Asterisks indicate the heterozygous G-to-A substitution that produces H²⁷⁴. **(B)** The location of R²⁷⁴ in relation to known functional domains and phosphorylation sites required for activation of AKT2. **(C)** R²⁷⁴ (in red and marked with an asterisk) is highly conserved across different AKT isoforms and diverse species. **(D)** A family pedigree demonstrating cosegregation of clinical phenotype (table S1) with the R²⁷⁴H mutation. All family members heterozygous for the mutation (+/–) are hyperinsulinemic, and three out of four have diabetes mellitus. All wild-type subjects (+/+) are normoinsulinemic and nondiabetic. Red denotes fasting hyperinsulinemia; green denotes diabetes mellitus.

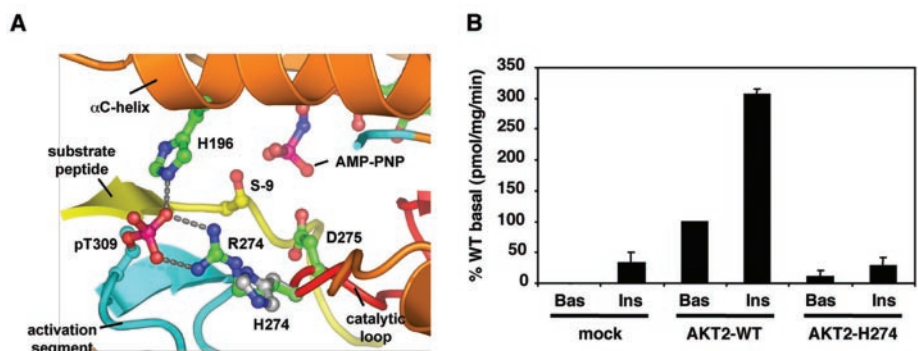


Fig. 2. The substitution of R²⁷⁴ by histidine disrupts the kinase domain and abolishes AKT2 kinase activity. **(A)** Proposed effects of R²⁷⁴H on a structural model of the AKT2 kinase domain. In the wild-type protein, R²⁷⁴ contacts phosphoT309 (pT309), organizing the activation segment to place the substrate peptide correctly for catalysis. Substitution of H for R²⁷⁴ is predicted to disrupt the conformation of both the activation segment and the catalytic loop. **(B)** Hemagglutinin-tagged HA-AKT2 and HA-AKT2H274 were immunoprecipitated from lysates of appropriately transfected CHO-T cells. Top: Enzyme activity was determined by an in vitro kinase assay. Mock-transfected cells received empty vector only. Serum-starved cells in the basal state were treated without (Bas) or with (Ins) 100 nM insulin for 10 min before lysis. Data are mean ± SEM of four experiments. Crossside was used as the substrate. WT, wild-type. Bottom: Duplicate immunoprecipitates were also immunoblotted with an antibody to AKT2, to demonstrate equal expression of the wild-type and mutant proteins. IP, immunoprecipitate; WB, Western blot.

domain that is invariant in AKT isoforms in all species and is also highly conserved within the protein kinase family (Fig. 1C) (6). The RD motif includes the invariant D residue (D²⁷⁵ of AKT2) that performs an essential catalytic function in all protein kinases.

R²⁷⁴ is positioned in the core of the catalytic domain, forming critical hydrogen bonds with the phosphate moiety of phosphoT309 in the activation segment permitting correct positioning of the substrate peptide relative to the catalytic base and adenosine triphosphate (ATP) (7). A model of the mutant protein AKT2H274 (Fig. 2A) indicates that this mutation would disrupt the conformation of both the activation segment and the catalytic loop, misaligning the substrate peptide relative to catalytic residues and ATP and hence ablating catalytic activity. Consistent with these predictions, unlike the wild-type AKT2, AKT2H274 was unable to phosphorylate a peptide substrate based on the AKT target sequence of glycogen synthase kinase 3 in an in vitro kinase assay (Fig. 2B).

To examine the effect of the R²⁷⁴H mutation on the signaling ability of AKT2, we studied the regulation of the FOXA2 transcription factor, a substrate of AKT2 (8). FOXA2 activity is inhibited by phosphorylation, which leads to its exclusion from the nucleus. Treatment of HepG2 human liver cells with insulin induced the translocation of endogenous FOXA2 from the nucleus to the cytosol (Fig. 3A). Overexpression of wild-type AKT2 mimicked this effect (Fig. 3A). In contrast, in cells transfected with mutant AKT2H274, FOXA2 remained entirely nuclear (Fig. 3A). Consistent with this, although cotransfected wild-type AKT2 ablated FOXA2-mediated transcription from the phosphoenolpyruvate carboxykinase (PCK1) promoter in HepG2 cells, AKT2H274 had no effect (Fig. 3B). AKT2H274 also prevented wild-type AKT2 from inhibiting FOXA2-driven transcription from the PCK1 promoter, in a dose-dependent manner, when both AKT2 proteins were coexpressed (Fig. 3C). In the same assay, AKT2H274 also inhibited the effect of wild-type AKT1, which also contributes substantially to AKT activity in the liver (Fig. 3D) (9).

AKT overexpression in preadipocytes augments differentiation to adipocytes (10). We therefore examined the effect of AKT2H274 on adipocyte differentiation. 3T3-L1 mouse preadipocytes overexpressing wild-type AKT2 showed increased accumulation of lipid during adipogenesis (Fig. 3E), whereas cells overexpressing the mutant AKT2H274 showed markedly decreased lipid accumulation. The fatty acid transport protein aP2 is a well-defined marker of adipogenesis (11). Expression of aP2 was augmented by expression of wild-type AKT2 but reduced by expression of AKT2H274 (Fig. 3F). Thus,

AKT2H274 may also exert dominant-negative effects over endogenous AKT proteins during adipocyte differentiation.

To allow closer examination of the in vivo consequences of the AKT2H274 mutation, the proband underwent a two-step hyperinsulinemic/euglycemic clamp. This revealed extreme insulin resistance (fig. S1), with the glucose infusion rate remaining very low even in the second step, despite an insulin concentration of 7346 pmol/l. Examination of the effects of insulin on hepatic glucose production and peripheral glucose use revealed severe insulin resistance in this subject in both the liver and peripheral tissues (supporting online text).

Analysis of the proband's body composition revealed a ~35% difference in total body fat compared to that predicted for her height and weight, consistent with both the ability of AKT2H274 to impair adipogenesis and the recently reported observation that *AKT2* knockout mice develop lipoatrophy as they age (12).

This family provides an example of a monogenic inherited defect in post-receptor insulin signaling that leads to human insulin resistance and diabetes mellitus. Genetic variants in *IRS1* and *PIK3R1* have been previously reported in subjects with insulin resistance and/or type 2 diabetes mellitus, but they have not clearly segregated with insulin resistance in a pedigree or seriously disrupted signal transduction (13, 14). Dominant-negative mutations in peroxisome proliferator-activated receptor gamma (*PPARγ*) are associated with autosomal, dominant, severe insulin resistance and diabetes mellitus. However, the mechanisms whereby they result in insulin resistance are unclear and are unlikely to be due to simple impairment of insulin signal transduction (15).

AKT2^{-/-} mice show resistance to insulin's effects on glucose metabolism in muscle and liver, and a subset of these mice go on to develop frank diabetes (12, 16). In view of the abnormal fat distribution in our proband, it is of interest that atrophy of adipose tissue with age has been described in one strain of these mice (12, 16). *AKT2*^{+/-} mice show little metabolic phenotype, and even in (-/-) animals, the degree of insulin resistance is only moderate. This contrasts with the extreme hyperinsulinemia and insulin resistance seen in humans heterozygous for the AKT2H274 mutation. This may result from this mutant inhibiting other coexpressed AKT isoforms in a dominant-negative manner or from interference with other functions of upstream kinases such as PDK1.

Apart from lipodystrophy, there are no other overt structural or functional alterations in AKT2-expressing tissues from patients with this mutation. This suggests that, at least

in those tissues where AKT2 is highly expressed, insulin signal transduction represents the major role for AKT. Frank diabetes

mellitus developed in three of the four human carriers of the AKT2 mutation. In the fourth, a middle-aged male, marked hyperinsulin-

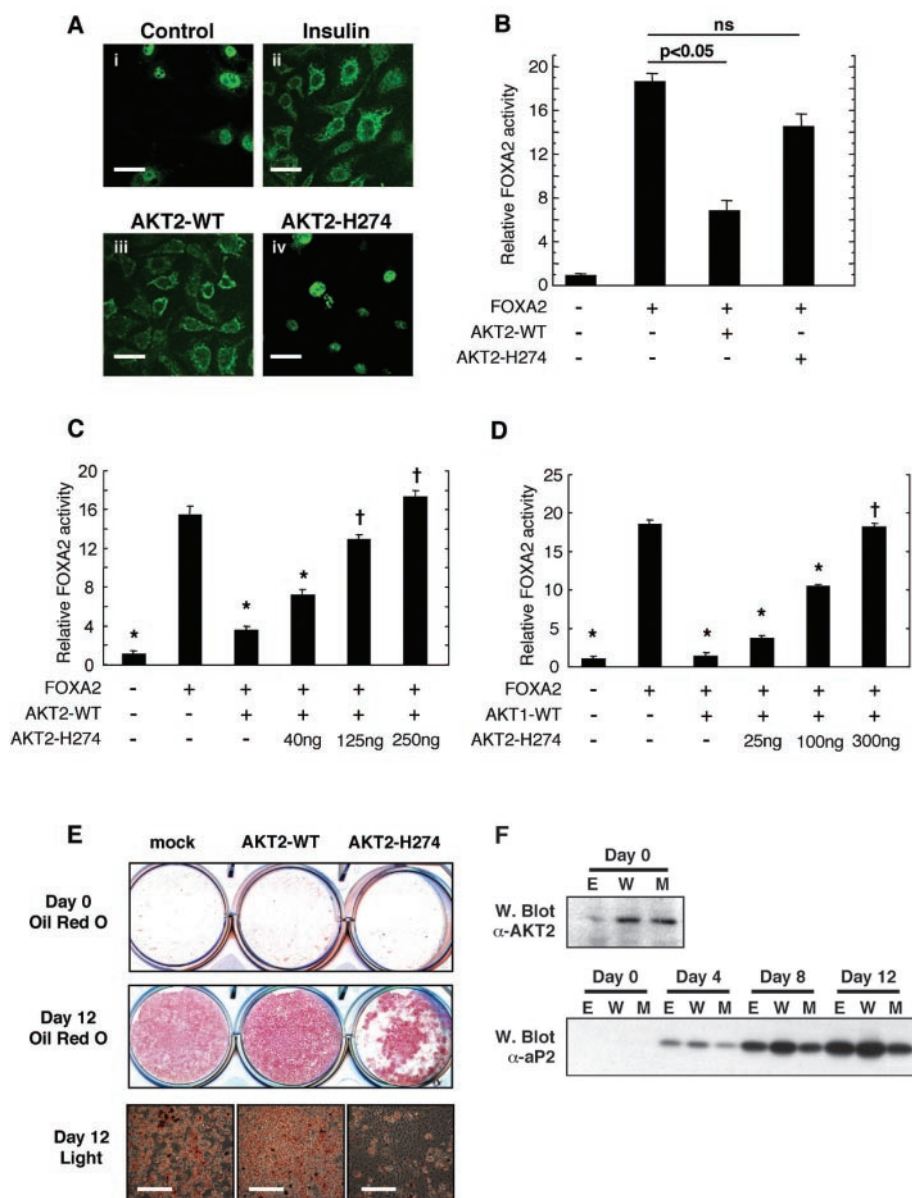


Fig. 3. Functional properties of wild-type AKT2 and AKT2H274 in cultured human liver and rodent fat cells. (A) HepG2 cells were treated in the (i) absence or (ii) presence of 50 nM insulin, then fixed and probed with antibodies to FOXA2 to determine intracellular localization (8). The same assay was performed with cells transfected with (iii) wild-type AKT2 or (iv) AKT2H274. Scale bars, 40 μm. (B) Luciferase activity was determined in extracts of HepG2 cells transfected with the phosphoenolpyruvate carboxykinase reporter construct, pPCK1, with or without FOXA2 and in the absence or presence of wild-type AKT2 or AKT2H274. ns, no significant difference. (C and D) Alternatively, HepG2 cells were transfected with the pPCK1 reporter construct with or without FOXA2 and either (C) wild-type AKT2 (125 ng) or (D) wild-type AKT1 (100 ng). In each case, AKT2H274 was cotransfected in increasing quantities as indicated. *, a significant difference from activity in cells transfected with FOXA2 alone ($P < 0.05$); †, no significant difference in this comparison. In all cases, data are mean ± SD of four experiments. All data were normalized to coexpressed β-galactosidase activity. (E) 3T3-L1 preadipocytes were stably transfected with empty vector (mock), wild-type AKT2, or AKT2H274. Top: Two-day post-confluent cells (Day 0) or cells induced to differentiate for 12 days were fixed and stained with oil red O to assess lipid accumulation. Bottom: Images of day 12 differentiated cells were obtained by light microscopy; scale bars, 200 μm. (F) Lysates were prepared from cells transfected with empty vector (E), wild-type AKT2 (W), or AKT2H274 (M) at 2 days post-confluence (Day 0) or at various intervals up to day 12 after the induction of differentiation. Day 0 samples were Western blotted (W.) for AKT2 expression, and all lysates were Western blotted for aP2 expression.

emia occurred simultaneously with normal glucose tolerance. Moreover, in our proband, severe hyperinsulinemia preceded diabetes by many years. Although we cannot exclude an effect of the AKT2 mutation on beta-cell function, it is clear that the major effect of this mutation was on insulin action.

Germline loss-of-function mutations in genes that encode intracellular signaling kinases are being increasingly recognized as causes of human inherited disease. Thus, *JAK3* mutations cause severe combined immunodeficiency disease (17), *RPS6KA3* mutations cause Coffin Lowry Syndrome (18), and *WNK4* mutations cause an inherited form of hypertension (19). The kindred described here demonstrate that AKT2 can be added to this list, the R²⁷⁴H mutation in this enzyme causing a rare form of human diabetes due to a post-receptor defect in insulin signaling. Although AKT2 mutations are unlikely to explain most common forms of diabetes, this mutant uniquely demonstrates the critical role

of AKT signaling in maintaining insulin sensitivity in humans.

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Supporting Online Material

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S-Nitrosylation of Parkin Regulates Ubiquitination and Compromises Parkin's Protective Function

Kenny K. K. Chung,^{1,2} Bobby Thomas,^{1,2} Xiaojie Li,^{1,2} Olga Pletnikova,³ Juan C. Troncoso,^{2,3} Laura Marsh,^{2,4} Valina L. Dawson,^{1,2,5,6} Ted M. Dawson^{1,2,5*}

Parkin is an E3 ubiquitin ligase involved in the ubiquitination of proteins that are important in the survival of dopamine neurons in Parkinson's disease (PD). We show that parkin is S-nitrosylated in vitro, as well as in vivo in a mouse model of PD and in brains of patients with PD and diffuse Lewy body disease. Moreover, S-nitrosylation inhibits parkin's ubiquitin E3 ligase activity and its protective function. The inhibition of parkin's ubiquitin E3 ligase activity by S-nitrosylation could contribute to the degenerative process in these disorders by impairing the ubiquitination of parkin substrates.

Parkinson's disease (PD) is a common neurodegenerative disorder that leads to the progressive loss of dopamine (DA) neurons (1). The majority of PD is sporadic and is thought to be due in part to oxidative stress through derangements in mitochondrial complex-I activity (1–3). There are also rare familial causes of PD due to mutations in α -synuclein, parkin, and DJ1 (4–6). Several indices of oxidative stress are also present in PD, including increased nitrotyrosine immunoreactivity (7, 8), reduced glutathione and ferritin levels, increased lipid peroxidation, and increased levels of iron (1, 3, 8).

Parkin is a ubiquitin E3 ligase that is responsible for the addition of ubiquitin on specific substrates (9–11). Mutations in parkin that lead to a loss of parkin's ubiquitin E3 ligase activity are the most common cause of hereditary PD (12). Parkin has a number of putative substrates, and the failure to ubiquitinate some of these substrates in the absence of functional parkin protein may play an important role in the demise of DA neurons (13–17). In addition, parkin may play a more general role in the ubiquitin proteasomal pathway by participating in the removal and/or detoxification of abnormally folded or damaged proteins (18). The observation that there are increased markers of nitrosative stress in PD (7, 8) prompted our investigation of whether parkin could be modified by nitric oxide (NO). Here, we show that parkin is S-nitrosylated and that this S-nitrosylation markedly diminishes parkin's E3 ligase activity and protective function.

Baculovirus recombinant parkin protein (BV-parkin) and human embryonic kidney (HEK) 293 cells transfected with myc-parkin were treated with S-nitrosoglutathione (GSNO), and both samples were subjected to the S-nitrosylation biotin switch assay (Fig. 1A) (19, 20). HEK293-expressed parkin was readily S-nitrosylated, whereas S-nitrosylation of BV-parkin was not detectable. The absence of S-nitrosylated parkin in samples treated with glutathione devoid of NO (GSH) demonstrated the specificity of this modification of parkin. A similar result was observed when we used another NO donor, NOC18, with NOC18 depleted of NO [NOC18(–NO)] as a control (Fig. 1B).

To further control for the specificity of the S-nitrosylation of parkin, we examined whether myc- α -synuclein, which contains no cysteines, or myc-ITCH-1, an unrelated ubiquitin E3 ligase, are S-nitrosylated. Under conditions that lead to S-nitrosylation of parkin, neither α -synuclein (Fig. 1C) nor ITCH-1 (Fig. 1D) were S-nitrosylated. Because parkin expressed in HEK293 cells was S-nitrosylated but BV-parkin was not, we wondered whether the cellular extract contained a factor that facilitated the S-nitrosylation of parkin. Accordingly, we subjected BV-parkin to the S-nitrosylation biotin switch assay in the presence or absence of HEK293 cell lysate (Fig. 1E). BV-parkin was S-nitrosylated only in the presence of cell lysate. The factor is likely to be proteinaceous, because denaturing the cell lysate by boiling completely blocked the S-nitrosylation of BV-parkin (Fig. 1E). To further confirm that parkin is S-nitrosylated, we used the 2,3-diaminonaphthalene (DAN) assay on HEK293 cells transfected with myc-parkin (21). This assay showed that parkin was S-nitrosylated, whereas ITCH-1 and α -synuclein were not (Fig.

¹Institute for Cell Engineering, Departments of ²Neurology, ³Pathology, ⁴Psychiatry, ⁵Neuroscience, and ⁶Physiology, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

*To whom correspondence should be addressed. E-mail: tdawson@jhmi.edu