

## RESEARCH ARTICLE

# Parkin Maintains Robust Pacemaking in Human Induced Pluripotent Stem Cell-Derived A9 Dopaminergic Neurons

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**ABSTRACT: Background:** The degeneration of nigral (A9) dopaminergic (DA) neurons results in cardinal motor symptoms that define Parkinson's disease (PD). Loss-of-function mutations in parkin are linked to a rare form of early-onset PD that is inherited recessively.

**Objective:** We generated isogenic human A9 DA neurons with or without parkin mutations to establish the causal relationship between parkin mutations and the dysfunction of human A9 DA neurons.

**Methods:** Using TALEN (transcription activator-like effector nuclease)- or CRISPR/Cas9-mediated gene targeting, we produced two isogenic pairs of naivetropic induced pluripotent stem cells (iPSCs) by repairing exon 3 deletions of parkin in iPSCs derived from a PD patient and by introducing the PD-linked A82E mutation into iPSCs from a healthy subject. The four lines of isogenic iPSCs were differentiated

to A9 DA neurons, which fired spontaneous pacemaking action potentials (AP) dependent on L-type Ca<sup>2+</sup> channels.

**Results:** The frequency of the pacemaking APs was significantly reduced by parkin mutations introduced to normal neurons. Consistent with this, isogenic repair of parkin mutations significantly increased the frequency from that observed in patient-derived neurons.

**Conclusions:** The results show that parkin maintains robust pacemaking in human iPSC-derived A9 DA neurons. The function is critical to normal DA transmission required for controlling voluntary locomotor activities. © 2023 International Parkinson and Movement Disorder Society.

**Key Words:** Parkinson's disease; induced pluripotent stem cells; A9 dopaminergic neurons; pacemaking; isogenic

Parkinson's disease (PD) is the most common movement disorder. The age-dependent progressive loss of nigral (A9) dopaminergic (DA) neurons leads to its defining motor symptoms that include bradykinesia, resting tremor,

rigidity, and postural instability.<sup>1</sup> Approximately 85% of PD cases are sporadic, whereas the remaining 15% have a family history. Identification and functional studies of monogenic mutations linked to PD have shed significant insights into the molecular and cellular mechanisms of PD.<sup>2,3</sup> Among these genes, loss-of-function mutations in parkin (*PRKN*) are frequently found in autosomal recessive early-onset PD.<sup>4</sup> Parkin is a ubiquitin E3 ligase<sup>5</sup> that controls dopamine utilization in human midbrain DA neurons by limiting dopamine-induced oxidative stress and enhancing the precision of DA transmission.<sup>6</sup> The importance of parkin in maintaining DA transmission is evidenced by the oscillatory neuronal activities observed in human midbrain neurons differentiated from induced pluripotent stem cells (iPSCs) of PD patients with parkin mutations.<sup>7</sup> The oscillatory neuronal activities, which parallel those found in the PD brain,<sup>8</sup> are rescued by the overexpression of parkin.<sup>7</sup>

Nigral DA neurons are autonomous pacemakers that spontaneously fire action potentials (APs) in the range of 2 to 10 Hz.<sup>9,10</sup> These pacemaking APs ensure the

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continuous release of dopamine to striatal neurons. In response to increased excitatory inputs, nigral DA neurons exhibit phasic firing, which is superimposed on the tonic firing pattern and at a much higher frequency.<sup>11</sup> Both tonic and phasic firings are important for maintaining the neural computation needed to control voluntary movements.<sup>12,13</sup> The pacemaking APs are not affected when excitatory inputs are blocked; they are dependent on L-type  $\text{Ca}^{2+}$  channels, as shown in animal nigral DA neurons<sup>14</sup> and in iPSC-derived human A9 DA neurons.<sup>15</sup>

The lack of robust PD phenotypes in parkin knock-out mice<sup>16</sup> and rats<sup>17</sup> is in sharp contrast to the situation in PD patients with parkin mutations, which makes it necessary to establish the causal relationship between parkin mutations and the dysfunction of human A9 DA neurons. Genomic editing based on TALEN (transcription activator-like effector nuclease)- or CRISPR/Cas9-facilitated homologous recombination enables the generation of isogenic pairs of iPSCs that have the same genetic background and differ only in the engineered genetic change.<sup>18</sup> The efficiency of gene targeting is further enhanced in naive iPSCs, which are in a naive-like state resembling the mouse embryonic stem cells and thus have a much higher single-cell survival rate than primed state human iPSCs do.<sup>19</sup> By performing TALEN- and CRISPR/Cas9-facilitated gene targeting in naive iPSCs, we repaired the parkin mutations in a PD patient with homozygous deletion of exon 3, which is the most frequent mutation in parkin, occurring in 10% of index patients.<sup>20</sup> To establish that parkin mutations are sufficient in producing a phenotype, we introduced the PD-linked A82E missense mutations to a control iPSC line. We differentiated the two pairs (four lines) of isogenic iPSCs to A9 DA neurons using the method that we have developed recently.<sup>15</sup> These human A9 DA neurons expressed the appropriate markers, such as GIRK2 and ALDH1A1,<sup>21</sup> but largely not the A10 DA neuron marker CALBINDIN.<sup>22</sup> They fired pacemaking APs that were blocked by the L-type  $\text{Ca}^{2+}$  channel antagonist nimodipine. We found that parkin mutations were necessary and sufficient to significantly reduce the frequency of pacemaking APs. The results demonstrate the causal relationship between parkin mutations and the disruption of robust pacemaking in human A9 DA neurons, which is critical for controlling voluntary locomotor activities.

## Patients and Methods

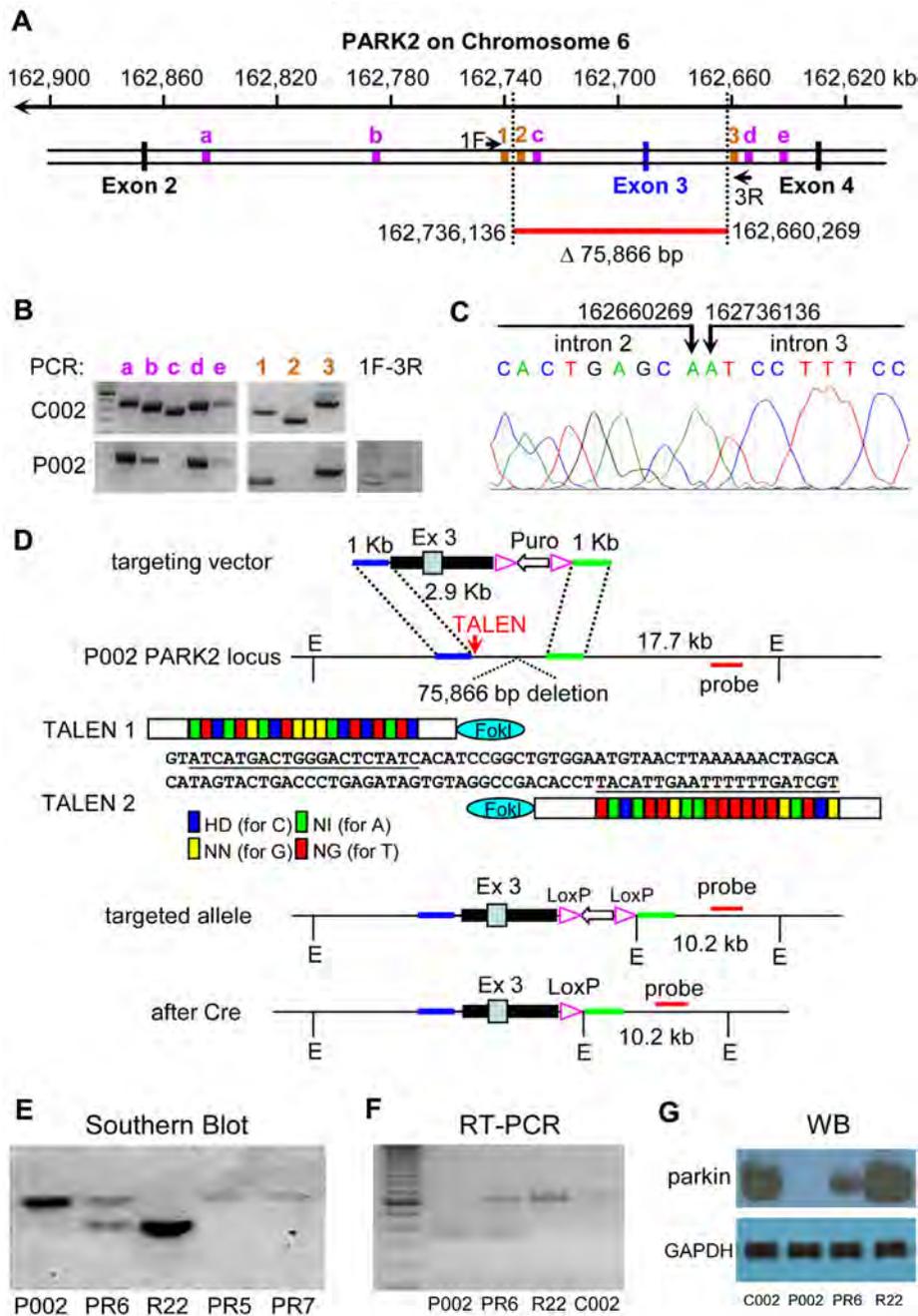
### TALEN-Mediated Repair of Exon 3 Deletions in P002 Naivetropic iPSCs

This study modified and analyzed iPSCs generated in a study<sup>6</sup> previously approved by the Health Sciences Institutional Review Board of the State University of

New York at Buffalo, which has determined that such a study does not require ongoing review. A pair of TALENs were designed according to a well-established method<sup>23</sup> and assembled using the golden gate protocol (Addgene TALEN Kit 100000024).<sup>24</sup> Detailed sequences of the TALENs are shown in Figure 1D. One million P002 naive iPSCs suspended in the Human Nucleofactor Stem Cell Kit 1 (Lonza) buffer were nucleofected with 5  $\mu\text{g}$  of linearized targeting vector and 4  $\mu\text{g}$  of each of the two TALEN plasmids using Nucleofection Program A23 following the manufacturer's protocol. Cells were subsequently plated on mouse embryonic fibroblasts (MEF) feeders in naive iPSC medium supplemented with the ROCK inhibitor. Puromycin (0.2 mg/mL) was applied 48 hours later. Individual colonies were manually picked and expanded 7 to 10 days after the puromycin selection. Polymerase chain reaction (PCR) was performed on genomic DNA isolated from each colony to screen for homologous recombinants. Positive clones were further confirmed by Southern blotting of genomic DNA using the external probe. After expansion, a confirmed positive clone was nucleofected with 10  $\mu\text{g}$  of pCAGCre:GFP (Addgene plasmid 13776) for transient expression to remove the puromycin resistance cassette by manual picking of GFP<sup>+</sup> clones.

### CRISPR/Cas9-Mediated Genomic Editing of C002 Naivetropic iPSCs

Human codon-optimized Cas9 and chimeric guide RNA (gRNA) expression backbone plasmid pSpCas9 (BB)-2A-GFP (PX458) were obtained from Addgene (plasmid 48138). A pair of annealed oligos targeting exon 3 of parkin (forward strand: CACCGGTCAAGAAATGAATGCAAC; reverse strand: AAACGTTGCATTCATTTCTTGACC; underlined sequences correspond to the gRNA sequence) were cloned into the gRNA construct. The oligos were designed based on the target-site sequence (20 bp) and were flanked at the 3' end by a 3-bp NGG PAM sequence as described.<sup>25</sup> One million C002 naive iPSCs were trypsinized into single cells, which were resuspended in the Human Nucleofactor Stem Cell Kit 1 buffer and mixed with 1  $\mu\text{g}$  of single-stranded DNA (ssDNA) containing the C to A mutation (Ala82Glu) and 5  $\mu\text{g}$  of parkin pSpCas9 (BB)-2A-GFP. Nucleofection was performed using Program A23 according to the manufacturer's protocol. The sequence of the ssDNA was acctgtgctcccaaacagAATTGTGACCTGGATCAGCAGAGCATTGTTACATTGTGCAGAGACCGTGGAGAAAAGGTCAAGAAATGAATGAAACTGGAGGCGACGACCCCAGAAACGCGGGGGAGGCTGTGAGCGGGAGCCCCAGAGCTTGACTCGG-GTGGACCTCAGCAGCTCAGTCCT (181 nt), with lowercase letters representing intronic sequences,



**FIG. 1.** TALEN (transcription activator-like effector nuclease)-mediated repair of parkin exon 3 deletions in P002 naivetropic iPSCs (induced pluripotent stem cells). **(A–C)** Using primer pairs (a–e and 1–3) at different locations flanking exon 3 of the human PARK2 gene **(A)**, we performed PCR (polymerase chain reaction) on genomic DNA from a healthy subject (C002) and a PD patient with homozygous exon 3 deletion of parkin (P002). **(B)** The two breakpoints in P002 were between primer pairs 1 and 3 and can be cloned using 1F and 3R primers. **(C)** The PCR product was sequenced to reveal the locations of the two breakpoints, which were merged in P002. **(D)** A TALEN-mediated gene-targeting strategy was designed to insert a 2.9-kb fragment containing exon 3 into P002 by homologous recombination. The 17.7-kb EcoRI fragment in P002 became 10.2 kb after homologous recombination. **(E)** Genomic DNA from P002 and individual clones of naive iPSCs after puromycin selection was digested with EcoRI and Southern blotted with the external probe indicated in **(D)**. **(F)** RT-PCR (reverse transcription-polymerase chain reaction) amplification of parkin using total RNA isolated from the indicated naivetropic iPSCs. **(G)** Western blotting of parkin in cell lysates of neuroepithelial cells differentiated from the indicated iPSCs. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

uppercase letters representing the sequence in exon 3, underlined sequence corresponding to the gRNA sequence, and bold-italic *A* representing the mutation. Cells were subsequently plated on MEF feeders in the

naivetropic iPSC medium supplemented with the ROCK inhibitor for 24 hours. Dissociated GFP<sup>+</sup> cells were sorted on a flow cytometer at 24 to 48 hours after nucleofection and plated on MEF feeders.

Individual colonies were picked and expanded 7 to 10 days after cell sorting. The genomic region surrounding the parkin CRISPR target site was amplified by PCR and sequenced to identify targeted clones.

### Reversion of Naivetropic iPSCs to the Primed State

Naivetropic iPSC colonies were picked manually and plated on MEF feeders in the human embryonic stem cells (hESC) medium with basic fibroblast growth factor (bFGF) in 21% O<sub>2</sub> as previously described.<sup>19</sup> Typical primed state colonies appeared 7 to 10 days later. These reverted iPSCs were passaged every 5 to 7 days using dispase (1 mg/mL) in the hESC medium with bFGF.<sup>19</sup>

### Directed Differentiation of Reverted iPSCs to A9 DA Neurons

Reverted iPSCs were differentiated to A9 DA pacemakers using the protocol that we have developed recently.<sup>15</sup> Briefly, iPSC colonies were detached from MEF feeders with dispase to produce embryoid bodies (EBs) in suspension culture in DMEM/F12 and Neurobasal (1:1) containing N2 (1:100), B27 without vitamin A (1:50), ascorbic acid (0.2 mM), SB431542 (10 μM), dorsomorphin (5 μM), purmorphamine (3 μM), and CHIR99021 (0.8 μM). On day 4, the EBs were attached on matrigel-coated plates and cultured in the same medium until day 16 to generate midbrain floor plate (mFP) progenitors. SB431542 and dorsomorphin were removed on day 6. On day 16, the progenitor cells were dissociated with accutase to single cells, which were plated on polyornithine/matrigel-coated plates at a density of 5000 to 10,000 cells/cm<sup>2</sup> in the same medium as previously but with 1 μM purmorphamine instead. On day 18, the medium was changed to Neurobasal with N2, B27 without vitamin A, brain-derived neurotrophic factor (20 ng/mL), glial cell line-derived neurotrophic factor (20 ng/mL), and TGFβ3 (1 ng/mL). On day 20, dibutyl-*c*-AMP (dcAMP) (0.5 mM) and N-[N-(3, 5-difluorophenacetyl)-*l*-alanyl]-*s*-phenylglycine-*n*-butyl ester (DAPT) (1 μM) were added to the medium. The ROCK inhibitor Y27632 (10 μM) was added during the first 48 hours. From day 24, cells were cultured in the differentiation medium without purmorphamine and CHIR99021. The medium was half-changed every 2 days up to day 140.

### Immunostaining

Cells were cultured in matrigel-coated 24-well plates. They were fixed in 4% paraformaldehyde (Sigma, St Louis, MO, USA) for 20 minutes, permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature, blocked in 3% bovine serum albumin for 60 minutes at room temperature, and then incubated

with primary antibody overnight at 4°C and by the secondary antibody for 2 hours at room temperature. The primary antibodies (all from Millipore, St Louis, MO, USA) used in this study were TH (1:1000), NANOG (1:1000), OCT4 (1:1000), FOXA2 (1:1000), LMX2 (1:1000), OTX2 (1:1000), CORIN (1:1000), GIRK2 (1:1000), ALDH1A1 (1:1000), and CALB (1:1000). The secondary antibodies used were Alexa Fluor 488 and 594 (1:1000; Invitrogen, Carlsbad, CA, USA).

### Electrophysiology

Neurons on days 110 to 140 were used to record spontaneous APs. Whole-cell current-clamp recordings were performed using the internal solution containing (in mM) 125 K-gluconate, 10 KCl, 10 N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 0.5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 3 Na<sub>2</sub>ATP, 0.5 Na<sub>2</sub>GTP, and 12 phosphocreatine at pH 7.25 and 280 mOsm. The cells were perfused with artificial cerebrospinal fluid, and membrane potentials were kept at -55 to -65 mV. Spontaneous APs were recorded without current injection. Data analyses were performed using Clampfit (Axon Instruments, Burlingame, CA, USA) and KaleidaGraph (Albeck Software, Reading, PA, USA).

### Statistical Analysis

All data are expressed as mean ± standard error of measurement. Unpaired 2-tailed Student's *t* tests were performed to evaluate whether the two groups were significantly different from each other. Statistical significance was set at *P* < 0.05.

## Results

### TALEN-Mediated Repair of Parkin Exon 3 Deletions in P002 Naivetropic iPSCs

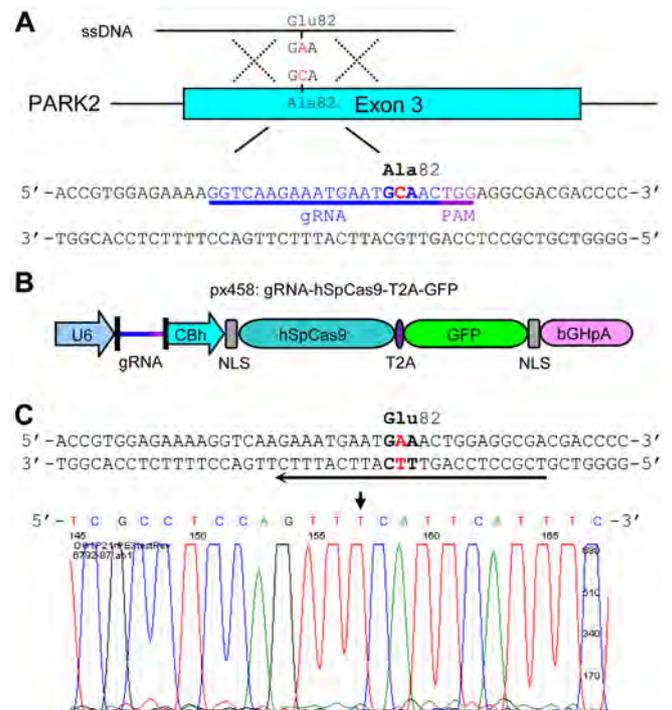
The PD patient P002 has homozygous deletions of exon 3 of parkin.<sup>6</sup> To repair the mutations by homologous recombination, we first performed a series of PCRs to locate the breakpoints of exon 3 deletions. We amplified 400 to 500 bp products at (a) 160 kb, (b) 80 kb, and (c) 40 kb upstream or (d) 30 kb and (e) 40 kb downstream of exon 3 (Fig. 1A). Fragment c was not amplified from the P002 genomic DNA, in contrast to the situation when the genomic DNA of the healthy subject C002 was used (Fig. 1B). It indicated that the fragment was deleted in P002. We designed additional primers at (1) 54 kb and (2) 50 kb upstream and (3) 21 kb downstream of exon 3 (Fig. 1A) and found that only fragment 2 was deleted in P002 (Fig. 1B). The sequences of all primers and the sizes of the PCR products are presented in Table S1. Using primers 1F and 3R, we amplified a 2-kb band from P002 (Fig. 1B), cloned it into the TA vector, and sequenced 10 individual clones. When the sequence was aligned with

the reference sequence of the human genome, a deletion of 75,866 bp was identified in the P002 genome (Fig. 1C). All 10 TA clones had the same sequence, indicating that both alleles had the same deletion. P002 is an offspring of a consanguineous marriage.

Based on the sequence flanking the breakpoints in P002, we constructed a gene-targeting vector to insert a 2.9-kb genomic DNA fragment containing exon 3 into the P002 genome. To increase the efficiency of homologous recombination, we generated a pair of TALENs to introduce a double-stranded cut 1188 bp upstream of the fused breakpoints (Fig. 1D) in naive iPSCs, which have significantly higher efficiency of homologous recombination than primed-state iPSCs do.<sup>19</sup> We introduced the targeting construct and the TALEN pair by nucleofection of P002 naive iPSCs.<sup>19</sup> Southern blotting with an external probe (Fig. 1D) showed that R22 was a homozygously targeted clone, whereas PR6 was a heterozygously targeted clone (Fig. 1E). Both clones were transiently transfected with a Cre plasmid to remove the puromycin-resistance cassette. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of mRNA isolated from naive iPSCs showed that parkin mRNA in P002 was homozygously repaired to the same state as in the C002 healthy subject (Fig. 1F). Sequencing of the RT-PCR products confirmed that the mutations were repaired. The protein expression levels of parkin in naive iPSCs were too low for Western blot detection. Thus, we reverted naive iPSCs to primed iPSCs,<sup>19</sup> which were differentiated into neuroepithelial cells for Western blotting. Parkin expression was fully restored in R22 cells and partially restored in PR6 cells (Fig. 1G). This pair of isogenic iPSCs enabled us to study whether parkin mutations were necessary for a phenotype.

### CRISPR/Cas9-Mediated A82E Point Mutations of Parkin in C002 Naive iPSCs

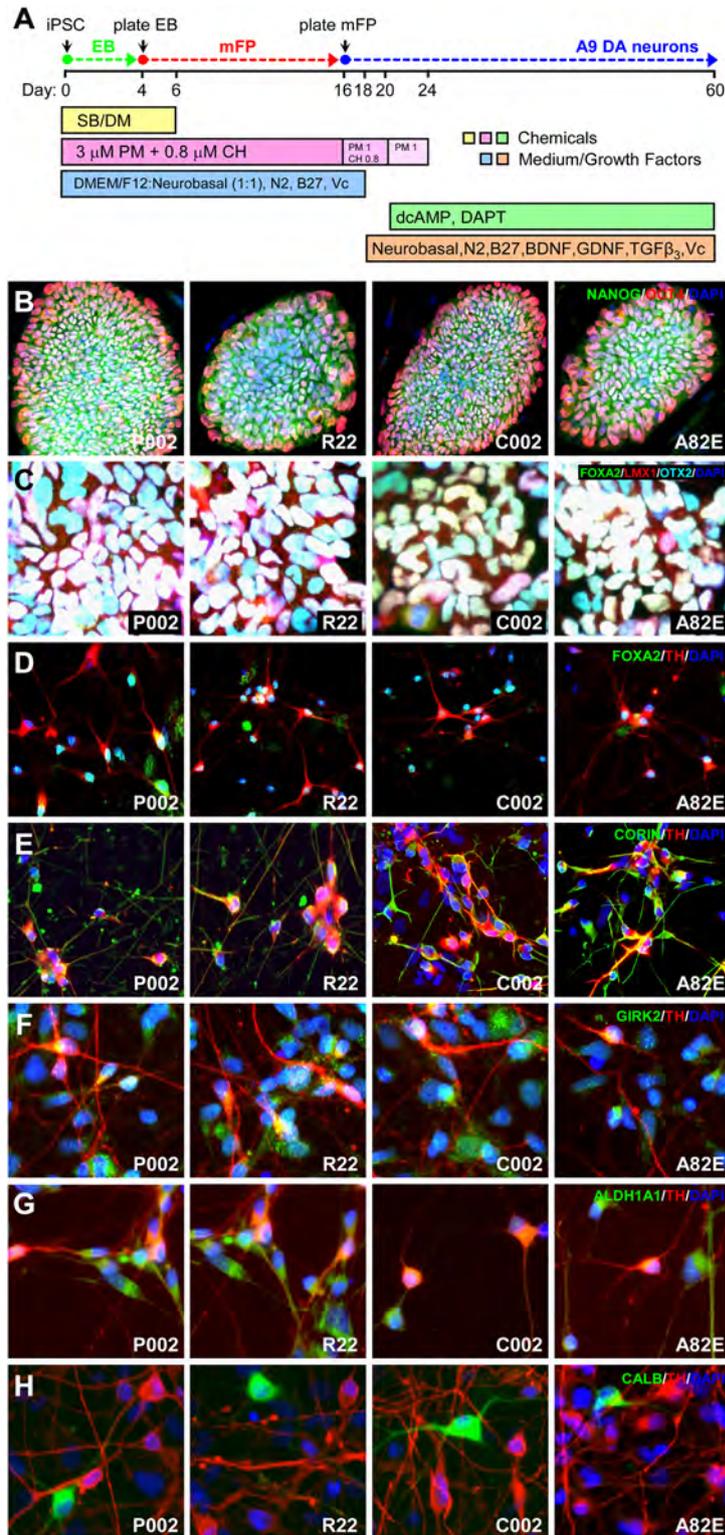
To establish that parkin mutations are sufficient to produce a phenotype, we introduced the PD-linked A82E point mutations in the normal C002 naive iPSCs that we have generated.<sup>19</sup> A gRNA was selected to introduce a double-stranded break near Ala82 in exon 3 of the human parkin gene (Fig. 2A). A 181-nucleotide ssDNA containing a C to A mutation, which corresponds to the A82E mutation, was used as the gene-targeting construct (Fig. 2A). Oligos corresponding to the gRNA sequence were annealed and cloned into the CRISPR/Cas9 construct with GFP for selection (Fig. 2B). We nucleofected the CRISPR/Cas9 construct and ssDNA into C002 naive iPSCs.<sup>19</sup> Then, we selected GFP<sup>+</sup> clones, expanded them, and extracted genomic DNA for PCR amplification of exon 3 of parkin. One clone contained homozygous mutations of A82E (Fig. 2C) and was named as A82E.



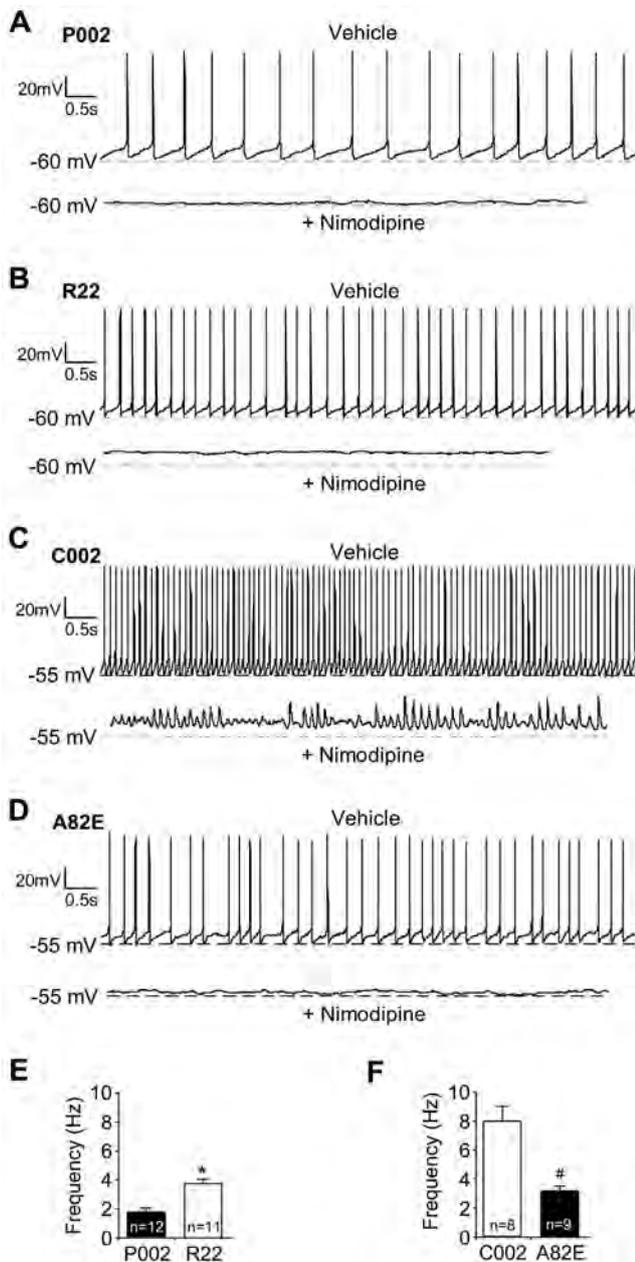
**FIG. 2.** Introducing A82E mutations to the parkin gene in naive iPSCs (induced pluripotent stem cells) from a healthy subject using CRISPR/Cas9. **(A)** A gene-targeting strategy to introduce A82E mutations (C to A in DNA) in exon 3 of parkin. The location and length of the single-stranded DNA (ssDNA) targeting construct are drawn to scale on top of exon 3. The guide RNA (gRNA) sequence in the CRISPR construct is shown below exon 3. **(B)** A single-vector CRISPR/Cas9 construct containing the gRNA sequence above was electroporated into naive iPSCs, which were picked for the transient expression of GFP. **(C)** After PCR (polymerase chain reaction) amplification of exon 3 of parkin from genomic DNA, a sequencing trace of a homozygously targeted clone (named A82E) showed the presence of A82E mutations. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

### Directed Differentiation of Isogenic Naive iPSCs to A9 DA Neurons

We reverted the two pairs of isogenic naive iPSCs (P002 and R22, C002 and A82E) to the primed state by switching the naive iPSC medium with 2i, LIF, and DOX to the standard hESC medium that contains bFGF.<sup>19</sup> Using the method that we have developed recently,<sup>15</sup> we differentiated the four lines of primed iPSCs into A9 DA neurons (Fig. 3A). Primed iPSCs, which showed very similar expression of pluripotency markers NANAG and OCT4 (Fig. 3B), were differentiated into mFP cells, which exhibited similar expression of FOXA2, LMX1, and OTX2 across the four lines on day 14 of differentiation (Fig. 3C). Further differentiation of mFP cells in neuronal maturation medium containing the Notch inhibitor DAPT, dcAMP, and various neurotrophic factors generated TH<sup>+</sup> neurons that coexpressed the midbrain marker FOXA2 (Fig. 3D) and the floor plate marker CORIN (Fig. 3E) on day 42. Immunostaining of these neurons on day 60 showed that



**FIG. 3.** Directed differentiation of human iPSCs (induced pluripotent stem cells) to A9 DA (dopaminergic) neurons. **(A)** The protocol to differentiate human iPSCs to embryoid bodies (EB), midbrain floor plate (mFP) cells, and then A9 DA neurons. We used the double SMAD inhibitors (SB, SB431542 at 10  $\mu$ M, and DM, dorsomorphin, 5  $\mu$ M) to drive the differentiation of iPSCs into neural lineages, appropriate levels of purmorphamine (PM, 3  $\mu$ M) to specify the dorsal–ventral position, and CHIR99021 (CH, 0.8  $\mu$ M) to determine the rostral–caudal location. **(B)** The resulting mFP cells were differentiated into A9 DA neurons with the indicated compounds and growth factors to enhance the maturation of neurons. The four lines of isogenic-primed iPSCs (P002 and R22, C002 and A82E) were co-stained for pluripotency markers NANOG and OCT4, along with DAPI. **(C)** mFP cells differentiated from the four lines of isogenic iPSCs coexpressed FOXA2, LMX1, and OTX2. **(D–H)** The iPSC-derived neurons coexpressed TH with **(D)** the midbrain marker FOXA2, **(E)** the floor plate marker CORIN, **(F)** the A9 markers GIRK2, **(G)** and ALDH1A1 but not **(H)** the A10 marker CALBINDIN (CALB). [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIG. 4.** Parkin mutations decreased the frequency of pacemaking action potentials. (A–D) Representative pacemaking action potential traces of neurons (on days 110–140) differentiated from (A) P002, (B) R22, (C) C002, or (D) A82E iPSCs (induced pluripotent stem cells). Application of the L-type  $\text{Ca}^{2+}$  channel blocker nimodipine (20  $\mu\text{M}$ ) abolished these APs (action potentials). (E) AP frequency significantly increased when parkin mutations in P002 neurons ( $n = 12$ ) were repaired in R22 neurons ( $n = 11$ , \* $P < 0.001$ ,  $t$  test). (F) AP frequency was significantly lower in A82E neurons ( $n = 9$ ) compared to that in the parental C002 neurons ( $n = 8$ , # $P < 0.01$ ,  $t$  test).

they coexpressed TH and markers for A9 DA neurons, such as GIRK2<sup>21</sup> (Fig. 3F) and ALDH1A1<sup>21</sup> (Fig. 3G), but largely not CALBINDIN (Fig. 3H), which is expressed in A10 DA neurons.<sup>22</sup> Separate channels of the merged images for Figure 3B–H are shown in Figure S1.

## Parkin Mutations Significantly Decreased the Frequency of Pacemaking APs in A9 DA Neurons

We performed electrophysiological recordings in mature A9 DA neurons differentiated from the four lines of iPSCs for 110 to 140 days. Spontaneous APs in the absence of current injections were recorded in all four lines of iPSC-derived A9 DA neurons (Fig. 4A–D). Application of the L-type  $\text{Ca}^{2+}$  channel blocker nimodipine (20  $\mu\text{M}$ ) abolished the APs (Fig. 4A–D), which confirms that these APs are pacemaking activities, as we have demonstrated recently.<sup>15</sup> The frequency of pacemaking APs in P002 neurons ( $1.79 \pm 0.17$  Hz,  $n = 12$  neurons) (Fig. 4A) significantly increased when the exon 3 deletion mutations of parkin were repaired in the isogenic R22 neurons ( $3.72 \pm 0.31$  Hz,  $n = 11$  neurons,  $P < 0.001$ ,  $t$  test) (Fig. 4B,E). Consistent with these results, the frequency of pacemaking APs in the healthy control C002 neurons ( $8.01 \pm 1.02$  Hz,  $n = 8$  neurons) (Fig. 4C) significantly decreased when parkin mutations were introduced in the isogenic A82E neurons ( $3.17 \pm 0.32$  Hz,  $n = 9$ ,  $P < 0.01$ ,  $t$  test) (Fig. 4D,F). The results from the two isogenic pairs of A9 DA neurons suggest that parkin mutations are necessary and sufficient in reducing the frequency of pacemaking APs.

## Discussion

In this study, we generated two pairs of isogenic naitropic iPSCs with or without parkin mutations and differentiated them into A9 DA neurons to study the impact of parkin mutations on the function of these neurons. The repair of parkin mutations in P002 neurons from a PD patient significantly increased the frequency of pacemaking APs, whereas the introduction of parkin mutations to C002 neurons from a control subject significantly decreased the frequency of pacemaking APs. The reciprocal experiments demonstrated that parkin mutations were necessary and sufficient to reduce the frequency of pacemaking APs.

There are at least two mechanisms that ensure the robust delivery of dopamine to the striatum by nigral DA neurons. One is the massive axon arborization of a single nigral DA neuron, which occupies 2.7% of the striatal volume in a rat.<sup>26</sup> This means that only 37 nigral DA neurons are needed in one hemisphere to achieve 1× innervation of the whole striatum on the same side.<sup>26</sup> The number of nigral DA neurons in a rat brain hemisphere is estimated to be 3500 to 7200,<sup>27,28</sup> which serves to innervate the striatum at more than 100× redundancy. The other mechanism is the tonic release of dopamine induced by pacemaking APs of nigral DA neurons.<sup>10,29</sup> Indeed, striatal dopamine concentration is not significantly

decreased until 80% of nigral DA neurons are lesioned by 6-hydroxydopamine in rats.<sup>30,31</sup> Thus, the reduction in the frequency of pacemaking APs in the absence of functional parkin may compromise the robustness of dopamine delivery only when there is a sufficient loss of nigral DA neurons, which is age dependent. This may explain why the strong effect that we observed in the culture dish, which may reflect the situation in the embryonic brain, results in clinical symptoms decades later. As parkin limits dopamine-induced oxidative stress<sup>32</sup> by suppressing the transcription of monoamine oxidases,<sup>33,34</sup> parkin mutations render human midbrain DA neurons much more vulnerable to oxyradicals produced in the routine catabolism of dopamine through oxidative deamination.<sup>6</sup>

The impact of parkin mutations on the frequency of pacemaking APs is not uniform on the genetic background of the patient (P002) and the healthy subject (C002). In A9 DA neurons without parkin mutations, the AP frequency was  $3.72 \pm 0.31$  Hz in R22 neurons (repaired from P002) and  $8.01 \pm 1.02$  Hz in C002 neurons (Fig. 4E,F). The two subjects have very different genetic characteristics. P002 is a female Japanese offspring of a consanguineous marriage, with substantially higher degrees of homozygosity in her genome. Her PD symptoms started at age 40 years, and she was sampled at age 45 years. C002 is a healthy Caucasian male sampled at age 60 years. The differential expression of many genes in A9 DA neurons derived from both subjects may contribute to the different frequencies of their pacemaking APs in the absence of parkin mutations. Future studies exploring the transcriptome and proteome of these isogenic A9 DA neurons under different genetic backgrounds may identify the genetic factors that contribute to the baseline difference in AP frequencies and how parkin interacts with these factors to influence AP frequency.

The main limitation of this study is the singular finding that pacemaking AP frequency is decreased by parkin mutations. The generation of mature A9 DA pacemakers from iPSCs required significant effort and time (up to 140 days), which substantially constrained exploratory studies on these neurons, particularly using electrophysiology. It is unknown what factors determine the frequency of pacemaking APs. Possible contributors may include various L-type  $\text{Ca}^{2+}$  channels, dopamine  $\text{D}_2$  autoreceptors, voltage-gated  $\text{Na}^+$  and  $\text{K}^+$  channels, K-ATP channels, and HCN channels. Separate studies, for example, on intracellular  $\text{Ca}^{2+}$  levels, K-ATP currents,  $I_h$  current, and  $\text{D}_2$  autoreceptor functions may uncover critical contributing factors. Once a key contributor is identified, it would then become feasible to study how parkin mutations affect the function of the key contributor. As parkin regulates mitophagy,<sup>35</sup> it is conceivable that mitochondrial dysfunction as a consequence of parkin mutation may affect the ability of mitochondria to produce ATP and buffer the rise of intracellular  $\text{Ca}^{2+}$ . Cellular

ATP levels regulate the activity of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, which critically affects membrane excitability. Intracellular  $\text{Ca}^{2+}$  concentration regulates  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from intracellular stores, such as the endoplasmic reticulum. It seems likely that parkin may regulate the frequency of pacemaking APs through complex and intertwined mechanisms that may not fit a simple qualitative narrative. A more quantitative understanding of the enzymatic property of parkin is needed to model its cellular functions in the context of human A9 DA neurons. Regardless of these cell biology questions, the physiological impact of parkin mutations on autonomous pacemaking of human A9 DA neurons is highly important to the pathogenesis of PD. Further studies on additional pairs of isogenic iPSC-derived A9 DA neurons with or without parkin mutation will confirm the consistent directionality of the changes. These studies would be most meaningful when we recruit subjects of different genetic backgrounds.

## Conclusion

The study generated two isogenic pairs of human A9 DA neurons with or without parkin mutation on the genetic backgrounds of a healthy subject and a PD patient. The frequency of pacemaking APs significantly decreased when parkin mutations were introduced in normal neurons and significantly increased when parkin mutations in PD neurons were repaired. The results established that parkin mutations were sufficient and necessary to reduce the robustness of autonomous pacemaking in human A9 DA neurons, a critical function required for motor control. ●

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## Data Availability Statement

All data are included in the manuscript.

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## Supporting Data

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J.P.: conception and design, data acquisition and analysis, drafting of the text. L.L.: conception and design, data acquisition and analysis, drafting of the text. H.J.: conception and design, data acquisition and analysis, drafting of the text. Z.H.: data acquisition and analysis. H.L.: data acquisition and analysis. Z.Y.: drafting and editing of the text. B.Z.: conception and design, drafting of the text. J.F.: conception and design, data analysis, drafting, editing, and revising of the text.