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Generation of RAB39B knockout isogenic human embryonic stem cell lines to model RAB39B-mediated Parkinson's disease

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Publication Details

Gao, Y., Wilson, G., Bozaoglu, K., Elefanty, A. G., Stanley, E. G., Dottori, M. & Lockhart, P. J. (2018). Generation of RAB39B knockout isogenic human embryonic stem cell lines to model RAB39B-mediated Parkinson's disease. Stem Cell Research, 28 161-164.

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Generation of RAB39B knockout isogenic human embryonic stem cell lines to model RAB39B-mediated Parkinson's disease

Abstract

Mutations in RAB39B are a known cause of X-linked early onset Parkinson's disease. Isogenic human embryonic stem cell lines carrying two independent deletions of RAB39B were generated using CRISPR/Cas9 genome editing tool. The deletions were confirmed by PCR and direct sequence analysis in two edited stem cell lines. Both cell lines showed pluripotency and displayed a normal karyotype. Further, they were able to form embryoid bodies in vitro, and express markers indicative of differentiation to the three germ layers.

Disciplines

Medicine and Health Sciences

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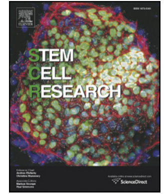
This journal article is available at Research Online: http://ro.uow.edu.au/ihmri/1207

Stem Cell Research 28 (2018) 161–164

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



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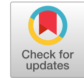

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A R T I C L E I N F O

Article history:
Received 24 January 2018
Received in revised form 14 February 2018
Accepted 20 February 2018
Available online 21 February 2018

A B S T R A C T

Mutations in *RAB39B* are a known cause of X-linked early onset Parkinson's disease. Isogenic human embryonic stem cell lines carrying two independent deletions of *RAB39B* were generated using CRISPR/Cas9 genome editing tool. The deletions were confirmed by PCR and direct sequence analysis in two edited stem cell lines. Both cell lines showed pluripotency and displayed a normal karyotype. Further, they were able to form embryoid bodies *in vitro*, and express markers indicative of differentiation to the three germ layers.

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Resource Table:

Unique stem cell lines identifier	SCSe001-A-1/MCRIe002-A-1; SCSe001-A-2/MCRIe002-A-2
Alternative names of stem cell lines	MEL-1 10.2; MEL-1 30.3
Institution	Murdoch Children's Research Institute
Contact information of distributor	paul.lockhart@mcri.edu.au
Type of cell lines	ESC
Origin	Human
Cell Source	N/A
Clonality	Clonal
Method of reprogramming	N/A
Multiline rationale	Isogenic clones
Gene modification	Yes
Type of modification	Gene deletion
Associated disease	Parkinson's Disease
Gene/locus	*RAB39B*/Xq28
Method of modification	CRISPR/CAS9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	October 2017
Cell line repository/bank	The parental cell line MEL-1 (SCSe001-A) is listed on the NIH Human Embryonic Stem Cell Registry (NIH approval number NIHhESC-11-0139).
Ethical approval	This study was approved by the ethics committee of the Royal Children's Hospital (HREC 28097).

1. Resource utility

The isogenic human embryonic stem cell (hESC) lines generated are valuable models for studying the pathogenic mechanisms underlying disease.

1.1. Resource details

Mutations in *RAB39B* are a cause of X-linked early onset Parkinson's disease (PD) (Wilson et al., 2014; Mata et al., 2015; Lesage et al., 2015; Shi et al., 2016). We generated isogenic hESC lines with a deletion of *RAB39B* to model this genetic form of PD. These lines will facilitate a better understanding of the pathogenesis of PD, in addition to improved understanding of the function of *RAB39B* and its therapeutic potential

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https://doi.org/10.1016/j.scr.2018.02.015
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in PD. The parental male hESC line MEL-1 (SCSe001-A) was utilised and the deletions were generated using two CRISPR guides targeted to either exon 1 or 2 of *RAB39B* (Table 3). Two of eight independent single colonies isolated were selected for further analysis based on cell morphology and successful genome editing, MEL-1 10.2 and MEL-1 30.3. Both hESC lines displayed a typical hESC morphology consistent with the unmodified parental line, including small and tightly packed cells, high nucleus to cytoplasm ratio and a prominent nucleoli (Fig. 1A, B, scale bar = 100 μM). Successful deletion of *RAB39B* was confirmed by PCR analysis. Further, Sanger sequencing analysis showed a 5 base pair deletion in exon 1 of *RAB39B* in MEL-1 10.2 (NM_171998.3: c.82_86del, p.Phe28Argfs*9), and a 148 base pair deletion spanning exons 1 and 2 of *RAB39B* in MEL-1 30.3 (NM_171998.3: c.87_234del, p.Glu30Thrfs*5) (Fig. 1C). The expression of pluripotency markers OCT4A, SOX2 and NANOG, were verified by immunofluorescence staining (Fig. 1D, E, scale bar = 50 μM). Further, flow cytometry analysis utilising the markers SSEA-4, CD9 and EPCAM was performed to quantify pluripotent cells. Mouse embryonic fibroblast (MEF) feeders negative for these pluripotent markers were present in the cultures, and were thus utilised in gating. >80% of viable cells sorted were positive

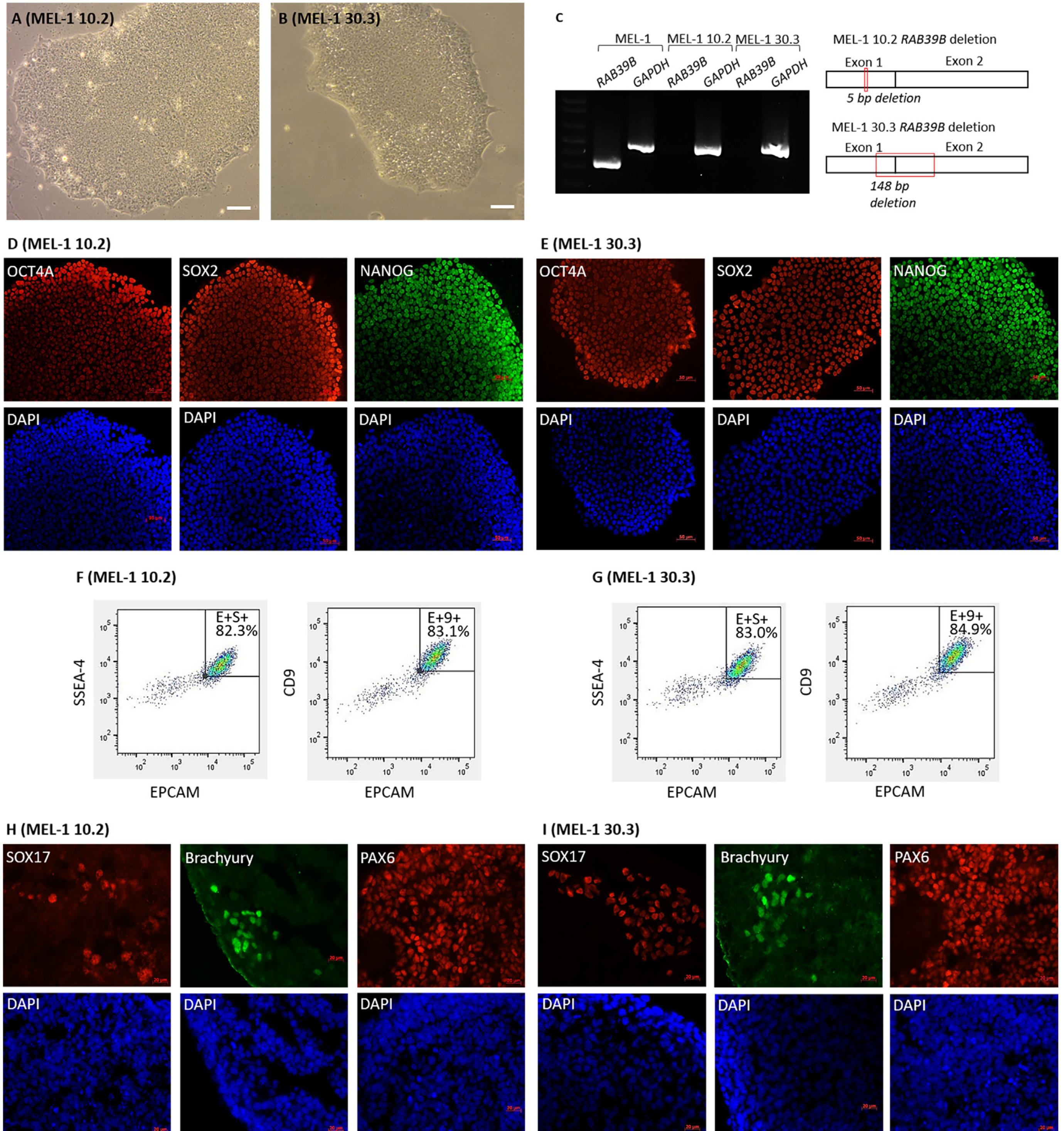

Fig. 1. Characterization of RAB39B-knockout pluripotent stem cells.

Table 1
Summary of lines.

hESC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
SCSe001-A-1/ MCRIe002-A-1	MEL-1 10.2	Male	N/A	European	NM_171998.3: c.82_86del	Parkinson's Disease
SCSe001-A-2/ MCRIe002-A-2	MEL-1 30.3	Male	N/A	European	NM_171998.3: c.87_234del	Parkinson's Disease

for both SSEA-4 and EPCAM expression, and for both CD9 and EPCAM expression (Fig. 1F, G). The hESC lines formed embryoid bodies *in vitro*, which expressed markers consistent with development of the three germ layers. This included SOX17 marking endoderm, Brachyury marking mesoderm and PAX6 marking ectoderm development (Fig. 1H, I, scale bar = 20 μM). In addition, the derived hESC RAB39B mutant lines (MEL-1 10.2 and MEL-1 30.3) showed a normal karyotype (46, XY) and an identical genotype with the parental MEL-1 cell line when analysed by array comparative genomic hybridisation (SNP array) confirming no alterations in genomic integrity during editing. In conclusion, we report the generation and characterization of novel stem cell models for *RAB39B*-mediated PD, which can be differentiated into neuronal models to study the pathogenic mechanisms underlying PD. (See Tables 1 and 2.)

2. Materials and methods

2.1. Genome editing of hESC

To genetically modify the hESC line SCSe001-A (MEL-1), cells were transfected with two CRISPR guides targeted to *RAB39B* (Table 3) by electroporation, using the Neon transfection system (Life Technologies). Briefly, ~1×10^6 cells were harvested for electroporation, after which the cells were immediately plated down onto MEF-coated plates in hESC medium containing DMEM/F12 media (Gibco/Invitrogen) supplemented with 20% knockout serum replacer (Gibco/Invitrogen), 1× non-essential amino acids (Gibco/Invitrogen), 1× GlutaMAX-I (Gibco/Invitrogen), 1 × 2-Mercaptoethanol (Gibco/Invitrogen) and 10 ng/ml basic fibroblast growth factor (PeproTech). After recovering for 2–3 days, the cells were single cell sorted into 96 well plates on the basis of GFP expression, expression of which was associated with uptake of the CRISPR guide expressing vectors (Ran et al., 2013). At ~2 weeks single hESC colonies began to appear and were picked for expansion and genotyping. Subsequently, successfully edited hESC lines were converted to feeder free bulk culture on plates coated with Vitronectin (Stemcell technologies), in E8 medium (Stemcell technologies) for continued culture. All cells were cultured at 37 °C with 10% CO_2.

2.2. cDNA synthesis and genotyping

Total RNA for cDNA synthesis was extracted from hESC pellets using the SV Total RNA Isolation System according to manufacturer's instructions (Promega). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit according to manufacturer's instructions (Roche). For genotyping, DNA was amplified using MyTaq DNA polymerase (Bioline) using specified primers (Table 3).

2.3. Immunofluorescence (IF)

hESC were cultured on sterilized glass coverslips for IF analysis. Cells were fixed with 4% Paraformaldehyde (PFA) for 10 min at room temperature, permeabilized in 0.2% Triton™ X-100 (Sigma) for 10 min at room temperature, and blocked in 20% Goat Serum (Life Technologies) for 60 min at room temperature. Cells were then incubated with primary antibodies at 4 °C overnight, followed by secondary antibodies for 60 min at room temperature (Table 3). The coverslips were mounted on slides with mounting media containing DAPI (VectorLabs). Images were captured with AXIO Imager M1 light microscope and Axiocam MRm camera (Carl-Zeiss).

2.4. Flow cytometry (FACs) analysis

hESC were harvested and filtered through a cell-strainer cap fettle to a FACs tube. Cells were then incubated with primary antibodies for 15 min on ice (Table 3), and stained with Propidium Iodide (Sigma) prior to acquisition (LSRFortessa, BD Bioscience).

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1A, B
Phenotype	Qualitative analysis (Immunofluorescence)	OCT4A, NANOG, SOX2	Fig. 1D, E
	Quantitative analysis (Flow cytometry)	SSEA-4, CD9, Ep-CAM	Fig. 1F, G
Genotype	Karyotype (SNP array)	arr(1−22)×2,(XY)×1 Resolution 0.50 Mb	Submitted in archive with journal
Identity	Genetic analysis	SNPduo comparative analysis performed to compare parental and both derived clones	Submitted in archive with journal
		Identical genotypes (>99.9%) for the entire genome, indicating derived cell lines are from the same individual	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing (PCR)	MEL-1 10.2 (NM_171998.3: c.82_86del) MEL-1 30.3 (NM_171998.3: c.87_234del)	Fig. 1C
	Southern Blot OR WGS	N/A	–
Microbiology and virology	Mycoplasma	Negative	Data with author
Differentiation potential	Embryoid body formation (Immunofluorescence)	Endoderm: SOX17 Mesoderm: Brachyury Ectoderm: PAX6	Fig. 1H, I
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	–
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	–
	HLA tissue typing	N/A	–

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Marker	Mouse anti-OCT4A	1:200	R and D Systems Cat# MAB17591, RRID: AB_10719296
Pluripotency Marker	Mouse anti-SOX2	1:200	R and D Systems Cat# MAB2018, RRID: AB_358009
Pluripotency Marker	Goat anti-NANOG	1:200	R and D Systems Cat# AF1997, RRID: AB_355097
Pluripotency Marker	SSEA-4 APC	1:100	BioLegend Cat# 330408, RRID: AB_1089200
Pluripotency Marker	CD9	1:20	BD Biosciences Cat# 555371, RRID: AB_395773
Pluripotency Marker	CD36 (Ep-CAM) PeCy7	1:100	BioLegend Cat# 324222, RRID: AB_2561506
Differentiation Marker	Goat anti-SOX17	1:200	R and D Systems Cat# AF1924, RRID: AB_355060
Differentiation Marker	Goat anti-Brachyury	1:200	R and D Systems Cat# AF2085, RRID: AB_2200235
Differentiation Marker	Mouse anti-PAX6	1:200	DSHB Cat# pax6, RRID:AB_528427
Secondary antibody	Goat anti-Mouse IgG, Alexa Fluor 594	1:1000	Thermo Fisher Scientific Cat# A-11032, RRID: AB_2534091
Secondary antibody	Donkey anti-Goat IgG, Alexa Fluor 488	1:1000	Thermo Fisher Scientific Cat# A-11055, RRID: AB_2534102
Secondary antibody	Donkey anti-Goat IgG, Alexa Fluor 594	1:1000	Thermo Fisher Scientific Cat# A-11058, RRID: AB_2534105
Primers			
	Target	**Forward/Reverse primer (5′-3′)**	
CRISPR guide	*RAB39B* Exon 1	5'-CTGATCCGCCGCTTCACCGA-3'	
CRISPR guide	*RAB39B* Exon 2	5'-CCATCACTCGCGCCTACTAC-3'	
Genotyping and targeted mutation sequencing	*RAB39B* Forward	5'-CTGATCCGCCGCTTCACCGA-3'	
Genotyping and targeted mutation sequencing	*RAB39B* Reverse	5'-CGAGTCACTTGCCTCTGTGT-3'	
House-Keeping gene	*GAPDH* Forward	5'-CAGCCGAGCCACATCGCTCAG-3'	
House-Keeping gene	*GAPDH* Reverse	5'-TGAGGCTGTTGTCATACTTCTC-3'	

2.5. Embryoid body (EB) formation

EBs were formed by spin-aggregation. Briefly, hESC were seeded in ultra-low adherence 96 well plates, aggregated by centrifugation and cultured in EB medium containing E8 medium (Stemcell technologies), 0.02% Gentamicin (Gibco/Invitrogen) and 0.5% polyvinyl alcohol (PVA) (Sigma). After 24 h, the cells were cultured in E8 medium supplemented only with 0.02% Gentamicin, and medium was changed every 2 days for 3 weeks before analysis. For IF analysis, EBs were fixed with 4% PFA for 30 min, then placed in a serial dilution of PBS-buffered sucrose solutions (10, 20 and 30% respectively) at room temperature. EBs were then embedded in Optimal cutting temperature compound (OCT), cryosectioned at 10 μm before IF staining was carried out as described above.

2.6. Karyotype analysis

We performed molecular karyotype analysis using the Infinium CoreExome-24 SNP array and compared the data to the human reference sequence hg19/GRCh37 (Feb 2009). Both derived RAB39B mutant lines (MEL-1 10.2 and MEL-1 30.3) showed a normal karyotype. To confirm the integrity of the clones, we performed Infinium CoreExome-24 SNP array analysis on the parental MEL-1 line. We then compared the SNP array data from all lines the using SNPduo comparative analysis (http://pevsnerlab.kennedykrieger.org/SNPduo/). This analysis demonstrated that the parental and mutant lines were identical, confirming no alterations to the genomic integrity of the mutant clones occurred during gene editing.

2.7. Mycoplasma detection

Absence of mycoplasma contamination was confirmed by real-time PCR by the commercial service provider Cerberus Sciences (Adelaide, Australia).

Author disclosure statement

There are no conflicts of interest.

Acknowledgements

The authors would like to thank the following laboratory and facility staff for their generous support and assistance: Tanya Labonne and Dr. Freya Bruveris (Blood Cell Development & Disease laboratory, Murdoch Children's Research Institute), Dr. Katerina Vlahos, Penny McDonald, Koula Sourris and Robyn Mayberry (iPSC Core Facility, Murdoch Children's Research Institute) and Dr. Matthew Burton (Flow Cytometry Facility, Murdoch Children's Research Institute). This study was funded in part by National Health & Medical Research Council (Australia) project grants (GNT1041860, GNT1144724) and the Michael J. Fox Foundation/Shake it up Australia grant (12173) to P.J.L. Additional infrastructure funding to the Murdoch Children's Research Institute was provided by the Australian Government National Health and Medical Research Council (NHMRC) Independent Research Institute Infrastructure Support Scheme and the Victorian Government's Operational Infrastructure Support Program. The MCRI iPSC Core Facility is supported by the Stafford Fox Medical Research Foundation. A.G.E. and E.G.S are Research Fellows of the NHMRC.

References

Lesage, S., Bras, J., Cormier-Dequaire, C., Condroyer, C., Nicolas, A., Darwent, L., Guerreiro, R., Majounie, E., Federoff, M., Heutink, P., Wood, N.W., Gasser, T., Hardy, J., Tison, F., Singleton, A., Brice, A., 2015. And For the French Parkinson's Disease Genetics Study Group (PDG) and the International Parkinson's Disease Genomics Consortium (IPDGC), Loss-of-function mutations in RAB39B are associated with typical early-onset Parkinson disease. Neurol. Genet. 1, e9.

Mata, I.F., Jang, Y., Kim, C.-H.H., Hanna, D.S., Dorschner, M.O., Samii, A., Agarwal, P., Roberts, J.W., Klepitskaya, O., Shprecher, D.R., Chung, K.A., Factor, S.A., Espay, A.J., Revilla, F.J., Higgins, D.S., Litvan, I., Leverenz, J.B., Yearout, D., Inca-Martinez, M., Martinez, E., Thompson, T.R., Cholerton, B.A., Hu, S.-C.C., Edwards, K.L., Kim, K.-S.S., Zabetian, C.P., 2015. The RAB39B p.G192R mutation causes X-linked dominant Parkinson's disease. Mol. Neurodegener. 10, 50.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., Zhang, F., 2013. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308.

Shi, C.-H.H., Zhang, S.-Y.Y., Yang, Z.-H.H., Yang, J., Shang, D.-D.D., Mao, C.-Y.Y., Liu, H., Hou, H.-M.M., Shi, M.-M.M., Wu, J., Xu, Y.-M.M., 2016. A novel RAB39B gene mutation in X-linked juvenile parkinsonism with basal ganglia calcification. Mov Disorders 31, 1905–1909.

Wilson, G.R., Sim, J.C., McLean, C., Giannandrea, M., Galea, C.A., Riseley, J.R., Stephenson, S.E., Fitzpatrick, E., Haas, S.A., Pope, K., Hogan, K.J., Gregg, R.G., Bromhead, C.J., Wargowski, D.S., Lawrence, C.H., James, P.A., Churchyard, A., Gao, Y., Phelan, D.G., Gillies, G., Salce, N., Stanford, L., Marsh, A.P., Mignogna, M.L., Hayflick, S.J., Leventer, R.J., Delatycki, M.B., Mellick, G.D., Kalscheuer, V.M., D'Adamo, P., Bahlo, M., Amor, D.J., Lockhart, P.J., 2014. Mutations in RAB39B cause X-linked intellectual disability and early-onset Parkinson disease with α-synuclein pathology. Am. J. Hum. Genet. 95, 729–735.