

Short communication

Cytoplasmic aggregates of dynactin in iPSC-derived tyrosine hydroxylase-positive neurons from a patient with Perry syndrome



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ABSTRACT

Background: Perry syndrome is a rare autosomal dominant disorder clinically characterized by parkinsonism with depression/apathy, weight loss, and central hypoventilation. Eight mutations in *DCTN1* gene have been reported. A novel disease model is required because the detailed pathogenesis remains unclear.

Methods: To develop a novel model, we generated induced pluripotent stem cells (iPSCs) from a Perry syndrome patient with F52L mutation in *DCTN1*, and describe clinical and neuroimaging investigations. We differentiated iPSCs into tyrosine hydroxylase (TH)-positive neurons. Immunocytochemistry analyses of control and mutant were performed.

Results: The patient displayed levodopa responsive parkinsonism. Dopamine transporter single photon emission tomography showed markedly decreased uptake in the striatum, and meta-iodobenzylguanidine cardiac scintigraphy also showed decreased uptake. Perry syndrome TH-positive neurons showed dynactin aggregates in cytoplasm.

Conclusions: TH-positive neurons from Perry syndrome iPSCs recapitulated an aspect of the disease phenotype of Perry syndrome.

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1. Introduction

Perry syndrome is a rare genetic disorder associated with parkinsonism, depression/apathy, weight loss, and central hypoventilation [1]. Histology showed neuronal loss and gliosis in the substantia nigra, and transactive response DNA-binding protein 43 (TDP-43) and dynactin-positive inclusions in the neurons of the basal ganglia and brainstem including substantia nigra [1]. Eight

point mutations have been identified: F52L, G71A, G67D, G71R, G71E, T72P, Q74P, and Y78C located in exon 2 of *DCTN1*. The clinical presentations caused by each mutation are almost the same, but F52L mutation occurs later in the disease course [2]. In this study, we successfully obtained induced pluripotent stem cells (iPSCs) from a patient with F52L and differentiated them into tyrosine hydroxylase (TH)-positive neurons.

2. Methods

2.1. Participant

The subject of this study was a Japanese man whose genetic presentation has already been briefly described. He was a IV-2 patient in a previous report [2]. He provided written informed consent. We report the clinical course and imaging study of this

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patient.

2.2. Standard protocol approval

This study was approved by the Institutional Review Boards of Kyoto University and Fukuoka University.

2.3. Generation of iPSCs and TH-positive neuron differentiation

Peripheral blood mononuclear cells were reprogrammed by inducing the episomal vectors carrying OCT3/4, SOX2, KLF4, L-MYC, LIN28, EBNA1, and p53 carboxy-terminal dominant-negative fragment [3]. We used a feeder-free culture system to establish iPSCs [4]. We produced control iPSCs from an unrelated healthy individual and Perry syndrome patient iPSCs. We characterized TH-positive neurons by quick embryoid body-like aggregate method [5] and dual SMAD inhibition by modifying previous procedures [6]. Briefly, a floating culture of cell aggregates was introduced, and LDN193189, A83-01, purmorphamine, CHIR99021, fibroblast growth factor 8, brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, dibutyryl cyclic AMP, and ascorbic acid were added. TH-positive neurons were evaluated 6–9 weeks later *in vitro*.

2.4. HEK293T cells with of p150^{glued} -GFP overexpression

Dynactin consists of many subunits, with p150^{glued} protein, encoded by *DCTN1* gene, being one of them. HEK293T cells were transiently transfected with GFP-tagged wild-type (WT) and mutant F52L p150^{glued} (c.156T > A) using Lipofectamine LTX (Thermo Fisher Scientific) according to the manufacturer's protocol. Analysis was performed 24 h after transfection.

2.5. Immunocytochemistry

HEK293T cells transfected with GFP-tagged WT and mutant F52L p150^{glued}, and TH-positive neurons derived from the control and patient iPSCs were assessed by immunocytochemistry.

Cells were fixed with 4% paraformaldehyde and blocked with PBS containing 5% fetal bovine serum or Blocking One Histo (Nacalai Tesque). DAPI (4', 6-diamidino-2-phenylindole) (Life Technologies) was used to label nuclei. Fluorescence imaging was performed using BIOREVO (Keyence), IN CELL Analyzer 6000 (GE Healthcare), and Delta Vision (Applied Precision). The following primary antibodies were used: NANOG (REPROCELL, 1:500), SSEA-4 (Millipore, 1:1,000), SOX-17 (R&D Systems, 1:300), α SMA (DAKO, 1:3,000), Tuj1 (Covance, 1:2,000), TH (Millipore, 1:600), p150^{glued} (abcam, 1:200), TDP-43 (Proteintech, 1:250), ubiquitin (DAKO, 1:1,000), α -tubulin (SIGMA-ALDRICH, 1:1,000), p50 (Santa Cruz Biotechnology, 1:50), p62 (Santa Cruz Biotechnology, 1:50), TOM20 (Santa Cruz Biotechnology, 1:50), LAMP2 (abcam, 1:100), Calnexin (Enzo Life Sciences, 1:200). We quantified the number of p150^{glued} aggregate-positive cells in HEK293T or iPSC-derived TH-positive neurons. We defined the p150^{glued} aggregates as fluorescent accumulations, observed in the cytosol of iPSC-derived TH-positive neurons by using Z-stacked slices. Detection of aggregates was performed blindly. Results represent quantitation from randomly selected fields for each mutation or clone in a blinded manner. Results are representative of three experiments for HEK293T cells or four experiments for iPSC-derived TH-positive neurons.

2.6. Statistics

T-test was performed. Significance was set at $P < 0.05$.

3. Results

3.1. Case report

The patient (Fig. 1A) was a 61-year-old man. He had a family history of parkinsonism in his mother and aunt. He presented with depression at the age of 53. At age 55, he developed progressive bradykinesia, walking difficulty and mild tremor in his hands. At that time, neurological examination revealed bradykinesia, rigidity, postural instability, and postural tremor in his hands. He was treated with levodopa/carbidopa, which provided moderate benefit. Subsequently, he experienced unexpected weight loss and constipation. The patient's cognition was normal during the follow-up of two years, with a Mini-Mental State Examination score of 29/30 at age 57. On admission, the patient's postural instability had profoundly deteriorated. Brain MRI showed mild frontotemporal atrophy (Fig. 1C). ¹²³I-metaiodobenzylguanidine cardiac scintigraphy showed decreased uptake (heart/mediastinum [H/M] ratio: 1.82 early phase and 1.65 late phase) (Fig. 1B). ¹²³I-FP-CIT-single photon emission computed tomography revealed markedly low uptake (Fig. 1D) in the striatum (specific binding ratio: Rt = 0.27 and Lt = 0.11).

3.2. Generation of iPSC and TH-positive neuron differentiation

One clone from control iPSCs and one clone from the patient iPSCs were analyzed. These iPSCs exhibited morphology similar to human embryonic stem cells and were positive for NANOG and SSEA4 staining (Fig. 1E). These iPSCs were able to differentiate into cells of all three germ layers *in vitro* (Fig. 1F) and had normal karyotypes (Fig. 1G). Genomic analysis showed the presence of a point mutation in *DCTN1* only in Perry syndrome iPSCs (Fig. 1H).

To confirm that cells derived from patient iPSCs and control were TH-positive neurons, we performed immunofluorescence staining with both TH and Tuj1. The Tuj1-positive cells coexpressed TH (Fig. 1I).

3.3. Cellular distribution of p150^{glued} in control and F52L

Histological studies of postmortem brain tissue have shown dynactin and TDP-43-positive inclusions in Perry syndrome. To recapitulate Perry syndrome pathology [1], we first used HEK293T cells transiently transfected with GFP-tagged WT and mutant F52L p150^{glued}. HEK293T overexpressing GFP-tagged WT p150^{glued} showed thread-like cytoplasmic distribution. By contrast, those with GFP-tagged mutant F52L p150^{glued} showed diffuse cytoplasmic distribution with various sizes of aggregates (Fig. 2A, B, E, G, Supplementary Fig. 1A, D, Supplementary Fig. 2A, C). We next analyzed control and Perry syndrome iPSCs and TH-positive neurons derived from each of the iPSCs. We provided positive control of p150^{glued} antibody using HEK293T cells (Supplementary Fig. 1A). We detected various sizes of p150^{glued} aggregates in cytoplasm and neurites only in Perry syndrome patient iPSC-derived TH-positive neurons (Fig. 2C, D, F, H, Supplementary Fig. 1B, E, Supplementary Fig. 2B, D). Control and Perry syndrome patient iPSCs did not present aggregates (Supplementary Fig. 1C). In both HEK293T cells and TH-positive neurons, p150^{glued} aggregates were not positive for TDP-43 (Fig. 2E, F). We examined the characteristics of the aggregates by immunocytochemistry. A small proportion of aggregates of the Perry syndrome patient iPSC-derived TH-positive neurons was partially positive for ubiquitin but not for those of HEK293T overexpressing GFP-tagged F52L p150^{glued} (Fig. 2G, H). p50 and p62, other subunits of dynactin, partially co-localized with p150^{glued} aggregates in HEK293T cells and TH-positive neurons (Supplementary Fig. 1D, E). We conducted additional staining

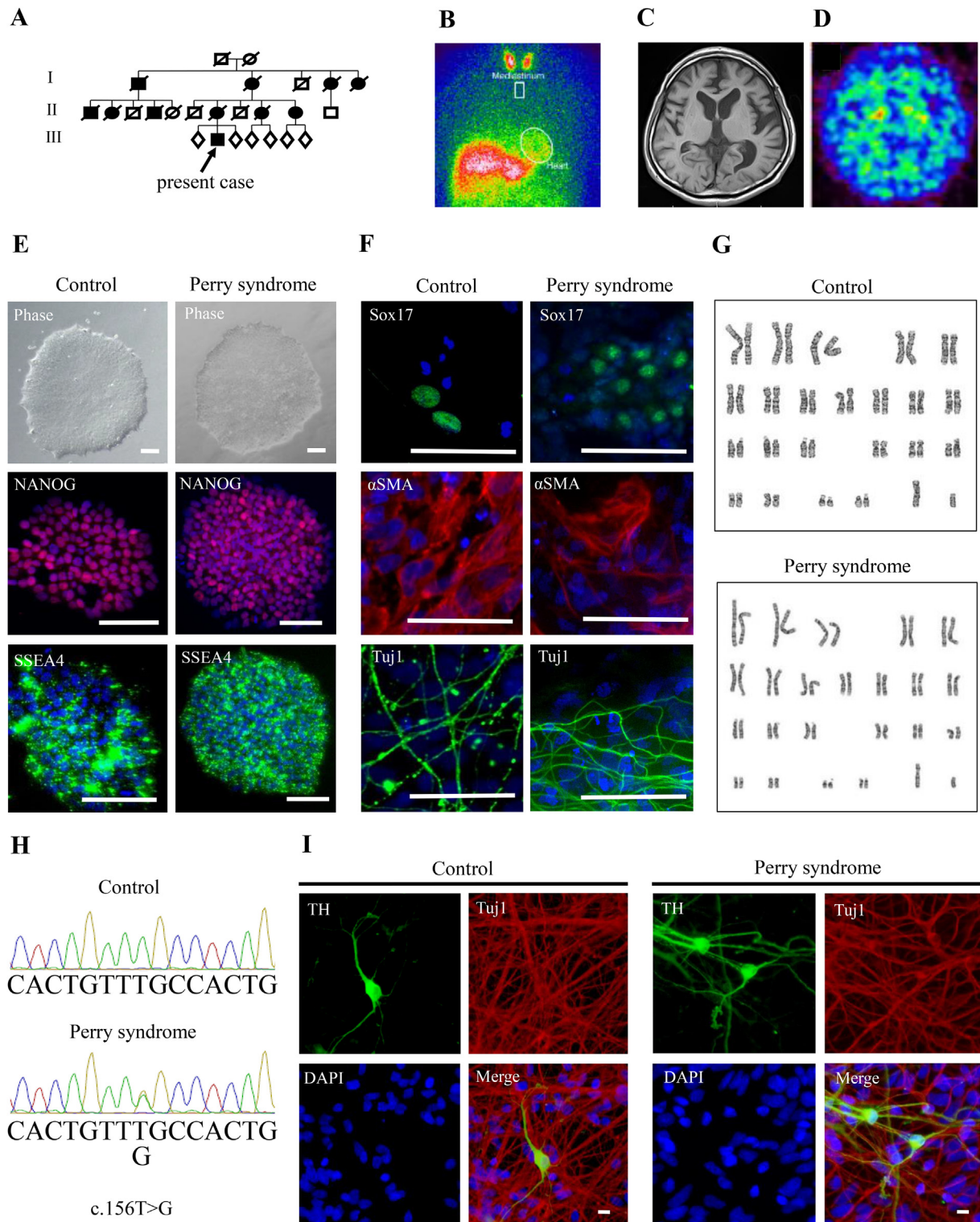


Fig. 1. (A) Pedigree of F52L mutation. Circles indicate females; squares indicate males; solid symbols indicate affected members; diagonal lines indicate deceased individuals. Diamonds were used to disguise gender. The arrow indicates the patient. (B) ^{123}I -metaiodobenzylguanidine cardiac scintigraphy of the patient. (C) T1-weighted MRI of the patient. (D) ^{123}I -FP-CIT-single photon emission tomography of the patient. (E) Generation of control and Perry syndrome patient iPSCs. Images of human embryonic stem cell-like colonies. Immunocytochemistry for NANOG and SSEA4. Scale bar: 100 μm . (F) *In Vitro* differentiation of iPSCs to three germ layers: Sox17 (endoderm), αSMA (mesoderm), and Tuj1 (ectoderm). Scale bar: 100 μm . (G) Karyotype analysis of control and Perry syndrome patient iPSCs. (H) Mutational analysis of control and Perry syndrome patient iPSCs. (I) Generation of tyrosine hydroxylase (TH)-positive neurons from control and patient iPSCs. Scale bar: 10 μm .

experiments with other markers of subcellular organelles. p150^{glued} aggregates partially co-localized with mitochondria, lysosomes and ER in HEK293T cells and TH-positive neurons

(Supplementary Fig. 2A, B). To investigate the microtubule alteration, we stained α -tubulin. F52L p150^{glued} in HEK293T cells showed p150^{glued} aggregates with a scattered translocation along

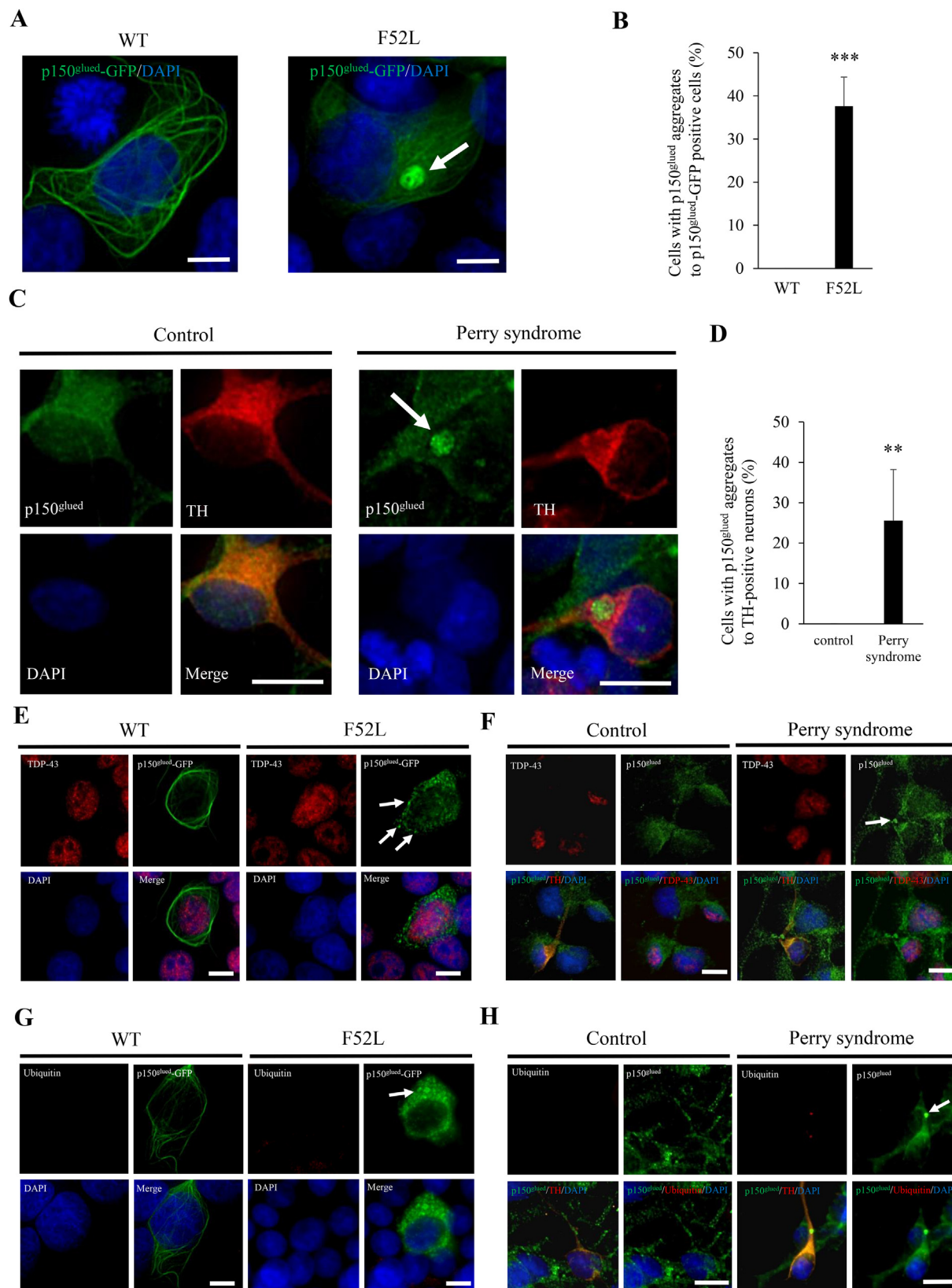


Fig. 2. (A) HEK293T cells transfected with GFP-tagged WT and mutant F52L p150^{glued}. Arrow indicates a p150^{glued} aggregate. Scale bar: 10 μ m. (B) Percentage of p150^{glued}-GFP-positive cells containing aggregates. $P < 0.001$ by t -test. Error bar indicates standard deviation. Statistics are from three experiments. (C) Control and Perry syndrome patient iPSC-derived TH-positive neurons immunostained for p150^{glued}. Arrow indicates a p150^{glued} aggregate. Scale bar: 10 μ m. (D) Percentage of TH-positive neurons containing p150^{glued} aggregates. $P < 0.01$ by t -test. Error bar indicates standard deviation. Statistics are from four experiments. (E) HEK293T cells transfected with GFP-tagged WT and mutant F52L p150^{glued} immunostained for TDP-43. Arrows indicate p150^{glued} aggregates. Scale bar: 10 μ m. (F) Control and Perry syndrome patient iPSC-derived TH-positive neurons immunostained for TDP-43 and p150^{glued}. Arrow indicates a p150^{glued} aggregate. Scale bar: 10 μ m. (G) HEK293T cells transfected with GFP-tagged WT and mutant F52L p150^{glued} immunostained for ubiquitin. Arrow indicates p150^{glued} aggregates. Scale bar: 10 μ m. (H) Control and Perry syndrome patient iPSC-derived TH-positive neurons immunostained for ubiquitin and p150^{glued}. Arrow indicates a p150^{glued} aggregate. Scale bar: 10 μ m.

α -tubulin as compared with WT p150^{glued} (Supplementary Fig. 2C). In TH-positive neurons, patient TH-positive neurons had p150^{glued} aggregates, but there was no alteration in the immunostaining pattern of α -tubulin compared with control cells (Supplementary Fig. 2D). These findings, suggesting a crucial role of p150^{glued}, were compatible with a previous study [7].

4. Discussion

Perry syndrome is a devastating disease, leading to death within 5 years from disease onset. This poor prognosis is mainly caused by rapidly progressive parkinsonism and sudden respiratory failure with a central origin. However, the mechanism details are still poorly understood due to a lack of appropriate disease models. iPSC technology has the great potential of revealing disease mechanisms by providing the opportunity to study disease-affected cells from patients [8]. In this study, we found a phenotype in TH-positive neurons derived from Perry syndrome patient iPSCs.

Intracellular abnormal protein inclusion is a hallmark of neurodegenerative disease; TDP-43 and dynactin-positive inclusions are a key pathological feature of Perry syndrome [1]. We have reported a diffuse cytoplasmic distribution in F52L p150^{glued} in HEK293T cells, but no evident aggregates [2]. We used a GFP-tagged p150^{glued} to detect the clear structure of dynactin in HEK293T cells, and detected p150^{glued}-positive aggregates in HEK293T cells overexpressing GFP-tagged F52L p150^{glued}, as seen in another study of *DCTN1* mutations [9]. This result was generated by exogenous fluorescently-tagged p150^{glued}. Thus, we next analyzed the cytoplasmic distribution of p150^{glued} in iPSC-derived TH-positive neurons with the physiological expression of p150^{glued} levels. We successfully detected cytoplasmic aggregates of p150^{glued} in iPSC-derived TH-positive neurons from the Perry syndrome patient.

In spite of the potential utility of applying iPSC-derived neurons as described, we also have to address the limitations of our study. First, our result cannot completely recapitulate the pathology of the brain of a Perry syndrome patient. This is consistent with a previous report that cytoplasmic aggregates of p150^{glued} did not merge with TDP-43 in mutant p150^{glued} overexpressing cells [9]. We could not detect TDP-43 aggregates in patient iPSC-derived TH-positive neurons, although there are a couple of reports of TDP-43 aggregates in familial ALS patient iPSC-derived models with TDP-43 mutation [8,10]. We speculated that TDP-43 aggregates may be a secondary event and take a long time after dynactin aggregates in Perry syndrome, and thus it was difficult to detect TDP-43 aggregates in the limited number of TH-positive neurons with dynactin aggregates. There is limited knowledge about the impact of aging factor. Reprogramming somatic cells results in an embryonic-like state of iPSCs and their derivatives, thus hampering the modeling late-onset disorders [11]. We were able to partially recapitulate a phenotype in iPSC-derived TH-positive neurons despite Perry syndrome with F52L p150^{glued} being late-onset [2]. We speculate that, as dopaminergic and serotonergic dysfunction in *DCTN1* mutation carriers *in vivo* has been reported [12], damage in neuronal cells may have already begun before disease onset. In the study of p150^{glued}-overexpressing cells, the phenotype of G59S mutation-linked familial motor neuron disease is considerably severe [9]. The clinical phenotype of the patient with F52L p150^{glued} in the present study was milder than other mutations. Although we did not compare this mutation and other mutations including G59S linked to familial motor neuron disease, this will be required for further elucidation of the pathophysiology. In spite of these limitations, we think that this approach could be useful for the study of Perry syndrome pathogenesis.

In conclusion, our results demonstrate that Perry syndrome

patient iPSCs could be used to investigate the disease phenotype.

Authorship

(1) The conception and design of the study: H Inoue; acquisition of data: T Mishima and T Ishikawa; analysis and interpretation of data: T Mishima, T Ishikawa, K Imamura, T Kondo, and H Inoue.

(2) Drafting the article: T Mishima; revising it critically for important intellectual content: T Ishikawa, K Imamura, T Kondo, Y Koshiba, R Takahashi, J Takahashi, A Watanabe, N Fujii, Y Tsuboi, and H Inoue.

(3) Final approval of the version to be submitted: H Inoue.

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The authors declare no financial disclosures.

Conflict of interest

The authors declare no potential conflicts of interest with respect to research, authorship, and publication of the article.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.parkreldis.2016.06.007>.

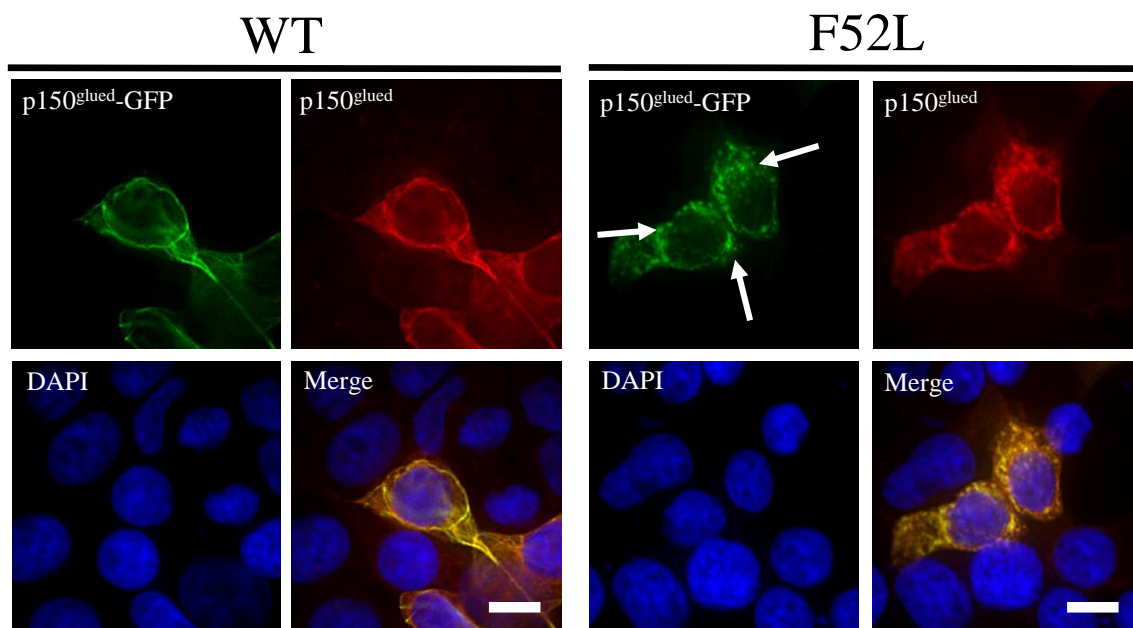
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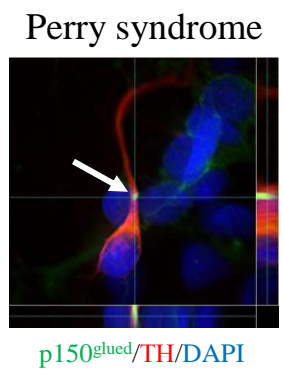
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Supplementary Fig.1.

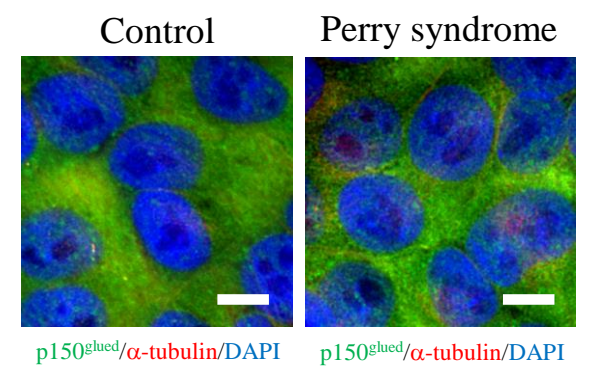
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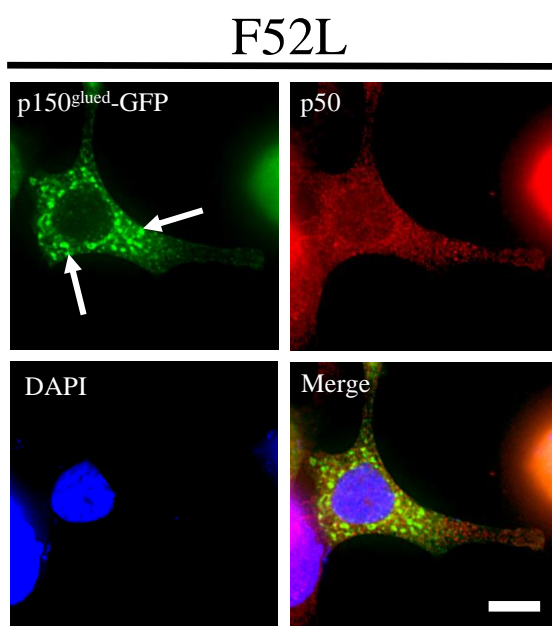
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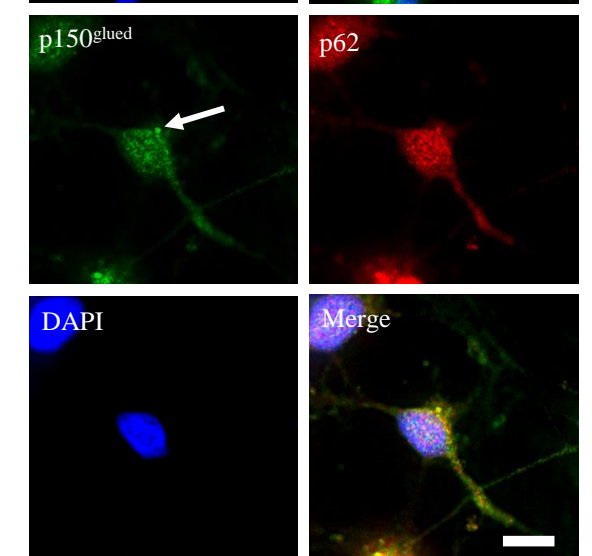
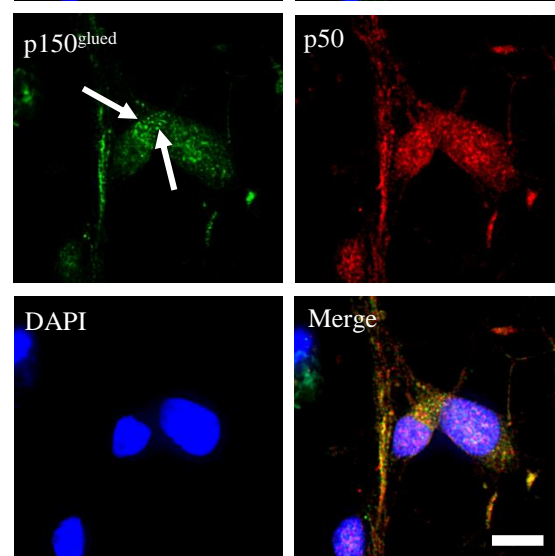
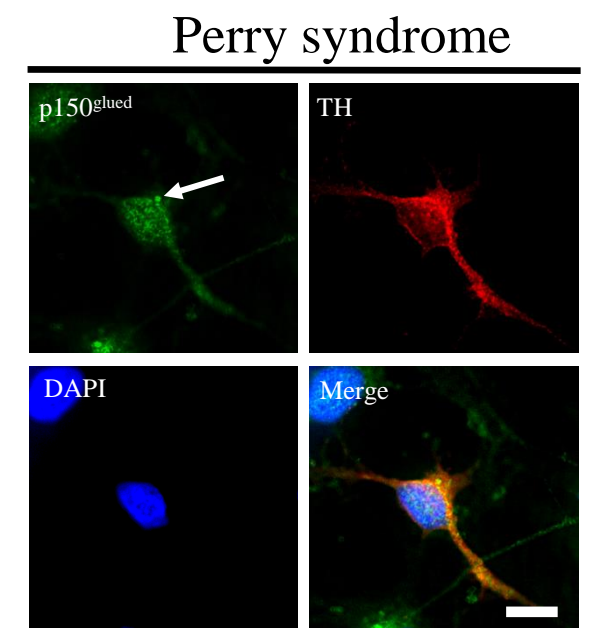
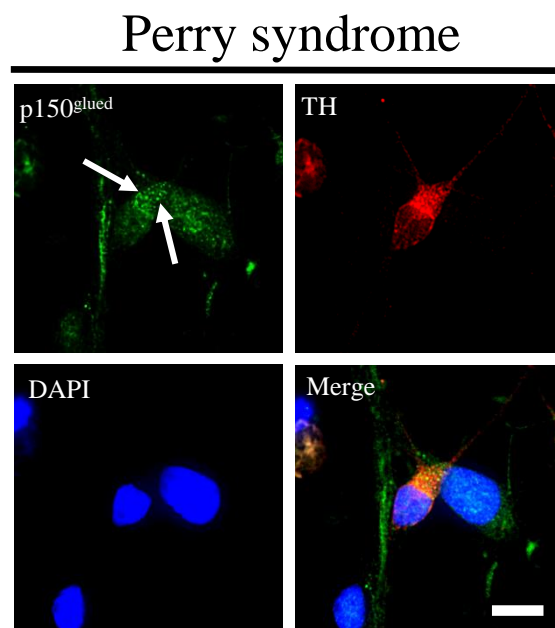
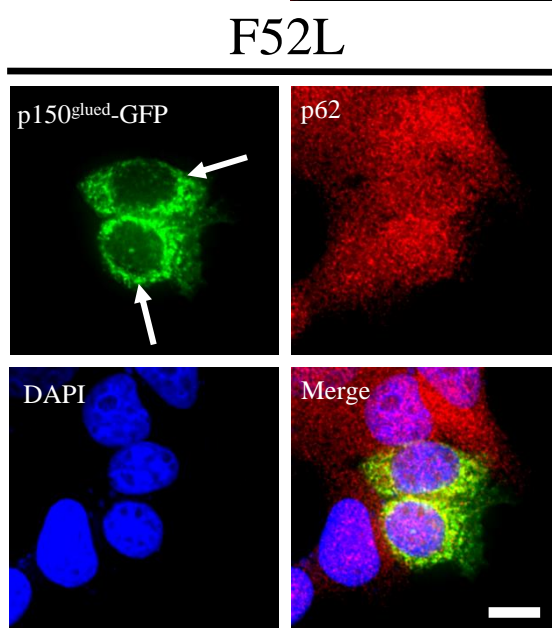
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Supplementary Fig.2.

