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TITLE: Determining the Pathogenesis of Dystonia in Reprogrammed Human Neurons

PRINCIPAL INVESTIGATOR: Baojin Ding

CONTRACTING ORGANIZATION: University of Louisiana at Lafayette

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14. ABSTRACT The limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research. We have successfully generated human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). We found that dystonia neurons show developmental delay, thickened nuclear lamina, disrupted nuclear morphology, and impaired nucleocytoplasmic transport. Furthermore, we have uncovered that nuclear lamina protein LMNB1 is upregulated in DYT1 neurons and exhibits abnormal subcellular distribution in a cholinergic motor neuron (MN)-specific manner. Interestingly, downregulation of LMNB1 can largely ameliorate all the cellular defects in DYT1 MNs. These results reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 pathology.					
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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Dystonia is the third most common movement disorder and characterized by sustained or intermittent muscle contractions, leading to abnormal movements and postures. Veterans are particularly at risk due to the frequent occurrence after head trauma and potentially irreversible side effects of medications used to treat PTSD (post-traumatic stress disorder). Because the clinical characteristics and underlying causes are very heterogeneous, the pathological mechanisms of dystonia remain largely unknown and the diagnosis and etiological definition of this disorder remain a challenge. However, the limited access to patient neurons greatly impedes the progress of research in dystonia. The major purpose of this project is to develop a novel cellular model system for dystonia research and examine the cellular and molecular pathogenesis of this disease. We will generate human patient-specific neurons through either direct conversion of patients' skin fibroblasts or differentiation of induced pluripotent stem cells (iPSCs). These patient-specific neurons will be extensively examined and compared with healthy control cells at cellular morphology, neurodevelopment, survival, nuclear transport and functions. Expected results emanating from this study will provide novel insights into dystonia pathology and potentially lead to molecular targets for therapeutic interventions.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Dystonia, Human Neurons, Motor Neurons, Reprogramming, TOR1A

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major goals:

1. To establish the protocol of directly reprogramming human neurons from adult fibroblasts.

Milestones achieved:

1.1 Obtained all research materials, including fibroblast cell lines are used in this project.
80% completion.

1.2 Established the protocol of reprogramming human neurons from adult fibroblasts.
80% completion. We are able to generate patient-specific motor neurons.

1.3 Verified the neuronal identity, electrophysiology and maturation of reprogrammed neurons.
50% completion. Electrophysiological analysis needs to be done.

1.4 Successfully generated dystonia neurons.
50% completion. Other neuronal subtypes will be generate, including from adulthood-onset patients.

2. To examine the nuclear morphology and nucleocytoplasmic transport in dystonia neurons.

Milestones Achieved:

2.1 Optimized conditions of immune-staining reprogrammed neurons.
100% completion.

2.2 Acquired plenty of confocal micrographs for data analysis.
50% completion. More target proteins will be analyzed.

2.3 Optimized protocols for EM sample preparation.
30% completion. The fixation and embedding condition need to be optimized.

2.4 Acquired plenty of EM data of reprogrammed neurons.
20% completion. More EM samples will be prepared and examined.

3. To examine nucleocytoplasmic transport (NCT) in dystonia neurons.

Milestones Achieved:

3.1 Optimized Fluorescence in situ hybridization (FISH) condition for nuclear mRNA export assay in reprogrammed neurons.
60% completion. The protocol for measuring total mRNA distribution has been established. Next will set up protocols for measuring gene-specific transcripts.

3.2 Optimized condition for dual reporter assay.
100% completion.

3.3 Acquired plenty of confocal micrographs of FISH data and dual reporter data.
70% completion. More samples will be analyzed.

3.4 Quantified the micrographs by ImageJ.
70% completion. More samples will be analyzed.

3.5 Organized data and prepared manuscript (s).
This project has generated four peer-reviewed papers so far, including 3 research papers and 1 invited perspective. (See appendix A-D)

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

During this reporting period (March 15, 2021, to March 14, 2022), this project achieved the following accomplishments.

1. We have systematically summarized the regulatory mechanisms of nucleocytoplasmic transport and the implications in neurological diseases.

Previously, we have developed two systems to measure the nucleocytoplasmic transport (NCT) of both mRNA and proteins cargos. Using these two systems, we examined NCT activity at the single-cell level by measuring mRNA subcellular distribution and protein transport in directly induced motor neurons (diMNs) from adult human fibroblasts. We found the impaired NCT activities in dystonia neurons (*Ding et al. 2021. JNeurosci*). As impaired NCT has been shown to be implicated in many neurological diseases (such as Alzheimer's disease, Parkinson's disease etc.), we systematically studied related literatures and wrote a comprehensive review paper with the focus on the regulatory mechanisms of nucleocytoplasmic transport and the implications in neurological diseases. This comprehensive review has been published in the *International Journal of Molecular Sciences (IJMS)* (See Appendix A) and reprinted as a Book Chapter in *Prime Archives in Molecular Sciences: 2nd Edition* (See Appendix E).

2. We have generated and characterized the induced pluripotent stem cells (hiPSC) for modeling dystonia.

We have generated two sets of DYT1 dystonia iPSC lines, including a GAG deletion in the *TOR1A* gene from WT-iPSCs and a GAG knock-in to correct patient iPSCs. In the first set, we identified two clones of heterozygous and one clone of homozygous GAG deletion as well as two wild-type isogenic controls. In the correction set, we obtained two clones of corrected lines, in which GAG deletions have been restored. All clones were verified by DNA sequencing. The pluripotency of these iPSC lines was further verified by Alkaline Phosphatase (AP) staining, the highly expressed pluripotency markers, and in vitro differentiation of iPSCs to embryoid bodies (EBs), which highly expressed trilineage markers. Using these dystonia iPSCs, we could generate a large number of patient-specific neurons to decipher the pathogenesis of the disease via biochemical approaches. The information about generation and characterization of these hiPSC lines has been published in *Stem Cell Research* (See Appendix B).

3. We have established the protocol of directly reprogramming human neurons from adult fibroblasts.

We developed the protocol of directly reprogramming motor neurons from adult fibroblasts via lentiviral delivery of transcription factors. Using this protocol, we have successfully generated human neurons from fibroblasts of dystonia patients and healthy controls. This protocol has been published in *Star Protocols* (See Appendix C).

4. Our publication was selected as JNeurosci Spotlight 2021.

We have first reported the disease-dependent cellular deficits in dystonia using patient-specific neurons (*Ding et al. 2021. JNeurosci*). These deficits include disrupted neurodevelopment, thickened nuclear lamina, deformed nucleus, impaired nucleocytoplasmic transport, and the most unexpected finding of the dysregulation of nuclear lamina protein LMNB1. This publication was selected as JNeurosci Spotlight 2021. **JNeurosci's Annual Spotlight features articles that received the highest marks for both methodological merit and significance.** See the [papers selected](#) for this year's recognition.

5. Invited perspective for novel insights into the pathogenesis of dystonia.

Given that we have made a breakthrough in understanding the pathogenesis of dystonia using patient-specific neurons, I was invited to write a perspective by the *Neural Regeneration Research*. The invited perspective entitled ***Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons*** has been published recently. (See Appendix D).

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

During this reporting period, this project provided 3 Graduate Research Assistant (GRA) positions (person semester) and research opportunity for 1 undergraduate researcher. Through participating this project, graduate students learned the basic skills of cell culture, immunostaining, imaging and the advanced techniques of reprogramming human neurons from adult fibroblasts. Undergraduate student worked with graduate students and learned the basic experimental skills and how to analyze the confocal images using ImageJ software.

The PI attended two conferences and presented the new findings of this project.

1. Keystone Symposia: Neurodegenerative Diseases: Genes, Mechanisms and Therapeutics | EK48 (June 7, 2021. Virtual).

Presentation title: *Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons* (Appendix F).

2. Neuroscience 2021 50th Annual Meeting (Nov 8-11, 2021. Virtual).

Presentation title: *Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia* (Appendix G).

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The new findings from this project were published in prestigious peer-reviewed journals and the PI also presented the new findings to scientific communities in scientific conferences.
(See appendix A-G)

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

1. Generate more human neurons from dystonia patients and healthy controls.

Using both direct conversion and iPSC-based approach to generate different neuronal subtypes, including motor neurons, cholinergic interneurons and generic neurons.

2. Examine the cellular defects in DYT1 neurons.

We will examine the nuclear morphology of DYT1 neurons by TEM, and analyze the nuclear lamina and nuclear pore complex.

3. Optimize protocols for transcriptomic and proteomic studies.

We will optimize protocols to generate a large number of patient-specific neurons from newly generated iPSC lines and optimize the conditions to extract RNA and proteins for transcriptomic and proteomic studies.

4. Organize the data and prepare more manuscripts.

The new findings will be organized and published in peer-reviewed journals.

- 4. IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and

research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have developed and further optimized the techniques for generation of human neurons from patient skin fibroblasts. These techniques overcome the limited access to human brain neurons and provide valuable materials in dystonia research. Using patient-derived neurons, we first reported the disease-dependent cellular deficits, including dysregulation of nuclear protein LMNB1. When we used a technique to bring down the LMNB1 protein level and the cellular defects in dystonia neurons were corrected. These findings reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia. Additionally, we have generated two sets of iPSC lines for modeling DYT1 dystonia, providing valuable research materials in dystonia research.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Besides the impact on neuroscience and disease modeling, the findings from this project have a broad impact on other disciplines. For example, the regulations of nucleocytoplasmic transport involves the Cell Biology, Molecular Biology and Biochemistry. Dysregulation of LMNB1 in dystonia neurons advanced our understanding of the impact of nuclear lamina on nuclear envelope morphology and nucleocytoplasmic transport.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

The findings from this project reveal high value of disease modeling with human patient-specific neurons and indicate that dysregulation of nuclear LMNB1 may constitute a major molecular mechanism underlying DYT1 dystonia pathology. Downregulation of LMNB1 could be a novel target for developing therapeutic interventions in DYT1 dystonia.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Nothing to Report

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Because of the unprecedented crisis of Covid-19, the research and the expenditure of the fund was significantly delayed.

In January 2022, the PI and his team relocated to LSU Health Shreveport, where excellent research core facilities and neuroscientists are available on campus. The new lab has been established and the research activities are fully resumed. The strong support at the new institution will significantly facilitate the proposed studies of this project.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The pandemic of CovID-19 has a significant impact on the expenditures. During university closure, we had to stop or minimize our research activities and collaborations. After university reopen, the lab was only partly functional. The overall expenditure during this reporting period was significantly delayed. Additionally, the PI and his team relocated to LSU Health Shreveport in early 2022. The relocation also delayed the expenditure.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. **Ding B*** and Sepehrimanesh M. (2021). Nucleocytoplasmic transport: regulatory mechanisms and the implications in neurodegeneration. *Int. J. Mol. Sci.* 22 (8): 4165. (See Appendix A).
Published, acknowledgement of federal support (yes).
2. Akter M, Cui H, Chen Y-H and **Ding B***. (2021). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous GAG deletion in TOR1A gene from a healthy hiPSC line. *Stem Cell Research.* 56 (2021)102536 (See Appendix B).
Published, acknowledgement of federal support (yes).
3. Sepehrimanesh M., Akter M. and **Ding B***. (2021). Direct conversion of adult fibroblasts into motor neurons. *STAR Protocols.* 2 (4) 17 December 2021, 100917 (Cell Press, Invited protocol). (See Appendix A).
Published, acknowledgement of federal support (yes).
4. **Ding B***. (2022). Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons. *Neural Regen Res* 17(3):561-562. (Invited Perspective) (See Appendix A).
Published, acknowledgement of federal support (yes).

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Book Chapter. (Reprint Appendix A)
Prime Archives in Molecular Sciences: 2nd Edition
Ding B* and Sepehrimanesh M. (2021). Nucleocytoplasmic transport: regulatory mechanisms and the implications in neurodegeneration. (Appendix E).
Published, acknowledgement of federal support (yes).

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Presentation:

*Poster presentation 1. Keystone Symposia: Neurodegenerative Diseases: Genes, Mechanisms and Therapeutics | EK48 (June 7, 2021. Virtual).

B. Ding. *Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons (Appendix F)*

Poster presentation 2. Neuroscience 2021 50th Annual Meeting (Nov 8-11, 2021. Virtual).

B. Ding. *Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia (Appendix G)*

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

The publication (Ding et al. Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. J Neurosci. (2021) 41 (9): 2024-2038.) was selected as **JNeurosci Spotlight 2021**.

JNeurosci's Annual Spotlight features articles that received the highest marks for both methodological merit and significance. See the [papers selected](#) for this year's recognition.

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Developed and optimized protocols for generation human neurons from adult fibroblasts. These methods have been shared via publications in peer-reviewed journals (see appendix B and C).

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance

progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Research materials:

We have generated two sets of DYT1 dystonia iPSC lines. The information about the generation and characterization of these cell lines has been published as a lab resource paper in *Stem Cell Research* (see Appendix B).

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name:

Mary Smith

Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.
Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name	Baojin Ding	Masuma Akter	Haochen Cui	Masood Sepehrimanesh
Project role	PI	Graduate Student	Graduate Student	Postdoc
ORCID ID	0000-0002-2149-2599	N/A	N/A	0000-0002-6300-2906
Nearest person month worked	4	5	5	3
Contribution to Project	Supervision for the project, including design experiment, train the graduate students and prepare manuscripts and presentations for scientific meetings.	Performed experiments, including culture cells, reprogramming human neurons, and neuronal survival assay.	Performed experiments, including culture cells, plasmid purification, lentivirus preparation.	Performed experiments, including culture cells and lentivirus preparation. Helped PI oversee the lab.
Funding support	Start-up and NIH	University fellowship	RA position from this Award	Start-up and NIH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

As the PI and his team relocated to a new institution in January 2022, the PI received Start-up fund to build a new laboratory.

Faculty Start-up (PI) ; 2022-2025

Source: LSU Health Shreveport

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

Nothing to Report

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

Nothing to Report

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix A-Journal article

Ding B* and Sepehrimanesh M. (2021). Nucleocytoplasmic transport: regulatory mechanisms and the implications in neurodegeneration. *Int. J. Mol. Sci.* 22 (8): 4165.

Appendix B-Journal article

Akter M, Cui H, Chen Y-H and **Ding B***. (2021). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous GAG deletion in TOR1A gene from a healthy hiPSC line. *Stem Cell Research.* 56 (2021)102536 <https://doi.org/10.1016/j.scr.2021.102536>

Appendix C-Journal article

Sepehrimanesh M., Akter M. and **Ding B***. (2021). Direct conversion of adult fibroblasts into motor neurons. *STAR Protocols.* 2 (4) 17 December 2021, 100917 (Cell Press, Invited protocol).

Appendix D-Journal article

Ding B*. (2022). Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons. *Neural Regen Res* 17(3):561-562. (Invited Perspective)

Appendix E- Book Chapter. (Reprint Appendix A)

Prime Archives in Molecular Sciences: 2nd Edition

Appendix F Poster presentation 1. Keystone Symposia: Neurodegenerative Diseases: Genes, Mechanisms and Therapeutics | EK48 (June 7, 2021. Virtual).

B. Ding. *Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons*

Appendix G-Poster presentation 2. Neuroscience 2021 50th Annual Meeting (Nov 8-11, 2021. Virtual).

B. Ding. *Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia*

Appendix H-the PI's updated CV



Review

Nucleocytoplasmic Transport: Regulatory Mechanisms and the Implications in Neurodegeneration

Baojin Ding * and Masood Sepehrimanesh

Department of Biology, University of Louisiana at Lafayette, 410 East Saint Mary Boulevard, Lafayette, LA 70503, USA; masood.sepehrimanesh@louisiana.edu

* Correspondence: Baojin.Ding@Louisiana.edu

Abstract: Nucleocytoplasmic transport (NCT) across the nuclear envelope is precisely regulated in eukaryotic cells, and it plays critical roles in maintenance of cellular homeostasis. Accumulating evidence has demonstrated that dysregulations of NCT are implicated in aging and age-related neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Alzheimer's disease (AD), and Huntington disease (HD). This is an emerging research field. The molecular mechanisms underlying impaired NCT and the pathogenesis leading to neurodegeneration are not clear. In this review, we comprehensively described the components of NCT machinery, including nuclear envelope (NE), nuclear pore complex (NPC), importins and exportins, RanGTPase and its regulators, and the regulatory mechanisms of nuclear transport of both protein and transcript cargos. Additionally, we discussed the possible molecular mechanisms of impaired NCT underlying aging and neurodegenerative diseases, such as ALS/FTD, HD, and AD.

Keywords: Alzheimer's disease; amyotrophic lateral sclerosis; Huntington disease; neurodegenerative diseases; nuclear pore complex; nucleocytoplasmic transport; Ran GTPase



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1. Introduction

As a hallmark of eukaryotic cells, the genetic materials are separated from the cytoplasmic contents by a highly regulated membrane, called nuclear envelope (NE), which has two concentric bilayer membranes, the inner nuclear membrane (INM), and outer nuclear membrane (ONM). The ONM is continued by the endoplasmic reticulum (ER). There are some large specific protein structures across the NE, such as nuclear pore complex (NPC) that controls nucleocytoplasmic transport (NCT). NPC consists of special proteins, named nucleoporins (Nups), which play critical roles in regulation of the transport of most macromolecules back and forth across the NE [1]. NCT is a complex mechanism with involvement of many protein-protein interactions and recognitions, regulators and signaling pathways [2]. The transport across NE consists of import and export of protein and transcript cargos. All transcripts are transcribed in the nucleus and must be exported to the cytoplasm for protein synthesis, while certain proteins such as polymerases, histones, and transcription factors have to be imported into the nucleus to fulfill their functions [3,4]. Accumulating evidence revealed that impaired NCT is a fundamental pathological factor in aging [5] and aging-related neurological diseases [6,7], such as amyotrophic lateral sclerosis (ALS) [8,9], frontotemporal dementia (FTD) [10,11], Huntington's disease (HD) [12,13], Alzheimer's disease (AD) [14], Parkinson's disease (PD) [15], Ataxia [16], and dystonia [17]. Understanding the regulation of NPC-mediated nuclear transport is critical to decipher the pathogenesis underlying impaired NCT and identify molecular targets for therapeutic interventions. In this paper, we comprehensively reviewed the components of NCT machinery and its regulatory mechanisms, including nuclear envelope and nuclear pore complex, importins and exportins, Ran GTPase and its regulators, and the regulations of nuclear transport of both protein and transcript cargos. Moreover, we discussed the possible mechanisms that link the impaired NCT to neurodegeneration.

2. Nucleocytoplasmic Transport (NCT)

2.1. Components of NCT Machinery

2.1.1. Nuclear Pore Complex (NPC)

The NPC is the principal gateway between the nucleus and cytoplasm. There are more than a hundred to more than a thousand NPCs on the NE of a yeast cell [18] and a human cell [19], respectively. NPCs are huge protein complexes penetrating and bridging the inner and outer nuclear membrane [4,20]. A fully assembled NPC in vertebrates consists of multiple copies of about 30 different Nups with an estimated molecular mass of 120 MDa [1]. The three-dimensional structure of NPCs shows an eight-fold rotational symmetry and contains several major domains, such as cytoplasmic filaments, nuclear basket, central transport channel, and a core scaffold that supports the central channel [20,21]. The exact place of each Nup in an NPC plus their structure and functions are summarized in Figure 1 and Table 1.

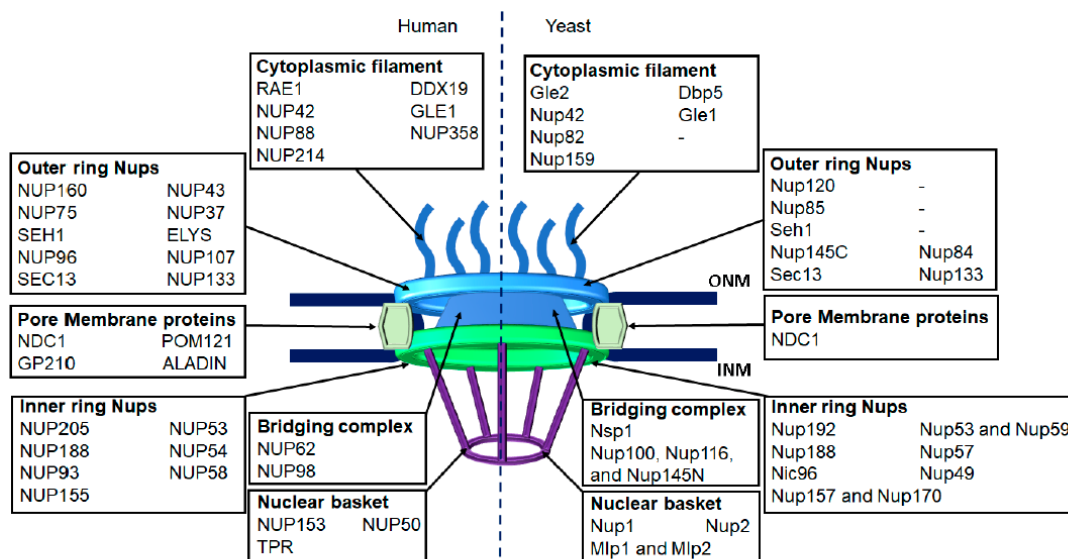


Figure 1. Nuclear pore complex structure and nucleoporins. Nuclear pores are embedded in the nuclear envelope and across the inner nuclear membrane (INM) and outer nuclear membrane (ONM). Each nuclear pore consists of outer ring, inner ring, pore membrane proteins, cytoplasmic filament, bridging complex and nuclear basket. Each part contains multiple copies of different nuclear pore subunits (nucleoporins). See Table 1 for more information about different nucleoporins.

Table 1. Characteristics of human nucleoporins and their homologous in *Saccharomyces cerevisiae*.

Nups	AA	Structure	<i>S. cerevisiae</i>
Outer ring Nups			
Nup160	1436	Unstructured region, β -propeller domain, and α -helical solenoid	Nup120
Nup75	656	Domain invasion motif and α -helical solenoid	Nup85
Seh1	360	β -propeller domain	Seh1
Nup96	936	Unstructured region and α -helical solenoid	Nup145C
Sec13	322	β -propeller domain	Sec13
Nup107	925	Unstructured region and α -helical solenoid	Nup84
Nup133	1156	Unstructured region, β -propeller domain, and α -helical solenoid	Nup133
Nup43	380	β -propeller domain	-
Nup37	326	β -propeller domain	-
ELYS	2266	Unstructured region, β -propeller domain, and α -helical solenoid	-

Table 1. Cont.

Nups	AA	Structure	<i>S. cerevisiae</i>
Inner ring Nups			
Nup205	2012	α -helical solenoid	Nup192
Nup188	1749	α -helical solenoid	Nup188
Nup93	819	Unstructured region, Rec-A-like domain, and α -helical solenoid	Nic96
Nup155	1391	Unstructured region, β -propeller domain, and α -helical solenoid	Nup157 and Nup170
Nup53	326	Unstructured region, Rec-A-like domain, and RNA recognition motif	Nup53 and Nup59
Nup54	507	FG-repeat region and coiled-coil region	Nup57
Nup58	599	FG-repeat region and coiled-coil region	Nup49
Bridging complex			
Nup62	522	FG-repeat region and coiled-coil region	Nsp1
Nup98	880	FG-repeat region, Gle2-binding sequence, unstructured region, and auto-proteolytic domain	Nup100, Nup116, and Nup145N
Cytoplasmic filament			
Rae1	368	Unstructured region and β -propeller domain	Gle2
Nup42	423	FG-repeat region, Zinc finger region, and Gle2-binding sequence	Nup42
Nup88	741	Unstructured region, β -propeller domain, and coiled-coil region	Nup82
Nup214	2090	Unstructured region, β -propeller domain, coiled-coil region, and FG-repeat region	Nup159
DDX19	479	Unstructured region and Rec-A-like domain	Dbp5
Gle1	698	Unstructured region, coiled-coil region, and α -helical region	Gle1
Nup358	3224	α -helical solenoid, unstructured region, Zinc finger region, E3 ligase domain, Ran-binding domain, and cyclophilin domain	-
Nuclear basket			
Nup153	1475	Unstructured region, Zinc finger region, and FG-repeat region	Nup1
Nup50	468	Unstructured region, FG-repeat region and Ran-binding domain	Nup2
Tpr	2363	FG-repeat region and coiled-coil region	Mlp1 and Mlp2
Pore membrane proteins			
Ndc1	674	Unstructured region, transmembrane helices, Rec-A-like domain, and α -helical region	NDC1
NUP210	1886	B-strand region, coiled-coil region, and unstructured region	-
POM121	1249	Coiled-coil region, and unstructured region	-
ALADIN	546	β -propeller domain	-

Nups contain phenylalanine-glycine (FG) motifs or repeats which create a permeability barrier against passive diffusion of larger cargo molecules (>60 KDa) [22,23]. FG repeats are intrinsically disordered domains [24], and they directly function in mediating the passage of the soluble transport receptors pass through the NPC [2,25,26]. Despite of small molecules such as ions that pass the nuclear membrane via passive diffusion, all other macromolecules including RNAs and proteins need specific molecules called nuclear transport receptors (NTR), such as importins and exportins to achieve the specific nuclear trafficking [27,28].

2.1.2. Importins and Exportins

Both importins and exportins belong to a protein family called Karyopherins (Kaps) [29]. These Kaps mediate transportation through binding to the nuclear localization sequence (NLS) or nuclear export sequence (NES) in the protein cargos and simultaneously bind to the FG repeats of Nups in the NPCs. In this way, Kaps also associate with Ras-related nuclear protein (Ran), a small regulatory Ras-related GTPase that controls both nuclear export and import (Figure 2). There are several human homologous Kaps with low sequence homology but similar biochemical properties such as molecular masses, isoelectric points, and architecture. Each of them specifically transports a set of proteins. Some of the Kaps just export or import the cargos and called exportins or importins, while some of them work as bidirectional transporter and called transportins. They recognize NES and/or NLS and help the protein and transcript cargos to pass across the NPC (Table 2).

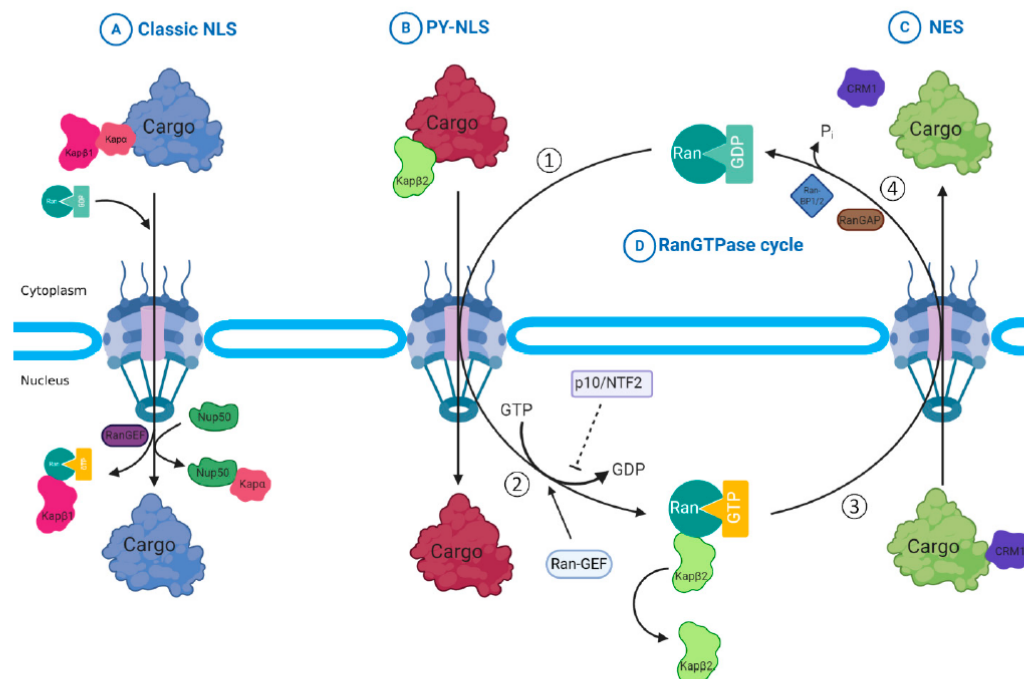


Figure 2. A Ran-dependent mechanism of protein nuclear transport. **(A)** Nuclear import of protein cargos containing classic nuclear localization sequence (NLS) is mediated by Kap α and Kap β 1. Import complex contains cargo-NLS, importin and RanGDP is formed in cytoplasm and imported into nucleus through a nuclear pore complex (NPC). The cargo is released from the complex after RanGEF exchanges the binding of RanGDP to RanGTP. **(B)** Nuclear import of cargos with the signal of proline-tyrosine amino acid pairing (PY-NLSs) is mediated by Kap β 2 in a similar mechanism as A. **(C)** CRM1 mediates the nuclear export of protein cargos containing nuclear export sequence (NES). Export complex contains cargo-NES, exportin (CRM1) and RanGTP is formed in the nucleus and exported into cytoplasm through a NPC. The cargo is released from the complex after the hydrolysis of GTP to GDP by RanGTPase, which is stimulated by RanGAP (Ran GTPase-activating protein). **(D)** RanGTPase cycle. ① RanGDP binds import complex in cytoplasm and it will be transported into nucleus. ② Ran-GEF exchanges the GDP to GTP and disassembles the complex in nucleus, resulting in the release of imported cargos. ③ RanGTP binds the export complex in the nucleus and it will be exported to cytoplasm. ④ RanGAP activates the GTPase activity of Ran and hydrolyze GTP to GDP, resulting in the release of exported cargos. In this cycle, another regulator Ran-GPI (p10/NTF2) could keep Ran at an inactivated form (RanGDP) by inhibiting the dissociation of GDP from Ran. Ran and Ran regulatory proteins generate and maintain the RanGTP-RanGDP gradient across the nuclear envelope and provide the driving force for the nuclear transport of protein cargos.

Table 2. Nomenclature and functions of human importins and exportins.

Human Kaps	Other Names	Functions	References
Importin- α	Kap α	Nuclear import of proteins containing either a simple or bipartite NLS motif.	[30]
Importin- β 1	Kap β 1 Importin-90 NF-p97 PTAC97	Nuclear import of ribosomal proteins, H1 histone, HIV-1 Rev, SNAI1 and PRKCI; In vitro, nuclear import of other histones	[31–35]
Importin-4	Imp-4b RanBP4	Nuclear import of ribosomal protein, RPS3A; In vitro, nuclear import of human cytomegalovirus UL84 by recognizing a non-classical NLS	[36]
Importin-5	Kap β 3 Imp- β 3 RanBP5	Nuclear import of ribosomal proteins and HIV-1 Rev and reverse transcription complex (RTC) integrase; In vitro, nuclear import of H2A, H2B, H3 and H4 histones; Nuclear import of CPEB3 after neuronal stimulation.	[31,32,37,38]
RanBP6		Act as a nuclear transport receptor	[39]

Table 2. Cont.

Human Kaps	Other Names	Functions	References
Importin-7	RanBP7	Nuclear import of ribosomal proteins and H1 histone; In vitro, nuclear import of other histones.	[31,32,37,38]
Importin-8	RanBP8	In vitro mediates the nuclear import of SRP19	[40]
Importin-9	RanBP9	Nuclear import of ribosomal proteins, actin, and histone H2A and H2B; Prevents the cytoplasmic aggregation of RPS7 and RPL18A by shielding exposed basic domains.	[36]
Importin-11	RanBP11	Nuclear import of UBE2E3, and of RPL12	[41]
Importin-13	Kap13 RanBP13	Nuclear import of UBC9, the RBM8A/MAGOH complex, PAX6 and probably other members of the paired homeobox family; Nuclear export of eIF-1A, and the cytoplasmic release of eIF-1A is triggered by the loading of import substrates onto IPO13.	[42,43]
Transportin-1	Kap β 2 Imp- β 2	Nuclear import of ribosomal proteins ADAR/ADAR1 isoform 1 and isoform 5 in a RanGTP-dependent manner; In vitro, nuclear import of H2A, H2B, H3 and H4 histones, and SRP19.	[31,38,40,44–46]
Transportin-2 Isoform 2	Kap β 2b	Nuclear export of mRNA and import of HuR.	[47–49]
Transportin-2		Same as Transportin-2 Isoform 2	[47,48]
Transportin-3	Trn-SR Imp-12	Nuclear import of splicing factor serine/arginine (SR) proteins, HIV-1 pre-integration complex (PIC)	[50–60]
Transportin-SR2	Isoform 2 of Trn-SR	Nuclear import of phosphorylated SR proteins	[52]
Exp-t	Xpo-t	Binds to RanGTP and cooperatively export mature tRNA (Figure 3)	[61]
Exportin-1	CRM1	Nuclear export of unspliced or incompletely spliced viral RNAs and proteins	[62–67]
Exportin-2	CAS CSE-1	Nuclear export of importin alpha	[68]
Exportin-4		Nuclear export of different protein cargos	[69,70]
RanBP17		Nuclear export activity by binding to the GTP and RanGTPase	[71]
Exportin-5	RANBP21	Nuclear export of proteins bearing a double-stranded RNA-binding domain (dsRBD) and double-stranded RNAs (cargos), isoform 5 of ADAR/ADAR1, micro-RNA precursors, synthetic short hairpin RNAs, deacylated and aminoacylated tRNAs, and adenovirus VA1 dsRNA.	[72–76]
Exportin-6	RANBP20	Nuclear export of actin and profilin-actin complexes in somatic cells.	[77]
Exportin-7	RANBP16	Nuclear export of different protein cargos	[71,78]

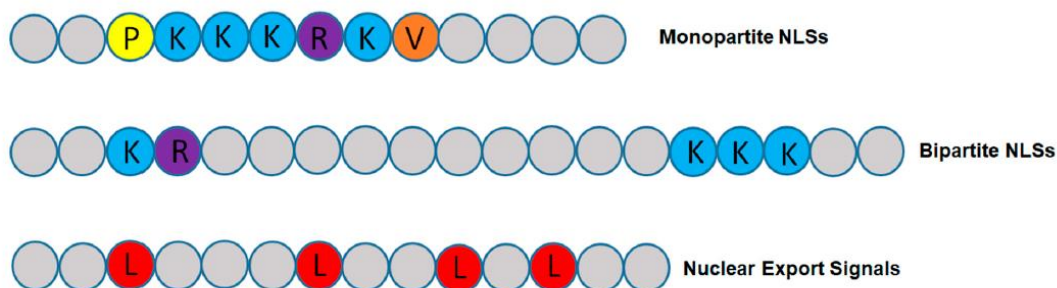


Figure 3. Conserved sequences of monopartite NLS, bipartite NLS and the mostly common form of NES. Each circle with different color is a different amino acid. P, proline; K, lysine; R, arginine; V, valine; L, leucine.

2.2. NCT Regulations

2.2.1. RanGTPase and Its Regulators

The NCT of most protein and transcript cargos (except mRNAs) depends on RanGTP-RanGDP gradient across the nuclear membrane, which provides a driving force and is generated by RanGTPase and its regulators [79]. The nucleus contains a high concentration

of Ran that binds to GTP to form RanGTP, while the RanGDP is more concentrated in the cytoplasm. There is a mechanism for attaching/detaching of cargos from importins/exportins (Figure 2). Importins bind to the cargo in the cytoplasm in the presence of RanGDP and release cargo in the nucleus in the presence of RanGTP and exactly opposite manner occurs for exportins. In these processes, GTPase activity of Ran, the binding of Ran to GTP or GDP, and their subcellular distributions are tightly controlled by a set of regulators, such as Ran-binding proteins (RanBPs), Ran GTPase-activating protein (Ran-GAP), Ran guanine nucleotide exchange factor (Ran-GEF), and Ran-GDP dissociation inhibitor (Ran-GDI).

Ran has a low GTPase activity that is increased by attaching of other molecules including RanBPs and RanGAP. RanBPs work as scaffolding proteins that bind Ran and RanGAP. As RanBPs are anchored in the cytoplasmic side of the nuclear membrane, efficient conversion of RanGTP to RanGDP will occur only in the cytoplasm, resulting in a nuclear/cytoplasm ratio of RanGTP of approximately 200:1 [80]. Nuclear converting of RanGDP to RanGTP through regulator of chromosome condensation 1 (RCC1, also called Ran-GEF) further strengthens this RanGTP-RanGDP gradient [79,81]. Another regulator, Ran-GDIs, such as nuclear transport factor p10/NTF2 that inhibit the dissociation of GDP from Ran and keep Ran in an inactivated form of RanGDP until Ran-GEFs trigger the exchange of GDP to GTP [82] (Figure 2). After nuclear entering of importin α/β -attached cargo, binding of nuclear RanGTP to importin β causes nuclear releasing of the cargo, and then the complex of importin β /RanGTP will be dissociated and transported separately. With the help of RanBPs, RanGAP1 dissociates importin β from RanGTP by stimulating GTPase activity. During cargo export, the complex of exportin, RanGTP, and protein cargo is exported via NPC. RanGDP is transported from cytoplasm into the nucleus and converted to the RanGTP by the chromatin-bound Ran-GEF, forming the RanGTPase cycle [83] (Figure 2D).

NCT regulations consist of importing and exporting processes of protein and transcript cargos. On the protein side, they must contain a peptide signal known as NLS for importing to the nucleus and/or another specific signal called NES for exporting from the nucleus to the cytoplasm. NLS and NES signal peptides can be recognized by importins and exportins, respectively.

2.2.2. Nuclear Import of Proteins with NLS

NLS is a sequence of positively charged amino acids including lysine and/or arginine. It usually localizes on the surface of the protein and can be recognized by importins (Table 2). In bipartite NLS, two positively charged amino acid sequences are separated by a spacer sequence and find in nucleoplasm, while monopartite just has one positively charged amino acid sequence such as SV40 T-antigen and c-Myc (Figure 3). Other types of discovered but uncommon NLS include mostly as proline-tyrosine amino acid pairing (PY-NLSs), and with lower frequency as the acidic M9 domain of hnRNP A1, the KIPK sequence in yeast Mat α 2, a transcription repressor, and the complex signals of U snRNPs. Also, some specific NLS motifs are used to bind DNA and can be found near the DNA-binding region [84]. However, we can categorize common NLSs as two distinct types, basic classical-NLSs which described above and the PY-NLSs. Both types of NLSs must be recognized by importins. The heterodimer Imp α -Imp β 1 recognize most of the classical NLS. In molecular view, acidic part of Imp α binds directly to the basic NLS side chains via electrostatic and polar interactions, its tryptophan residues hydrophobically interact with aliphatic parts, and its asparagine residues make hydrogen-bonds with the NLS main chains [85]. However, Transportin-1, which is a Kap β 2 protein, binds to PY-NLS [29]. This categorization of PY-NLSs is based on their loose N-terminal sequence motifs [86], which are either hydrophobic PY-NLSs containing hydrophobic residues of Glycine/Alanine/Serine or basic PY-NLSs enriched with basic residues. However, the c-terminal has a unique sequence as Arginine/Lysine/Histidine-sequence of 2 to 5 residues-Proline-Tyrosine motifs [87] (Figure 3).

In the process of protein import, protein cargos containing NLS are recognized by importins (such as Kap α and Kap β 1) in the cytoplasm and then bind RanGDP to form import complex (Figure 2). Importins will guide the complex to physically associate with Nups of NPC and then pass through the NPC with the driving force of RanGTP-RanGDP gradient [88,89]. In the nucleus, Ran-GEF exchanges the binding of GDP to GTP and disassembles the complex and releases the cargo in the nucleus. Importins will be exported to the cytoplasm and to be used for another run of protein import. RanGTP will participate the process of nuclear export [27].

2.2.3. Nuclear Export of Proteins with NES

NES is a short signal peptide in the proteins which need to go out of the nucleus and contains four to five hydrophobic amino acids. This signal directs the protein out of the nucleus through the NPC by the help of nuclear transporter (Table 2). The diversity of NESs is very high. However, the typical NES has an L-X-X-X-L-X-X-L sequence in which L is a hydrophobic amino acid, often Leucine, but also can be Valine, Isoleucine, Phenylalanine, or Methionine, and X can be any other amino acids (Figure 3). The intervening amino acids routinely are negatively charged, polar, or small amino acids [90]. Any proteins that have this signal and also RNA molecules that bind to these proteins and form ribonucleoproteins (RNPs) can export from the nucleus (Figure 2D). Chromosome region maintenance 1 protein (CRM1) or Exportin-1 (XPO1) is the most important karyopherin that helps to export of most protein cargos with NES [91]. There are ten classes of NES including 1a, 1b, 1c, 1d, 2, 3, 1a-R, 1b-R, 1c-R, and 1d-R which bind to the hydrophobic pockets of CRM1 [92,93]. Most of NESs have low affinity for CRM1 and need another molecule with RanGTP-binding ability in the nucleus called RanBP3 [94]. There are no exact reasons for this low affinity, but it has been reported that bioengineered NES with high CRM1 affinity cannot pass through the NPCs due to firm binding to the NPCs [95].

In the process of protein export, exportin CRM1 binds protein cargos containing NES and RanGTP to assemble the export complex in the nucleus (Figure 2C). CRM1 mediates the nuclear export of the complex to the cytoplasm through NPCs. RanGTP-RanGDP gradient across the NE provides the driving force for the export. When the complex reaches the cytoplasmic side, RanGAP activates the RanGTPase activity and hydrolyzes the GTP to GDP to disassemble the complex and release the protein cargo into the cytoplasm [2,79]. Some RanBPs play critical roles in the export and the cargo release [96]. For example, Ran-Binding Protein 1 (RanBP1) induces a conformation change in the export complex, and promotes the disassembly of the complex and cargo release by increasing the RanGTPase hydrolysis that is activated by Ran-GAP1 [97,98]. RanBP2, also known as Nup358, is a large protein that constitutes the cytoplasmic filaments of NPCs [99]. It works as a scaffolding protein and provides a specific docking site for the nuclear export factor CRM1 [100]. RanBP2/Nup358 also exhibits a Small Ubiquitin-like Modifier (SUMO) E3 ligase activity [99,101], suggesting that sumolation at cytoplasmic filaments of the NPC may play an important role in the regulation of nuclear transport, at least for some substrates.

2.2.4. Transcripts Export

The export of transfer (t) RNAs, micro (mi) RNAs, small nuclear (sn) RNAs and ribosomal (r) RNAs is also governed by the exportins of karyopherin family (Table 2) and the Ran-dependent NCT mechanism (Figure 2) [102]. However, the export of mRNA uses a non-karyopherin transport receptor and does not directly depend on the RanGTP-RanGDP gradient, making the mRNA export is mechanistically different from proteins and other RNAs [3,79] (Table 3 and Figure 4). Newly synthesized mRNAs are packed as large messenger ribonucleoprotein (mRNP) complexes, in which a single mRNA is associated with RNA-binding proteins (RBPs) that have functions in processing, capping, splicing and polyadenylation [21]. Thus, this exporting process involves three steps: (1) synthesis of pre-mRNA in the nucleus, processing, and packaging mRNP complexes; (2) targeting

and translocation of mRNPs via NPCs or an NPC-independent mechanism called NE budding [103]; (3) intracytoplasmic release of the mRNPs for translation [104] (Figure 4).

Table 3. Nuclear export of different types of RNAs.

RNAs Type	Key Factors	Functions in Nuclear RNA Export	References
mRNAs	NXF1/nxf1 (TAP/p15)	A transport receptor heterodimer. Bridges the interaction between mRNPs and FG Nups to facilitate transport of mRNPs through the NPC	[105,106]
	CRM1 (exportin 1)	Another major transport receptor, export a subset of endogenous mRNAs and HIV mRNA via adaptor proteins	[107]
tRNAs	Los1p/exportin-t, Msn5p/exportin-5, Nup116p	Export tRNA from the nucleus to the cytoplasm occurs through nucleopores, in an energy-dependent mechanism proceeds via the Ran pathway.	[108,109]
rRNAs	CRM1/Exp-t	rRNA export depend on Imp β family	[110]
snRNAs	Mex67 and Xpo1/Crm1	pre-snRNAs immediately exported into the cytoplasm upon binding of the export receptor Mex67-Mtr2 and the karyopherin Xpo1/Crm1	[111]
miRNAs	Exportin-5	Exportin-5 (Exp5) mediates efficient nuclear export of short miRNA precursors (pre-miRNAs)	[73]

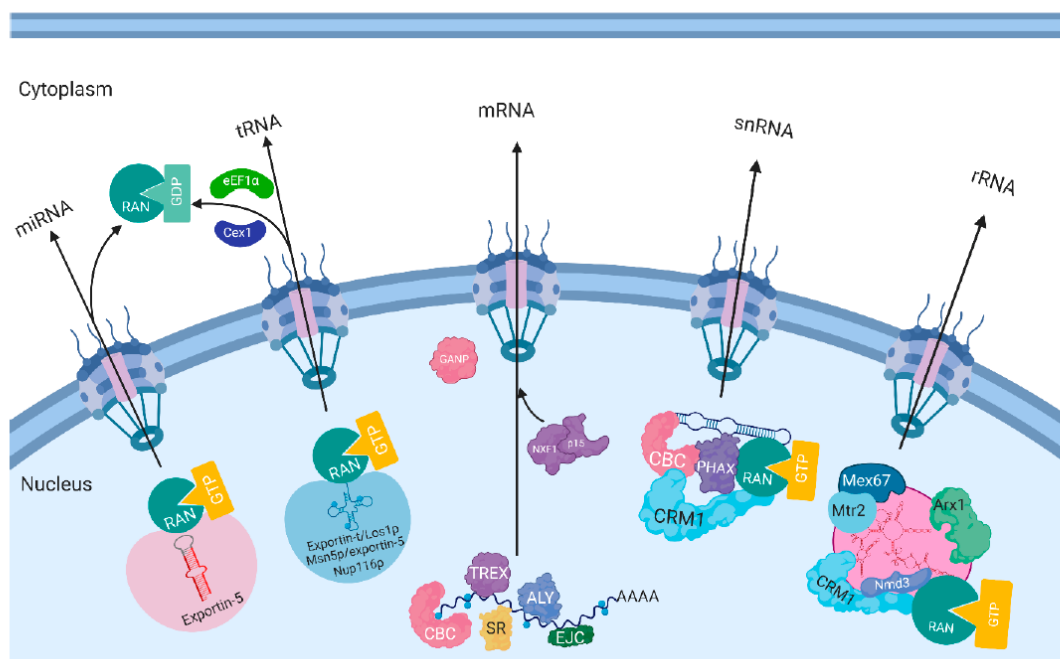


Figure 4. Nuclear export of different types of RNAs. RanGTP-RanGDP gradient provides driving force for nuclear export of miRNAs, tRNAs, snRNAs, and rRNAs, but not mRNAs. The export of mRNAs uses a non-karyopherin transport receptor and does not directly depend on the RanGTP-RanGDP gradient. The export receptors are exportin-5 for miRNAs, exportin-t for tRNAs, NXF for mRNAs, and CRM1 for snRNAs and rRNAs.

The processing of nascent mRNA transcripts including 5' capping [112,113], splicing [114], 3' end cleavage, and polyadenylation [115] has direct influence on mRNA export. In human, there are about 30 heterogeneous nuclear ribonucleoproteins (hnRNPs), which bind to the nascent mRNA transcripts during transcription elongation and make mRNAs

ready for further processing including packaging, export, and translation. Some of hnRNPs contain nuclear retention signals and should be removed before the export of mRNP. However, most of hnRNPs are attached to mRNA during export and come back to the nucleus after releasing in the cytoplasm [116].

In human, the NXF1-NXT1 (TAP-p15) heterodimer (1:1) functions as a general export receptor of mature mRNPs [117,118]. Like karyopherins, the NXF1-NXT1 complexes can physically interact with FG Nups and mediate RNP cargos pass through the nuclear membrane via NPCs. Like in the protein export process, RanBP2/Nup358 also provides a major binding site for NXF1-NXT1 dimers at the NPCs and functions in nuclear mRNA export [119]. However, once part of the mRNP reaches the cytoplasmic face of the NPC, the transport receptor NXF1-NXT1 heterodimer will be released in an ATP-dependent manner, rather than by GTP hydrolysis [120,121]. On the other hand, the mRNA export receptor NXF1 also plays important roles in coordinating transcriptional dynamics, 3' end processing, and nuclear export of long three prime untranslated region (3' UTR) transcripts [122]. The protein export receptor CRM1/Xpo1 also participates in the nuclear export of certain types of mRNAs, such as unspliced and partially spliced viral mRNAs [123]. Interestingly, besides the NPC-dependent pathway for nuclear mRNA export, another mechanism was identified in *Drosophila* body wall muscles called NE budding. In this mechanism, ultra-large ribonucleoprotein (megaRNP) granules containing mRNAs exit the nucleus by budding through the nuclear membranes independent of NPCs [103]. This NE budding pathway is proposed to be more prominent in particular biological processes or at certain growth stages that require high levels of protein synthesis, such as the rapid growth in early development or in response to stimuli [124,125].

Nuclear RNA export could be disrupted by any alterations in the exporting process, such as the changes in the NE including any alterations in the Nups which make NPCs, dysregulations of Ran gradient and its regulatory proteins, and variations in the proteins which attach to the RNAs and assemble RNPs [121,126]. Accumulating evidence indicates that RNA transport is impaired in several neurodegenerative diseases (NDs), which will be discussed in the following sections.

3. Impaired NCT in Neurodegenerative Diseases and Aging

3.1. Common Features of NDs

3.1.1. Mislocalized and Aggregated Proteins Are Hallmarks in NDs

A typical pathological hallmark of NDs is the abnormal intracellular and/or extracellular protein accumulation in affected brain regions. It is believed that this dysregulation is directly involved in neurotoxicity, neurodegeneration, and finally causes clinical manifestation of the disease [127]. The intracellular inclusions detected in the brain of patients with a ND usually contain aggregates of mislocalized and misfolded disease-specific proteins. For example, aggregated β -amyloid peptide and intracellular neurofibrillary tangles (NFTs) containing aggregated and hyperphosphorylated tau protein are often found in Alzheimer's disease (AD) [128,129]. TAR DNA-binding protein 43 (TDP-43)- or tau-positive inclusions can be detected in patients with ALS/FTD [130,131]. Intracellular Lewy bodies containing aggregated α -synuclein is a pathological feature in Parkinson's disease (PD) [132]. The aggregates of superoxide dismutase (SOD) in motor neurons can be seen in ALS [133]. In Huntington's disease (HD), the intranuclear inclusions of aggregated huntingtin protein containing polyglutamine (polyQ) expansion can be detected [134]. The exactly molecular mechanisms that trigger the formation of protein aggregates are not clear. Genetic mutations, environmental factors, and different stress conditions have been suggested to induce protein misfolding and aggregation in these diseases [127].

3.1.2. Impaired NCT Is One Fundamental Pathogenesis of NDs

Accumulating evidence indicates that alterations of NCT are the fundamental pathological factors underlying these NDs (Table 4). Under normal NCT conditions (Figure 5A), the protein transport mechanisms enable each protein cargo to reach and retain in ap-

appropriate compartments, nucleoplasm, or cytoplasm. The right subcellular localization of proteins and the proper protein-protein interactions are foundations underlying their physiological functions. Similarly, in a healthy neuron, the majority of mRNAs should be exported to the cytoplasm for protein synthesis [79,135], including some RNPs that are delivered to axons or dendrites for localized translation, through which the cells are able to achieve the spatiotemporal regulation of gene expression with extraordinary precision [136]. The appropriate distributions of transcripts are extremely important in the regulation of gene expression and as well as in the maintenance of the hemostasis of local protein reservoir [137,138].

Table 4. Neurodegenerative diseases with impaired NCT and possible mechanisms.

ND Diseases	Mutations	Related NCT Defect	Mechanisms
ALS/FTD	C9orf72 [139], SOD1 [140], FUS [141,142], TARDBP [143,144], CHCHD10 [145,146], UBQLN2 [147], SQSTM1 [148,149], OPTN [150,151], VCP [152,153], TBK1 [154], MAPT [155], GRN [156], CYLD [157]	Impairment of Nup35, Nup50, Nup54, Nup58, Nup62, Nup88, Nup93, Nup98, Nup107, Nup153, Nup155, Nup160, Nup188, Nup205, Nup214, Nup358, Kap β 1, RanGAP1, importin- α/β , Xpo5, Gle1, Nxf1 [158–164].	Cytoplasmic aggregates of DPRs, SOD1, TARDBP, FUS and tau [130,131,133,165–167].
HD	HTT [168]	Impairment of RanGAP1, Rae1, Nup Sec13, Nup62, Nup88, Gle1 [12,13,169]	Nuclear accumulation of mutant Huntingtin (mHTT), disrupts NE architecture and NPCs, sequesters NCT factors (Gle1 and Ran-GAP1) [12,13,134].
AD	APP [170], Presenilins 1 [171], Presenilins 2 [171], ABCA7 [172] and SORL1 [173]	Impairment of Nup62, Nup98, importin- α 1, Accumulation of NTF2 [14,128,174]	Accumulation of amyloid plaques and neurofibrillary tangles [128,129,175]. Tau directly interacts with nucleoporins of NPCs and affects their structural and functional integrity [14].

ALS: Amyotrophic lateral sclerosis; FTD, Frontotemporal dementia; HD, Huntington's disease; AD, Alzheimer disease.

Dysregulations of protein NCT process will interfere with the normal distributions of protein cargos, leading to protein mislocalization (Figure 5B). Protein mislocalization could occur in nucleus or cytoplasm in diseased or aged neurons. Subsequently, the hemostasis of local protein reservoir could be disrupted, leading to abnormal protein-protein interactions, and triggering the formation of protein aggregates [176,177]. On the other hand, these aggregated proteins may have deleterious effects on neuronal functions by sequestering factors that are critical in signaling pathways or the NCT machinery. For example, by interfering with the NPCs, TDP-43 aggregates or poly-dipeptides encoded by C9orf72 repeat expansion in ALS/FTD and tau proteins in Alzheimer's disease disrupt the NCT activities in diseased neurons [8,158,178]. These disruptions will further aggravate the impaired NCT activities and cause more severe protein and transcript mislocalization.

If nuclear transcript export mechanisms are impaired in a neuron (Figure 5C), the normal distribution of RNAs will be disrupted, such as nuclear RNA accumulation and mRNA mislocalization. These abnormal subcellular distribution of RNAs will dysregulate the protein synthesis, such as localized mRNA translation in axons and dendrites, leading to abnormal compartmental protein reservoirs [2,179,180]. Finally, the effects of impaired NCT, no matter the dysregulated protein transport or the disrupted nuclear RNA export, will be converged to neuronal toxicity and dysfunction, resulting in neurodegeneration and clinical manifestation of the disease (Figure 5D).

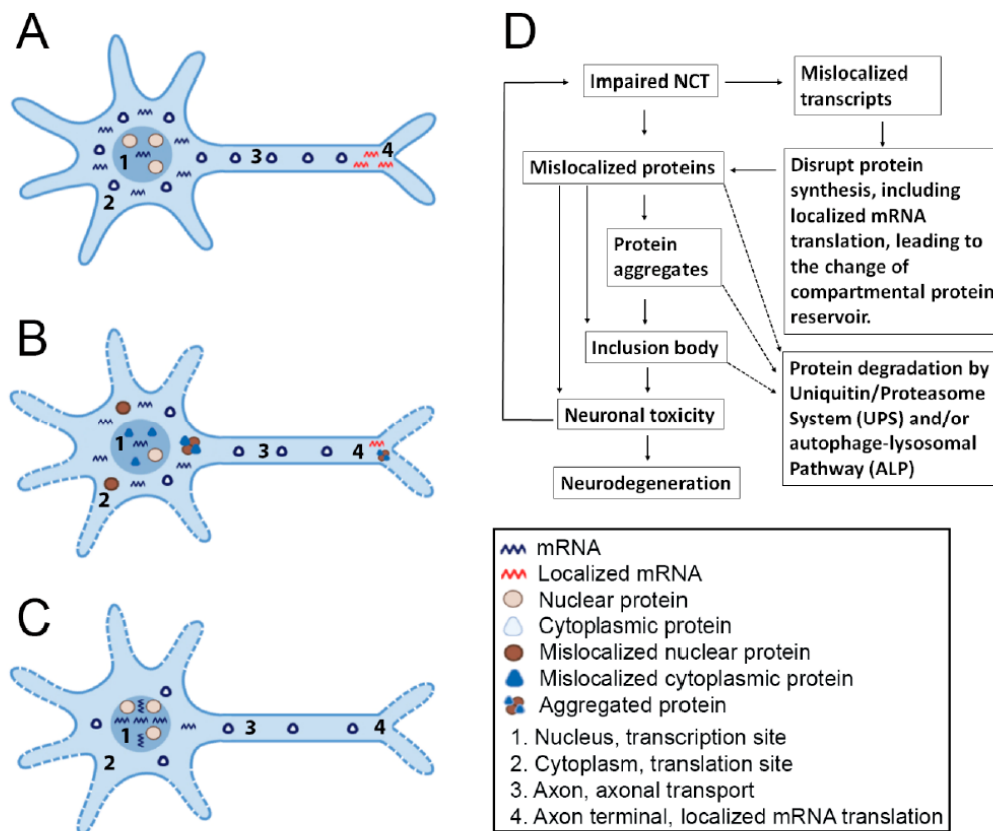


Figure 5. The linkages between impaired NCT and neurodegeneration. (A) A neuron possesses normal NCT activities. Transport mechanisms enable both protein and transcript cargos to reach and retain in appropriate compartments at subcellular locations of nucleus (1), cytoplasm (2), axons (3), and axon terminals (4), at which some localized mRNAs are translated into proteins. The majority of mRNAs localize in the cytoplasm. (B) A neuron with dysregulated protein NCT. The normal distributions of protein cargos are disrupted and cause protein mislocalization in either nucleus (1) or cytoplasm (2). Abnormal protein-protein interactions and protein aggregates (3 and 4) could disrupt neuronal functions and finally lead to neurodegeneration. (C) A neuron with impaired nuclear mRNA export. Nuclear mRNA accumulation and mislocalization (1) will occur and disrupt the protein synthesis, leading to the change of compartmental protein reservoir (2 and 3). Localized mRNA translation at axons and dendrites could be diminished (4). (D) Linkages between impaired NCT and neurodegeneration. Impaired NCT causes protein and transcript mislocalization, which will disrupt the homeostasis of local protein reservoirs and cause abnormal protein-protein interactions and/or protein aggregation, which may lead to neuronal toxicity, including interfering with NCT factors and signaling pathways, and finally causes neurodegeneration.

Neurons are specialized cellular subtypes that possess unique features and functions, including long processes and actively transport between soma and axon terminals, complex synaptogenesis, and synaptic connections. These features make neurons more vulnerable to the impairment of intracellular transport. The implication of defective NCT in NDs has been revealed by a variety of research model systems, including *Drosophila* [181,182], mouse [12,183], patient-derived induced pluripotent stem cells (iPSCs) [17,79,159,184–186], and autopsy brain of patients [187]. The reported NCT-defective diseases, related mutations and possible mechanisms were summarized in Table 4. In the following section of this review, we will take some diseases as examples to discuss how impaired NCT contributes to the pathogenesis of these diseases.

3.2. Impaired NCT in NDs

3.2.1. Amyotrophic Lateral Sclerosis (ALS)

ALS is one of motor neuron diseases (MNDs) with excessive weight loss and muscles wasting even in the tongue, emotional lability, and cognitive dysfunction. Several pathophysiological mechanisms are involved in ALS including glutamate excitotoxicity, dysregulated interactions between neurons and glial cells, intracytoplasmic and intranuclear aggregation of certain proteins and RNAs, impairment of NCT and axonal transport, and changes in the axon terminals and neuromuscular junction (NMJ). Accumulating evidence indicates that defective NCT is a key mechanism in the pathophysiology of ALS. Sometimes, irregular nuclear morphology is seen in the ALS neurons. Irregularity of full lack of Nup62 has been reported in some cases of sporadic and familial ALS with SOD1 mutation [160] as well as mice model with mutations of SOD1 [188,189]. In addition, disruption of nuclear staining of Nup62 and Kap β 1 are reported in spinal motor neurons of sporadic ALS with clear mislocalization of TDP-43 [160]. Moreover, Rocznik-Ferguson and Ferguson reported a significant decrease in Nup188 protein level in human TDP-43 knockout cells which is contributed to the abnormal nuclear pore morphology [161]. Besides the dysregulations in NPCs and Nups, NCT defects are also related to the disruptions of Ran gradient, which provides the drive force for the nuclear transport of most transcript and protein cargos. For example, the hexanucleotide GGGGCC repeat RNA of C9orf72 could directly interact with RanGAP and cause its mislocalization [11,158]. By using fly model and the mammalian cells, Freibaum and coworkers reported that di-peptide repeat proteins (DPRs), which translated from six reading frames in either the sense or antisense direction of the hexanucleotide repeat [190], caused a loss of function in the Nup50 and changed the function of Nup153 and transportins, which help Nup50 and Ran in NCT [162]. Recently, Coyne and collaborators also found that GGGGCC repeat RNA of C9orf72 reduced eight Nups which initiated by reduction in POM121 and subsequently this reduction decreased the expression of other seven Nups and altogether affected the localization of Ran GTPase [191].

There are some controversies about the roles of these DPRs in the pathophysiology of ALS. Shi and collaborators reported that poly-PR dipeptides could disrupt NCT by attaching to the central channel of nuclear pores and locking the FG repeats of Nup54 and Nup98 [178]. In another study, Khosravi et al. reported that the non-coding region of C9orf72 gene repeat expansion related to poly-GA aggregations inhibited the nuclear import of a reporter containing NLS but did not affect a non-classical PY-NLS. In addition, they found that these poly-GA aggregations prohibited the nuclear transport of p65, which is induced by TNF α possibly through the impairment of importin- α / β -dependent pathway [163]. However, by using of Hela Kyoto cells that expressing the shuttling reporter NLSSV40-mNeonGreen2x-NESpki, Vanneste et al. demonstrated that poly-GR and poly-PR did not directly impair NCT. The exact molecular mechanisms underlying the impaired NCT by DPRs in ALS needs more investigations [192].

Mutations in the profilin1, a small actin-binding protein that regulates actin polymerization, cause NCT impairment in motor neurons in familial ALS. In the motor neurons with mutant profilin1, Ran, RanBP2 and RanGAP1 were misdistributed in the cytoplasm instead of normal NE localization. Additionally, profilin1 mutations changed the nuclear membrane structure and clearly decreased nuclear import [9]. On the other hand, TDP-43 aggregates in cytoplasm contain certain NCT components including Nups, such as Nup35, Nup58, Nup62, Nup88, Nup93, Nup98, Nup107, Nup153, Nup155, Nup160, Nup205, Nup214, and Nup358, and transport factors, such as Xpo5, Gle1, Nxf1, RanGap1, and Ran [158,159]. Furthermore, in ALS with C9orf72 mutations, nuclear pore integrity could be rescued by modulation of actin polymerization, which plays an important role in connection of cytoskeleton and NCT [9]. Moreover, Bennett and collaborator found that sentaxin may play a role in the pathophysiology of ALS [193]. Sentaxin is a RNA-binding protein that regulates transcription but not its termination in higher eukaryotes [194]. They showed that TDP-43 is aggregated in the neuronal cytoplasm of mice with sentaxin mutations.

Also, by immunocytochemistry for two NCT proteins including Ran and RanGAP1, they confirmed that the nuclear membrane was deformed and nuclear import was impaired in motor neurons of mutated mice [193]. Furthermore, a proline to serine conversion in the position of 56 in the vesicle-associated membrane protein-associated protein B and C (VAPB) has been reported in ALS patients, in which the transport of Nups and emerin to the NE was blocked and causes a NE defect [195]. Finally, as a lateral mechanism, it has been reported that motor neurons devoid of adenosine deaminase acting on RNA2 (ADAR2) are abnormally permeable to Ca^{2+} through the abnormal AMPA receptors. This intracellular increase of Ca^{2+} activates calpain, a Ca^{2+} -dependent protease that cleaves TDP-43 into aggregation-prone fragments and also disrupts NCT by cleaving molecules involved in nuclear transport, including Nups [196]. Thus, nuclear envelope, NPC and Nups, Ran and Ran regulators, and other transport factors could be disrupted by mutations in ALS, leading to impaired NCT.

3.2.2. Frontotemporal Dementia (FTD)

FTD is a clinical neurodegenerative disease and first described by Arnold Pick in 1892 [197]. It is characterized by progressive behavior deficits, executive function deficiency, language diminution, aphasia, lobar atrophy, and presenile dementia. FTD includes three clinical variants, behavioral variant FTD, non-fluent variant primary progressive aphasia, and semantic-variant primary progressive aphasia [198]. Less than 13% of patients with behavioral variant FTD and about 40% of patients with FTD show motor neuron disease [199]. In FTD, neuronal degeneration occurs mostly in the frontotemporal lobe and is distinguished by loss of neurons, gliosis, and certain alterations in the microvacuolar structure of frontal lobes [200]. The microtubule-associated protein tau (MAPT), the transactive response DNA-binding protein (TARDBP), and fused in sarcoma (FUS) are the most important affected proteins in FTD patients. Moreover, it has been reported that numerable cases of FTD with frontotemporal lobar degeneration had some inclusion bodies containing ubiquitin, neural precursor cell-expressed and developmentally down-regulated 8 (NEDD8) or p62, which are not FTD specific and can be seen in other ubiquitinated inclusions [200,201]. The most important mutations that seen in 35–65% of familial FTD patients include C9orf72, MAPT and GRN, while mutations in other genes are rarely seen [202]. Like ALS, a noncoding hexanucleotide repeat expansion in C9orf72 is reported as the most common genetic cause of FTD [203,204]. It is believed that the toxic gain of functions including translational repression, dysfunctions in mitochondria and nucleolus, and NCT disruption through the expression of DRPs are the main pathophysiological mechanisms [187,205].

NCT defects in FTD by DPRs occur through several mechanisms. Similar to ALS, DPRs disturbed the activity of Nup50, Nup153, and transportins as essential proteins in nuclear import [162]. In addition, the hexanucleotide repeat RNA of C9orf72 mislocalized RanGAP and disrupted its activity in the NCT [11]. Moreover, DPRs are neurotoxic and can cause neurodegeneration in cell culture and in animal models. By two unbiased genetic screens in a yeast model, overexpression of certain karyopherins including MTR10 and KAP104 did not change the mislocalization of DPRs in the rescue of DPRs toxicity. Also, in the 70–80% of induced neurons from patients with C9orf72, mislocalization of RCC1, also known as RanGEF, was occurred and just a very weak or no nuclear RCC1 signal was detected [10]. The MAPT which is encoded by MAPT gene on chromosome 17q21, stimulates tubulin polymerization into microtubules and stabilizes microtubules [206]. By using a model of MAPT mutation-induced FTD in human stem cells, Paonessa and colleagues found that cell body and dendrite mislocalization and hyperphosphorylation of tau in cortical neurons. This mislocalization apparently misshapes the nuclear membrane and directly influenced NCT which could be reversed by microtubule depolymerization [207].

3.2.3. Huntington's Disease (HD)

HD is a progressive neurodegenerative disease caused by a trinucleotide (CAG) expansion in exon 1 of the Huntingtin (HTT) gene, resulting in the expression of an expanded, mutant huntingtin protein (mHTT) [168]. In this disease, HTT protein has a long polyglutamine tract in the N-terminal. The most common cytological finding in HD is the aggregation of cleaved HTT protein in nucleus, cytoplasm, dendrites, and axon terminals. These aggregates are toxic and cause neuron death, mostly occur in striatal neurons in the basal ganglia [134]. The possibilities of selective neuronal degeneration in the striatal neurons could be due to the lack of brain-derived neurotrophic factor (BDNF) support, glutamate excitotoxicity [208,209], and expression of Rhes protein as a mediator of mHTT cytotoxicity in the striatal area [210].

Accumulating evidence indicates that the impaired NCT is another pathogenic factor of HD and mHTT could disrupt NCT by interfering with NPCs, Nups and other transport factors. For example, proteomic investigations confirmed that wild type HTT preferably bind to the nuclear import receptors importin- β 1, 4, 7, and 9, while mHTT interacts with RanGAP1, the mRNA export factor Rae1, and the Sec13, revealing the abnormal protein-protein interactions between mHTT and NCT factors [169]. Nuclear accumulations of polyA-mRNA were seen in certain HD animal models and human patients [12,177], suggesting that the nuclear mRNA export was impaired in HD. Grima and collaborators evaluated different models of HD, including fly, mice, mHTT transgenic neuron, HD iPSC-derived neuron, and cadaver brain tissues, and found that mislocalization and aggregation of Nups and the NCT defects in these models. In their mouse model with the expression of exon 1 of human HTT with 125–160 CAG repeats, nuclear co-aggregation of mHTT with RanGAP1 and Nup62 was detected. They further demonstrated that the FG repeats in Nup62 and RanGAP1 attach to the mHTT [13]. Another study indicated that these inclusion bodies also contain Nup88 and Gle1, which are important components for mRNA export [12]. Consistently, the nuclear level of RanGAP1 decreased over the time in the disease and its diminishing was recovered by anti PIAS1 miRNA that decreased the mHTT level [13]. These findings suggested that mHTT comprehensively influence the components of NTC machinery.

Woerner and collaborators reported that just cytoplasmic aggregation, not nuclear and perinuclear inclusions, interfered with NCT of proteins and RNAs [177]. This is consistent with the finding that specific inhibition of CRM1 which leads to inhibition of nuclear export is neuroprotective and reverse NCT defect [13], suggesting that the toxicity of mHTT mainly occur in cytoplasm. The researchers also evaluated another heterogeneous mouse model that expresses human HTT exon 1 sequence containing 193 CAG repeat instead of the mouse HTT exon 1 and found that co-aggregation and localization of mHTT with RanGAP1 and Nup88 [13]. They also confirmed that the aggregation and mislocalization of RanGAP1 and cytoplasmic and intranuclear mislocalization of Nup62 also occur in the brain tissue of HD patients. More evidence of impaired NCT can be noticed in HD iPSC-derived neurons, including a significant decrease in the nuclear to cytoplasmic ratio of endogenous Ran, and an increase of the nuclear concentration of microtubule-associated protein 2 (MAP2) that normally localize in the cytoplasm, and the decrease of both RanGAP1 and Nup62 expression [13]. Moreover, a rodent cortical neuron transfected with HTT 82Q showed a clear cytoplasmic mislocalization of Ran that indicated the impairment of NCT in HD [13].

HD repeat-associated non-AUG translation (HD-RAN) proteins are novel homopolymeric expansion of poly(Ala) and poly(Ser) from the sense transcript, and poly(Leu) and poly(Cys) from the antisense transcript [211]. Despite repeat polyglutamine, the neuronal accumulation and toxic effects of HD-RAN proteins in the HD human autopsy brains were reported [212]. These HD-RAN proteins can also impair both active and passive NCT [13]. Furthermore, modification of Nup62 via O-linked N-acetylglucosamine (O-GlcNAc) transferase could be related to the HD pathology because of using an inhibitor of O-GlcNAc transferase in the primary mouse mHTT-expressing cortical neurons can rescue the NCT defects [213].

3.2.4. Alzheimer's Disease (AD)

AD is one of the important and most concerning neurodegenerative diseases worldwide especially in the elderly and contributes an estimation of 60–80% of dementia cases. It has been predicted that the number of people with AD grows to 13.8 million in the United States by 2050 [214]. The molecular pathophysiology of AD is closely related to the metabolism of the amyloid beta precursor protein (APP), a transmembrane protein that can be cleaved by α , β , and γ secretases. Just sequential cleavages by β secretase and γ secretase create two amyloid proteins of 40 and 42 amino acids. $A\beta$ 42 aggregates as fibrillary amyloid protein in meningeal, cerebral vessels, and gray matter. This $A\beta$ aggregation results in phosphorylation and aggregation of tau, a protein that stabilizes axonal microtubules, as twisted paired helical filaments of NFTs. These two aggregations cause neuronal degeneration and neuronal death in AD. On the genetic side of view, it has been reported that mutations in the APP gene on the chromosome 21, Presenilin1 (PSEN1) on the chromosome 14 and PSEN2 on the chromosome 1 that regulate the activity of γ secretase, and sortilin (SORT1) on the chromosome 1 that mediates surface APP transport to intracellular Golgi-ER complex are associated with AD. Additionally, an SNP (rs5848) in the GRN gene that leads to reduced PGRN levels and activates neuroinflammatory, pathological, and cognitive-based disease, is linked to the increased risk of AD [215]. Recently, Eftekharzadeh et al. reported that somatodendritic accumulation of tau enhanced the perinuclear tau concentration that consequently interacted with certain Nups including Nup98 and Nup62. These interactions resulted in the missorting of Nups into the cytosol and led to impaired NCT. They also found that nuclear leak and Ran mislocalization occurred in relation to the phospho-tau accumulation, which could ruin both active neuronal nuclear transport [14]. In AD, NFTs cause nuclear irregularity that can be seen simultaneously with irregular nuclear distribution of Nup62 and cytoplasmic aggregations of NTF2 [128]. These findings suggested that nuclear pores and Nups are disrupted in AD.

More evidence also indicated that other regulatory factors of NCT machinery were dysregulated in AD. Neuronal TDP-43 positive inclusions were detected in near 26% of confirmed AD patients [216]. Hippocampus of AD patients showed Hirano bodies, the intracytoplasmic inclusions that have been shown to contain importin- α 1 [128,174]. NRF2, the nuclear transport of which is mediated by importin- α 5 and β 1, normally found in both cytoplasm and nucleus, but it is accumulated in cytoplasm in AD patients [217]. In AD mice model, importin- α 1 expression was reduced and nuclear ultra-architecture continuum was disturbed [218]. Despite Nups and transportins, disrupted nuclear lamina [219] and nuclear localization of phospho MAPK/ERK kinase 1 in AD cases has been reported [220]. In both animal model and human brain, tau accumulation can reduce lamina protein levels and cause nucleoplasmic reticulum expansion, resulting in a nuclear invagination [219]. On the other hand, affected neurons in AD with neurofibrillary tangle showed proximity of paired helical filaments to the NE and NPC [221,222]. By using the transgenic mouse model, Ke and colleagues found that tau can deplete splicing factor proline- and glutamine-rich (SFPQ) from nucleus and induce its accumulation in cytoplasm of the amygdala [223]. Moreover, by evaluation of brain biopsies of patients with early and late stages of AD using electron microscope, it has been found that fascicles of paired helical filaments oppose the NE, NPC and the perinuclear polysomes and disrupted the crosstalk between nucleus and cytoplasm [221]. All these disrupted factors and pathways could contribute to the AD pathogenesis via the dysregulations of NCT.

3.3. Impaired NCT in Aging

Aging is an irreversible physiological process that is characterized by the progressive alterations in the metabolism of cells, impaired self-regulation, degeneration, and structural and functional changes [224]. Aging itself is the primary risk factor for most neurodegenerative diseases, including ALS/FTD, HD and AD that have been discussed above. The molecular mechanisms underlying the aging process and lead to NDs are still mysterious. It has been shown that the nuclear transport activities were compromised during aging

due to dysregulations of NPCs, Nups and other transport factors, such as Ran-binding proteins [5,187]. Oxidative damage of long-lived scaffold Nups during aging is associated with defects in nuclear transport and the breakdown of the nuclear permeability barrier [225], suggesting the correlation between impaired NCT and aging process. This notion was further supported by the identification of long-lived proteins that include Nups [226]. Toyama et al. have conducted a system-wide identification of proteins with exceptional lifespans in the rat brain. They found that NPCs are maintained over a cell's life through slow but finite exchange of even its most stable subcomplexes. This maintenance is limited and these proteins are inefficiently replenished after damage or protein levels decrease during aging, providing a rationale for age-dependent deterioration of NPC function and NCT [226]. Accelerated leakiness during aging and oxidative damages of Nups in old cells were reported. Nup93, as the establisher of the NPC diffusion barrier, was lost in old *C. elegans* cells but no changes were seen in Nup107 and Pom121 [225]. Aged-human fibroblast-derived neurons exhibited an age-dependent decline in the nuclear transport receptor RanBP17 that led to a loss of nucleocytoplasmic compartmentalization [5]. Furthermore, age-dependent decrease in the NPC density of dentate gyrus neurons in the rat brain was confirmed by electron microscopy [227]. Even if the number of NPCs stays constant like in the CA1 pyramidal cells of aged rat brain, accumulated NPC damage may still cause a lack of performing their functions [225]. Consistently, diffused protein aggregations were seen in aged *C. elegans* neurons [228], because Nups play a critical role in the prevention of protein aggregation in the neuronal cells [229]. Additionally, HeLa cells contained Hutchinson Gilford progeria syndrome (HGPS) mutation showed mislocalization of Nup62 and Nup153, decreased the lamina dynamics, and the impaired nuclear import [230]. Other studies include an age-related diminish in NCT [12] and age-induced accumulation of misfolded proteins [177] that caused NCT defect. These findings further demonstrated that impaired NCT may constitute a major molecular mechanism underlying aging process.

Indeed, NCT decreased and simultaneously protein aggregation increased with aging [231]. Oxidative damage is one of the possible links between aging and NPC defects. In young cells, Lamin B1 recruits the antioxidant transcription factor OCT1 and protects cells against oxidative stress [232]. Age-dependent down-regulation of lamin B1 increases cellular sensitivity to oxidative stress [233]. In aged neurons, this oxidative stress damaged NPC [225] and disrupted NCT [234]. Reactive nitrogen species (RNS) induce nitrosative stress that is associated with age-related neurodegeneration. For instance, the S-nitrosylation of CDK5 by nitric oxide stimulates nuclear lamina dispersion [235,236]. On the other hand, accumulation of a truncated lamin A mutant, progerin, caused impairment of nuclear import and decreased nuclear sumoylation due to oxidative or nitrosative stresses [237]. Nitric oxide can inactivate CRM1 via S-nitrosylation and impair CRM1-Ran-cargo export [238]. It will be interesting to further evaluate the association of nitrosative stress and affected NCT in neurons. Although the recent studies have greatly advanced our understanding of the aging biology, extensive studies are still required to reveal the exact pathophysiological mechanisms underlying impaired NCT in aging process, especially to dissect the causes and consequences between impaired NCT and aging.

4. Conclusions

Here we highlighted the major components of nucleocytoplasmic transport, the regulatory mechanisms and its linkage to neurodegenerative diseases and aging. Despite the identified impairments of NCT in different neurodegenerative diseases and aging, the exact molecular pathological mechanisms underlying these impaired NCT and the contributions to the clinical syndromes are not fully understood. In this emerging research field, at least the following directions should be emphasized in the future research. First, the regulatory mechanisms of NCT under physiological condition. The elucidation of detailed molecular mechanisms of NCT regulation is the prerequisite to understand the impaired NCT under diseased conditions, including the specific signaling pathways in the regulation of different cargos. Second, to develop reliable and efficient methods to screen NCT-defective condi-

tions. In cultured cells, florescent reporters fused with NLS or NES are widely used for protein transport assay, and fluorescence in situ hybridization (FISH) is usually employed to measure the nuclear transcripts export [5,17,79]. However, these approaches work poorly at specificity and efficiency in brain tissues. Some biomarkers need to be identified and developed to reveal defective NCT activities in clinical samples. Third, to decipher the pathogenesis of defective NCT underlying diseased neurons and identify the molecular targets for therapeutic interventions. These molecular targets could be fundamental factors that regulate NCT process or some factors or pathways that are specifically involved in certain diseased conditions. The progress of neurodegeneration could be decelerated or even ameliorated if the intervention approaches are able to effectively rescue the defective NCT activities.

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Abbreviations

AD	Alzheimer disease
ALS	amyotrophic lateral sclerosis
APP	amyloid beta precursor protein
BDNF	brain-derived neurotrophic factor
CRM1	Chromosome region maintenance 1 protein
DPRs	di-peptide repeat proteins
ER	endoplasmic reticulum
FG repeats	phenylalanine-glycine repeats
FISH	fluorescence in situ hybridization
FUS	fused in sarcoma
FTD	frontotemporal dementia
hnRNPs	heterogeneous nuclear ribonucleoproteins
HAP1	huntingtin associated protein 1
HD-RAN	HD repeat-associated non-AUG translation
HTT	Huntingtin
INM	inner nuclear membrane
Kap	Karyopherin
MAP2	microtubule-associated protein 2
MAPT	microtubule-associated protein tau
mHTT	mutant huntingtin
MNDs	motor neuron diseases
mRNP	messenger ribonucleoprotein
NCT	nucleocytoplasmic transport
NDs	neurodegenerative diseases
NE	nuclear envelope
NEDD8	neural precursor cell-expressed and developmentally down-regulated 8
NES	nuclear export sequence
NFTs	neurofibrillary tangles
NLS	nuclear localization sequence

NMJ	neuromuscular junction
NPC	nuclear pore complex
NTR	nuclear transport receptors
Nups	nucleoporins
O-GlcNAc	O-linked N-acetylglucosamine
ONM	outer nuclear membrane
PSEN1	presenilin1
PY-NLSs	proline-tyrosine amino acid pairing
RAN	Ras-related nuclear protein
RanBP1	Ran-binding protein 1
RanBP2/Nup358	Ran-binding protein 2/nucleoporin 358
RanBP3	Ran-binding protein 3
RanGAP	Ran GTPase-activating protein
RanGEF	Ran Guanine nucleotide Exchange Factor
RBP	RNA-binding proteins
RCC1	regulator of chromosome condensation 1
RNPs	ribonucleoproteins
RNS	Reactive nitrogen species
SFPQ	splicing factor proline- and glutamine-rich
SOD1	superoxide dismutase-1
SORT1	sortilin
TARDBP	transactive response DNA-binding protein
XPO1	Exportin-1

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Lab Resource: Multiple Cell Lines



Generation of two induced pluripotent stem cell lines with heterozygous and homozygous GAG deletion in TOR1A gene from a healthy hiPSC line

Masuma Akter^a, Haochen Cui^a, Yi-Hsien Chen^b, Baojin Ding^{a,*}^a Department of Biology, University of Louisiana at Lafayette, 410 East Saint Mary Boulevard, Lafayette LA 70503, USA^b Genome Engineering and iPSC Center (GEIC), Washington University at St Louis, MO 63110, USA

ABSTRACT

A typical DYT1 dystonia is caused by a heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (ΔE , p.Glu303del) and the pathogenesis is not clear. In this study, human induced pluripotent stem cell (hiPSC) lines carrying the heterozygous or homozygous GAG deletion in *TOR1A* gene were generated by genetic modification of a healthy hiPSC line (WTC11, UCSF001-A). These hiPSC lines showed the normal stem cell morphology and karyotype, expressed the same pluripotency markers as their parental line, and had the capacity to differentiate into three germ layers, providing a valuable resource in determining the pathogenesis of human DYT1 dystonia.

1. Resource Table

Unique stem cell lines identifier	ULLi002-A-49 ULLi002-A-51
Institution	University of Louisiana at Lafayette, LA USA
Contact information of the reported cell line distributor	Baojin Ding (Baojin.Ding@Louisiana.edu)
Type of cell lines	iPSC
Origin	Human
Additional origin info (applicable for human ESC or iPSC)	Age: 30 YR Sex: Male Ethnicity: Asian
Cell Source	Skin fibroblasts.
Method of reprogramming	N/A
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT/q-PCR
Cell culture system used	Serum-free and feeder-free medium
Type of Genetic Modification	Induced mutation
Associated disease	DYT1 dystonia
Gene/locus	<i>TOR1A</i> c.907_909delGAG (p.Glu303del)/9q34.11
Multiline rationale	Isogenic clones carrying heterozygous or homozygous of the same gene mutation.
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporated with a 4D-Nucleofector (Lonza) using CA-137 program.
All genetic material introduced into the cells	gRNA vector MLM3636 (Addgene #43860) Cas9 vector p3s-Cas9HC (Addgene #43945)

(continued on next column)

(continued)

Unique stem cell lines identifier	ULLi002-A-49 ULLi002-A-51
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	4/29/2021
Cell line repository/bank	https://hpscereg.eu/cell-line/ULLi002-A-49 https://hpscereg.eu/cell-line/ULLi002-A-51
Ethical/GMO work approvals	Genetic modification was performed at Genome Engineering and iPSC Center (GEIC) at Washington University in St. Louis.
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

2. Resource utility

Although rodent models of DYT1 dystonia provide insights into disease mechanisms, significant species-dependent differences exist because animals with the identical heterozygous mutation fail to show pathology. These hiPSC lines will provide a valuable resource to develop human cellular systems in modeling DYT1 dystonia. [Table 1](#)

* Corresponding author.

E-mail address: Baojin.Ding@Louisiana.edu (B. Ding).<https://doi.org/10.1016/j.scr.2021.102536>

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3. Resource details

Dystonia is a common movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and/or postures. Because the clinical characteristics and underlying causes of dystonia are very heterogeneous, the pathological mechanisms of dystonia remain largely unknown (Ding, 2022). The diagnosis and etiological definition of this disorder remain challenging. The childhood-onset DYT1 dystonia represents the most frequent and severe form of dystonia, providing an excellent example to understand the pathogenesis of this disease. The typical DYT1 dystonia is caused by a heterozygous GAG deletion (c.907–909) in the *TOR1A* gene (ΔE , p. Glu303del) (Ozelius et al., 1997). Interestingly, mice with the identical *Tor1a* mutation as a heterozygote failed to show any pathological phenotypes (Goodchild et al., 2005), suggesting that the significant species-dependent differences exist between DYT1 mouse models and human patients. However, the limited access to patient neurons and the lack of *in vitro* human neuronal systems greatly impede the progress of research in dystonia. Generation of hiPSC lines containing disease-causing mutations and their isogenic controls has been shown to be a powerful tool in modeling human neurological diseases (Sepehrmanesh and Ding, 2020; Ding et al., 2021). In this study, we genetically modified a well-characterized hiPSC line (WTC11, UCSFi001-A) with CRISPR/Cas9 method and obtained two iPSC clones carrying the heterozygous (ULLi002-A-49, or UCSFi001-A-49) and homozygous (ULLi002-A-51, or UCSFi001-A-51) GAG deletion in the *TOR1A* gene, respectively. These hiPSC lines and their isogenic controls provide a valuable resource in modeling DYT1 dystonia.

Both lines showed a typical pluripotent stem cell morphology with a high nucleus/cytoplasm ratio (Fig. 1A). Sanger DNA sequencing of polymerase chain reaction (PCR) products (Fig. 1B) and deep sequencing analysis (Supp. Fig. S1A and S1B) confirmed that these iPSC lines

contain heterozygous (ULLi002-A-49) and homozygous (ULLi002-A-51) GAG deletion (c.907–909) in the *TOR1A* gene. Chromosomes from iPSCs were harvested and analyzed using the GTW banding method. Of 20 metaphase cells examined of each clone, all cells are characteristic of a chromosomally normal male karyotype, 46, XY (Fig. 1C). Short tandem repeat (STR) analysis at 18 loci indicated that ULLi002-A-49 and ULLi002-A-51 clones completely matched the parental hiPSC (UCSFi001-A) identity (Supp. Fig. S1C). Immunostaining indicated that these iPSCs highly expressed pluripotency markers of *OCT4*, *NANOG*, *SOX2*, and *SSEA4* (Fig. 1D). Quantitative RT-PCR analysis demonstrated that the pluripotency markers of *OCT4*, *SOX2*, *NANOG*, and *KLF4* were expressed at similar levels as their parental line (UCSFi001A) (Fig. 1F). Following spontaneous differentiation, embryoid bodies (EBs) (Fig. 1E) derived from ULLi002-A-49 and ULLi002-A-51 displayed dramatic upregulation of markers of the ectoderm (*PAX6*, *OTX1*), mesoderm (*DCN*, *IGF2*, *GATA2*) and endoderm (*SOX7*, *SOX17*) lineages. The expression levels of these trilineage markers were consistent with their parental line UCSFi001-A and much higher than undifferentiated iPSCs (Fig. 1G). PCR screening demonstrated that ULLi002-A-49 and ULLi002-A-51 were negative for mycoplasma (Fig. S1 D).

4. Materials and methods

4.1. Generate and culture *TOR1A* mutant iPSCs

Human *TOR1A* mutant clones were generated from the WTC11 iPSC line (Gladstone institute, UCSFi001-A) by the Genome Engineering and iPSC center (GEIC) at Washington University in St. Louis. Briefly, approximately 1×10^6 single cells were resuspended in P3 primary buffer (Lonza) with gRNA/Cas9 ribonucleoprotein (RNP) complex (200 pmol synthetic gRNA and 80 pmol SpCas9 protein) and h*TOR1A* mutant single-stranded oligo donor (ssODN) (Table 2). Subsequently, cells were

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Typical primed pluripotent human stem cell morphology.	Fig. 1 Panel A
Pluripotency status evidence for the described cell line	Qualitative analysis	Immunocytochemistry showed expression of pluripotency markers: <i>OCT4</i> , <i>SOX2</i> , <i>NANOG</i> , <i>SSEA4</i> .	Fig. 1 Panel D
	Quantitative analysis	Compared to DAPI, % of positive cell (ULLi002-A-49, ULLi002-A-51) <i>OCT4</i> : 98%, 98%; <i>SOX2</i> : 99%, 98%; <i>NANOG</i> : 97%, 95%; <i>SSEA-4</i> : 97%, 98%. RT-PCR showed highly express <i>OCT4</i> , <i>SOX2</i> , <i>NANOG</i> , <i>KLF4</i> .	Fig. 1 Panel D and F
Karyotype	Karyotype (G-banding) and resolution	46, XY, Resolution 400	Fig. 1 Panel C
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site and deep sequencing analysis	Heterozygous or homozygous GAG deletion (c.907–909) in <i>TOR1A</i> gene.	Fig. 1 Panel B and Supplementary Fig. S1A and B.
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	Off Target Analysis of gRNA showed 100% minus a weighted sum of off-target hit-scores in the target genome.	N/A
Parental and modified cell line genetic identity evidence	STR analysis, microsatellite PCR (mPCR) or specific (mutant) allele seq	STR analysis of 18 loci, all matched.	Supplementary Fig. S1C
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	The sequencing results of genomic DNA all matched with the parental line.	Fig. 1 Panel B and Supplementary Fig. S1A and B
	PCR-based analyses	The sequencing results PCR products all matched with the parental line.	Fig. 1 Panel B and Supplementary Fig. S1A and B
Off-target nuclease analysis	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	N/A
	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	N/A	N/A
Specific pathogen-free status	Mycoplasma	Tested by MycoAlert PLUS kit: Negative	Fig. S1D
Multilineage differentiation potential	Embryoid body formation, RT-PCR	Upregulation of trilineage markers <i>PAX6</i> , <i>OTX1</i> (ectoderm), <i>DCN</i> , <i>IGF2</i> , <i>GATA2</i> (mesoderm), and <i>SOX7</i> , <i>SOX17</i> (endoderm).	Fig. 1 Panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

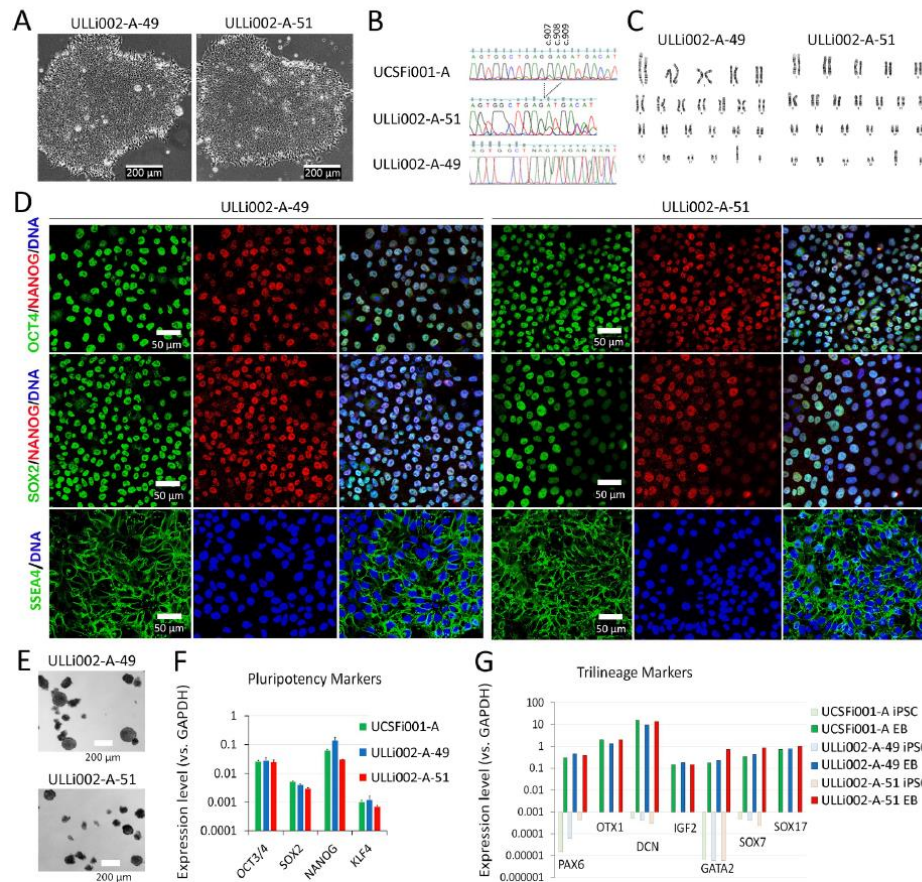


Fig. 1. Characterization of ULLi002-A49 and ULLi002-A51 iPSC lines.

electroporated with a 4D-Nucleofector (Lonza) using CA-137 program. Following nucleofection, the editing efficiency was confirmed by targeted deep sequencing using primer sets specific to target regions, and then the pool was single-cell sorted. Single cell clones were screened with targeted deep sequencing analysis. All iPSCs were cultured with mTeSR Plus (STEMCELL Technology) on matrigel-coated plates at 37 °C in a humidified, 5% CO₂ incubator and passage at a 1:6 ratio using gentle cell dissociation reagent (Versene, Gibco).

4.2. Embryoid bodies (EB) formation

hiPSCs were dissociated with Versene and transferred to low attachment 10-cm petri dishes in KOSR medium (DMEM/F12 medium containing 20% KnockOut Serum Replacement, 1% GlutaMax, 1% non-essential amino acids, 50 μ M β -mercaptoethanol, and 1% penicillin-streptomycin) supplemented with 10 μ M Y-27632. The medium was changed every other day and EBs gradually formed. After 7 days of suspension culture, EBs were digested with 0.25% Trypsin and cultured on gelatin coated plates with KOSR medium for another 7 days. The total RNAs were extracted for RT-PCR analysis.

4.3. Immunostaining and confocal microscopy

Briefly, cultured iPSCs were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature followed by incubation in blocking buffer (3% bovine serum albumin in PBS) with (for nuclear

markers) or without (for cell surface marker SSEA4) 0.2% Triton X-100 for 1 h. Cells were then incubated with primary antibodies (Table 2) in blocking buffer at 4 °C overnight and then followed by washing and incubation with fluorophore-conjugated corresponding secondary antibodies at room temperature for 2 hrs. Nuclei were stained with Hoechst 33342 (Invitrogen). Images were obtained with a Leica SP5 confocal microscope.

4.4. Quantitative PCR analysis

iPSCs and EBs were collected from cultured plates and lysed in TRIzol (Invitrogen). Total RNAs were isolated by the phenol/chloroform extracting method, and then were reverse-transcribed into cDNAs with the SuperScriptTM III Reverse Transcriptase (Invitrogen). Quantitative PCR analysis was performed using SYBR Green PCR Master Mix (Applied Biosystems) and run on StepOne qPCR machine (Applied Biosystems). The gene expression data were analyzed using the $\Delta\Delta C_T$ method and the values were normalized to the expression of the GAPDH housekeeping gene (Fig. 1F and G). Primers used in this study were listed in Table 2.

4.5. Karyotyping

Chromosomes from iPSC clones were analyzed using the GTW banding method at GEIC at Washington University in St. Louis.

Table 2
Reagents details.

Antibodies used for immunocytochemistry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Mouse anti-OCT4	1:200	Santa Cruz Cat# sc-5279, RRID: AB_628051
	Mouse anti-SOX2	1:200	Santa Cruz Biotechnology Cat# sc-365823, RRID:AB_10842165
	Mouse anti-SSEA4	1:200	Abcam Cat# ab16287, RRID:AB_778073
	Rabbit anti-Nanog	1:100	Abcam Cat# ab21624, RRID:AB_446437
Secondary antibodies	Donkey anti-Mouse IgG (H + L), Alexa Fluor 488	1:500	Jackson ImmunoResearch Labs Cat# 715-545-150, RRID:AB_2340846
	Donkey Anti-Rabbit IgG (H + L), Alexa Fluor 594	1:500	Jackson ImmunoResearch Labs Cat# 711-585-152, RRID:AB_2340621
Nuclear stain	Hoechst33342	1 µg/mL	Invitrogen Cat # H3570. RRID: NOT FOUND
Site-specific nuclease			
Nuclease information	Cas9		Cas9 vector p3s-Cas9HC (Addgene #43945)
Delivery method	electroporation		4D-Nucleofector (Lonza, Cat # AAF-1002B)
Selection/enrichment strategy	sorted into 96-well plates with one cell per well		Single cell clones were screened and expanded
Primers and Oligonucleotides used in this study			
	Target	Forward/Reverse primer (5'-3')	
Pluripotency marker	OCT 3/4	CGAGAGGATTTTGGGCTGC/CGAGGAGTACAGTGCAGTGA	
Pluripotency marker	SOX 2	AGGATAAGTACACGCTGCC/TTTCATGTGGCGCTAACTGTC	
Pluripotency marker	NANOG	TGCTTCTGCTGAGATGCCT/CAGAAGTGGGTTGTTTGCT	
Pluripotency marker	KLF4	TCTCAATTGCTGACCCAT/CGGATCGGATAGGTGAAGCT	
Differentiation marker	PAX6	GGGCGGAGTTATGATACCTACA/ATATCAGGTTCACTTCCGGGAA	
Differentiation marker	OTX1	TACGCCCTCCTCTTCTACT/GCATGTGGGTGGTGATGATG	
Differentiation marker	DCN	CTGAAGAACCCTTCACGCATTGA/GGCAATTCTTCAGCTGATTCT	
Differentiation marker	IGF2	CAATATGACACCTGGAAGCAGT/GTAGAGCAATCAGGGGACGG	
Differentiation marker	GATA2	ACCTGTTGTGCAAAATTGTGAGA/ATCCCTTCTTCTTCATGGTCA	
Differentiation marker	SOX7	ACTCCACTCCAACCTCCAAG/TTCAATTGGGATCCATGTCCC	
Differentiation marker	SOX17	ATCGGGACATGAAGGTGAA/TCCTTAGCCACACCATGAA	
House-Keeping Genes	GAPDH	CAAATTCATGGCACCCTCA/GGACTCCACGACGTACTCAG	
Genotyping-PCR	TOR1A	ACAGCAGCTTAATTGACCGGA/ATCATGAGCCCTGCGATGAG	
Sequencing	TOR1A	GTGTATCCGAGTGGAAATGC	
hTOR1A gRNA (IDT)	TOR1A	TGAAGACATTGTGAAGCAGAG	
hTOR1A ssODN (IDT)	TOR1A	AATGTGTATCCGAGTGGAAATGCAGTCCCGAGGCTATGAAATT	
		GATGAAGACATTGTGAAGTAGAGTGGCTGAGATGACATTTTCC	
		CCAAAGAGGAGAGAGTTTCTCAGATAAAGGCTGCA	

4.6. STR analysis

Short tandem repeat (STR) analysis of 18 loci (Fig. S1C) were performed at GEiC at Washington University in St. Louis.

4.7. Mycoplasma test

Mycoplasma test (Fig. S1D) was performed by MycoAlert PLUS kit (Lonza) at GEiC at Washington University in St. Louis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

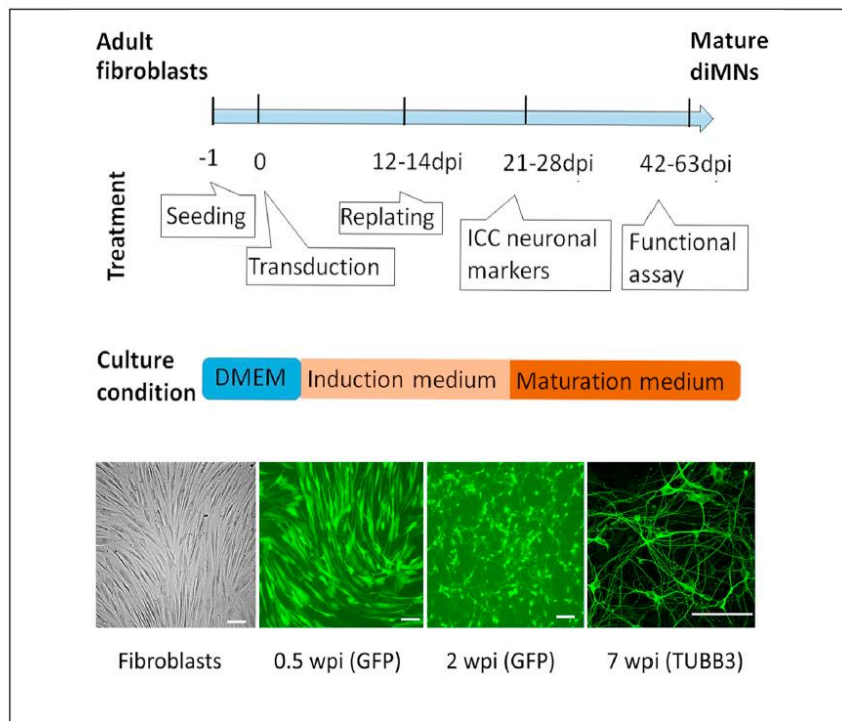
Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2021.102536>.

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Protocol

Direct conversion of adult fibroblasts into motor neurons



Generation of patient-derived neurons provides an unprecedented approach in modeling neurological diseases. Here, we describe the direct conversion of adult fibroblasts into motor neurons via lentiviral delivery of transcription factors. Compared with iPSC-based approach, directly converted neurons from donors retain features associated with age, making them ideal systems for modeling age-related neurological diseases. Low yield is the major challenge of this protocol. High quality lentiviruses and optimized cell culture conditions are critical to improve the final yield.

Masood

Sepehrmanesh,
Masuma Akter,
Baojin Dingbaojin.ding@louisiana.
edu**Highlights**

Detailed protocol to generate MNs from human adult fibroblasts

Protocol assesses the identity and efficiency of MN generation *in vitro*

Descriptions of troubleshooting common problems during the reprogramming process

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Protocol

Direct conversion of adult fibroblasts into motor neurons

Masood Sepehrimanesh,¹ Masuma Akter,¹ and Baojin Ding^{1,2,3,*}

¹Department of Biology, University of Louisiana at Lafayette, 410 East Saint Mary Boulevard, Lafayette, LA 70503, USA

²Technical contact

³Lead contact

*Correspondence: baojin.ding@louisiana.edu
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SUMMARY

Generation of patient-derived neurons provides an unprecedented approach in modeling neurological diseases. Here, we describe the direct conversion of adult fibroblasts into motor neurons via lentiviral delivery of transcription factors. Compared with iPSC-based approach, directly converted neurons from donors retain features associated with age, making them ideal systems for modeling age-related neurological diseases. Low yield is the major challenge of this protocol. High quality lentiviruses and optimized cell culture conditions are critical to improve the final yield. For complete details on the use and execution of this protocol, please refer to Ding et al. (2020), Ding et al. (2021), and Liu et al. (2016)

BEFORE YOU BEGIN

⌚ Timing: 24–48 days

For more information about the protocols, please refer to (Ding et al., 2020, 2021; Liu et al., 2016) for direct reprogramming procedure.

1. Use the exact reagents mentioned in the [key resources table](#).
2. Prepare all stock solutions listed below and make the necessary working aliquots.
3. Order the antibodies found in the [key resources table](#) for quality control.

Note: All types of cell culture media are good for up to 2 weeks at 4°C. It is recommended to prepare the medium when needed at the specific step of the protocol.

⚠ **CRITICAL:** All procedures are performed in a BSL-2 certified laboratory using a Laminar Airflow Hood with standard aseptic techniques. Cultures are grown and maintained in a humidified incubator at 37 °C with 5% CO₂.

KEY RESOURCES TABLE

REAGENT and RESOURCES	SOURCE	IDENTIFIER
Experimental models: Cell lines		
Human embryonic kidney (HEK) 293T cells	American Type Culture Collection	ATCC Cat# CRL-3216, RRID:CVCL_0063
Healthy skin fibroblasts	Coriell Institute for Medical Research	Coriell Cat# GM04506, RRID:CVCL_7413
Healthy skin fibroblasts	Coriell Institute for Medical Research	Coriell Cat# GM03652, RRID:CVCL_7397
Healthy skin fibroblasts	Coriell Institute for Medical Research	Coriell Cat# GM08400, RRID:CVCL_7483
Mouse Primary Astrocytes	N/A	N/A

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Continued

REAGENT and RESOURCES	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Fetal Bovine Serum (FBS)	Corning	Cat# 35-010-CV
Penicillin/Streptomycin (Pen/Strep)	Thermo Fisher Scientific	Cat# 15140122
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific	Cat# 11995073
DMEM/nutrient mixture F-12 (DMEM-F12) with HEPES; without L-glutamine	Cytiva	Cat# SH30126.01
Neurobasal medium	Thermo Fisher Scientific	Cat# 21103049
Gelatin	Bio-Rad	Cat# 118860A
Matrigel	Corning	Cat# 354230
B-27 Supplement (50 ×)	Invitrogen	Cat# 17504044
N-2 Supplement (100×)	Invitrogen	Cat# 17502001
Forskolin (FSK)	Sigma Aldrich	Cat# F6886
Dorsomorphin (DM)	EMD Millipore	Cat# 171260
Heat Stable Recombinant Human Basic Fibroblast Growth Factor (bFGF)	Gibco	Cat#PHG0367
Brain Derived Neurotrophic Factor (BDNF)	PeproTech	Cat# 450-02
Glial-Derived Neurotrophic Factor (GDNF)	PeproTech	Cat# 450-10
Neurotrophin-3 (NT3)	PeproTech	Cat# 450-03
Bovine serum albumin (BSA)	Thermo Fisher Scientific	Cat# bp9706
Hoechst 33342 (HST)	Thermo Fisher Scientific	Cat# H3570
Triton X-100 (TX-100)	Thermo Fisher Scientific	Cat# 85111
Paraformaldehyde (PFA)	Sigma Aldrich	Cat# 158127
Trypsin-EDTA (0.25%)	Thermo Fisher Scientific	Cat# 25200056
Polyethyleneimine (PEI), 25 kDa linear	Polysciences	Cat# 23966-2
Polybrene (Hexadimethrine bromide)	Sigma Aldrich	Cat# H9268-10g
Ara-C (Arabinosylcytosine)	Sigma Aldrich	Cat# C1768
Antifade Mounting Medium	Vector Laboratories	Cat#H-1400-10
Na ₂ HPO ₄	Sigma Aldrich	Cat# S-0876
NaCl	Thermo Fisher Scientific	Cat# S271-10
KCl	Thermo Fisher Scientific	Cat# P-217
K ₂ HPO ₄ ·3H ₂ O	Sigma Aldrich	Cat# P-5504
PVA mountain medium	N/A	N/A
DMSO, Dimethyl Sulfoxide	Thermo Fisher Scientific	Cat# D1391
Antibodies		
Rabbit anti-tubulin Beta 3 Class III (TUBB3), monoclonal (1:2000)	BioLegend	BioLegend Cat# 801201, RRID:AB_2313773
Chicken anti-microtubule-associated protein 2 (MAP2) (1:10,000), polyclonal	BioLegend	Cat# 822501, RRID:AB_2564858
Goat anti-CHAT, monoclonal (1:200)	Millipore	Millipore Cat# AB144P, RRID:AB_2079751
Rabbit anti-SYN1, monoclonal (1:200)	Cell Signaling Technology	Cat# 5297
Mouse anti-HB9, monoclonal (1:500)	DSHB	Cat# 81.5C10-c
Rabbit anti-Isl1 (ISL1), monoclonal (1:250)	Abcam	Cat# ab109517; RRID: AB_10866454
Donkey anti-Mouse IgG (H + L), Alexa Fluor 488 (1:500)	Jackson ImmunoResearch	Cat# 715-545- 150, RRID: AB_2340846
Sheep anti-Mouse IgG (H + L), Alexa Fluor 594 (1:500)	Jackson ImmunoResearch	Cat# 515-515-062, RRID:AB_2340331
Donkey Anti-Rabbit IgG (H+L), Alexa Fluor 488 (1:500)	Jackson ImmunoResearch	Cat# 711-545-152, RRID:AB_2313584
Donkey Anti-Rabbit IgG (H+L), Alexa Fluor 594 (1:500)	Jackson ImmunoResearch	Cat# 711-585- 152, RRID:AB_2340621
Recombinant DNA		
pRSV-Rev	Addgene	RRID:Addgene_12253
pMDLg/pRRE	Addgene	RRID:Addgene_12251
pMD2.G	Addgene	RRID: Addgene_12259
pCSC-NGN2-IRES-GFP-T2A-Sox11	Addgene	RRID:Addgene_90214
pCSC-ISL1-T2A-LHX3	Addgene	RRID:Addgene_90215
Others		
15 mL polystyrene Conical tube	Thermo Fisher Scientific	Cat# 14-959-53A
50 mL polystyrene Conical tube	Thermo Fisher Scientific	Cat# 14-432-22

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Continued

REAGENT and RESOURCES	SOURCE	IDENTIFIER
24-well cell culture plates	Thermo Fisher Scientific	Cat#142485
10 cm Cell Culture Dishes	Thermo Fisher Scientific	Cat# 130182
0.45 μ m Sterile Filter Unit (PVDF membrane)	EMD Millipore	Cat# SLHVM33RS
0.22- μ m vacuum filter	Millipore	Cat# SLGP033RB

MATERIALS AND EQUIPMENT

All equipment and materials should be sterile or autoclaved. Some liquids that cannot be autoclaved need to be filtered with 0.22 μ m filters. All materials to be prepared freshly and stored at 4°C for a short-term storage no longer than two weeks. Scaling up and down according to the amount of medium needed.

HEK293 cell medium	Final concentration	Amount
Dulbecco's Modified Eagle Medium (DMEM, 1 x)	N/A	44.5 mL
Fetal bovine serum (FBS)	10%	5 mL
Penicillin/Streptomycin (100x)	1%	0.5 mL
Total	N/A	50 mL

⚠ **CRITICAL:** Store HEK293 cell medium at 4°C up to 2 weeks.

Fibroblast medium	Final concentration	Amount
DMEM	N/A	42 mL
Fetal bovine serum (FBS)	15%	7.5 mL
Penicillin/Streptomycin (100x)	1%	0.5 mL
Total	N/A	50 mL

⚠ **CRITICAL:** Store fibroblast medium at 4°C up to 2 weeks.

Astrocyte medium	Final concentration	Amount
DMEM	N/A	42 mL
Fetal bovine serum (FBS)	15%	7.5 mL
Penicillin/Streptomycin (100x)	1%	0.5 mL
Total	N/A	50 mL

⚠ **CRITICAL:** Store astrocyte medium at 4°C up to 2 weeks.

Neural induction medium	Final concentration	Amount
DMEM:F12 (1:1)	N/A	24 mL
Neurobasal media	N/A	24 mL
Penicillin/Streptomycin (100x)	1%	0.5 mL
N2 (100x)	1x	0.5 mL
B27 (50x)	1x	1 mL
FSK (20 mM)	10 mM	25 μ L
DM (10 mM)	1 mM	5 μ L
bFGF (200 μ g/mL)	10 ng/mL	2.5 μ L
Total	N/A	50 mL

△ CRITICAL: Prepare all stock aliquots of FSK, DM and bFGF at indicated concentration and keep them in -80°C freezer. Store the complete medium at 4°C up to 2 weeks.

Neural maturation medium	Final concentration	Amount
DMEM:F12 (1:1)	N/A	24 mL
Neurobasal media	N/A	24 mL
Penicillin/Streptomycin (100×)	1%	0.5 mL
N2 (100×)	1×	0.5 mL
B27 (50×)	1×	1 mL
FSK (20 mM)	5 mM	12.5 μL
BDNF (20 $\mu\text{g/mL}$)	10 ng/mL	25 μL
GDNF (20 $\mu\text{g/mL}$)	10 ng/mL	25 μL
NT3 (20 $\mu\text{g/mL}$)	10 ng/mL	25 μL
Total	N/A	50 mL

△ CRITICAL: Prepare all stock aliquots of FSK, BDNF, GDNF, and NT3 at indicated concentration and keep them in -80°C freezer. Store the complete medium at 4°C up to 2 weeks.

PEI solution stock	Final concentration	Amount
PEI (transfection grade, 25 kDa, Linear)	1 $\mu\text{g}/\mu\text{L}$	50 mg
MilliQ water	N/A	50 mL
Total	N/A	~50 mL

△ CRITICAL: Dissolve PEI in sterile distilled water. Incubate the tube at 55°C in a water bath and vortex until PEI is completely dissolved. Adjust pH to 7.0 if necessary. Cool PEI solution to room temperature ($\sim 25^{\circ}\text{C}$) and sterilize the solution using a $0.22\text{ }\mu\text{m}$ vacuum filter. Aliquot and store at -80°C for up to 1 year.

Gelatin stock	Final concentration	Amount
Gelatin	1 mg/mL	0.5 g
MilliQ water	N/A	500 mL
Total	N/A	500 mL

△ CRITICAL: Autoclave stock and keep it at room temperature or at 4°C for up to 6 months..

10× PBS stock	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	27 mM	2.0 g
Na_2HPO_4	100 mM	14.4 g
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	18 mM	2.4 g
MilliQ water	N/A	~950 mL
Total	N/A	1000 mL

△ CRITICAL: Adjust pH to 7.4 with HCl. The autoclaved 10× PBS stock can be stored at room temperature for 1 year. Before use, dilute 10× PBS stock using MilliQ water to make 1× PBS working solution.

STEP-BY-STEP METHOD DETAILS

Lentivirus production and titration

⌚ Timing: 14 days (5–7 days for step 1; 4 days for step 2; 4 days for step 3)

Lentiviruses can infect both proliferating and non-proliferating cells. The genetically modified lentiviral vectors lack dispensable genes from the HIV-1 genome and separate cis-acting sequences from trans-acting factors required for viral particle production, infection, and integration. A third-generation packaging system provides maximal biosafety but requires the transfection of four separate plasmids, including three helper plasmids and one lentiviral vector expressing genes of interest (Dull et al., 1998). To ensure the high reprogramming efficiency, it is critical to prepare high-quality supercoiled plasmids and make lentiviruses with excellent titers. For detailed information about lentivirus preparation, titration and applications, please see our previous studies (Ding et al., 2013, 2016, 2018; Ding and Kilpatrick, 2013).

1. Expanding HEK293T cells.

- Culture HEK293T cells in HEK culture medium one week before starting the preparation of viruses. Pass them at about 80% confluency twice.
- For each 10 cm plate, seed about 8×10^6 HEK cells in 10 mL medium and gently mix well to let cells distribute evenly on the surface. The next day, the cell density should be about 80% confluency for transfection (Figure 1A).

2. Transfection and lentivirus collection

Before transfection, prepare high quality supercoiled plasmids (Ding and Kilpatrick, 2013). Lentiviral plasmid DNA will be delivered by the Polyethyleneimine (PEI)-mediated transfection method.

- We use the third-generation lentiviral packaging system which consists of three helper plasmids, including pRSV-Rev (3rd generation lentiviral packaging plasmid contains Rev), pMDLg/pRRE (3rd generation lentiviral packaging plasmid contains Gag and Pol), and pMD2.G (VSV-G envelope expressing plasmid). Two lentiviral vectors expressing reprogramming factors are plasmids pCSC-SP-PW-CMV-Ngn2-IRES-GFP-T2A-mSOX11 (Plasmid#1) and pCSC-ISL1-T2A-LHX3 (Plasmid#2).
- For each 10 cm plate, add 4 μ g of plasmid#1 or plasmid#2; 2 μ g of pMDLg/pRRE plasmid; 1 μ g of pMD2.G plasmid and 0.5 μ g of pRSV-Rev plasmid to 1 mL DMEM (-FBS) media and mix well.
- Add 22.5 μ L transfection reagent PEI (1 μ g/ μ L) to plasmids mixture and immediately mix well by gently pipetting up and down 5 times or vortexing for 15 s. Incubate at room temperature for 15–20 min.

Note: The ratio of PEI to the mixture of total DNA is 1:3. For example, the total amount of DNA is 7.5 μ g for a 10 cm plate. Add 22.5 μ L of PEI (equivalent to 22.5 μ g) into the plasmid mixture in 1 mL DMEM.

- Add 1 mL of transfection dropwise to each 10 cm plate of HEK293 cells and gently mix to distribute transfection reagent evenly.
- After 8 h transfection or overnight, replace with 10 mL HEK culture medium for each 10 cm plate.
- Collect lentiviral supernatant completely (around 10 mL) at 48 h post-transfection and add 10 mL fresh culture medium. Store this first collection of lentiviral supernatant at 4°C.
- Another 24 h later (72 h post-transfection), collect lentiviral supernatant again and store at 4°C. Discard the plates in a biohazard bag and trash it after autoclave.

Note: Transfection efficiency can be examined under a fluorescence microscope if the lentiviral vector contains GFP reporter (e.g. plasmid #1). At lentivirus collection, most HEK cells (>90%) should be GFP (+) (Figure 1B).

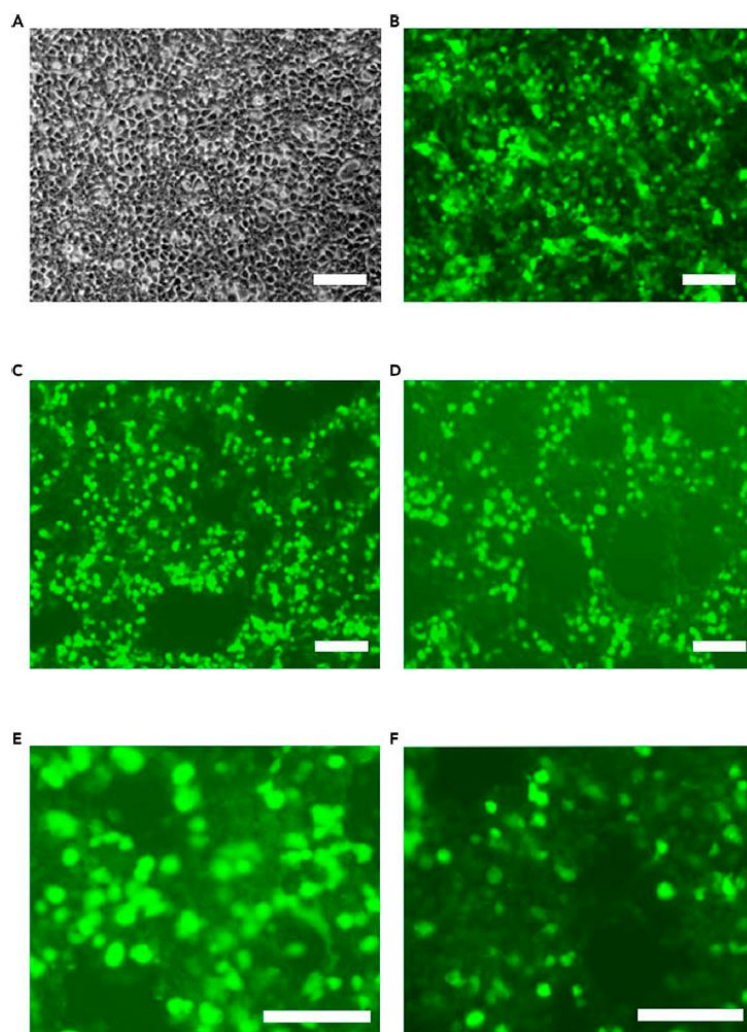


Figure 1. Lentivirus preparation and titration

(A) HEK cells at transfection with ~80% confluency.
 (B) At virus collection (72 h post-transfection) almost all HEK cells are transfected positive (plasmid #1 with co-expressed GFP).
 (C) Virus titration with undiluted virus (#1) at 72 h post-transduction (> 90% cells are GFP positive).
 (D) Virus titration with 1:10 diluted virus (#1) at 72 h post-transduction (~40% cells are positive).
 (E) Virus titration of undiluted virus (#2) at 72 h post-transduction with immunocytochemistry (ICC) of ISL1 (~90% cells are positive).
 (F) Virus titration with 1:10 diluted virus (#2) at 72 h post-transduction with ICC of ISL1 (~40% cells are positive). From A to F, scale bars, 100 μ m.

h. Pool two collections of the same lentivirus together and add 6 μ g/mL polybrene. Filter the viral supernatants with 0.45 μ m vacuum filters. Store viral supernatants at 4°C until ready to use (within two weeks) or freeze at –80°C for longer term storage.

⚠ **CRITICAL:** The ratio of PEI to plasmids should be 4:1 to 3:1. Lesser or higher ratios decrease the efficiency of virus production. When you add transfection mixture to

HEK293 cells, just gently move the plate on the base up-down and left-right for 10 times.
Do not move circular to prevent pooling transfection mixture in the center.

3. Titration to verify the quality of lentivirus.
 - a. The virus can be titrated based on GFP reporter (plasmid#1) (Figures 1C and 1D) or immunocytochemistry (ICC) of lentivirus expressed factors (plasmid#2) (Figures 1E and 1F) as previously reported (Ding and Kilpatrick, 2013).
 - b. Seed HEK cells in a 24 well plate at a density of $7-10 \times 10^4$ cells/well/0.5 mL medium. Cells will reach ~70 % confluency the next day at the time of titration.
 - c. Make a serial dilution (e.g., 1:10, 1:100 etc.) of viral supernatant with HEK culture medium.
 - d. Add 50 μ L of undiluted and diluted lentivirus to desired wells.

Note: Before addition of lentivirus, do not need to remove original culture medium from 24-well plate. Set one well as a negative control without transduction.

- e. Change the medium after 8 h or overnight (~16 h) exposure to lentivirus.
- f. Check the percentage of positive cells after 48–72 h.

Note: For lentivirus expressing GFP reporter, examine GFP (+) cells directly under a fluorescence microscope in living cells. For lentivirus that does not express fluorescent reporters, cells need to be fixed and perform ICC to examine lentivirus-expressed factors.

- g. Routinely, transduced positive cells should be more than 90% in undiluted wells and more than 30% in 10-fold diluted wells (Figures 1C–1F).

△ CRITICAL: A good titer of lentiviral supernatants should be no less than 1×10^6 IFU/mL. (IFU, Infectious Unit).

Human fibroblast culture

⌚ Timing: 5–10 days

4. Fibroblast culture and expansion. If starting to culture fibroblast from a frozen stock, it is recommended to split cells twice before seeding on Matrigel coated plates for reprogramming.

Note: You can freeze fibroblasts in DMEM contains 15% FBS and 10% DMSO using cryotubes at a concentration of 1×10^6 /mL/vial.

Note: Passage number of fibroblasts is important for conversion. Cells with high passage numbers (>15) show slow growth and the low conversion rate.

- a. Quickly thaw a frozen stock of human fibroblast in a 37°C water bath.
- b. When there is just a small bit of ice left in the vial, immediately transfer cells into a 15 mL tube containing 5 mL of fibroblast culture medium and gently mix.
- c. Centrifuge at 200 g for 3 min to collect cells.
- d. Remove the supernatant and resuspend cell pellet into 10 mL fibroblast culture medium and seed cells in one 10 cm plate.
- e. Change media every two or three days until close to full confluency.
- f. Split fibroblast. Wash the cells with 1× PBS and detach them by trypsinization with 0.05% trypsin (make 1:5 dilution of 0.25% trypsin with 1× PBS) at 37°C for 4 min.
- g. When cells reach about 80% confluency, they will be ready for setting direct reprogramming.

⚠ **CRITICAL:** The growth speed varies dramatically between different cell lines. Some aged or patient fibroblasts may grow slowly. During culture of these slow-growth cells, the medium must be changed regularly every two or three days to facilitate the cell growth and maintain antibiotics effective in the culture. The splitting ratio could be from 1: 2 to 1: 5 depending on the growth.

Fibroblast transduction and motor neurons induction

⌚ **Timing:** 15–20 days (4–16 h for step 5; 24 h for step 6; Timing: 14 days for step 7)

5. Prepare Matrigel-coated plates.
 - a. Dilute Matrigel at 1:100 with cold DMEM medium.
 - b. Add 5 mL of diluted Matrigel to each 10 cm plate and gently shake to ensure to cover all the bottom surface of the plate.
 - c. Incubate at 37°C more than 3 h or overnight.

Note: Matrigel is sensitive to temperature. When working with Matrigel, place the aliquot of Matrigel on ice or ice pack within the biosafety cabinet.

6. Seed proper amount of fibroblasts onto Matrigel-coated plates.
 - a. Fibroblasts at about 90% confluency will be harvested by trypsinization.
 - b. Seed $\sim 1 \times 10^6$ cells for a Matrigel-coated 10 cm plate.

Note: A 10 cm plate with $\sim 90\%$ confluency usually give rise to ~ 3 million fibroblast cells. It is enough for seeding three 10 cm plates. The seeding numbers should be optimized according to cell growth to ensure that the cell density will be around 60%–80% confluency in next day at transduction.

7. This step describes the induction of directly reprogrammed motor neurons from fibroblasts. The neural induction media is used at this stage.
 - a. The day after seeding fibroblast onto the Matrigel-coated plate, cell density should be around 60%–80% confluency at transduction (Figure 2).
 - b. Mix the desired volume of two lentiviruses (1:1) in the presence of 6 $\mu\text{g/mL}$ polybrene.
 - c. Add proper amount of lentiviral supernatants to the plate to transduce fibroblasts. For a 10 cm plate, add ~ 20 mL of total lentivirus expressing 4 reprogramming factors.

Note: The lentiviral dosage should be optimized based on the viral titer and the growth of the cell line. Do not need to remove original culture medium before addition of lentiviruses to the plate.

- d. Cells are exposed to lentiviruses overnight (~ 16 h) in incubator.
- e. The next morning, replace the media with fresh fibroblast medium. This is the first day post-viral infection (1 dpi).
- f. The next day (2 dpi), change the neural induction medium.
- g. Change half volume of neural induction medium every the other day at 4, 6, 8, 10, 12 dpi.

Note: For a good transduction, most ($>90\%$) fibroblast cells are GFP (+) and then gradually become neuron-like cells within 2 weeks post-viral infection (wpi) (Figure 3).

⚠ **CRITICAL:** Addition of proper amount of high-quality lentiviruses is very important to ensure good reprogramming efficiency. The proper MOI (Multiplicity of Infection) could be used are 5–8 (Figure 2). As the properties of cells (such as growth speed) will influence the transduction efficiency, different cell lines may need different dosages to obtain

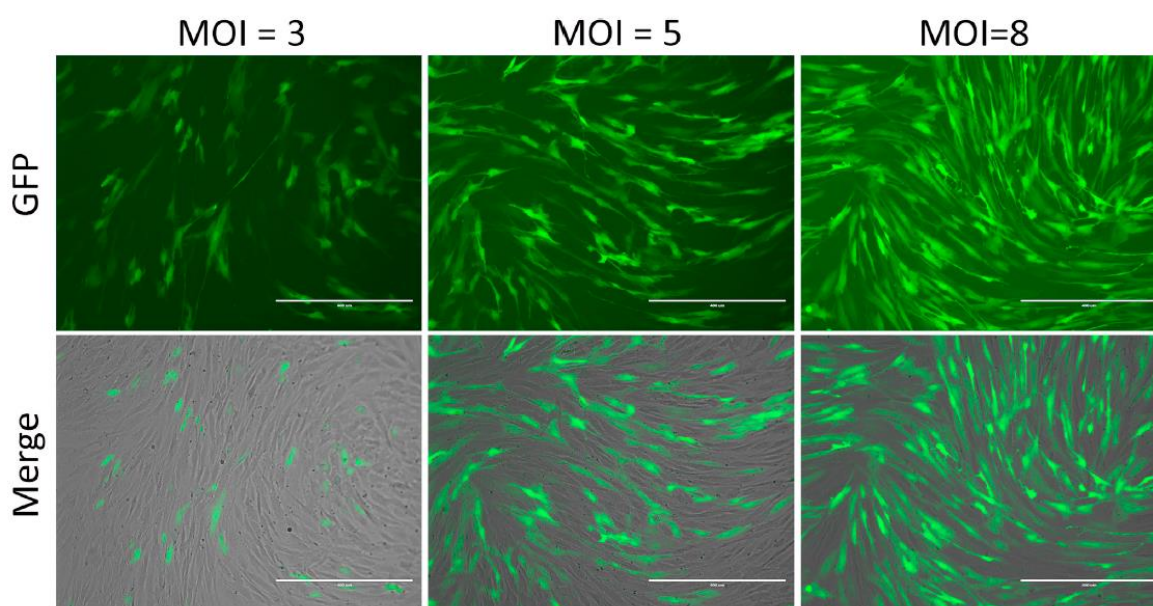


Figure 2. Transduction of human fibroblasts with proper amount of lentiviruses
Micrographs of fibroblast cells (GM04506) transduced with lentivirus expressing reprogramming factors with indicated MOI (Multiplicity of Infection) at 3 days post-viral infection (dpi). The transduced positive cells can be identified with co-expressed GFP. Scale bars: 400 μ m.

optimized transduction efficiency. However, too much viruses could cause more cell death. Transduction efficiency and cell survival should be balanced.

Purify reprogrammed neurons by replating

⌚ Timing: 5–7 days (3 h for step 9)

This step describes purification of reprogrammed neurons from fibroblasts by replating cells with gelatin coated plates. The attachment of fibroblast cells will occur earlier and more tightly than reprogrammed neurons. Purified neurons will be seeded onto coverslips with a monolayer of astrocytes for long-term culture (2–10 wpi) in neural maturation medium. For primary astrocytes isolation and monolayer astrocyte preparation, please refer (Schildge et al., 2013).

8. Culture astrocyte cells. Isolate mouse astrocytes from postnatal day 1 (P1) pups and culture them in DMEM with 15% FBS. Prepare frozen stocks at $\sim 1 \times 10^6$ cells/vial. Start culturing of astrocytes at least 5 days before replating.

a. Thaw astrocytes frozen stocks and culture them in astrocyte culture media in 10 cm cell culture plates.

Note: Seed cells from one vial of frozen stock (1×10^6 cells) on one 10 cm plate. It will take about 3 days to reach around 90% confluency.

b. Coat 24-well plates contain cover slips with Matrigel (1:100) at least for 3 h in 37°C for seeding astrocytes.

c. When astrocytes reach $\sim 90\%$ confluency in the 10 cm plate, trypsinize astrocytes with 0.025% trypsin (make 1:10 dilution of 0.25% trypsin with $1 \times$ PBS) for 3 min at 37°C.

d. Add astrocyte medium to neutralize trypsin and detach cells.

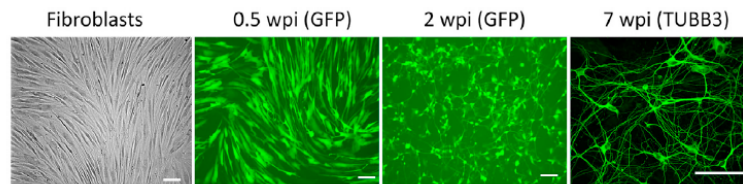


Figure 3. Representative morphology of fibroblasts and directly induced motor neurons (diMNs) at indicated weeks post-viral infection (wpi)

Transduced cells are identified with co-expressed green fluorescent protein (GFP) at early time points (0.5 and 2 wpi). Transduced fibroblasts gradually become neuron-like morphology within 2 wpi. More complicated branches and very long axons can be detected at late developmental stages with immunostaining of tubulin Beta 3 Class III (TUBB3). Scale bars: 100 μ m. Adapted from (Ding et al., 2020) with permission.

- e. Transfer cell suspension to a 15 mL tube and centrifuge at 200 g for 3 min to collect cells.
- f. Remove supernatant and resuspend the cell pellet into 12 mL astrocyte medium.
- g. Seed astrocytes onto coverslips in a 24-well plate at 0.5 mL/well.
- h. Change medium every two days until astrocytes form monolayer on coverslips.

Note: One full 10 cm plate of astrocytes is enough for seeding a 24-well plate. If astrocytes reach full confluency on coverslips before neurons are ready for replating, add 10 μ M Ara-C in culture medium to inhibit cell amplification.

9. Infected fibroblasts (GFP +) at 14 dpi will show typical neuron-like morphology (Figure 3). These cells could be directly used for early developmental analysis or replated onto monolayer astrocytes for long-term culture.
 - a. Coat culture plates with 5 mL of 0.1% gelatin for 30 min at 37°C.

Note: For one 10 cm plate of reprogrammed cells, need two 10 cm plates for replating to ensure efficient purification.

- b. Remove culture medium from culture plates with induced neurons and wash once with PBS.
- c. Add 3 mL of 0.025% trypsin (make 1:10 dilution of 0.25% trypsin with 1 $\times$ PBS) to a 10 cm plate and incubate for 3 min at 37°C to detach the cells.
- d. Add 7 mL fibroblast culture medium to quench trypsin activity. Gently pipet up and down to break cell aggregates.
- e. Transfer cell suspension to gelatin-coated plates (about 5 mL each).

Note: Remove gelatin just before use. Avoid air drying coated plates.

- f. Incubate plates at 37°C and keep checking the attachment of cells under microscope.

Note: It will take 15–30 min to see differential adhesion which means most fibroblasts attach at the bottom and the reprogrammed neurons (GFP+) are still floating.

- g. Collect cell suspensions and transfer to a 15 mL tube. Pool cell suspensions from two plates together.
- h. Centrifuge at 300 g for 3 min to collect cells.
- i. Remove the supernatant and gently resuspend the cell pellets into neural maturation medium with desired volume based on the yield and the following experimental plans.

Note: The neurons generated from one 10 cm plate are enough for seeding a 24-well plate with the cell density $\sim 2 \times 10^4$ cells/well.

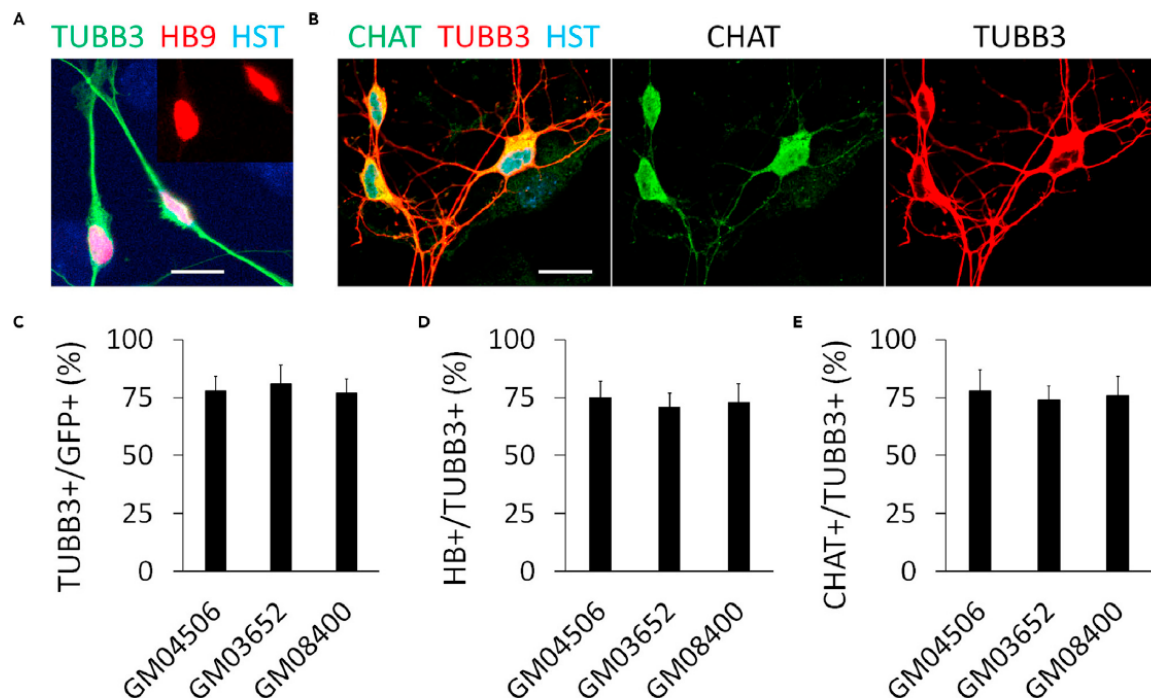


Figure 4. Neuronal identity verification
(A) Confocal micrograph shows the highly expressed nuclear HB9 (motor neuron and pancreas homeobox 1, the early MN marker) at 2 wpi. The inset shows the nuclear HB9 alone. DNA dye hoechst33342 (HST) stained nuclei. Scale bar: 20 μ m.
(B) Confocal micrographs show the robust expression of the mature MN marker CHAT (choline acetyltransferase) at 8 wpi. Scale bar: 20 μ m.
(C) Quantification of the reprogramming efficiency was shown as the percentage of GFP (+) cells expressed TUBB3.
(D) Fractions of diMNs among the reprogrammed neurons was shown as the percentage of TUBB3 (+) cells expressed HB9.
(E) Fractions of diMNs among the reprogrammed neurons was shown as the percentage of TUBB3 (+) cells expressed CHAT. From C to E, data are presented as mean $\pm$ SEM. More than 300 cells of each line were counted from triplicates.
Adapted from (Ding et al., 2020, 2021) with permission.

⚠ CRITICAL: The proper length of incubating time is important during replating. Too short, most fibroblasts are still floating, and the purity of neurons is low. If incubation time is too long, more neurons could also attach at the bottom and may lead to poor yield. The best time point is that the majority of fibroblasts attached at the bottom and few neurons attached.

Neuronal identity verification

⌚ Timing: 3 days

10. This step describes how to validate the MN identity of reprogrammed neurons using ICC of different markers.

a. Take coverslips from 24-well plates at desired time points.

Note: As early as 2 wpi, the generic neuronal markers of TUBB3 and early MN marker HB9 could be examined. At 4 wpi or later, MAP2 and mature MN marker CHAT could be examined (Figure 4).

b. Fix cultured cells with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature.

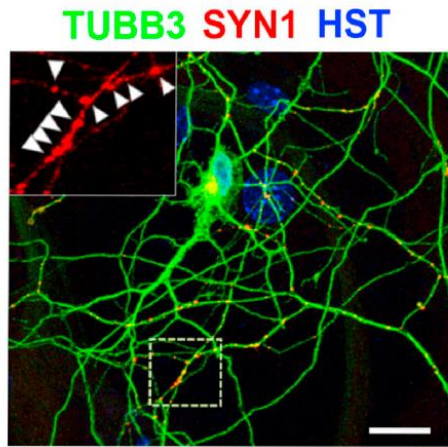


Figure 5. Micrograph shows the robust expression of the presynaptic marker synapsin 1 (SYN1) at 9 wpi. The inset shows discrete SYN1-positive puncta (indicated by arrow heads). DNA dye hoechst33342 (HST) stained nuclei. Scale bar: 50 μ m. Adapted from (Ding et al., 2021) with permission.

- c. Wash twice with PBS.
- d. Incubate fixed cells in blocking buffer (PBS containing 0.2% Triton X-100 and 3% BSA) for 1 h at room temperature for permeabilization and blocking.
- e. Cells are then incubated with primary antibodies in blocking buffer at 4°C overnight (~16 h).

Note: The antibodies include anti-TUBB3 and anti-MAP2 as neuronal markers, anti-HB9 as an early MN marker, anti-CHAT as a late MN marker, and anti-SYN1 as a presynaptic marker.

- f. After removal of primary antibodies, wash coverslips with PBS for 10 min $\times$ 4.
- g. Incubated with corresponding fluorophore-conjugated secondary antibodies in dark at room temperature for 2 h.
- h. Wash coverslips with PBS for 10 min $\times$ 3.
- i. The nuclei were stained with Hoechst 33342 (1:3000) in PBS at room temperature for 15 min.
- j. Briefly wash coverslips once with PBS.
- k. Mount coverslips with mounting medium on microslides.
- l. Air dry the coverslips and visualize cells under a confocal microscope.

△ CRITICAL: The examination of different markers need to use samples at different time points. The best time frame for staining nuclear HB9 is 2–3 wpi, and the best time for staining CHAT is 4 wpi or later (Figures 4). For staining presynaptic markers and electrophysiology assay, need reprogrammed MNs reach the full maturation at 6 wpi or later (Figure 5). For the changes of properties at different developmental stages during the reprogramming process, please refer to our previous report (Ding et al., 2020).

EXPECTED OUTCOMES

Fibroblasts are transduced with reprogramming factors can be identified with co-expressed GFP at 3 days post-viral infection (dpi) (Figure 3), and they gradually become neuron-like morphology (Figure 4). These neurons robustly express generic neuronal marker TUBB3 (Figures 3, 4, and 5), early MN marker nuclear HB9 (Figure 4), late MN marker CHAT (Figure 4), and presynaptic marker synapsin 1 (SYN1). About 80% transduced positive cells can be successfully converted into neurons (TUBB3+/GFP+) (Figure 4C). Among these neurons, about 75% cells express MN markers (HB9+/TUBB3+ and CHAT+/TUBB3+) (Figures 4D and 4E). At a late stage, such as 9 wpi, neurons show complicated branches and presynaptic markers, such as synapsin 1 (SYN1) (Figure 5).

LIMITATIONS

For aged or diseased fibroblast cell lines, the final yield could be poor due to the slow growth of cells and the low transduction efficiency. Preparation of lentiviruses with high quality and optimization of cell culture conditions are critical to improve the yield.

TROUBLESHOOTING

Problem 1

Detachment of cells during reprogramming (steps 5–7).

Potential solution

Coat culture plates with freshly prepared Matrigel (up to 1:50) at least 3 h and seed fibroblast at proper density. The confluency of fibroblast should be around 60%–80% at transduction.

Problem 2

Low quality of viruses (steps 1–3).

Potential solution

When performing the viral preparation, we recommend using low passage (less than 20) of HEK293T cells and checking on the quality of plasmids and transfection reagents. Proper ratio of helper plasmids, lentiviral vectors, and transfection reagent will improve lentivirus titers.

Problem 3

Low transduction efficiency (step 7).

Potential solution

Prepare the high quality of virus ($> 1 \times 10^6$ IFU/mL) and add proper amount of virus (MOI 5–8). Add 6 μ g/mL of polybrene to the lentiviral supernatant and mix well. Add the lentiviral mixture directly to cultured fibroblasts without removal of culture medium. Let cells exposed to virus for 8 h or overnight.

Problem 4

Low yield of differentiated neurons (steps 8 and 9).

Potential solution

Increase the transduction efficiency and the starting materials. Use 10 cm plates instead of smaller plates. The reprogrammed neurons from multiple plates could be pooled together at replating step to increase the total yield.

Problem 5

Poor survival of reprogrammed neurons (step 10).

Potential solution

Culture proper amount of neurons onto Matrigel coated coverslips with a monolayer of astrocytes. Given substantial neurons will die within 72 h after replating, seed proper amount of neurons in each well (about 2×10^4 cells/well of 24-well plate) is important to improve the neuronal survival. Co-culture with a monolayer of astrocytes is required for long-term survival. We have cultured reprogrammed neurons up to 10 weeks.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by Dr. Baojin Ding (Baojin.Ding@Louisiana.edu).

Materials availability

The majority of materials required in this protocol are commercially available.

Data and code availability

This study did not generate/analyze any data sets or code that were submitted to any repositories.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, funding acquisition, and supervision, B.D.; investigation and writing, B.D., M.S., and M.A.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Perspective

Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons

Baojin Ding*

Dystonia is a common movement disorder characterized by sustained or intermittent muscle contractions causing abnormal movements and/or postures (Keller Sarmiento and Mencacci, 2021). The dystonic syndromes are classified as primary dystonia (dystonia is the only motor feature without tremor) and the secondary dystonia (dystonia is combined with other movement disorders, such as Parkinsonism). Based on the age of onset, dystonias are also dichotomously classified as childhood onset or adulthood onset. The distribution of affected body parts may change over time and progressively spread of dystonia to previously uninvolved sites. Because the clinical characteristics and underlying causes of dystonia are very heterogeneous, the pathological mechanisms of dystonia remain largely unknown. The diagnosis and etiological definition of this disorder remain challenges. The current therapies, such as anticholinergics, intramuscular botulinum toxin injection and deep brain stimulation, are largely symptom-based and only partially satisfactory (Balint et al., 2018). The childhood-onset torsion dystonia, also called DYT1 dystonia, represents the most frequent and severe form of hereditary primary dystonia, providing an excellent example to understand the pathogenesis of this disease (Gonzalez-Alegre, 2019; Keller Sarmiento and Mencacci, 2021).

Most cases of typical DYT1 dystonia are caused by a heterozygous 3-bp deletion in exon 5 of the TOR1A gene, resulting in the loss of one of the two adjacent glutamate residues (E302 or E303) in the carboxy-terminal region of TOR1A protein (ΔE) (Keller Sarmiento and Mencacci, 2021). Protein TOR1A is a member of conserved AAA* (ATPases Associated with diverse cellular Activities) ATPase, which typically use the energy of ATP hydrolysis to disassemble protein complexes in diverse biological processes, such as membrane trafficking, cytoskeleton dynamics, vesicle fusion, response to stress, and the biogenesis of nuclear pore complex (Laudermilch and Schlieker, 2016; Rampello et al., 2020). In humans, TOR1A is widely distributed throughout the central nervous system with high expression in cerebral cortex, striatum, substantia nigra pars compacta, hippocampus, cerebellum and spinal cord, underscoring its critical roles in neural development and functions. At the cellular level, most TOR1A proteins are embedded in the lumen of the endoplasmic reticulum and the endomembrane space of nuclear

envelope. The activity of TOR1A depends on the association with one of two transmembrane cofactors, LAP1 (lamina associated polypeptide 1, also named TOR1AIP1) and LULL1 (luminal domain like LAP1, also named TOR1AIP2), suggesting that TOR1A works in a membrane-spanning machinery (Laudermilch and Schlieker, 2016). Although ΔE has been shown to be a loss-of-function mutation, the actual functions of the TOR1A protein in DYT1 dystonia and how it leads to changes in the nervous system to cause dystonia remain poorly understood. While rodent models provide insights into disease mechanisms, significant species-dependent differences exist because animals with the identical heterozygous mutation fail to show pathology (Gonzalez-Alegre, 2019). However, inaccessibility to patient neurons greatly impedes our understanding of the pathological mechanisms for dystonia.

It is fascinating that reprogramming of human neurons from adult fibroblasts provides an unprecedented approach to deciphering the molecular pathogenesis underlying neurological diseases. Recently, we modeled DYT1 by using human patient-specific cholinergic motor neurons (MNs) that are generated through either direct conversion of patients' skin fibroblasts (Ding et al., 2020) or differentiation of induced pluripotent stem cells (iPSCs) (Sepehrmanesh and Ding, 2020; Ding, 2021). These human MNs naturally express the heterozygous TOR1A mutation and display cellular pathology (Ding et al., 2021). Compared to healthy MNs, DYT1 MNs had significantly shorter neurite and many fewer branches, deformed nuclear morphology, fewer nuclear pore complex, and impaired nucleocytoplasmic transport (NCT) that leads to mislocalization of mRNAs and proteins. This is the first demonstration of cellular deficits in a heterozygous state of patient-specific neurons, unravelling important differences between human and mouse models (Ding et al., 2021).

The distinguished features of human DYT1 neurons are deformed nuclear envelope (Figure 1A and B) and dysregulations of nuclear LMNB1 at both expression (Figure 1D and E) and subcellular localization (Figure 1C). Protein LMNB1 is a major component of the nuclear lamina and it contributes to the structural integrity of the nuclear envelope. Overexpression of LMNB1 has been shown to increase nuclear rigidity. An abnormal extra copy of LMNB1 gene causes autosomal dominant leukodystrophy, which is characterized by loss of myelin in the brain

and the spinal cord, leading to movement problems (Kohler et al., 2018). Similarly, I think the increased LMNB1 also contributes to these cellular abnormalities in DYT1 neurons. Consistently, overexpression of LMNB1 indeed disrupts nuclear morphology (Figure 1F). Downregulation of LMNB1 can largely ameliorate all the cellular deficits in DYT1 MNs, including neurodevelopment, nuclear morphology, NCT activities (Figure 1G). These results indicate that dysregulation of LMNB1 may constitute a major molecular mechanism underlying DYT1 pathology. Importantly, this study describes the first cellular model system for human Dystonia using techniques of direct conversion and iPSC system, revealing the great value of disease modeling with human patient-derived neurons.

Traditionally, dystonia has been considered a disorder of the basal ganglia, a subcortical nuclei involved in diverse functions including motor control and motor learning. Later studies suggest that dystonia is a network disorder, with involvement of a basal ganglia–cerebello-thalamo-cortical circuit (Balint et al., 2018). Interestingly, recent reports also indicated that the cerebellum and the spinal cord could be other major sites of dysfunction in Tor1a mutant mice (Zhang et al., 2017). The findings that deficits in presynaptic inhibition caused by impaired GABAergic neurons in the spinal cord suggest that the abnormalities of proprioceptive and/or muscle spindle feedback contribute to the DYT1 dystonia (Zhang et al., 2017). Using patient-derived MNs, our study further provides evidence that lower MNs are implicated in the pathogenesis of dystonia (Ding et al., 2021). In healthy MNs, nuclear morphology, neurite outgrowth and NCT activities are normal. The majority of mRNAs localize in the cytoplasm (Figure 1H). In DYT1 MNs, upregulated LMNB1 causes thickening of nuclear lamina, thereby leading to abnormal nuclear morphology and impaired NCT, resulting in nuclear accumulation of mRNAs and mislocalized proteins (Figure 1H). Progressive LMNB1 accumulation and subcellular mislocalization are critical pathological features of DYT1 MNs, providing a novel molecular target for therapeutic intervention.

To further understand the dysregulation of LMNB1 underlying the pathogenesis of DYT1 dystonia, the following questions need to be addressed in future studies (Figure 1H). First, to examine the neuronal subtype specificity of LMNB1 dysregulation in DYT1 cells. Dysregulation of LMNB1 has been recapitulated by either ectopic expression of the mutant TOR1A gene or shRNA-mediated downregulation of endogenous TOR1A in healthy control MNs, suggesting that LMNB1 mislocalization are caused by TOR1A hypoactivity (Ding et al., 2021). In non-MNs that were generated by spontaneous differentiation of DYT1 neural progenitors and differentiation of human SH-SY5Y neuroblasts with ectopic

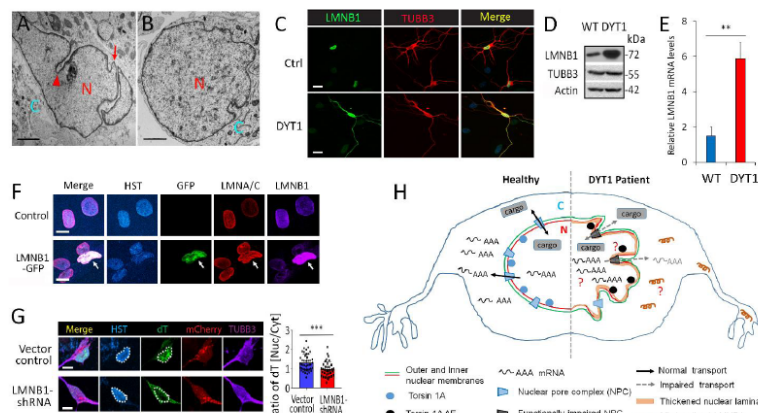


Figure 1 | Dysregulation of nuclear LMNB1 in DYT1 patient-derived neurons.

(A) Representative electron micrograph of DYT1-iMNs at 4 weeks post-infection (wpi) shows deformed nuclear morphology. Arrowhead indicates a deep invagination; arrow indicates sharp angles. C: Cytoplasm; N: nucleus. Scale bar: 5 μ m. (B) Representative electron micrograph of control-iMNs at 4 wpi. N: Nucleus; C: cytoplasm. Scale bar: 5 μ m. (C) Representative confocal images of iMNs of healthy control (Ctrl) and DYT1 at 6 wpi. Scale bars: 20 μ m. (D) Western blots for LMNB1 proteins in WT- and DYT1 iMNs. TUBB3 and Actin are loading controls. (E) Relative LMNB1 mRNA levels analyzed by qRT-PCR in iMNs. $n = 3$, $**P < 0.01$. (F) Confocal micrographs of fibroblasts with or without overexpression of LMNB1 (fused with GFP). Arrow indicates a cell with LMNB1 overexpression. Scale bars: 10 μ m. (G) Fluorescence in situ hybridization (FISH) analysis shows LMNB1 downregulation ameliorated mRNA nuclear export in DYT1 iMNs at 3 wpi. Oligo-dT probes are used to detect mRNAs. shRNA-expressing cells were identified by the co-expressed mCherry. n (neuron) > 50 from triplicate. $***P < 0.001$. Scale bars: 10 μ m. (H) A working model for DYT1 pathogenesis. The healthy MNs have normal nuclear morphology and nuclear transport activities. The majority of mRNAs localize in the cytoplasm. In DYT1 MNs, LMNB1 upregulation leads to thickened nuclear lamina, cytoplasmic localization, abnormal nuclear morphology, and impaired nucleocytoplasmic transport, which subsequently leads to mislocalization of mRNAs and proteins and dysfunction of neurons. Question marks indicate important questions to be addressed, including (1) identification of nuclear accumulated transcripts, (2) the mechanism underlying LMNB1 dysregulation in DYT1 neurons, and (3) how mislocalized LMNB1 contributes to the disease. Adapted from Ding et al. (2021). FISH: Fluorescence in situ hybridization; GFP: green fluorescent protein; HST: Hoechst 33342; iMNs: induced motor neurons; qRT-PCR: quantitative real time polymerase chain reaction; TUBB3: Tubulin Beta 3 Class III; wpi: weeks post-infection; WT: wild-type.

expression of ΔE , no mislocalized LMNB1 can be noticed (Ding et al., 2021), suggesting that LMNB1 mislocalization could be MN-specific. A recent report that neural increase in LMNB1 also contributes to nuclear dysfunction in Huntington's disease (Alcala-Vida et al., 2021). Other DYT1 neural subtypes should be examined to test whether TOR1A hypoactivity causes LMNB1 mislocalization in a MN-specific manner. Second, to verify these abnormalities in clinical samples. If these abnormalities identified using *in vitro* cellular system also present in clinical samples, these findings will lead to potential therapeutic strategies for DYT1 dystonia. Third, to identify nuclear accumulated transcripts in DYT1 neurons. Impaired nuclear export activity in DYT1 neurons causes the nuclear accumulation of mRNAs (Figure 1H). Are these mRNAs proportional to all transcripts or do they represent some particular subset that are involved in certain neural functions? Do the retained mRNAs share common sequences or secondary structures? These questions could be addressed by biochemical approaches of RNA isolation together with the next-generation sequencing to identify mislocalized transcripts. Fourth, to decipher the molecular mechanisms of how TOR1A ΔE causes dysregulation of LMNB1 and how mislocalized LMNB1 contributes to DYT1 dystonia. Protein co-immunoprecipitation

coupled with mass spectrometry analysis could be used to identify ΔE -disrupted factors and mislocalized LMNB1-interacting proteins in DYT1 MNs. The abnormal protein-protein interactions and/or disrupted signaling pathways could provide novel insights into the dysfunctions of DYT1 neurons. No matter RNAseq assay or co-immunoprecipitation coupled with mass spectrometry analysis, generation of patient-specific MNs with high purity and yield is absolutely required. Excitingly, we have developed a protocol by which highly pure patient-specific MNs could be generated from hiPSC (Sepehrmanesh and Ding, 2020; Ding, 2021), providing a solid foundation to address these questions using biochemical approaches.

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Baojin Ding*

Department of Biology, University of Louisiana at Lafayette, Lafayette, LA, USA

*Correspondence to: Baojin Ding, MD, PhD, Baojin.Ding@Louisiana.edu.

https://orcid.org/0000-0002-2149-2599 (Baojin Ding)

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Book Chapter

Nucleocytoplasmic Transport: Regulatory Mechanisms and the Implications in Neurodegeneration

Baojin Ding* and Masood Sepehrimanesh

Department of Biology, University of Louisiana at Lafayette,
USA

***Corresponding Author:** Baojin Ding, Department of Biology,
University of Louisiana at Lafayette, 410 East Saint Mary
Boulevard, Lafayette, LA 70503, USA

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Defense (DoD) Peer Reviewed Medical Research Program (PRMRP) Discovery Award (W81XWH2010186 to B.D.).

Appendix F - Poster presentation 1

4/16/2021

Mail - Baojin Ding - Outlook

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June 7-9 | 9:00AM EDT | 1:00PM UTC | 3:00PM CEST | #VKSNeuroDegen21

Baojin Ding, MD, PhD, University of Louisiana at Lafayette	POSTER SESSION 1, FLOOR 1: Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons
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Appendix G - Poster presentation 2

7/14/2021

cOASIS, The Online Abstract Submission System



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Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia

AUTHOR BLOCK: *B. DING;

Univ. of Louisiana At Lafayette, Lafayette, LA

Abstract:

DYT1 dystonia is a hereditary neurological movement disease. It is caused by a heterozygous deletion in *TOR1A* gene, resulting in the loss of one glutamate residues in the carboxy-terminal region of TOR1A protein (ΔE). Although ΔE has been shown to be a loss-of-function mutation, the mechanisms underlying ΔE leads to changes in the nervous system to cause dystonia remain poorly understood. While rodent models provide insights into disease mechanisms, significant species-dependent differences exist because animals with the identical heterozygous mutation fail to show pathology. Excitingly, reprogramming of human neurons from adult fibroblasts overcomes the limitation of inaccessibility to patient

neurons and provides an unprecedented approach in deciphering the molecular pathogenesis underlying neurological diseases. In this study, DYT1 neurons have been generated via two strategies: (1) direct conversion from patient fibroblasts using lentiviral delivery of transcription factors, and (2) induced pluripotent stem cells (iPSCs)-based induction and differentiation. Using different combinations of reprogramming factors, different neuronal subtypes could be generated, including generic neurons (GNs), motor neurons (MNs), and cholinergic interneurons (ChINs). Directly converted MNs (diMNs) and iPSC-derived MNs (iMNs) have been systematically examined and compared in modeling DYT1 dystonia. Diseased neurons generated by both approaches showed similar phenotypes in impaired neurodevelopment, abnormal nuclear morphology, disrupted nucleocytoplasmic transport and dysregulation of nuclear lamin B1. Interestingly, diMNs showed more severe deficits than iMNs. Thus, diMNs are excellent materials for pathogenesis study based on immunocytochemistry. Compare to diMNs, iMNs possess unique advantages, such as much higher purity and yield, and the shorter culture time to reach the mature stage (3 weeks vs 6 weeks). Generation of large amount of highly pure neurons via iPSC-based approach makes it possible to biochemically identify dysregulated factors in diseased neurons.

Author Disclosure Information:

B. Ding: None.

Linking Group (Complete): None selected

Theme and Topic (Complete): A.03.a. Pluripotent stem cells – Disease models ; A.03.b. Pluripotent stem cells – Differentiation and reprogramming

Linking Group Information (Complete):

Keyword (Complete): MOTOR NEURON ; IPSC ; MODELING

Support (Complete):

Support: Yes

Grant/Other Support: : NIH/NINDS grant NS112910

Grant/Other Support: : DoD grant W81XWH2010186

Special Requests (Complete):

How do you plan to participate in the annual meeting?: Virtual only

Religious Conflict?: No Religious Conflict

Additional Conflict?: No

Is the presenting author of this abstract a high school or undergraduate student?: None

Do you think media would be interested in your abstract and are you willing to share it with them?: Yes

Special Needs Accommodations: : None

Status: Finalized



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Appendix H -The PI'S updated CV

CURRICULUM VITAE Baojin Ding M.D., PH.D.

TITLE AND CONTACT INFORMATION

Assistant Professor
Department of Biochemistry and Molecular Biology
LSU Health Shreveport
1501 Kings Highway, Shreveport, LA 71130

EDUCATION

- 2010** Ph.D. in Biochemistry and Molecular Biology of Veterinary Medical Sciences,
Louisiana State University, School of Veterinary Medicine, Baton Rouge, LA
- 2004** M.S. of Medicine in Clinical Laboratory
Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China
- 2001** Bachelor Degree of Medicine (M.D. equivalent)
Medical College of Qingdao University, Qingdao, Shandong, P. R. China

RESEARCH SUPPORT

Current

NIH/NINDS R21 (NS112910) (PI) 04/01/2020-09/30/2022

Title: *Determining the Pathogenesis of DYT1 Dystonia in Reprogrammed Human Neurons*

Source: NINDS Exploratory Neuroscience Research Grant [PA18-358]

Overall goal: To determine the pathogenesis of childhood onset DYT1 dystonia in patient-specific neurons that are generated by direct conversion and iPSC-based reprogramming and differentiation.

DoD (W81XWH2010186) Peer Reviewed Medical Research Program (PRMRP) Discovery Award (PI)
03/15/2020-3/14/2023

Title: *Determining the Pathogenesis of Dystonia in Reprogrammed Human Neurons*

Source: Department of Defense, Congressionally Directed Medical Research Programs (CDMRP)

Overall goal: To determine the pathogenesis of adulthood onset dystonia via directly reprogramming human neurons from patient fibroblasts.

Faculty Start-up (PI) 2022-2025

Source: LSU Health Shreveport

Professorship

MID-WINTER FAIR ASSOCIATION/BORSF 2020-2023

Pending

Molecular & Cellular Mechanisms of Dystonia Grant (PI) 2022-2024 Title:

Determining the pathogenesis of dystonia in patient-specific neurons

Source: Dystonia Medical Research Foundation (DMRF)

Completed

Lahaye Faculty Development Grant (PI) 2019-2020

This fund is intended to support undergraduate researchers working on biomedical studies.

Enhancement of Biology Research and Teaching through Personal Flow Cytometry (Co-PI)

06/01/2020-06/30/2021, _____

Source: Louisiana Board of Regents Support Fund, Departmental Enhancement

Goals: To improve the educational and research infrastructure in the department of Biology in UL Lafayette

NIH/NIA P30-12300-21 (PI) 2017 -2018

Title: *Nucleocytoplasmic Transport Defect in Alzheimer's Disease*

Source: The Friends of the Alzheimer's Disease Center and NIH Alzheimer's Disease Center

Major goal: to understand how nucleocytoplasmic transport defect contributes to Alzheimer's disease (AD) by using mouse primary neurons, mammalian cell lines and directly reprogrammed neurons (diNs) from fibroblasts of AD patients.

PROFESSIONAL EXPERIENCE**2022- Present Assistant Professor (Tenure track)**

Department of Biochemistry and Molecular Biology, LSU Health Shreveport, 1501 Kings

Highway, Shreveport, LA 71130

Research area: Biochemistry and Molecular Biology, Neuroscience and Neurological Diseases

2018- 2021 Assistant Professor (Tenure track)

Department of Biology, University of Louisiana at Lafayette, Lafayette, LA 70503

Research area: Cell Biology, Neuroscience and Neurological Diseases

2016- 2018 Assistant instructor

Department of Molecular Biology, Center for Regenerative Science and Medicine,

UT Southwestern Medical Center, Dallas, TX

Research interests: Nucleocytoplasmic transport in neural development and neurological disorders.

2010-2016 Postdoctoral Fellow

Departments of Physiology and Neurobiology, University of Massachusetts Medical School, Worcester, MA

Research topic: Molecular Mechanisms of Gene Expression in Neurodevelopment and nuclear mRNA export.

2005 —2010 Graduate Research Assistant

Department of Comparative Biomedical Science, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA

Research topic: The Molecular Mechanisms of Transcription Coupled DNA Repair in Eukaryotic cells. (Ph.D. Dissertation: http://etd.lsu.edu/docs/available/etd-04072010-012548/unrestricted/Ding_diss.pdf)

2004 —2005 Research Associate

Department of Biological Sciences, Louisiana State University, Baton Rouge, LA

Research topic: The Biogenesis and Functions of Iron-Sulfur Cluster proteins.

2002 —2004 Research Assistant

The Institute of Cellular and Molecular Medicine, Wenzhou Medical College, Wenzhou, Zhejiang, P. R. China

Research topic: Isolation, Purification and Screening of Effective Components from Natural Products.

2000 —2001 Intern Doctor

Affiliated Hospital of Medical College of Qingdao University, Qingdao, P. R. China

PUBLICATIONS (*Dr. Baojin Ding is the corresponding author)

<https://www.ncbi.nlm.nih.gov/myncbi/baojin.ding.1/bibliography/public/>

1. **Ding B***. (2022). Novel insights into the pathogenesis of DYT1 dystonia from induced patient-derived neurons. *Neural Regen Res* 17(3):561-562. (Invited Perspective)
2. Sepehrimanesh M., Akter M. and **Ding B***. (2021). Direct conversion of adult fibroblasts into motor neurons. *STAR Protocols*. 2 (4) 17 December 2021, 100917 (Cell Press, Invited protocol).
3. Akter M, Cui H, Chen Y-H and **Ding B***. (2021). Generation of two induced pluripotent stem cell lines with heterozygous and homozygous GAG deletion in TOR1A gene from a healthy hiPSC line. *Stem Cell Research*. 56 (2021)102536 <https://doi.org/10.1016/j.scr.2021.102536>
4. **Baojin Ding*** and Masood Sepehrimanesh. Nucleocytoplasmic Transport: Regulatory Mechanisms and the Implications in Neurodegeneration. In: Letizia Giampietro, editor. **Prime Archives in Molecular Sciences: 2nd Edition**. Hyderabad, India: Vide Leaf. 2021. (Book Chapter)
5. **Ding B*** and Sepehrimanesh M. (2021). Nucleocytoplasmic transport: regulatory mechanisms and the implications in neurodegeneration. *Int. J. Mol. Sci.* 22 (8): 4165.
6. **Ding B***, Tang Y, Ma S, Akter M, Liu ML, Zang T, Zhang CL.(2021). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. *J Neurosci*. 41 (9): 2024-2038.
Featured Article, <https://www.jneurosci.org/content/41/9/1846>
Selected as **JNeurosci's Annual Spotlight**
7. **Ding B***. (2021). Generation of patient-specific motor neurons in modeling movement diseases. *Neural Regen Res* 16(9):1799-1800.
8. **Baojin Ding***, Yu Tang, Shuaipeng Ma, Meng-Lu Liu, Tong Zang, Masuma Akter and Chun-Li Zhang (2020). Disease modeling with human neurons reveals LMNB1 dysregulation underlying DYT1 dystonia. *bioRxiv*. doi: <https://doi.org/10.1101/2020.08.11.246371>
9. Sepehrimanesh M, and **Ding B*** (2020). Generation and Optimization of Highly Pure Motor Neurons from Human Induced Pluripotent Stem Cells via Lentiviral Delivery of Transcription Factors. *Am J Physiol Cell Physiol*. 319: C771–C780.
10. **Ding B***, Akter M, and Zhang C-L. (2020). Differential Influence of Sample Sex and Neuronal Maturation on mRNA and Protein Transport in Induced Human Neurons. *Front Mol Neurosci*. 2020 Apr 3; 13: 46.
11. Selvam K, **Ding B**, Sharma R and Li S. (2019). Evidence that moderate eviction of Spt5 and promotion of error-free transcriptional bypass by Rad26 facilitates transcription coupled repair. *J Mol Biol*. 2019 Feb 18. doi: 10.1016
12. **Ding B**, Dobner PR, Mullikin-Kilpatrick D, Wang W, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2018). BDNF Activates an NFI-Dependent Neurodevelopmental Timing Program By Sequestering NFATc4. *Mol Biol Cell*. 2018 Apr 15; 29(8):975-987
13. **Ding B.**, Mirza A.M., Alshley J. Budnik V. and Munson M. (2017). Nuclear Export Through Nuclear Envelope Remodeling in *Saccharomyces cerevisiae*. *bioRxiv* 224055; doi: <https://doi.org/10.1101/224055>
14. Leto K, Arancillo M, Becker EB, Buffo A, Chiang C, **Ding B**, Dobyns WB, Dusart I, Haldipur P, Hatten ME, Hoshino M, Joyner AL, Kano M, Kilpatrick DL, Koibuchi N, Marino S, Martinez S, Millen KJ, Millner TO, Miyata T, Parmigiani E, Schilling K, Sekerková G, Sillitoe RV, Sotelo C,

- Uesaka N, Wefers A, Wingate RJ, Hawkes R. (2016). Consensus Paper: Cerebellar Development. **Cerebellum**. Dec;15(6): 789-828.
15. Li Y, Hassinger L, Thomson T, **Ding B**, Ashley J, Hassinger W and Budnik V. (2016). Lamin Mutations Accelerate Aging via Defective Export of Mitochondrial mRNAs through Nuclear Envelope Budding. **Curr Biol**. 2016 Aug 8;26(15):2052-9
 16. **Ding B**, Cave HW, Dobner PR, Kilpatrick DM, Bartsokis M, Zhu H, Chow CW, Gronostajski RM and Kilpatrick DL. (2016) Reciprocal Auto-Regulation by NFI Occupancy and ETV1 Promotes the Developmental Expression of Dendrite-Synapse Genes in Cerebellar Granule Neurons. **Mol Biol Cell**. 2016 May 1;27(9):1488-99
 17. **Ding B***. (2015) How does a 1.5-Fold Increase in Gene Dosage in Chromosome 21 Cause the Pleiotropic Phenotypes in Down Syndrome? **J Down Syndr Chr Abnorm 1**: 1: e101. doi:10.4172/jdsca.1000e101 (Editorial)
 18. **Ding B***. (2015) How to Assist Parents of Children with Autism Spectrum Disorders in Rural Area? **J Neurosci Rural Pract**. 6 (4), 465-6 (Editorial)
 19. Packard M, Jokhi V, **Ding B** and Budnik V. (2015) Nucleus to Synapse Nesprin Railroad Tracks Direct Synapse Maturation through RNA localization. **Neuron**. 86(4):1015-28).
 20. **Ding B***. (2015) Gene Expression in Maturing Neurons: Regulatory Mechanisms and Related Neurodevelopmental Disorders. **ACTA PHYSIOLOGICA SINICA (Sheng Li Xue Bao)**. 67(2):113-33. (Invited Review)
 21. **Ding B**, Wang W, Selvakumar T, Xi HS, Zhu H, Chow CW, Horton JD, Gronostajski RM and Kilpatrick DL. (2013) Temporal Regulation of Nuclear Factor One Occupancy by Calcineurin/NFAT Governs a Voltage-Sensitive Developmental Switch in Late Maturing Neurons. **J Neurosci**. 33(7):2860-2872.
 22. **Ding B** and Kilpatrick DL. (2013) Lentiviral Vector Production, Titration, and Transduction of Primary Neurons. **Methods Mol Biol**. 1018:119-31. Chapter 12.
 23. **Ding B** and Kilpatrick DL. (2013). Chromatin Immunoprecipitation Assay of Brain Tissue Using Percoll Gradient-Purified Nuclei. **Methods Mol Biol**. 1018:199-209. Chapter 19.
 24. **Ding B**, Lejeune D and Li S. (2010) The C-terminal Repeat Domain of Spt5 Plays an Important Role in Suppression of Rad26-independent Transcription Coupled Repair. **J Biol Chem**. 285 (8): 5317-5326.
 25. Chen X, **Ding B**, Lejeune D, Ruggiero C and Li S. (2009) Sumoylation of Rpb1 in Response to UV Radiation or Impairment of Transcription Elongation in Yeast. **PLoS One**. 4 (4) e5267.
 26. Lejeune D, Chen X, Ruggiero C, Berryhill S, **Ding B** and Li S. (2009) Yeast Elc1 Plays an Important Role in Global Genomic Repair but not in Transcription Coupled Repair. **DNA repair (Amst)**. 8(1): 40-50.
 27. **Ding B**, Ruggiero C, Chen X and Li S. (2007) Tfb5 is Partially Dispensable for Rad26 Mediated Transcription Coupled Nucleotide Excision Repair in Yeast. **DNA Repair (Amst)**. 6 (11): 1661-1669.
 28. Li S, **Ding B**, LeJeune D, Ruggiero C, Chen X and Smerdon MJ. (2007) The Roles of Rad16 and Rad26 in Repairing Repressed and Actively Transcribed Genes in Yeast. **DNA Repair (Amst)**. 6 (11): 1596-1606.
 29. Li S, **Ding B**, Chen R, Ruggiero C and Chen X. (2006) Evidence that Transcription Elongation Function of Rpb9 is Involved in Transcription Coupled DNA Repair in *Saccharomyces cerevisiae*. **Mol Cell Biol**. 26 (24): 9430-9441.

30. Li S, Chen X, Ruggiero C, **Ding B** and Smerdon M. (2006) Modulation of Rad26 and Rpb9 Mediated DNA Repair by Different Promoter Elements. **J. Biol. Chem.** 281(48): 36643-36651.
31. **Ding B**, Smith ES, and Ding H. (2005) Mobilization of Iron Center in IscA for Iron-sulfur Cluster Assembly in IscU. **Biochem. J.** 389 (Pt 3):797-802.
32. **Ding H**, Clark RJ and **Ding B**. (2004) IscA Mediates Iron Delivery for Assembly of Iron-Sulfur Clusters in IscU under the Limited Accessible Free Iron Conditions. **J. Biol. Chem.** 279 (36): 37499 – 37504.
33. **Ding B**, Jin L and Lv J. (2004) Biological Activity Research Progresses of Polysaccharides. **Chin Pharm J.** 39 (8):561-564.
34. **Ding B**, Jin L and Lv J. (2004) The Effect of Polysaccharides from *Paecilomyces tenuipes* on TNF- α of PBMC. **Chinese Journal of Biochemical Pharmaceutics.** 25 (5): 268-270.
35. **Ding B**, Jin L and Lv J. (2004) Determination of Polysaccharides in *Paecilomyces tenuipes* by Spectrophotometry of Phenylhydrate-sulfuric acid. **Journal of Wenzhou Medical College.** 34(1): 15-17.
36. **Ding B** and Qiu X. (2004) The Immunoregulation of Fungi from *Paecilomyces*. **Chin J Clin Pharmacol Ther.** 9(1):17-20.
37. Qiu X and **Ding B**. (2003) Research Progress and Application Prospect of Nitrogen Monoxide Donor Medicine. **Chin J Clin Pharmacol Ther.** 8(1): 118-120.

TEACHING

- **Fall Semester (2018- 2021)**

BIOL-457(G), Advanced Cell Biology, CRN 224133, 3 Credits Lecture

This course aims to examine life at its most fundamental level, including mechanisms and pathways responsible for membrane transport, metabolism, gene expression, protein synthesis and secretion, membrane trafficking, cytoskeleton dynamics, and cell signaling.

BIOL-458(G), Advanced Cell Biology Lab, 2 Credits, 4 Hours Lab

This class is intended to immerse students in the process of performing scientific experiments, through which students will master some experimental skills, acquire the capabilities for data analysis and result interpretation. The model system will be cultured mammalian cells and/or fixed cells on slides.

BIOL-410, 1-6 Credit(s) Individual Project

Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques

- **Spring Semester (2019-2021)**

BIOL-472(G), Neurophysiology, 3 Credits Lecture

This course aims to understand neurophysiological activities at cellular and molecular levels and their linkage to human neurological diseases.

BIOL-473(G), Neurophysiology Lab, 1 Credit Lab

BIOL-410, 1-6 Credit(s) Individual Project

Cellular and Molecular Neuroscience - from Basic Knowledge to Advanced Techniques

- **Teaching Assistant (2002-2003)**

Wenzhou Medical University, Wenzhou, Zhejiang, China

Course: Molecular Biology

STUDENTS/POSTDOCS MENTORED (*co-authors of publication)

Postdoc Researcher/Visiting Scholar Mentored

1. *Dr. Masood Sepehrimanesh (Ph.D., D.V.M., Shiraz University of Medical Sciences, Shiraz, Iran. Postdoctoral Research Associate, 03/2020- present)
2. Dr. Ameneh Zare-Shahabadi (M.D. Tehran University of Medical Sciences, Iran. Research Assistant, 2018-2019, UL Lafayette. Current: Medical Doctor, Cincinnati, OH)

Graduate Students (Major Advisor)

1. *Abir Hosain (PHD, 01/2021-, LSUHS)
2. *Haochen Cui (PHD, 01/2020-, LSUHS)
3. *Casey Cottee (MSci, 08/2020-, UL Lafayette)
4. *Masuma Akter (PHD, 08/2019-, UL Lafayette)
5. Jacob Stagrays (PHD, 08/2019-06/2021, UL Lafayette)
6. Makaila Mitchell (MSci, 01/2019-12/2019, UL Lafayette)

Graduate Students (Committee Member)

1. Santosh Paudel (PhD, UL Lafayette, 2018-)
2. Brittany M. Pitruca (MSc, UL Lafayette, 2018-2021)
3. Chukwunonso Chukwudozie (MSc, UL Lafayette NIRC, 2018-2020)
4. Alex Esteve, (MSc. UL Lafayette, 2018-2021)
5. Jachie Barua, (PhD, UL Lafayette, 2019-)

Undergraduate students

1. Catherine Wertz (Scholarship, Biology major, UL Lafayette. 01/2020-12/2021)
2. Bennett Garbarino (Scholarship, Biology major, UL Lafayette. 08/2018-12/2020)
3. Matthew Authement (Scholarship, Biology major, UL Lafayette. 08/2018-05/2020)
4. Casey A. Coutee (Undergraduate Researcher, Biology major, UL Lafayette. 01/2019-05/2020)
5. Bao Doan (BIOL410, Biology major, UL Lafayette. Fall 2019, Spring 2020)
6. Audrey Lumpki (BIOL410, Biology major, UL Lafayette. Fall 2019)
7. Erin Miller (BIOL410, Biology major, UL Lafayette. Fall 2019)
8. Joy Aniede (STARS Summer Research Program, 2017, UT Southwestern Medical Center)
9. Adrienne Lemieux (Worcester State University, 2016, UMass Medical School)
10. Alexandra D'Ordine (Worcester Polytechnic Institute, Class of 2017; 2015 at UMass Medical School)
11. Emily Vancor (NIH, MIT 2014; 2013 at UMass Medical School) *Ms. Emily Vancor obtained the second place of the summer research presentation. This research experience made her stand out from many applicants and got acceptance by Yale School of Medicine in 2014.*
12. *Marina Bartzokis (2012-2014, UMass Medical School)
13. Salvador Esparza (NIH, MIT 2014, 2012 at UMass Medical School)
14. Robert Lumley (The 2011 Summer Research Fellowship Program at UMass Medical School)

15. Amy Corron (The 2011 Summer Research Fellowship Program at UMass Medical School)
16. Tatenda Mujeni (HHMI, Bennett College For Women, 2009, Louisiana State University)

HONORS AND AWARDS

South Louisiana Mid-Winter Fair Association/BORSF Professorship (2020-2023)
DoD Peer Reviewed Medical Research Program (PRMRP) Discovery Award (2020)
Lahaye Faculty Development Grant (2019, 2020)
Alzheimer's Disease Research Award from Friends of the Alzheimer's Disease Center (2017)
American Association of Anatomists (AAA) Travel Award (2013, 2014)
American Society for Microbiology Postdoctoral Research Fellowship Program (2010, declined)
Guang Hua Scholarship (2002, 2003)
Undergraduate Scholarship (1997, 1998, 2000)
Excellent Student Award (1997, 1999)

PROFESSIONAL ACTIVITIES

Professional Services

Associate Editor

Frontiers in Molecular Neuroscience (2018-)

Editorial Board

American Journal of Psychiatry and Neuroscience (AJPN) (2020-);
Austin Neurology (2016-2018);
Journal of Autism & Related Disabilities (2016-2018);
Journal of Down Syndrome & Chromosome Abnormalities (2015-2017);
Frontiers in Molecular Neuroscience (2013-2018)

Grant Reviewer

DoD Scientific Reviewer Neurological Disorders (ND) Panel (2021)
European Research Council (ERC) grant review (2016)

Ad Hoc Reviewer: *Neurobiology of Disease*; *Scientific Reports*; *Journal of Cellular Biochemistry*;
Journal of Cellular and Molecular Medicine; *Frontiers in Molecular Neuroscience*, *Analytica Chimica Acta*, *Frontiers in Computational Neuroscience*, *Journal of Neurosciences in Rural Practice*, *Neural Regeneration Research*, *JoVE*, *Cells* etc.

Scientific Member

Member of American Society for Cell Biology (ASCB) (2017-)
Member of Society for Neuroscience (2014-)
Member of *American Association of Anatomists (AAA)* (2012-2016)
Member of *American Association for the Advancement of Science (AAAS)* (2009-2013)
Member of American Society for Microbiology (ASM) (2009-2012)
Member of Environmental Mutagenesis Society (EMS) (2009-2010)

University Services

Faculty Search Committee for Physiologist (2019-2020)
Pre professional Committee (2020- present)
-Review applicants for professional school and write letters of support.

Community services

Louisiana Region VI Science and Engineering Fair Judge 2019
Massachusetts State Middle School Science and Engineering Fair Judge (2014-2016)
Reuters Health
<http://www.psychcongress.com/article/tiny-brain-organoids-show-neuron-differentiation-autism-23405>

ORAL PRESENTATIONS AND INVITED TALKS

1. July 9, 2021. *Nucleocytoplasmic Transport and Its Linkage to Neurological Diseases*. Department of Anatomy, Howard University College of Medicine, Washington, D.C. (Host: Dr. Antonei Csoka)
2. May 3, 2021. *Nucleocytoplasmic Transport and Its Linkage to Aging and Neurological Diseases*. Department of Neuroscience & Regenerative Medicine, Medical College of Georgia at Augusta University. Augusta, GA (Host: Dr. Dave Blake)
3. April 28, 2021. *Nucleocytoplasmic Transport and Its Linkage to Aging and Neurological Diseases*. Sponsored by the Dept. of Biochemistry & Molecular Biology and the Oklahoma Center for GeroScience and Healthy Brain Aging. The University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City, OK. (Host: Drs. Ann Olson and William Sonntag)
4. February 23, 2021. *Nucleocytoplasmic Transport and Its Linkage to Neurological Diseases*. Department of Cell Biology and Anatomy, LSU Health Shreveport, Shreveport, LA (Host: Dr. Kevin McCarthy)
5. January 5, 2021. *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. Department of Biochemistry and Molecular Biology, LSU Health Shreveport, Shreveport, LA (Host: Dr. Stephan N. Witt)
6. January 23, 2020. *Impaired Nucleocytoplasmic Transport in Neurological Diseases*. Department of Biological Sciences, University of Texas at Dallas, Dallas, TX (Host: Dr. Heng Du)
7. July 19, 2019. *Nucleocytoplasmic Transport in Neurodegenerative Diseases*. National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan Province, China. (Host: Dr. Yu Tang)
8. May 7, 2018. *Spatiotemporal Regulation of Gene Expression*. Department of Biology, University of Louisiana at Lafayette, Lafayette, LA. (Host: Dr. Karl H. Hasenstein)
9. April 25, 2016. *Spatiotemporal Regulation of Gene Expression in Neurodevelopment and its Linkage to Neurological Diseases*. Department of Molecular Biology, UT Southwestern Medical Center, Dallas, TX (Host: Dr. Chun-Li Zhang)
10. January 4, 2016. *Nuclear Envelope Budding Pathway: from Drosophila to Mammals*. Department of Neurobiology, UMass Medical School, Worcester MA
11. April 26-30, 2014. *Auto-Regulatory Interactions Between NFI Occupancy and ETV1 Direct the Timing of Gene Expression in Late Maturing Neurons*. Experimental Biology Annual Meeting. San Diego, CA.
12. August 7, 2013. Department of Neurology, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Fen-Biao Gao)
13. July 30, 2013. Department of Microbiology & Immunobiology, New England Primate Research Center, Harvard Medical School. (Host: Dr. Min Dong)

14. July 17, 2013. Department of Neurobiology, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Vivian Budnik)
15. July 5, 2013. Gene Therapy Center, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Guangping Gao)
16. January 25, 2013. *Regulators of Nuclear Factor One Occupancy Control the Timing of Neuronal Maturation*. Department of Microbiology and Physiological Systems (MaPS), UMass Medical School, Worcester MA
17. February 10, 2012. *Nuclear Factor One Controls a membrane potential-sensitive switch program in developing neurons*. Department of MaPS, UMass Medical School, Worcester MA
18. April 12, 2010. Department of Physiology, University of Massachusetts Medical School. Worcester, Massachusetts (Host: Dr. Daniel L. Kilpatrick)
19. April 8, 2010. Stowers Institute for Medical Research, Kansas City, Missouri (Host: Dr. Jerry Workman)
20. April 5, 2010. Department of Experimental Radiation Oncology, University of Texas M.D. Anderson Cancer Center. Houston, Texas (Host: Dr. Junjie Chen)
21. March 30, 2010. *The Roles of Transcription Factors in Nucleotide Excision Repair in Yeast*. Department of Comparative Biomedical Science, LSU, Baton Rouge, LA
22. February 23, 2010. Department of Systems Biology, University of Texas M.D. Anderson Cancer Center. Houston, Texas (Host: Dr. Gabor Balazsi)
23. October 26, 2009. Department of Cell Biology and Physiology, Washington University in St. Louis. St. Louis, Missouri (Host: Dr. Helen Piwnicka-Worms)
24. January 20, 2009. *Transcription elongation factors Spt4 and Spt5 cooperatively suppress transcription coupled DNA repair through binding to RNA polymerase II*. Department of Comparative Biomedical Science, LSU, Baton Rouge, LA
25. November 20, 2008. *The Regulatory Mechanisms of Transcription Coupled Nucleotide Excision Repair (TC-NER) by Spt4/Spt5*. Department of Comparative Biomedical Science, LSU, Baton Rouge, LA
26. *The biological function of Polysaccharide in health care*. Clinical Nutriology Conference of Zhejiang Province. Nov. 2003, Shaoxing, P. R. China.

POSTER PRESENTATIONS

1. **Ding B.** *Similarities and differences of reprogrammed neurons via direct conversion and human induced pluripotent stem cells in modeling DYT1 dystonia*. Neuroscience 2021 50th Annual Meeting (Virtual: November 8-11, 2021).
2. **Ding B.** *Modeling Neurodegenerative Diseases via Generation of Patient-specific Neurons*. Virtual Keystone Symposia. Neurodegenerative Diseases: Genes, Mechanisms and Therapeutics. June 7-9, 2021.
3. **Ding B., Yu T. and Zhang C-L.** *Dysregulated nuclear LMNB1 and impaired nucleocytoplasmic transport in Dystonia patient-derived neurons*. The American Society for Cell Biology (ASCB) and Annual Meeting. December 2-16, 2020. ASCB/EMBO Virtual Meeting.

4. K Selvam, **B Ding**, R Sharma and S Li. *Promotion of error-free transcriptional bypass of DNA lesions is essential for Rad26 to facilitate transcription coupled DNA repair*. ENVIRONMENTAL AND MOLECULAR MUTAGENESIS 59, 71-71, 2018.
5. **Ding, B.**, Dobner PR. and Kilpatrick DL. *Nuclear Factor One (NFI)-Dependent Developmental Program Directs the Timing of Gene Expression in Maturing Neurons*. The American Society for Cell Biology (ASCB) Annual Meeting. December 2-6, 2017. Philadelphia, Pennsylvania.
6. **Ding B.**, Mirza A.M., Budnik V. and Munson M. *An alternative nuclear export pathway in Saccharomyces cerevisiae*. The American Society for Cell Biology (ASCB) Annual Meeting. December 3-7, 2016. San Francisco, California.
7. **Ding, B.**, Cave J. and Kilpatrick DL. *Auto-Regulatory Interactions Between NFI Occupancy and ETV1 Direct the Timing of Gene Expression in Late Maturing Neurons*. American Association of Anatomists (AAA) Annual Meeting. April 26-30, 2014. San Diego, California. (Also accepted as oral presentation)
8. **Ding, B.**, and Kilpatrick DL. *Nuclear factor one controls a voltage-sensitive developmental switch required for late neuronal maturation*. American Association of Anatomists (AAA) Annual Meeting. April 20-24, 2013. Boston, Massachusetts. (Postdoctoral Poster Presentation Award Finalist)
9. **Ding, B.**, LeJeune, D and Li, S. *Phosphorylation of Yeast Spt5 C-terminal Repeat by Bur1 Kinase Is Involved in the Suppression of Transcription Coupled DNA Repair*. Environmental Mutagen Society 40th Annual Meeting. October 24-28, 2009. St. Louis, Missouri.
10. **Ding, B.**, Chen X., Ruggiero, C., LeJeune, D. and Li, S. *Spt4 and Spt5 cooperatively suppress transcription coupled DNA repair through binding to RNA polymerase II in the absence of Rad26*. ASBMB (American Society for Biochemistry and Molecular Biology) annual meeting. April 18-22, 2009, New Orleans, Louisiana.
11. LeJeune, D., **Ding, B.** and Li, S. *PAF and Spt4 act in the same pathway to suppress transcription coupled repair in yeast*. DNA Repair and Mutagenesis: From Molecular Structure to Human Disease. May 30-June 5, 2009. Whistler, Canada.
12. Chen, X., **Ding, B.**, LeJeune, D., Ruggiero, C. and Li, S. *Sumoylation of Rpb1 restrains activation of DNA damage checkpoint by RNA polymerase II*. DNA Repair and Mutagenesis: From Molecular Structure to Human Disease. May 30-June 5, 2009. Whistler, Canada.
13. LeJeune, D., Chen X., Ruggiero, C., Berryhill, S., **Ding, B.** and Li, S. *Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair*. ASBMB (American Society for Biochemistry and Molecular Biology) annual meeting. April 18-22, 2009, New Orleans, Louisiana.
14. Li, S., Chen X., **Ding, B.** and LeJeune, D. *Rpb1 sumoylation in response to UV radiation or transcription impairment in yeast*. ASBMB (American Society for Biochemistry and Molecular Biology) annual meeting. April 18-22, 2009, New Orleans, Louisiana.
15. **Ding, B.**, Jin, L. and Lv, J. *The biological function of Polysaccharide in health care*. Clinical Nutriology Conference of Zhejiang Province. Nov. 2003, Shaoxing, P. R. China.