Form 3

Report for Joint/Usage Research Program for Endocrine/Metabolism (Fiscal Year 2022)

Date：(Year)/(Month)/(Day)

To Director of Institute for Molecular and Cellular Regulation, Gunma University

Principal Applicant	
Institution	Ajou University
Position	Associate professor
Name	Bum-HO, Bin

We report on the results of joint research in fiscal 2022 as below.

（Program No.　　）

1． Research Title	Golgi stress as a source of aging
2． Purpose and Significance of the research project	Anti-aging research has been conducted from various perspectives. This study proposes a new aging mechanism that has not been tried so far, suggests an anti-aging strategy based on this, and seeks to discover related materials. In detail, attention is paid to the aging of the Golgi apparatus, which acts as a major pathway of senescence-associated secretory phenotype (SASP), which is seen when cells are aged. We reveal whether the aging of the Golgi apparatus progresses during aging, and confirms the adverse effects on cells caused by aging of the Golgi apparatus. Furthermore, factors involved in Golgi aging are identified, and related genes/proteins are discovered to reveal the mechanism of Golgi aging. Based on the Golgi aging research method found in this way, we present a method for screening anti-aging materials, discover active ingredients, and observe toxicity and healthy lifespan extension in animal models. The ultimate goal of these studies is to contribute to extending the healthy lifespan of mankind. ● Although it is well known that senescent cells secrete numerous substances, there is no known Golgi aging model that is directly linked to extracellular secretion. ● The reliability and universal applicability of Golgi senescence are increased by linking experiments using cells/animals and related cells or transcriptomes of patients. ● The stacking change seen in aging of the Golgi apparatus can be related to the nutrient deficiency according to aging. So, it can be easily applied to the development of anti-aging foods by linking the actual life-long phenomenon with food. ● It is possible to utilize ZIP13 deficient mice produced and reported previously by paying attention to ZIP13, the Golgi zinc transporter, which we studied as a gene that regulates aging of the Golgi apparatus.
3． Period of The Program	April 1, 2022 ～ March 31, 2023

4． Project Members

Name	Age	Sex	Affiliation	Position	Role

(Principal Applicant) Bum-HO, Bin	41	M	University of Ajou, Department of biological sciences	Associate professor	Project director
(Research Collaborators) Byungsun, Cha	30	M	University of Ajou, Department of biological sciences	Ph. D. candidate	Cell analysis
※If additional space is required, please attach a separate sheet.					
5. Collaborating Researcher of IMCR	Name of Laboratory	Developmental Biology and Metabolism	Name	Ayako Fukunaka	

6. Research Plans

● Presenting a mouse / human Golgi aging model

-Golgi apparatus-related protein changes in aging mouse / human skin and liver: WB (Western blotting) and tissue staining analysis using GM130 antibody and TGN46 antibody

- Analysis of Golgi apparatus-related genes in aging mouse / human skin and liver: Q-PCR (Quantitative PCR) analysis using Arf4, Uap1/1, Hsp47, Mt1 primers

-Golgi apparatus-related genetic changes in passage-aged human fibroblasts: analysis of organelle-related genes in Golgi cells through transcriptome analysis

-Identification of aging-related genes using transcriptome data generated from multiple aging models. Specifically, published in Nature in 2020, produced and published single-cell transcriptome data from 23 tissues and organs in mouse models of different stages of aging. From this data through systems biology techniques, the expression dynamics of genes constituting the Golgi apparatus network and how Golgi stress changes in each tissue according to the aging process are traced (A single-cell transcriptomic atlas characterizes aging tissues in the mouse, Nature, 2021).

- Development of a Golgi apparatus prediction machine learning model using expression information of Golgi apparatus-related genes. To this end, key predictive variables for model development are selected from the transcriptome data of the model with reduced Golgi function from the open database, and the aforementioned single-cell transcriptome data are used as training and cross-validation data. The developed model was finally verified for its performance by utilizing the transcript data of other aging diseases that were published.

-Golgi apparatus protein changes in passage-aged human fibroblasts: WB analysis using GM130 antibody and TGN46 antibody

-Golgi body lipid changes in passage-aged human fibroblasts: FACS (Fluorescence-activated cell sorting) analysis after staining with BODIPY-TR ceramide

-Golgi body morphological changes in passage-aged human fibroblasts: Confocal microscopy/airy scan analysis using GM130 antibody and TGN46 antibody

-Changes in Golgi apparatus protease function of passage-aged human fibroblasts: WB analysis using CREB3 antibody for S1P-dependent processing of CREB3 after BFA treatment

-Changes in Golgi stress responsiveness of passage-aged human fibroblasts: Analysis of the activation of the Golgi stress-regulating transcription factor CREB3 after induction of Golgi stress with GCA, BFA, Momensine, etc. (WB: CREB3, Q-PCR: Arf4, Uap1/1, Hsp47, Mt1)

● Identification of the mechanism of abnormal polymerization of microtubules due to aging of the Golgi apparatus

-Analysis of microtubule polymerization of senescent cells: After treatment with Nocodazole, the degree of recombination, after fluorescence staining with GM130 antibody, TGN46 antibody, and alpha-tubulin antibody, image analysis using confocal microscopy

-Analysis of microtubule polymerization due to Golgi stress: After Golgi stress induction with GCA, BFA, Momensine, etc., Nocodazole treatment, recombination level, GM130 antibody, TGN46 antibody, alpha-tubulin antibody after fluorescence staining, image using confocal microscopy analyze

-Aging Golgi apparatus microtubule polymerization change: analysis of Golgi apparatus-microtubule complex using Tubulin polymerization assay kit (Cytoskeleton, Inc. BK006P)

-Changes in microtubules in the aged crude perinuclear fraction: WB analysis of proteins using GM130

antibody and TGN46 antibody in the non-solubilized fraction of surfactant NP40
-Changes in nuclear migration of microtubule-dependent epigenetic enzymes in senescent cells: WB analysis using antibodies for p300 and HDAC1/2 proteins that migrated in the nucleus after separating the cytoplasm and nucleus using a sucrose cushion
-Microtubule-dependent changes in nuclear migration of p53 in senescent cells: WB analysis using antibody-based p53 protein after separating the cytoplasm and nucleus using a sucrose cushion
-Changes in nuclear migration of the transcription factor SMAD2/3 involved in collagen production in aging fibroblasts: After TGF-beta treatment, fluorescence staining using SMAD2/3 antibody, and confocal microscopy/airy scan analysis

7. Research results:
Please describe the details of the contribution of the joint research with IMCR in obtaining the results.

<Result 1. Golgi stress increases during aging>

To investigate the effect of aging on the function of the Golgi apparatus, the expression levels of Golgi proteins and proteins that change during aging were initially analyzed. The expression levels of collagen, MT1 (a cellular zinc indicator), and histone 3 acetylation at lysine on position 18 (H3K18ac) and 27 (H3K27ac) were markedly reduced during senescence. However, the expression of the Golgi proteins GM130 and TGN46 increased (Figures 1A). In accordance, senescent cells showed expanded and loosely stacked trans-Golgi/TGN and cis-Golgi/CGN structures (Figure 1B) with increased volume (Figures 1C), indicating increased Golgi stress. Moreover, to evaluate Golgi enzyme activity, the CREB3-dependent stress response, which requires cleavage of the CREB3 protein by zinc metalloprotease S2P in the Golgi, was monitored after pharmacological disruption of the Golgi was induced by BFA. In senescent cells, CREB3 cleavage did not progress properly, indicating Golgi dysfunction (Figure 1D). Considering the loosely packed structure of the Golgi and reduced zinc levels in senescent cells, zinc-dependent Golgi stacking proteins GRASP55 and Golgin45 were monitored by confocal microscopy, which showed a decrease in the co-stained area in the dispersed Golgi cisterna of senescent cells (Figure 1E). These results indicate that Golgi stress increases during aging, and that aging, zinc deficiency, and Golgi stress are associated with a disturbed Golgi morphology.

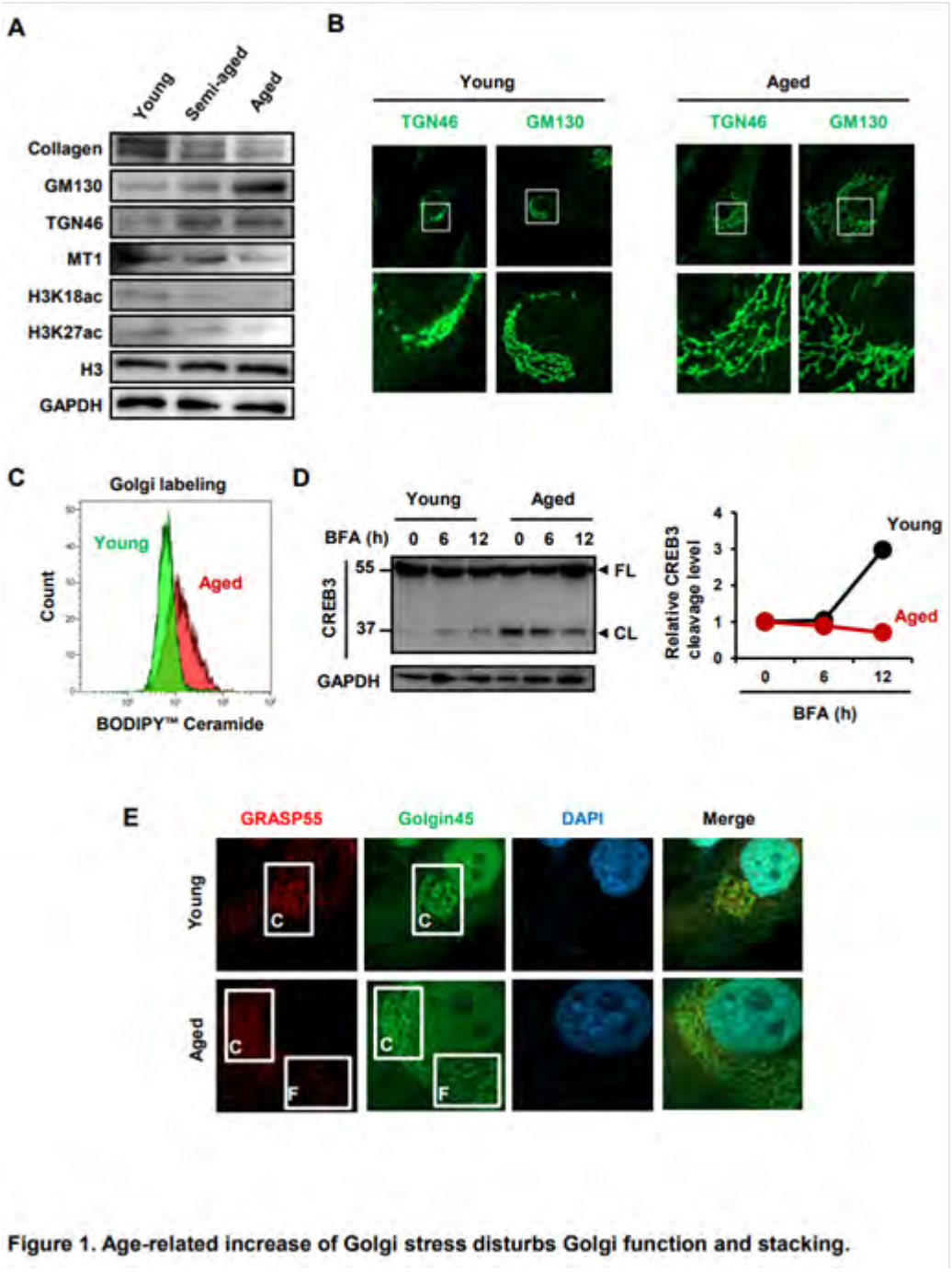

Figure 1. Age-related increase of Golgi stress disturbs Golgi function and stacking.

Figure 1. Aging-induced Golgi stress disturbs Golgi function and stacking.

(A) Western blotting of young (P6), semi-aged (P14), and aged (P32) human primary fibroblasts with each antibody. (B) Immunostaining of young and aged human primary fibroblasts with anti-TGN46 and anti-GM130 antibodies. (C) Golgi volume analysis of young and aged human primary fibroblasts stained with BODIPY™ TR Ceramide by FACS. (D) Western blotting of young and aged human primary fibroblasts with anti-CREB3 antibody after BFA treatment (3 μM) for

the indicated intervals. FL, Full-length; CL, Cleaved. Right panel indicates relative CREB3 cleavage level comparing to control. (E) Immunostaining of young and aged human primary fibroblasts with anti-GRASP55 and anti-Golgin45. Nuclei were stained with DAPI. C, Compact; F, Fragmented.

<Result 2. Golgi stress disrupts perinuclear microtubule assembly and impaired protein nuclear translocation>

The Golgi apparatus is a major non-centrosomal center for microtubule nucleation and anchorage. Golgi-derived microtubules are involved in stack organization after cell division, vesicle trafficking, cell migration, and regulation of durotaxis. Accordingly, we next isolated Golgi-associated microtubule complexes. Though the protein levels of tubulin were comparable in whole cell lysates, Golgi-associated microtubules were markedly decreased in aged cells, as well as in young cells treated with the Golgi stressors GCA and monensin (Figure 2A). In addition, analysis of the nuclear and perinuclear fraction revealed decreased protein levels of tubulin in aged cells and Golgi stressor-treated young cells (Figure 2B), indicating that Golgi stress leads to disruption of the structures of Golgi-associated perinuclear microtubules. Confocal microscopic analysis revealed a loosely packed and fragmented Golgi in both GCA-treated young and senescent cells, but not in young cells (Figure 2C). Furthermore, in cells treated with NOC to induce microtubule depolymerization, both GCA-treated and senescent cells showed impaired microtubule repolymerization after wash-off of NOC (NOC relief; Figure 2D), which is inconsistent with the results observed in young cells.

Since microtubules facilitate the nuclear translocation of proteins, we speculated whether Golgi stress-mediated microtubule misassembly would affect this event. Therefore, we analyzed the nuclear content of proteins that have been reported to be involved in nuclear translocation. The results showed that the levels of the tumor suppressor protein p53 and the epigenetic enzymes p300 and HDAC1/2 were evidently reduced in the nuclear fraction isolated from GCA-treated young cells (Figure 2E) and senescent cells (Figure 2F). To clarify the association between Golgi microtubules and nuclear translocation, CAMSAP2, a protein that stabilizes the minus ends of Golgi-associated microtubules in pigmented cells, was depleted by siRNA treatment in a melanocyte cell line to disrupt Golgi-associated microtubule structure.

Taken together, Golgi stress impairs the nuclear translocation of proteins by disrupting the

Golgi-associated perinuclear microtubule structures.

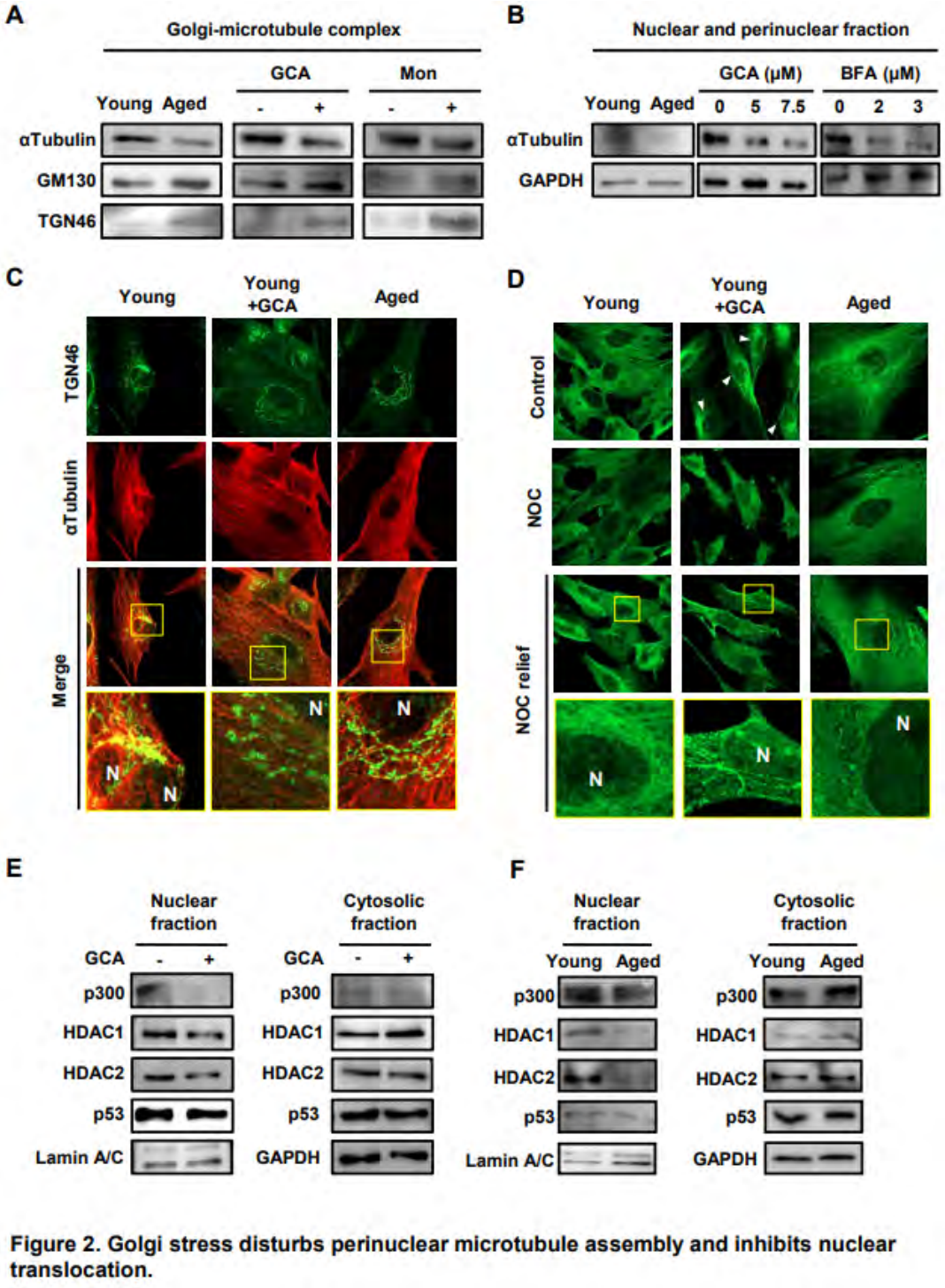

Figure 2. Golgi stress disturbs perinuclear microtubule assembly and inhibits nuclear translocation.

Figure 2. Golgi stress disrupts Golgi-associated microtubule assembly and inhibits nuclear translocation.

(A) Western blotting of Golgi-associated microtubule complex fractions isolated from young and

aged human primary fibroblasts, and young human primary fibroblasts treated with GCA (5 μM/12 h) and monensin (100 nM/12 h) for analysis of the indicated antibodies. (B) Western blotting of the nuclear and perinuclear fraction of young, young GCA- and BFA-treated, and aged human primary fibroblasts at the indicated concentrations for 12 h for analysis of anti-αTubulin. (C) Immunostaining of young, young GCA-treated (5 μM/12 h), and aged human primary fibroblasts with anti-αTubulin. N, Nucleus. (D) Immunostaining of young, young GCA-treated (5 μM/12 h), and aged human primary fibroblasts with anti-αTubulin. Cells were treated with nocodazole (NOC; 1 μM/1 h) and then washed with PBS (NOC relief; 15 min). N, Nucleus. (E and F) Western blotting of isolated cytosolic and nuclear fractions of young, young GCA-treated (5 μM/12 h), and aged human primary fibroblasts with the indicated antibodies.

<Contribution>

Animal experiments (IMCR + Ajou univ.)

Young (4 weeks old) and old (40 weeks old) C57BL/6JN mice were obtained from JA BIO (Suwon, Republic of Korea). *Zip13*$^{-/-}$ transgenic C57BL/6JN mice backcrossed for more than seven generations were provided by Gunma University, Japan. Mice were housed and handled at the Department of Meridian and Acupoint of the College of Korean Medicine, Semyung University (smecae 201001), according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC). All mice were housed in a specific pathogen-free barrier facility, maintained under a 12-h light-dark cycle, provided water *ad libitum*, and fed a standard AIN-76A rodent diet (Doo Yeol Biotech, Seoul, Republic of Korea). Because *Zip13*$^{-/-}$ mice showed hypodontia, they were fed a powdered diet (Doo Yeol Biotech) to provide appropriate nutrition. Mice in the zinc-deficient diet experiment were fed an AIN-76A Zn-deficient diet for 4 weeks (Doo Yeol Biotech).

Cell culture and materials (IMCR + Ajou univ.)

Human primary fibroblasts were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured until young (P6), semi-old (P14), or aged (P32). Mouse wild-type (*Zip13*$^{+/+}$) and *Zip13*-deficient (*Zip13*$^{-/-}$) dermal fibroblasts were obtained as previously described [55]. All cells were maintained in Dulbecco Modified Eagle Medium (DMEM)-high glucose culture medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; GenDEPOT, Katy, TX, USA) and 1% penicillin and streptomycin (P/S; Welgene, Gyeongsan, Korea). The cells were then incubated in an atmosphere of 5% CO_2 at 37 °C. N,N,N′,N′-Tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN), golgicide A (GCA), monensin (Mon), brefeldin A (BFA), nocodazole (NOC), etoposide (Eto), nutlin-3, doxorubicin (Dox) (Sigma-Aldrich, St. Louis, Missouri, USA), and H_2O_2 (Junsei Chemical Co., Tokyo, Japan)

were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and maintained at −36 °C until further use. Antisense LNA GapmeR in vivo Large Scale (LNA) (QIAGEN, Germantown, MD, USA) was used according to the manufacturer's instructions. Recombinant mouse TGF-β (R&D Systems, Minneapolis, MN, USA) was reconstituted in HCl containing bovine serum albumin (BSA), according to the manufacturer's instructions, and maintained at −80 °C. Cell irradiation experiments were performed using Stratalinker UV 1800 crosslinker (Stratagene, San Diego, CA, USA).

Western blotting (IMCR + Ajou univ.)

The cells were lysed with RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% sodium deoxycholate, 1% Triton X-100, and 1 mM EGTA) for 5 min, and the lysate was centrifuged at 13,000 rpm for 15 min. The supernatant was collected, and the sample protein concentration was determined using Bicinchoninic Acid (BCA) Protein Assay Kit (P8100BCA; GenDEPOT). Samples were prepared in sample buffer and subjected to polyacrylamide gel electrophoresis, and a wet transfer was performed. Blots were blocked with 1% BSA in Tris-buffered saline Tween 20 (TBST) for 1 h and rinsed thrice with TBST for 5 min each time. The membranes were incubated with the primary antibodies (Table S1) overnight at 4 °C. After rinsing thrice with TBST for 5 min each time, the membranes were incubated with HRP-conjugated polyclonal secondary antibodies (GenDEPOT) diluted at 1/10000 for 1 h at room temperature. Blot images were acquired using Fusion Solo S chemiluminescence imaging system (Vilber, Collégien, France).

Immunofluorescence staining of cells (Ajou univ.)

Cells were cultured on Lab-Tek chamber slides (Thermo Fisher Scientific), fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 in PBS containing 1% BSA for 5 min. Subsequently, the cells were stained with antibodies (Abcam,

Cambridge, UK) and 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). Secondary staining was performed using Alexa Fluor 488-conjugated F(ab')2 Fragment fluorescent dye (Invitrogen, Carlsbad, CA, USA). Fluorescence was detected using LSM 800 confocal microscope (Carl Zeiss, Jena, Germany).

Immunoprecipitation (Ajou univ.)

The assay was performed using MagListo™ Protein G Kit (Bioneer, Daejeon, Republic of Korea). Briefly, AccuNanoBead™ Protein G Magnetic Nanobeads were washed twice with Binding & Washing buffer using MagListo™-2 Magnetic Separation Rack (Bioneer). Subsequently, anti-Golgin45 antibody (ab155510; Abcam) was added to 500 µL of Binding & Washing buffer and incubated in a Roto-Minim rotator (Benchmark Scientific, Sayreville, NJ, USA) for 10 min at room temperature, and the supernatant was discarded. The cells previously cultivated in a 60-mm dish at 80% confluency were lysed with NP40 lysis buffer (150 mM NaCl, 1% NP40, and 50 mM Tris-HCl (pH 7.5)), centrifuged at 15,000 rpm for 5 min, and the supernatant was transferred to a 1.5-mL tube containing the bead complex. The samples were incubated in a rotator overnight at 4 °C. The following day, the tube was placed on the magnetic rack for 1 min and washed twice with 1 mL NP40 lysis buffer. Next, NP40 buffer and Elution buffer were added at a ratio of 1:1 to the bead complex, gently mixed, and incubated at 70 °C for 10 min. After removal of the magnetic beads, the eluted fraction was transferred to a new tube, and sample buffer was added.

Isolation of Golgi-associated microtubule complex fractions (Ajou univ.)

The assay was performed using the Microtubules/Tubulin In Vivo Assay Kit (Cat. #BK038; Cytoskeleton Inc., Denver, CO, USA), according to the manufacturer's instructions. Cells were lysed using LMS2 buffer at 37 °C, homogenized using Dounce homogenizer, and centrifuged at 3,000 rpm for 5 min at 37 °C. The pellets containing large complexes of microtubules attached to

the nucleus and Golgi apparatus were diluted in sample buffer.

Isolation of soluble and nuclear and perinuclear area fractions (Ajou univ.)

The cells were lysed using NP40 lysis buffer and centrifuged at 13,000 rpm for 15 min at 4 °C. The supernatant was collected as a soluble fraction. The pellet was collected as an insoluble fraction containing a crude nuclear and perinuclear fraction and sonicated for 5 min. Protein concentration was determined by BCA assay and samples were dissolved in sample buffer.

8. Present status of academic conference presentations and research papers associated with the results of the joint research, and exchange of information on the joint research with the collaborating researcher at IMCR.
(As much as possible, please state papers that include the names of the collaborating researcher at IMCR or papers stating that the research was supported by the Joint Research Program with IMCR.
Regarding papers, please send a PDF file together with the report to the email address of the general affairs section of the Institute.) Office of General Affairs: kk-msomu4@jimu.gunma-u.ac.jp

① Please list the publications that include the name of the collaborating researcher from IMCR and send a reprint of each publication to IMCR.
-In preparation

② Please list the publications that include a description that the research was supported by the Joint Research Program with IMCR and send a reprint of each publication to IMCR.
-In preparation

③ List up to 3 conferences (name of conference, date of conference, and title of the presentation).
-None

④ Exchange of information exchange with collaborating researcher from IMCR (please list main points of communication).
-Golgi stress increases during aging
-Golgi stress disrupts perinuclear microtubule assembly and impaired protein nuclear translocation