USP15 Regulates Neuromuscular Functions through the Control of RNA Splicing

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Abstract

Ubiquitin system controls various physiological functions in cells by modulating cellular processes such as protein degradation and signaling pathway. Recent studies have implied that deubiquitinating enzyme (DUB) responsible for the removal of ubiquitin from substrate is important as a regulator of neurological function. For example, dysfunction or deficiency of certain DUBs results in neurodegenerative and psychiatric disorders. Furthermore, it is also revealed that ubiquitin specific protease 15 (USP15), a member of large family of DUBs, is involved in several neurological disorders including autism, ataxia, Parkinson's disease and glioblastoma, providing a clue about the close relationship between USP15 and nervous system. However, the detailed molecular mechanism of how USP15 works on nervous system is yet to be elucidated.

Abbreviations

ADP	Adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
Cul	Cullin
DUB	Deubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EDTA	Ethylenediaminetetraacetic acid
FL	Flag
IB	Immunoblot
IP	Immunoprecipitation
JAMMs	JAB1/MPT/Mpv43 metalloenzymes
KLHL7	Kelch like protein 7
LSm	Like-Sm
MEF	Mouse embryonic fibroblast
NPM	Nucleophosmin
OUT	Ovarian tumor protease
PCR	Polymerase chain reaction
PD	Pull down
SART3	Squamous cell carcinoma antigen recognized by T cells 3
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SMA	Spinal muscular atrophy
snRNA	Small nuclear RNA
snRNP	Small nuclear ribonucleic particles
Ub	Ubiquitin
UCH	Ubiquitin C-terminal hydrolases
USP	Ubiquitin-specific proteases
WCL	Whole cell lysates
WT	Wild type

Introduction

Ubiquitin (Ub) system is responsible for regulating various cellular processes such as protein degradation, DNA transcription, signal transduction and protein quality control [1]. Ub is covalently attached to a target protein through a sequential action of Ub activating enzyme (E1), Ub conjugating enzyme (E2), Ub ligase (E3), and is removed from the target by deubiquitinating enzymes (DUBs). This reversible reaction, which governs a balance of ubiquitination status of target proteins, is also important for the control of nervous system functions including neurite growth [2], synaptic transmission [3-5], receptor turnover [6, 7] and synaptic plasticity [8, 9] and receive attention as a key regulator of nervous system functions.

DUBs have also attracted attention as therapeutic targets for neurodegeneration. The importance of DUB function at nervous system was first highlighted by specific mutation of DUB genes that link to several neurological disorders [10, 11]. Furthermore, it has been established that dysfunction or deficiency of DUBs results in disruption of synapse development and function, neurodegenerative disorders and psychiatric disorders [12], indicating that DUBs perform crucial functions in both the central and peripheral nervous system. DUBs are composed of five distinct subfamilies: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor protease (OTUs), Josephin proteases, and JAB1/MPT/Mpv43 metalloenzymes (JAMMs) [12]. One of this subfamily, USPs, which form a large family of deubiquitinating enzymes, is also

involved in several neurological disorders such as spinocerebellar ataxia type 1 [13], Down's syndrome [14, 15] and Parkinson's disease [16, 17].

USP15 is a member of the USP family. Recent studies have revealed that USP15 promotes oncogenesis in glioblastoma by activation of the TGF-β signaling pathway [18] and the expression levels of both USP15 mRNA and protein decreases in mouse models of spinocerebellar ataxia type 3 and Huntington's disease [19]. These suggest that USP15 may play important roles in neuromuscular functions. However, the detailed molecular mechanism of how USP15 functions in nervous and muscle systems were yet to be elucidated.

Materials and Methods

Rotarod performance test

2-month old wild-type and USP15 deficient mice were placed on rod. Rotation speed was 5 r.p.m. at the beginning and then gradually accelerated to 40 r.p.m. The time to fall from the rod was measured for 240 seconds.

Plasmids

Antibodies

For immunoblot analyses, anti-USP15 (Bethyl, A300-923A), anti-SART3 (Proteintech, 180251AP), anti-Tubulin (SIGMA, DM1A), anti-FLAG (SIGMA, M2), anti-cMyc (Santa Cruz, 9E10), anti-GFP (MBL, 598) and anti-His (GE Helthcare,

27-4710-01) were used as primary antibodies. The peroxidase-conjugated anti-mouse and anti-rabbit antibodies (SeraCare) were used as secondary antibodies.

Anti-Calbindin (SIGMA, CB-955) was used for immunohistochemistry as primary antibodies. For immunofluorescence analyses, anti-Flag (SIGMA, M2), anti-Flag (SIGMA, F7425), anti-HA (Roche, Roche) were used as primary antibodies. Anti-NPM antibody was provided by Dr. Mitsuru Okuwaki [22]. Alexa Fluor 488 and 594 conjugated anti-rabbit, anti-rat and anti-mouse antibodies were used as secondary antibodies.

Cell culture

Wild type and USP15 deficient MEFs, HEK293, HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (high glucose) (WAKO) supplemented with 10% fetal bovine serum, 1% penicillin streptomycin (Gibco) in a 37°C incubator with 5% CO_2.

Histology

Brains and skeletal muscles of 3-month-old and brains of 9-month-old mice were perfused with 4% paraformaldehyde in phosphate buffered saline (PBS). Tissues were fixed in the same fixative for 48 hours and then embedded in paraffin. Sections were stained with Meyer's Hematoxylin and Eosin or subjected to immunohistochemical analyses.

Immunohistochemistry

Deparaffinized tissue specimens were immersed in 0.01 M citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) and boiled in microwave oven for 10 minutes. After antigen retrieval, tissue sections were blocked for an hour in 3% bovine serum albumin (BSA) in TBST (25 mM Tris-HCl (pH 7.5), 0.14 M NaCl, 0.1% TritonX-100) and incubated with a primary antibody, mouse anti-Calbindin antibody diluted 1/300 in TBST for overnight at 4°C. Following wash with TBST, tissue sections were incubated with a secondary antibody (Alexa Fluor 594 anti-mouse IgG) diluted 1/500 in TBST for an hour at room temperature. Tissue specimens were observed using a fluorescence microscope (Keyence, BIOREVO BZ-9000).

Immunoprecipitation

Cells were lysed with ice-cold lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT) and centrifuged at 14,000 r.p.m. for 10 minutes. The supernatant was subjected to immunoprecipitation with anti-USP15 or anti-cMyc antibodies and protein G agarose beads (Thermo Scientific), or anti-Flag M2-agarose beads (SIGMA). Samples were incubated at 4°C overnight. The beads were then washed with lysis buffer. The protein samples were added to 4 X SDS sample buffer (250 mM Tris-HCl (pH 6.8), 40% Glycerol, 10% SDS, 0.04% bromophenol blue, 20% β-mercaptoethanol), boiled for 3 minutes and subjected to immunoblot analysis.

His-Ub Pull down assay

Cells were washed with PBS and lysed in extraction buffer (6M guanidinium-HCl, 50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl and 5 mM imidazole). Cell lysates were sonicated for 30 seconds on ice, centrifuged and then incubated with Talon metal affinity resin (Clonetech) at 4°C overnight. The precipitants were washed with buffer (50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl and 5 mM imidazole) and subjected to immunoblot analysis.

Immunoblot

The protein samples after immunoprecipitation and his-tagged pull down assay were separated by SDS-PAGE, transferred to PVDF membranes (Millipore). Membranes were incubated overnight at 4°C with primary antibodies. The proteins on membrane were detected with HRP-conjugated secondary antibodies and chemiluminescence reagent (Amersham ECL Prime Western Blotting Detection Reagents, GE Healthcare).

Immunocytochemistry

HeLa cells were cultured on cover slips and after 18 hours, transfected with indicated plasmids. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. The cells on coverslips were blocked in 0.4% Triton X-100 in blocking solution (3% BSA in PBS) for 30 min at room temperature, and then incubated with primary antibodies diluted in blocking solution for an hour or overnight at 4 °C. After washing with PBS, the cells were incubated with secondary antibodies diluted in blocking solution for 30 minutes at room temperature. Nuclei were stained with

Hoechst 33342 (Life Technologies). The coverslips were then mounted onto slides using the Fluoromount Plus mounting solution (Diagnostic BioSystems). Images were obtained using a fluorescence microscope (Keyence model BZ-9000).

Quantitative real-time PCR (qPCR)

Total RNAs from wild-type and USP15 deficient cerebellum, cortex, skeletal muscle, liver, spleen, kidney, heart and MEFs were prepared by ISOGEN II (NIPPON GENE). The cDNA were synthesized by SuperScript III CellsDirect cDNA Synthesis Kit (Life Technologies) using random hexamer primer. qPCR was performed with THUNDERBIRD SYBR qPCR Mix (TOYOBO). The data were analyzed using Thermal Cycler Dice Real Time System Software (TAKARA).

Following Primers were used.

Exon array

One-month-old mice of wild type and USP15 deficient mice (n=3) were euthanized using carbon dioxide. Their cerebellum and skeletal muscles were then dissected and snap frozen in liquid nitrogen. Total RNAs were extracted from each tissue using ISOGEN II (NIPPON GENE) according to the manufacture's instructions. Fragmented and labelled total RNA of each sample were hybridized on Affymetrix

GeneChip mouse exon 1.0 ST arrays. After hybridization, each probe array was washed and stained with Affymetrix GeneChip Fluidics Station 450 and scanned by Affymetrix GeneChip Scanner 3000. Data were analyzed with GeneSpring 12.6 Software and filtered by more than Splicing Index 0.5 and with *P*-value<0.05.

Splicing Index was calculated with following calculation:

$$\text{Splicing index} = log_2 (NI_{i1}/NI_{i2})$$

$$NI_{ij} = E_{ij}/G_j$$

NI_{ij} means normalized intensity (NI) for exon i in experiment j. E_{ij} is the estimated intensity level for exon i in experiment j. G_j is the estimated gene intensity.

Results

KLHL7 leads to relocation of TUT1 in nucleus.

In our preliminary study, KLHL7 is shown to interact with USP15 and several proteins involved in mRNA splicing machinery. KLHL7 is a component of Cul3-based ubiquitin ligase [21] and its mutations cause retinitis pigmentosa [29, 30].

To test this assumption, I analyzed TUT1 subnuclear localization. Flag-TUT1 was co-expressed with Myc-KLHL7 WT or disease-causative mutants (A153T, A153V and S150N) in HeLa cells, and stained with anti-Myc and anti-Flag antibodies. TUT1

was localized in nucleoplasm with expression of KLHL7 WT and S150N mutant. On the contrary, TUT1 still stayed in nucleolus with expression of KLHL7 A153T and A153V mutants (Figure 24). Given that KLHL7 A153 mutations inhibit Cul3 interaction but A150N does not [21], these data indicate that KLHL7 activity, as an ubiquitin ligase seems to be important for TUT1 translocation to nucleoplasm.

Discussion

Alternative mRNA splicing plays an important role in generating enormous proteomic diversity. It allows eukaryotic cell to produce a huge number of proteins from the limited number of genes (20,000~25,000 in the human genome) through selective elimination of introns and exon joining and may thereby contribute to tissue-specific functions. In brain, alternative splicing is involved in neuronal functions through the regulation of gene expression, which acts in the synapse such as neurotransmitter receptors, cation channels, adhesion and scaffold proteins [31]. Given the importance of alternative mRNA splicing in regulating neuronal functions, it is not surprising that disruption of RNA splicing leads to neuronal dysfunction, and thereby neurodegenerative disorder. Indeed, recent studies have indicated that disruption and misregulation of RNA splicing results in neuromuscular disorders such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) [32-34]. However, the biological mechanisms of how the failure in RNA splicing leads to neuromuscular diseases remains unclear.

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Table 1. Primers used in this study.

Primer name	Sequence (5' → 3')
Lsm1 forward	AGGATCCGCC ACCATGAACT ATATGCCTGG CACCG
Lsm1 reverse	GGGATCCTTA GTACTCATCA AGAGTATCTG CTCGAGG
Lsm2 forward	AGGATCCGCC ACCATGCTCT TCTATTCTTT TTTCAAGTCC CTTG
Lsm2 reverse	GGGATCCTCA CTGTTTCTGC TGCAGGGCTT CCTTCCTTGC
Lsm4 forward	AAGATCTGCC ACCATGTTGG TGGAGCTGAA AAATGGGGAG ACGTACAATG GACA
Lsm4 reverse	AAGATCTTCA CTGTTTGCCC GCCTGTCTGC CAGGCTTCTT C
Lsm6 forward	AGGATCCGCC ACCATGAGTC TTCGGAAGCA AACCCCTAGT GACTTC
Lsm6 reverse	GGGATCCTCA CATCCGTCTA AGATCTTCAC TGTTTGCCCG CCTGTCTGCC AGGCTTCTTC
Lsm7 forward	AGGATCCGCC ACCATGGCGG ATAAGGAGAA GAAGAAAAAG GAGAGCATCT TGGAC
Lsm7 reverse	GGGATCCCTA GGCGTCCTGC TGCTGGATGA AGGGGTTGGG GATGGCC
SART3 forward	AAGATCTGCC ACCATGGCGA CTGCGGCCGA AAC
SART3 reverse	AAGATCTTCA CTTTCTCAGA AACAGCTTGG CAAAATCG
$SART3^{1-649}$ reverse	AAGATCTGTT CTCGACCCTT CTGCGTTTG
$SART3^{278-649}$ forward	AAGATCTCCA GAGTCAGTAA TTCAGAAC
$SART3^{660-963}$ forward	AAGATCTGTA GAAGTAGCAG CAGGGCC
TUT1 forward	AAGATCTGCC ACCATGTCAC TTCCTATCGG ATCGGC
TUT1 reverse	AAGATCTTCA CTTGAGATGT CGAATTGCTT G

Table 2. USP15 interacting proteins identified by mass spectrometry.

Gene symbol	Gene title
LSm4 *	U6 snRNA-associated Sm-like protein LSm4
LSm5 *	U6 snRNA-associated Sm-like protein LSm5
LSm6 *	U6 snRNA-associated Sm-like protein LSm6
LSm7 *	U6 snRNA-associated Sm-like protein LSm7
LSm8 *	U6 snRNA-associated Sm-like protein LSm8
SART3 *	Squamous Cell Carcinoma Antigen Recognized By T-Cells 3
TUT1 *	U6 SnRNA-Specific Terminal Uridylyltransferase 1
PRS6	26S protease regulatory subunit 6B
PSR8	26S protease regulatory subunit 8
PSA1	Proteasome subunit alpha type 1
PSD3	26S proteasome non-ATPase regulatory subunit 3
PSD6	26S proteasome non-ATPase regulatory subunit 6
PSDB	26S proteasome non-ATPase regulatory subunit 11
PSDC	26S proteasome non-ATPase regulatory subunit 12
PSDD	26S proteasome non-ATPase regulatory subunit 13
ERdj5	ER-resident protein ERdj5
MycBP2	Myc binding protein 2
BIR4	Baculoviral IAP repeat-containing protein 4
UBIQ	Ubiquitin

* Proteins involved in RNA spliceosomal functions

Table 3. Genes with exon down-regulated in USP15 deficient cerebellum compared to wild type.

Splicing Index	Gene symbol	Gene title
-2.8161573	Usp15	ubiquitin specific peptidase 15
-1.174536	Mrps11	mitochondrial ribosomal protein S11
-1.1357758	Col5a3	collagen, type V, alpha 3
-1.0740294	Zfp286	zinc finger protein 286
-1.0740294	Wsb2	WD repeat and SOCS box-containing 2
-0.9656974	Sparcl1	SPARC-like 1
-0.9656974	Scpppq1	secretory calcium-binding phosphoprotein proline-glutamine rich 1
-0.9046491	Zfp422	zinc finger protein 422
-0.9019557	Morc2a	microrchidia 2A
-0.8996663	Rnf26	ring finger protein 26

Table 4. Genes with exon up-regulated in USP15 deficient cerebellum compared to wild type

Splicing Index	Gene symbol	Gene title
1.4664571	Dnase1l1	deoxyribonuclease 1-like 1
1.2240485	BC055324	cDNA sequence BC055324
1.2240485	Scyl3	SCY1-like 3 (S. cerevisiae)
1.0383224	Rab1b	RAB1B, member RAS oncogene family
1.0269091	Ccdc93	coiled-coil domain containing 93
0.96391046	4933426M11Rik	RIKEN cDNA 4933426M11 gene
0.9351945	Htra3	HtrA serine peptidase 3
0.8867378	Nop14	NOP14 nucleolar protein homolog (yeast)
0.8711058	Apeh	acylpeptide hydrolase
0.84356135	Hmha1	histocompatibility (minor) HA-1

Table 5. Genes with exon down-regulated in USP15 deficient skeletal muscle compared to wild type.

Splicing Index	Gene symbol	Gene title
-3.3602824	Usp15	ubiquitin specific peptidase 15
-1.2622142	Nmbr	neuromedin B receptor
-1.1337303	Tbx1	T-box 1
-1.1194124	Myl2	myosin, light polypeptide 2
-1.1038551	Hoxa5	homeobox A5
-1.0059121	Arhgap6	Rho GTPase activating protein 6
-0.9321963	Cryzl1	crystallin, zeta (quinone reductase)-like 1
-0.91569424	Nedd9	neural precursor cell expressed, developmentally down-regulated 9
-0.8909112	Ptdss1	phosphatidylserine synthase 1
-0.8685387	Sil1	endoplasmic reticulum chaperone SIL1 homolog (S. cerevisiae)

Table 6. Genes with exon up-regulated in USP15 deficient skeletal muscle compared to wild type

Splicing Index	Gene symbol	Gene title
0.9573728	Irgm2	immunity-related GTPase family M member 2
0.9573728	Gm12250	predicted gene 12250
0.9573728	Igtp	interferon gamma induced GTPase
0.9759165	Ankib1	ankyrin repeat and IBR domain containing 1
1.0177176	Tpd52l2	tumor protein D52-like 2
1.1769753	Grb14	growth factor receptor bound protein 14
1.4755071	Sln	sarcolipin
2.2558568	Mir208a	alphamicroRNA 208a
2.2558568	Myh7	myosin, heavy polypeptide 7, cardiac muscle, beta
2.2558568	Myh6	myosin, heavy polypeptide 6, cardiac muscle

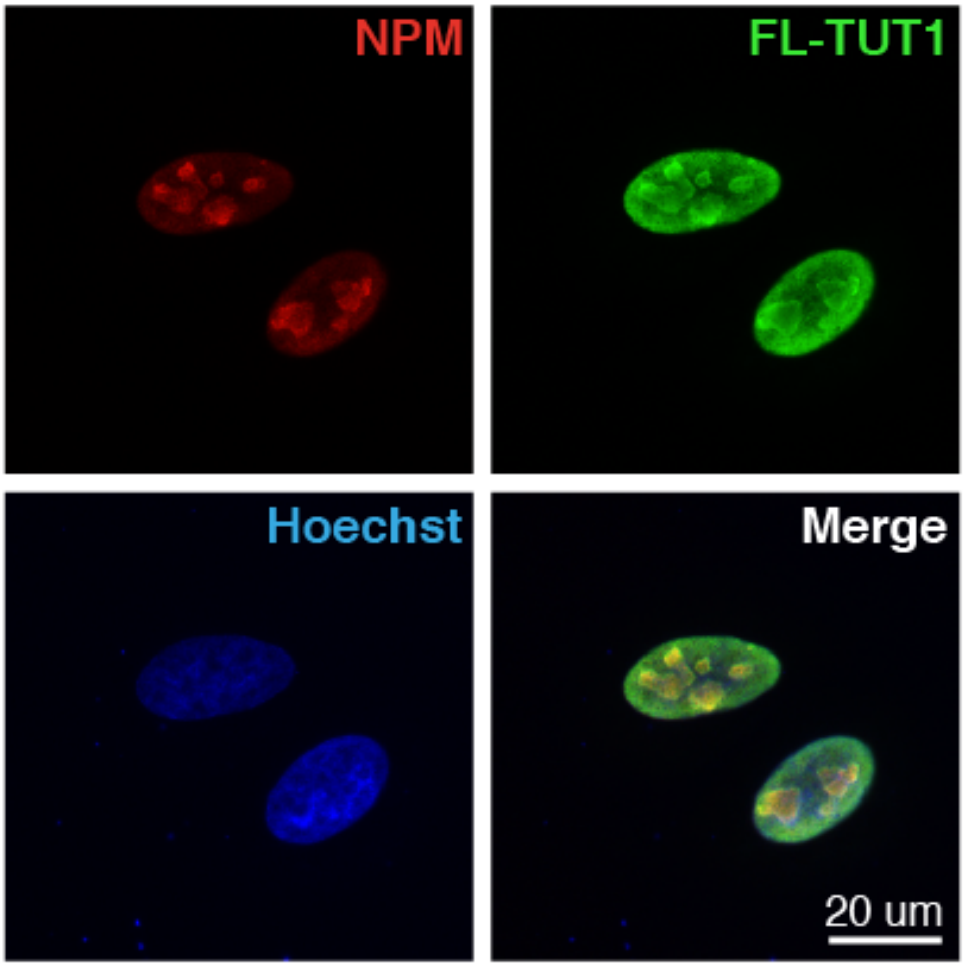

Figure 17. TUT1 is localized at nucleolus.

HeLa cells transfected with Flag-TUT1 were stained with anti-NPM (nucleophosmin) and anti-Flag antibodies and Hoechest 33342. Scale bar, 20 μm.

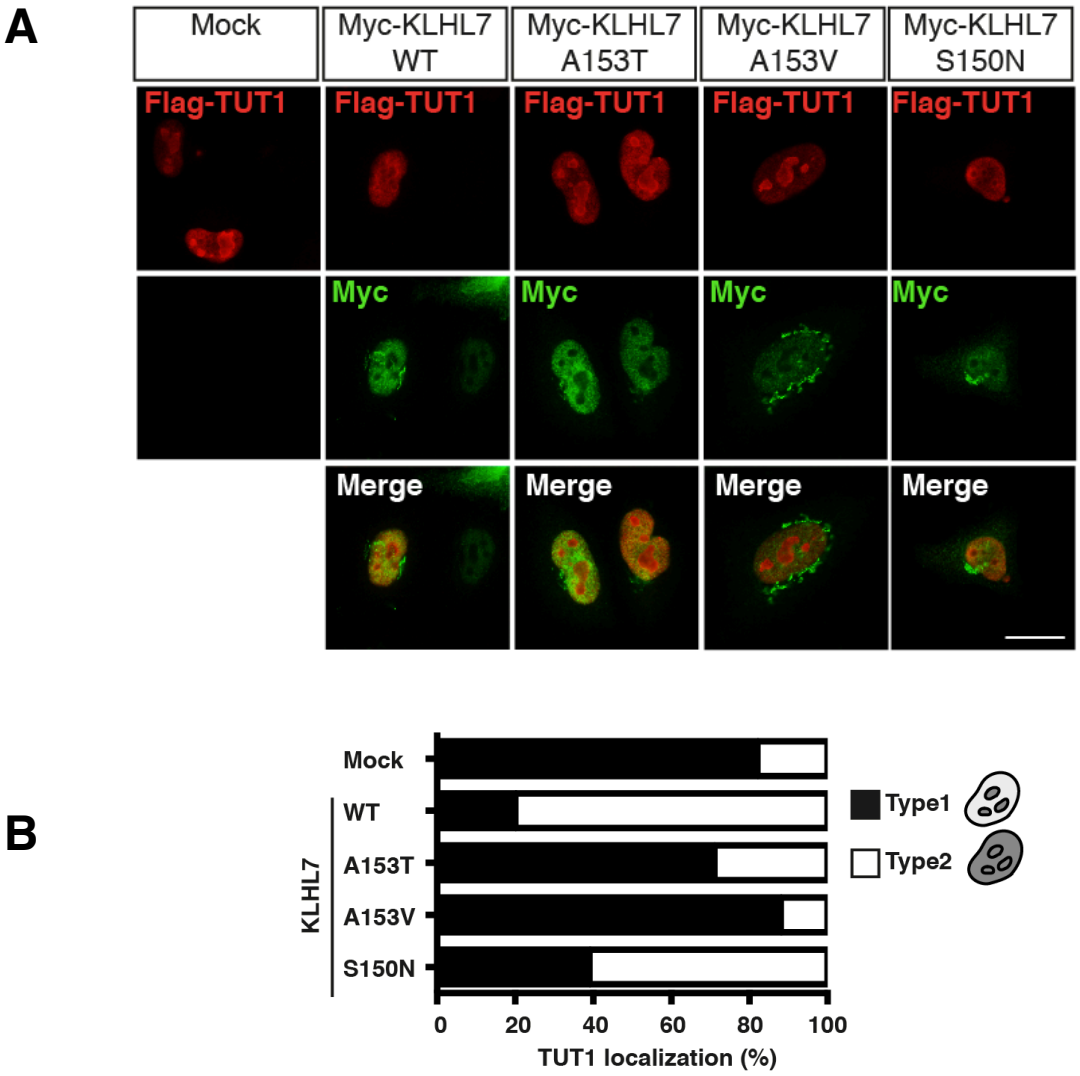

Figure 24. Overexpression of Wild-type KLHL7 and KLHL7 SN mutant induces relocation of TUT1 from nucleolus to nucleoplasm.

A, Fluorescent images of HeLa cells expressing Myc-TUT1 together with each KLHL7 wild-type and dieses causative mutant plasmids. Scale bar: 20 μm. B, Quantification of the data in (A). Subnuclear localization of Myc-TUT1 was examined. Cells that express Flag-TUT1 in nucleolus (Type 1) and in diffusely distribution (Type 2) were counted. n=30 ~ 34.