Title

A unique mouse model for investigating the properties of amyotrophic lateral sclerosis-associated protein TDP-43, by *in utero* electroporation

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Abstract

TDP-43 is a discriminative protein that is found as intracellular aggregations in the neurons of the cerebral cortex and spinal cord of patients with amyotrophic lateral sclerosis (ALS); however, the mechanisms of neuron loss and its relation to the aggregations are still unclear. In this study, we generated a useful model to produce TDP-43 aggregations in the motor cortex using *in utero* electroporation on mouse embryos. The plasmids used were full-length TDP-43 and C-terminal fragments of TDP-43 (wild-type or M337V mutant) tagged with GFP. For the full-length TDP-43, both wild-type and mutant, electroporated TDP-43 localized mostly in the nucleus, and though aggregations were detected in embryonic brains, they were very rarely observed at P7 and P21. In contrast, TDP-43 aggregations were generated in the brains electroporated with the C-terminal TDP-43 fragments as previously reported in *in vitro* experiments. TDP-43 protein was distributed diffusely—not only in the nucleus, but also in the cytoplasm—and the inclusion bodies were ubiquitinated and included phosphorylated TDP-43, which reflects the human pathology of ALS. This model using *in utero* electroporation of pathogenic genes into the brain of the mouse will likely become a useful model for studying ALS and also for evaluation of agents for therapeutic purpose, and may be applicable to other neurodegenerative diseases, as well.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a paralytic and fatal disorder caused by motor neuron degeneration in the brain and spinal cord. Familial ALS is represented in only 5-10% of all ALS patients, and most cases are sporadic. In familial ALS, mutations in superoxide dismutase 1 (*SOD1*), the TAR DNA-binding protein of 43 kDa (*TARDBP*, TDP-43), the fused in sarcoma protein (*FUS*, also known as the translocated in liposarcoma protein (*TLS*)), and *UBQLN2,* which encodes the ubiquitin-like protein ubiquilin 2, and the expansion of a hexanucleotide repeat in gene *C9orf72*, and in many other genes were reported (Deng et al. 2011, DeJesus-Hernandez et al. 2011). Although some mechanisms (Robberecht, Philips 2013), such as RNA editing deficiency, are suggested (Takuma et al. 1999), the detailed pathogenic mechanisms of familial and sporadic ALS remain unknown.

TDP-43 is a nuclear factor functioning in RNA processing, and has a 414-amino-acid protein with two RNA recognition motifs (RRM1 and RRM2) and a carboxyl-terminal glycine-rich domain (Lagier-Tourenne et al. 2010, Lee et al. 2011). This protein is identified as the major deposited protein in inclusion bodies in the brains of both familial and sporadic ALS, as well as frontotemporal lobar degeneration (FTLD) (Arai et al. 2006, Giordana et al. 2010, Mori et al. 2008). There are many mutations in TDP-43 (*TARDBP*) even in the sporadic cases (Mackenzie et al. 2010). In neuropathological examinations of ALS patients' neurons, TDP-43 is found in neuronal cytoplasmic inclusions, dystrophic neurites, and neuronal intranuclear inclusions. Three types of inclusions are

found in ALS, namely, skein-like inclusions, round inclusions, and dot-like inclusions (Arai et al. 2006, Giordana et al. 2010, Mori et al. 2008). The major disease-specific findings in ALS and FTLD include abnormal ubiquitination and phosphorylation of TDP-43, sarcosyl-insoluble TDP-43 inclusions, truncated 20-25 kDa TDP-43 C-terminal fragments (Arai et al. 2006, Arai et al. 2010, Hasegawa et al. 2008), and truncated N-terminal fragments (Yamashita et al. 2012). These abnormal features of TDP-43 and its fragments are purported to be associated with the mislocalization of the TDP-43 protein into the cytoplasm of motor neurons and loss of normal nuclear TDP-43 expression. Now TDP-43 aggregation, phosphorylation, truncation, and nuclear clearing of TDP-43 are considered hallmarks of ALS (Baloh 2011). However, the mechanisms of inclusion body formation and the pathological function caused by the mislocalization of TDP-43 into cytoplasm are unknown. To elucidate the problem, many researchers have established *in vitro* models. In mammalian cultured cells, TDP-43 remains predominantly soluble and localized in the nucleus, even though it is transfected by the full length TDP-43 protein (Baloh 2011). Moreover the neuronal cell death and neuronal cell loss were not confirmed in *in vitro* experiments. However in some experiments, TDP-43 inclusion bodies were observed in *in vitro* (Nonaka et al. 2009b), and it was necessary to mutate the nuclear localization signal (NLS), mutate the RNA binding domain, or express truncation mutants containing C-terminal domain in order to make pathological inclusions (Baloh 2011). In transfection experiments of C-terminal fragments or disruption of NLS of TDP-43 to primary neuron

or cultured cells, aggregations and inclusion bodies of TDP-43 were observed, and some of them were phosphorylated and ubiquitinated (Nonaka et al. 2009a).

Many types of rodent models for TDP-43 were generated, such as those overexpressing wild type or ALS-related mutant TDP-43 with a neuronal specific promoter, ubiquitous promoter, or inducible promoter (Tsao et al. 2012, Janssens et al. 2013). Few, however, reported pathological changes similar to human ALS patients. An accumulation of ubiquitin is observed in the neurons of most TDP-43 transgenic mice, and contrary to the results from humans, phosphorylated TDP-43 immunopositive cytoplasmic inclusions are rarely observed (Gendron, Petrucelli 2011). Only three transgenic mice (Wils et al. 2010, Xu et al. 2010, Igaz et al. 2011, Janssens et al. 2013) and one transgenic rat (Zhou et al. 2010) are reported to have shown phosphorylated TDP-43 inclusions in their neuronal cytoplasm.

In this study, we established an experimental model to express TDP-43 protein *in vivo* and to make pathological changes that are also detected in the human brain, such as inclusion bodies with ubiquitination and phosphorylation in the motor areas of the cerebral cortex by *in utero* electroporation of C-terminal fragments of TDP-43. Even though this method is much easier than making transgenic mice, it allows the targeted protein to be expressed *in vivo* and provides an analogue to human pathology.

2. Materials and methods

2.1. Plasmids

To generate full-length TDP-43 expression constructs, we first amplified the human TDP-43 coding region from human HeLa cell line cDNA using the primers of TDP43UP (5’-AAAAAAACGCGTGCCGCCATGTCTGAATATATTCGGGTAACCG-3’) and TDP43DW (5’-TATTACGCGTCTACATTCCCCAGCCAGAAGACTTAG-3’). After gel purification, PCR products were digested with *MluI* and inserted into the pCI vector (Promega, Madison, WI), then digested with the same restriction enzyme (pCI-hTDP-43). To construct N-terminally green fluorescent protein (GFP)-fused TDP-43 (pEGFP-C3-hTDP-43) and GFP-fused CTF-hTDP-43, a cDNA encoding full-length TDP-43 was amplified from pCI-hTDP-43, using the primers of GFP-C3-TDP-43UP (5'-AACCGCTCGAGATGTCTGAATATATTCGGGTAACCGAA-3') or GFP-C3-CTF-TDP-43UP (5'-AAAACTCGAGATGGTCTTCATCCCCAAGCCATTC-3') and GFP-C3-TDP-43DW (5'-AACGGGATCCCTACATTCCCCAGCCAGAAG-3'). The amplified fragments were digested with *XhoI/BamHI* cloned into the same cleavage sites of the pEGFP-C3 vector (Takara, Shiga, Japan). The pEGFP-C3-hTDP-43 and pEGFP-C3-CTF-hTDP-43 were digested with *NheI/BamHI*, and then a Klenow Fragment Kit (Takara) was used to unwind the fragments from the blunt-end duplexes. The pCX-EGFP (a gift from Prof. M. Okabe at Osaka University) was digested with *EcoRI,* and then unwind the fragments from the blunt-end duplexes. Either a GFP-hTDP-43 or a GFP-CTF-hTDP-43 fragment was cloned into the pCX vector. Among many mutations in the TDP-43 gene, we chose Met337 to Val (M337V), as we have dealt with a case

that exhibited this mutation (Tamaoka et al. 2010). We used a KOD Plus mutagenesis kit (TOYOBO, Osaka, Japan) with the primers of TDP-43_M337VUP (5'-GTATGGTGGGCATGTTAGCCAGCCA-3') and TDP43_M337VDW (5'-CCCAACTGCTCTGTAGTGCTGCCTG-3') for site-directed mutagenesis of the full-length hTDP-43 and the CTF fragment hTDP-43 to substitute for Met337 to Val (M337V). All constructs were verified by DNA sequencing.

2.2. Animals

Timed-pregnant ICR mice were purchased from SLC (Shizuoka, Japan). For *in utero* electroporation, the pregnant mice were anesthetized by intraperitoneal injection of sodium pentobarbital (70 mg/kg body weight). For transcardial perfusion, the mice were deeply anesthetized by intraperitoneal injection of an excess of sodium pentobarbital (100 mg/kg body weight). All the animal experiments were approved by the animal care and use committee of the University of Tsukuba and performed under its guidelines.

2.3. *In utero* Electroporation

Timed-pregnant mice on day 12.5 to 13.5 post coitum were anesthetized and the uterus was pulled out of the abdominal cavity. A glass capillary was inserted into the lateral ventricle of the embryo through the uterus, and 2.5 μl of a plasmid solution, pCX-GFP-hTDP43-wt or

pCX-GFP-hTDP43-M337V, pCX-GFP-hTDP-Ctf-wt, pCX-hTDP-Ctf-M337V (300 nM dissolved in 0.01% Fast Green FCF (Takara) in phosphate buffered saline (PBS)), or pCX-EGFP (150 nM), was injected by pressure (Okada et al. 2007, Saito 2006). Around 5 min after injection, the head of the embryo was held with a forceps-type electrode positioned in front of the parietal region and behind the posterior part of the cerebellum (CUY650P5, Unique Medical Imada, Miyagi, Japan), with the anode on the forebrain side, and five cycles of square electric pulses (35 V, 50 ms) with 950 ms intervals were delivered through the uterine wall using an electroporator, CUY21 (Nepa Gene, Chiba, Japan). The uterus was then repositioned in the abdominal cavity, and the abdominal wall and skin were closed to allow the embryos to continue normal development.

2.4. Brain preparation and Immunohistochemistry

The mouse embryos electroporated at embryonic day (E) 12.5 were removed from the pregnant mice at the stage of E15.5, and postnatal days (P) 7 or 21 mice were used. In the case of the E15.5 embryos, after their heads were cut off and immersion-fixed with 4% paraformaldehyde (PFA) in PBS at 4˚C overnight, the brains were removed. In the case of P7 and P21 mice, after transcardial perfusion with 4% PFA/PBS, the brains were removed from their head and immersed in the fixative at 4˚C overnight.

All the brains were incubated in 10%, 20%, and 30% sucrose/PBS at 4˚C overnight serially, and embedded in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan); 20-μm-tickness sections

were cut using a cryostat and mounted on slide glasses. The slides were washed with PBS with 0.1% Triton X-100 (PBS-T) to remove the OCT compound, and blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO)/PBS-T at room temperature for 1 hr. The slides were incubated with primary antibody at 4˚C for 20 hrs. For primary antibodies we used antibodies to GFP (1:10,000, A10262, Lifetechnologies, Carlsbad, CA), phosphorylated TDP-43 (pS409/410-1, 1:1,000; TIP-PTD-P01, Cosmo Bio, Tokyo, Japan), TDP-43 C-terminus (405-414, 1:1,000; TIP-TD-P09, Cosmo Bio), TARDBP (1:200, 10782-2-AP, ProteinTech), human specific TARDBP (1:100, 60019-2-Ig, Protein Tech) and ubiquitin (1:250, Z0458, DakoCytomation, Glostrup, Denmark). Alexa-Fluor-labeled antibody (A11039, A11012, Lifetechnologies) was used for the secondary antibodies and DAPI solution (DOJINDO, Kumamoto, Japan) was used for nuclear staining. Fluorescence microscopic studies were performed on a Zeiss Axioplan2 with Keyence VB-7000 CCD camera, or on a confocal laser scan Leica TCS-SP5 microscope. We examined five mice per experiment and counted in five random fields (220 μm x 165 μm) in two sections of each mouse.

To distinguish aggregation from non-aggregated distribution, we considered cells to have inclusions if the GFP was abnormally concentrated and differed from the diffuse nuclear or cytoplasmic pattern seen with GFP control vector (Bao 2002).

2.5. Cell culture and transfection

HEK293T cells were cultured in DMEM (Invitrogen (Life Technologies), Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen), in 5% CO_2 at 37˚C. HEK293T cells were transfected using Lipofectamine2000 reagents (Invitrogen) as per the manufacturer's instructions. The cells were cultured for 16 hrs after the transfection, and were then collected in PBS with a protease inhibitor cocktail (PI Complete, Roche-Applied Science, Mannheim, Germany). After centrifuging at 3,300 g at 4˚C for 5 min, the supernatant was removed, and the pellet was dissolved in 1% Triton X-100 in PBS with protease inhibitor. Then, the solution was mixed with same volume of 2x Tris-Glycine SDS Sample Buffer (LC2676, Invitrogen) and boiled for 5 min. Each 10 μl of the sample mixture was subjected to Western-blot analysis.

2.6. Western blot analysis

For immunoblotting, HEK293T cell extract was mixed with equal parts of Tricine Sample Buffer (161-0739, Bio-Lad, Hercules, CA) with dithiothreitol and boiled for 5 min. After transfer to PVDF membrane, the membrane was blocked with 1% BSA/PBS-T for 1 hr at room temperature. For primary antibodies, we used antibodies to GFP (1:1,000, A11122, Invitrogen), TDP-43 C-terminus (1:1,000, TIP-TD-P09 (405-414), Cosmo Bio) at 4˚C for 20 hrs. After letting this react with anti-Rabbit IgG, HRP-linked Whole Antibody (1:10,000, NA934, GE Healthcare Japan, Tokyo,

Japan) for 1 hr at room temperature, we got the signal using ECL Plus Western Blotting Detection System (RPN2124, GE healthcare).

2.7. Statistical analysis

ANOVA and Bonferroni collection analysis were conducted.

3. Results

3.1. Expression of disease-associated gene in the fifth layer of the cerebral cortex by *in utero* electroporation

In ALS, obvious motor neuron cell loss is observed in the fifth layer of the cerebral cortex of the motor area and in the ventral part of the spinal cord. As such, we set out to express the target gene in the motor cortex of the mice's brains for easy handling. We used constructs of both wild type (wt) and mutant M337V (m337) TDP-43 protein, for each full-length TDP-43 (FL-TDP) and C-terminal fragment of TDP-43 (219-414; Ctf-TDP). Neither Ctf-TDP-wt nor Ctf-TDP-m337 included NLS, and all types of TDP-43 were tagged with GFP (Fig. 1A). To confirm whether the transfected gene was expressed in the neurons of the fifth layer of the cortex, we at first transfected 150 nM pCX-EGFP into the cerebral cortex of E12.5 mouse embryos by *in utero* electroporation. To express the electroporated genes in the motor area of the cortex, we set the forceps-type electrode front-to-back, with the anode on the forebrain side and the cathode on the hindbrain side, because the

genes expressed in the lateral and medial area of the cortex when the electrode was set on either side of the mice's heads. GFP-protein was expressed in the pyramidal neurons in the fourth to fifth layer of the motor cortex (Fig. 1B). Generally in *in utero* electroporation, genes were introduced not only to motor cortex but also to sensory cortex and basal ganglia. But the gene-introduced region can be controlled in some extent by the age of embryo introduced and the direction of electrode. Next we electroporated GFP tagged TDP-43 genes. Four kinds of TDP-43 plasmids—FL-TDP-wt, FL-TDP-m337, Ctf-TDP-wt, and Ctf-TDP-m337—along with EGFP and pCX were transfected into HEK293T cells, and the expected size of TDP-43 proteins was confirmed by immunoblotting of the cell extract with anti-GFP and anti-C-teriminal-TDP-43 antibodies (Fig. 1C). The immunoblot with both antibodies showed that the full length TDP-43 proteins tagged with GFP (FL-TDP-wt and FL-TDP-m337) and the C-terminal fragments of TDP-43 (Ctf-TDP-wt and Ctf-TDP-m337) were properly expressed as expected, in positions around 69 kDa (Fig. 1C, arrowhead) and 46 kDa (Fig. 1C, arrow), respectively. With the anti-C-terminal-TDP-43 antibody (Fig. 1C right panel), weak signals of endogenous TDP-43 were detected at 43 kDa in the full length TDP-43 (both FL-TDP-wt and m337), EGFP, and pCX. In both Ctf-TDP-wt and Ctf-TDP-m337, the bands around this molecular weight in Ctf (Fig. 1C right, arrow) were stronger than in the full length TDP-43 (Fig. 1C right, *), because the C-terminal fragments of TDP-43 with GFP (46 kDa) and the endogenous full length TDP-43 (43 kDa) were almost same molecular weight. Furthermore we confirmed the expression of electroporated TDP-43 protein by immunostaining of mouse brains. Exogenous full

length TDP-43 was co-immunostained with anti-GFP antibody and human specific anti-TDP-43 antibody (Fig. 1D, arrow). As for the confirmation of the expression of C-terminal fragment of TDP-43 (219-414), we used anti-TDP-43 C-terminus (405-414) antibody because the epitope of human specific TDP-43 antibody was located at residues 203-209. Exogenous C-terminal fragment of TDP-43 that co-expressed with GFP showed much stronger fluorescence of TDP-43 (Fig. 1E, arrowhead) than that of GFP-negative cells, which means endogenous TDP-43 (Fig. 1E, asterisk).

3.2. Expression of TDP-43 associated with ALS related mutation

We next investigated the expression of TDP-43 *in vivo* at E15.5, P7 and P21 of the electroporated mice by immunohistochemistry. EGFP gene was electroporated as a control. As reported previously (Okada et al. 2007), GFP protein expressed efficiently and distributed extensively in cytoplasm and neurites of neuron (Fig. 2a-c). TDP-43 was tagged with GFP protein and the expression of TDP-43 was followed by the GFP expression with anti-GFP antibody. Exogenous full-length TDP-43 proteins (FL-TDP-wt and FL-TDP-m337) were mostly expressed in the nuclei in the cells located in the fifth layer of the cortex through all stages (Fig. 2, d-i). In the small number of neurons from each of five mice examined at E15.5, however, exogenous full length TDP-43 of both wild type and mutant M337V were diffusely distributed in the cytoplasm and generated small round aggregations (Fig. 3a-c, arrows), and the nuclei were free of exogenous TDP-43. Other cytoplasmic distributions were observed around the nuclei (Fig. 3d, arrowhead). Some of these aggregations around the nuclei

contained human TDP-43 (Fig. 3e, arrowhead) but not phosphorylated (Fig. 3f). These types of cytoplasmic aggregations were not observed at P7 and P21 of full length TDP-43 in either wild type or mutant M337V. No clear differences were seen in the distribution of full-length wild type and full-length mutant (M337V) TDP-43 proteins.

The distribution of both wild type (Ctf-TDP-wt) and mutant M337V (Ctf-TDP-m337) C-terminal fragments of the proteins was different from the expression pattern of full length TDP-43 (both FL-TDP-wt and FL-TDP-m337), and cytoplasmic inclusion bodies were clearly generated. Ctf-TDP-wt was expressed not only in the nucleus but also diffusely in the cytoplasm (Fig. 2j-l). C-terminal fragments of TDP-43 were diffusely distributed in the cytoplasm and neurites at E15.5 (Fig. 2j). At P21, small round inclusion bodies were detected in the cytoplasm and neurites in Ctf-TDP-wt (Fig. 2l, arrowheads). The expression pattern of Ctf-TDP-m337 (Fig. 2m-o) was similar to Ctf-TDP-wt, in that it was also diffusely expressed in the cytoplasm and neurites from E15.5 to P21, and aggregations were detected. This inclusion formation process suggests that over-expressed C-terminal fragments of TDP-43 may first be distributed in the cytoplasm, and then the widely spread TDP-43 may aggregate gradually to form the inclusion bodies.

3.3. Examination of inclusion bodies resulting from electroporation of C-terminal fragments of TDP-43 M337V

Immunohistochemistry of the electroporated brains with Ctf-TDP-m337 (P21) with anti-phospho-TDP-43 antibody and anti-ubiquitin antibody revealed that the TDP-43 protein within the inclusion bodies was phosphorylated (Fig. 4A) and ubiquitinated (Fig. 4B). It is known that the inclusion bodies in the neurons of ALS patients are in the cytoplasm, that they are ubiquitinated, and that the TDP-43 protein within them is phosphorylated and cleaved to the C-terminal fragments (Arai et al. 2010). From this point of view, our model with the electroporation *in utero* succeeded in reproducing human ALS pathology by C-terminal fragments electroporation; aggregation, phosphorylation, and truncation are considered to be the hallmarks of TDP-43 in ALS patients.

Next, we investigated the ratios of the aggregation-positive cells (Fig. 5D) and the phosphorylated TDP-43 positive cells (Fig. 5E) to the GFP-positive cells. Most of aggregations contained phosphorylated TDP-43, however, the other neurons which contained phosphorylated TDP-43 without aggregations (Fig. 5A and C, arrows) or non-phosphorylated TDP-43 aggregations (Fig. 5A and B, arrowheads) were observed at both E15.5 and P21. For the aggregation positive cells, at E15.5 and P21, cells into which wild type TDP-43 had been introduced showed a significantly higher aggregation ratio of C-terminal fragments of TDP-43 (Ctf-TDP-wt) than of full-length TDP-43 (FL-TDP-wt) (Fig. 5D; 4.9 ± 0.8% and 9.3 ± 1.1 for FL-TDP-wt and Ctf-TDP-wt, respectively at E15.5 ($p<0.01$), and 2.7 ± 1.2, 17.3 ± 4.4, respectively at P21 ($p<0.001$); mean ± SEM; n=5). In the comparison between wt and m337, there were no significant differences in the aggregation ratios. The phosphorylation ratio displayed a similar tendency to the aggregation ratio at E15.5 and P21 in

the wild type TDP-43 cells that showed the significantly higher ratio of C-terminal fragments of TDP-43 than of full-length TDP-43 (Fig. 5E; 4.9 ± 0.5% and 29.2 ± 5.4 for FL-TDP-wt and Ctf-TDP-wt, respectively at E15.5, $p<0.001$; and 5.3 ±1.6, 24.9 ± 6.4 at P21, $p<0.01$, $n=5$). The cells with aggregations at E15.5 might have been lost during development, and aggregations could have been growing within the surviving cells until P21. This could come from the over-expression of exogenous TDP-43, but the relationship between cell loss and aggregations needs to be confirmed. At P21, the phosphorylation ratio in the cells into which wild type C-terminal fragments of TDP-43 had been introduced was higher than those in into which M337V had been introduced (24.9 ± 6.4 and 8.1 ± 1.3, respectively, $p<0.05$).

4. Discussion

Since aggregation and modification of TDP-43 were first found in the tissue of ALS and FTLD patients, many researchers have been investigating the mechanisms of making aggregation and modification of TDP-43 and the effect of inclusion formation on the pathological pathway. TDP-43 is a RNA/DNA binding protein thought to regulate RNA splicing, translation, and RNA transport to cytoplasm (Lagier-Tourenne et al. 2010, Lee et al. 2011). The precise mechanisms by which neurons degenerate and cytoplasmic inclusions are generated in ALS patients' motor neurons in the cerebral cortex and the spinal cord have not yet been determined, but altered RNA metabolism may play a crucial role in the disease (Arnold et al. 2013). RNA metabolism can be affected by cellular

circumstances, and to address this issue, good models that reflect *in vivo* cellular conditions are essential. *In vitro* experiments reproduce such conditions only to a certain extent; somatically gene-engineered rodent models do better, but require a great deal of time and effort. In this study, we have clearly showed that our method, *in utero* electroporation, allows for the introduction of disease-related genes into the neurons of the target area, and the reproduction of the pathological ALS hallmarks of TDP-43, including cytoplasmic aggregation, nuclear clearing, and phosphorylation of the protein (Baloh 2011). As previously reported in *in vitro* experiments, C-terminal fragments of TDP-43 were diffusely localized mostly in the cytoplasm and distributed in the neurites, and produced dot-like and clear round inclusion bodies in this study. However some of C-terminal fragment of TDP-43 were distributed in the nucleus (Fig. 2j-o and Fig. 4) despite the lack of NLS. The similar nuclear distribution of C-terminal fragment of TDP-43 that lack NLS has shown in elsewhere with transgenic mice (Caccamo et al. 2012; Wils et al. 2010). It remains to be shown, but Wils et al. supposed that C-terminal fragment of TDP-43 passively transported to nucleus with binding to endogenous TDP-43 or other targets, or in dimeric state with full-length TDP-43 (Wils et al. 2010). In human pathology, we could not find precise data for the number of neurons that include phosphorylated TDP-43, but about 20% of neurons show ubiquitin-positive aggregates and about 12% TDP-43 (not only phosphorylated) aggregates in sporadic ALS patients (Giordana et al. 2010). In the reported transgenic rodent models, only a “few” or “rare” phosphorylated TDP-43 aggregations were observed. Xu et al. reported that 15% of motoneurons had phosphorylated

TDP-43 aggregations in the nucleus and the cytoplasmic aggregations were “far fewer” (Wils et al. 2010, Xu et al. 2010, Igaz et al. 2011, Janssens et al. 2013), and Igaz et al. reported only 1% of neurons as having phosphorylated TDP-43 aggregations (Wils et al. 2010, Xu et al. 2010, Igaz et al. 2011, Janssens et al. 2013). Our model showed much more TDP-43 and phosphorylated TDP-43 aggregations in the neurons. Phosphorylation of the inclusion bodies of TDP-43 means that our model is capable of reflecting human ALS pathology. This method requires attentive manipulations, but the cost and time are not so different from what is needed for *in vitro* preparations. Our model has the benefit of *in vivo* circumstances, as well as having the advantage over transgenic rodent models, in that it allows for more expeditious results. However, in our preparation, obvious decrease of the number of neurons by introducing any exogenous protein was not observed, and we did not quantify the neuronal death and the toxicity of TDP-43 in this study.

Our study also indicated that in some neurons that over expressed full-length TDP-43 showed diffuse distribution of TDP-43 in the cytoplasm, and small round, nuclear and perinuclear inclusions in the early stage after electroporation (E15.5, Fig. 3a-d). These aggregations were not observed at P7 and P21. The full-length construct used here was tagged with GFP at the N-terminal end, so these aggregations may have been composed of full-length TDP-43 itself or the N-terminal fragments of TDP-43 that were detected in the former study (Yamashita et al. 2012).

In our *in vivo* experiments, we had expected that full-length mutant TDP-43 would be more likely to aggregate than full-length wild type, or to be cut into short fragments. However, the distribution

patterns of the transfected TDP-43 were shown to be the same regardless of wild type or mutant in full length TDP-43. Tsao et al., in a review of transgenic rodent models, reported the same distribution pattern in the transgenic mice (Tsao et al. 2012). In our study, the wild-type C-terminal fragments of TDP-43 resulted in more phosphorylation than the full-length TDP-43 (Fig. 5E). Furukawa et al. showed that a TDP-43 C-terminal fragment basically becomes less soluble and more phosphorylated when the truncation site was more C-terminal in an RRM2 domain (Furukawa et al. 2011). They also indicated that the M337V mutation of TDP-43 did not affect the insolubility and the phosphorylation. However, wild-type C-terminal fragments of TDP-43 were more easily phosphorylated than mutant M337V at P21 in our study. It may be that the M337V mutation of TDP-43 affects the fragmentation step rather than the phosphorylation step, and it may related to the tendency of the aggregation ratio at P21, which wild-type C-terminal fragments of TDP-43 were more easily aggregated than mutant M337V (Fig. 5D). It could be that wild-type TDP-43 is tightly regulated in its expression in the nuclei and that over-expressed TDP-43 is released into the cytoplasm where it forms aggregations and is phosphorylated. In our study we detected phosphorylated TDP-43 without aggregations and non-phosphorylated TDP-43 aggregations (Fig. 5A-C). As a whole, there were just phosphorylated (non-aggregated) TDP-43 more than only aggregated. This might suggest that the phosphorylation of TDP-43 occurred before aggregation or phosphorylation reduce to be aggregated. As for mutation effect, if phosphorylation would be

protective from aggregation of TDP-43, the mutation might reduce its protective effect. However, these conjectures require future study.

Dormann et al. suggested that in FUS/TLS, formation of cytoplasmic inclusions comprises two steps: cytosolic distribution of the FUS/TLS protein and clustering of the protein (Dormann et al. 2010). FUS/TLS and TDP-43 resemble each other in their RNA/DNA binding properties, which suggests that TDP-43 cytoplasmic aggregation may also require two steps. If this is the case, the cytosolic distribution step would likely be performed by the C-terminal fragmentation of TDP-43, and this truncated protein would form aggregations in the cytoplasm in the second step. Our *in utero* electroporation system can introduce not only one gene or oligo-nucleotide, but many of them at once. This is advantageous in studies involving TDP-43, FUS/TLS, and other such proteins.

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Figure Legends

Figure 1. Expression of TDP-43 plasmids *in vitro* and *in vivo*

A. Constitution of electroporated plasmid. Both wild type (wt) and mutant M337V (m337) of TDP-43 protein, for each full-length TDP-43 (FL-TDP) and C-terminal fragment of TDP-43 (219-414; Ctf-TDP) were tagged with GFP. Neither of the C-terminal fragments of TDP-43 included a nuclear localization signal domain (NLS). RRM, RNA recognition motifs; GRR, glycine-rich region. B. Gene expression in the neurons of the fifth layer of the cortex by *in utero* electroporation of GFP-protein. The right panel shows an enlarged view of the region inside the square. C. Immunoblotting of proteins from transfected HEK293T cells by anti-GFP antibody (left) and anti-C terminal TDP-43 antibody (right). Arrowheads indicate the bands from full-length TDP-43 tagged with GFP, and arrows indicate C-terminal fragment of TDP-43 tagged with GFP. Endogenous full-length TDP-43 was detected in the right panel (*) and a GFP-tagged C-terminal fragment of TDP-43 was detected (arrow) in almost the same size. lane 1; Mock (pCX), lane 2; cells transfected from GFP expression plasmid, lane 3; FL-TDP-43-wt, lane 4; FL-TDP-m337, lane 5; Ctf-TDP-wt, lane 6; Ctf-TDP-m337. D. Expression of exogenous TDP-43 protein in the full length TDP-43 electroporated mouse brain (P7). Exogenous human TDP-43 was expressed only in GFP-positive cells (arrow). green, GFP; red, human specific TDP; blue, DAPI. Leica TCS-SP5, bar = 25 μm. E. Expression of exogenous TDP-43 protein in the C-terminal fragment of TDP-43 electroporated mouse brain (P21). Exogenous C-terminal fragment of TDP-43 was co-expressed with GFP and

showed stronger fluorescence (arrowhead) than endogenous TDP-43 (asterisk). green, GFP; red, TDP; blue, DAPI. Leica TCS-SP5, bar = 25 μm.

Figure 2. The distribution of each type of exogenous TDP-43 protein in the developmental stages. GFP-tagged TDP-43 was immunostained by anti-GFP antibody. GFP gene was electroporated as a control. GFP proteins expressed efficiently and distributed extensively in cytoplasm and neurites of neuron (a-c). Note that exogenous full-length TDP-43 proteins were expressed in the nuclei during all stages (d-i), and the C-terminal fragments of the TDP-43 proteins had cytoplasmic inclusion bodies (arrow heads in l and o). C-terminal fragments of TDP-43 were expressed not only in the nucleus but also diffusely in the cytoplasm (j-o). GFP (green) and DAPI (blue). Leica TCS-SP5, bar = 25 μm

Figure 3. Aggregations observed in the rare case of full length TDP-43 electroporation (E15.5). Various patterns of aggregations were observed at E15.5 in some neurons into which full length TDP-43 wild-type had been introduced. Small round nuclear and perinuclear inclusions were observed (a, b and c, arrow), and a halo-like distribution was also observed (d, arrowhead). Some of these aggregations were positive for human specific anti-TDP antibody (e, arrowheads), but not for anti-phosphorylated TDP antibody (f). GFP (green) and DAPI (blue). Leica TCS-SP5, bar = 5 μm.

Figure 4. Composition of the inclusion bodies in C-terminal fragments of TDP-43 introduced into mouse brains at P21

Brain sections from Ctf-TDP-m337 transfected *in utero* mouse brains were immunostained by anti-phosphorylated TDP-43 antibody (A), and anti-ubiquitin antibody (B). The introduced TDP-43 formed aggregations in the cytoplasm (arrows) and they were positive for each antibody. Leica TCS-SP5, bar = 25 μm

Figure 5. The ratio of the cells with aggregations or phosphorylated inclusions

The non-phosphorylated/aggregation-positive cells were shown in A and B (arrowheads), and the phosphorylated/ aggregation-negative cells were shown in A and C (arrow). Wild type C-terminal fragments of TDP-43 electroporated mouse brains were examined at E15.5 (A) and at P21(B and C), green (GFP), red (phosphorylated TDP-43, pS409/410). Bar = 100 μm. D. The ratio of aggregation-positive cells to GFP-positive cells at each stage. E. The ratio of phosphorylated TDP-43 positive cells to GFP-positive cells. The gray columns show the ratio from brains into which full-length TDP-43 was introduced, and the black columns show the ratio from those into which C-terminal fragments of TDP-43 were introduced. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

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Figure1

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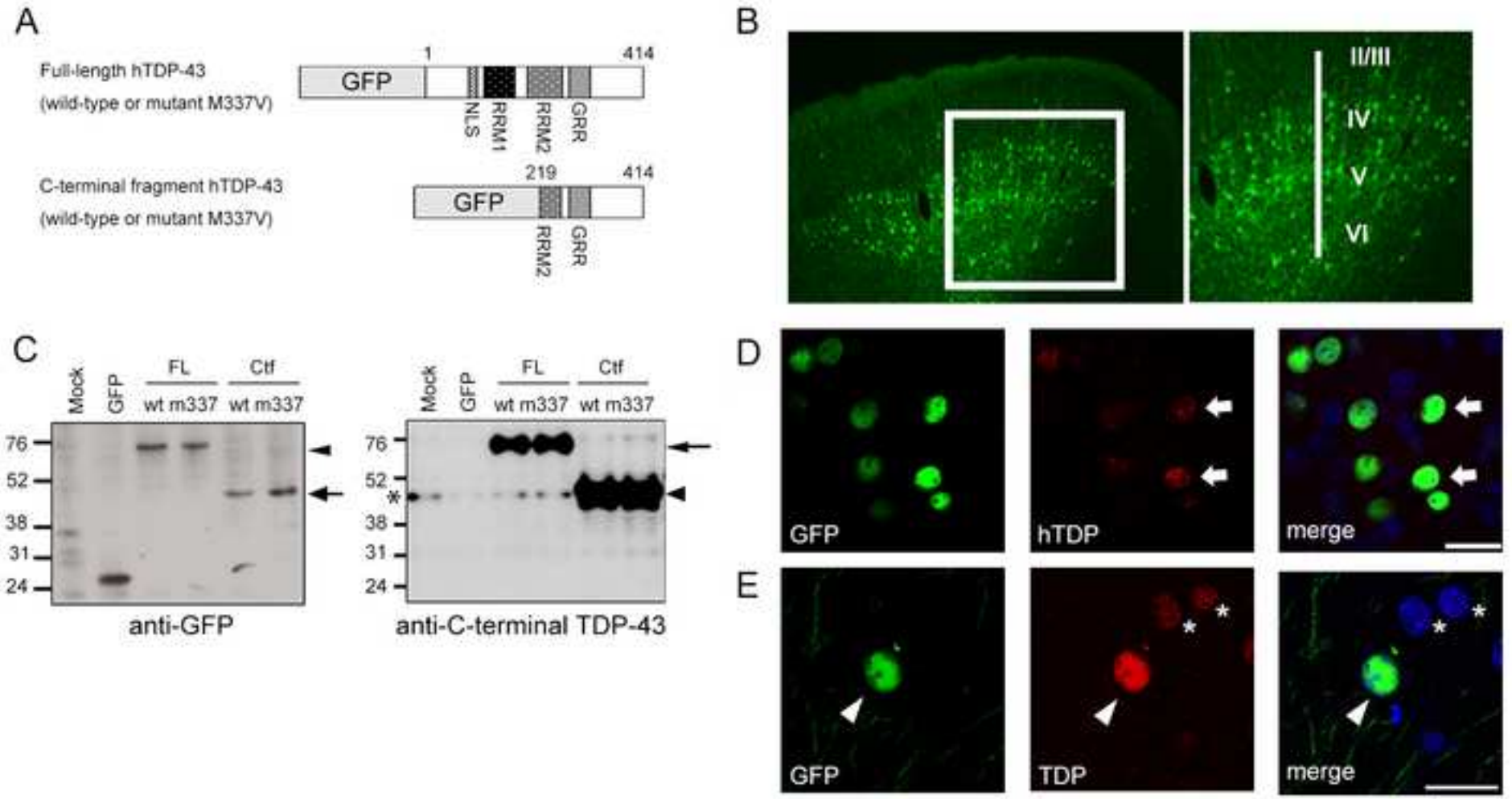

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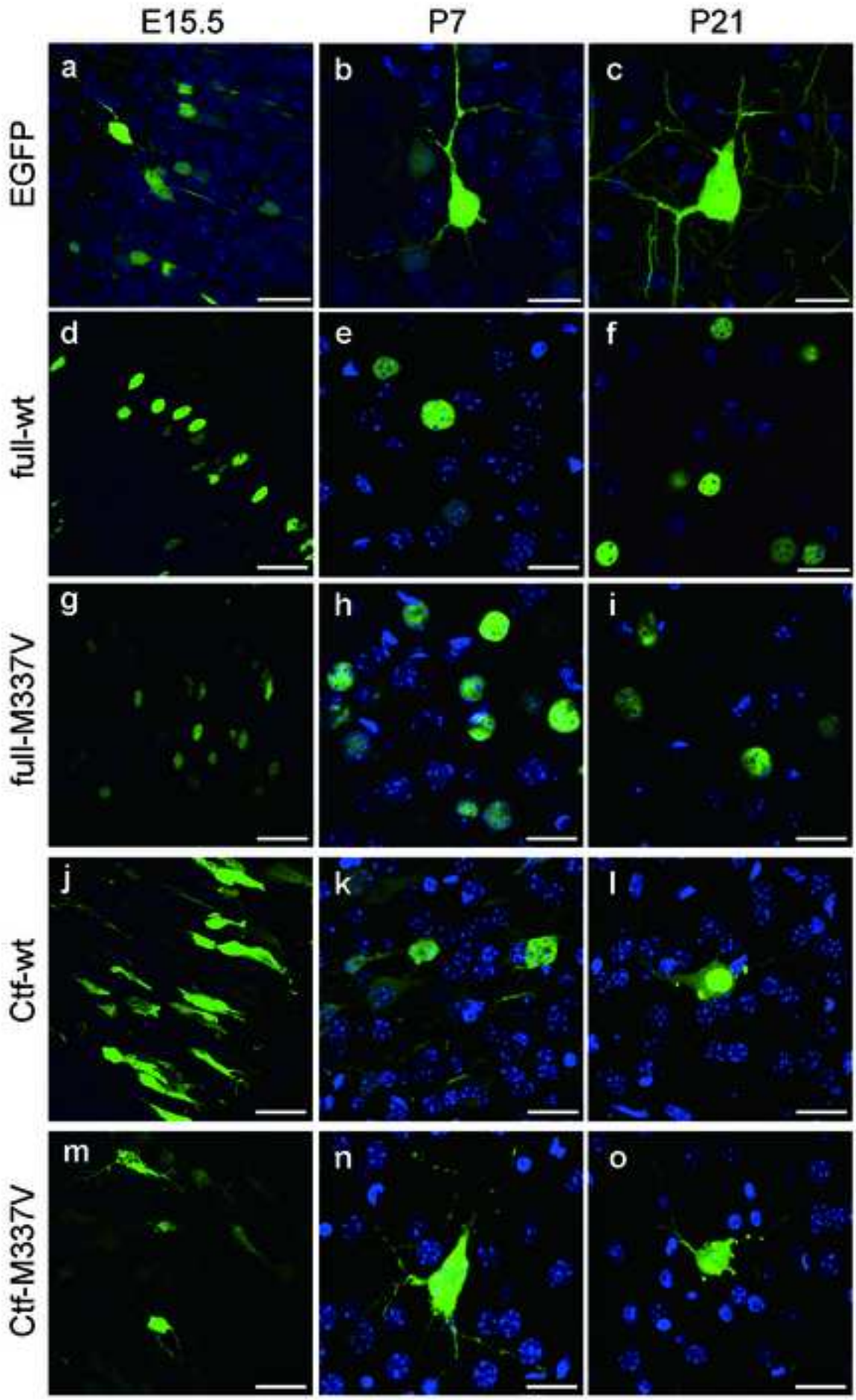

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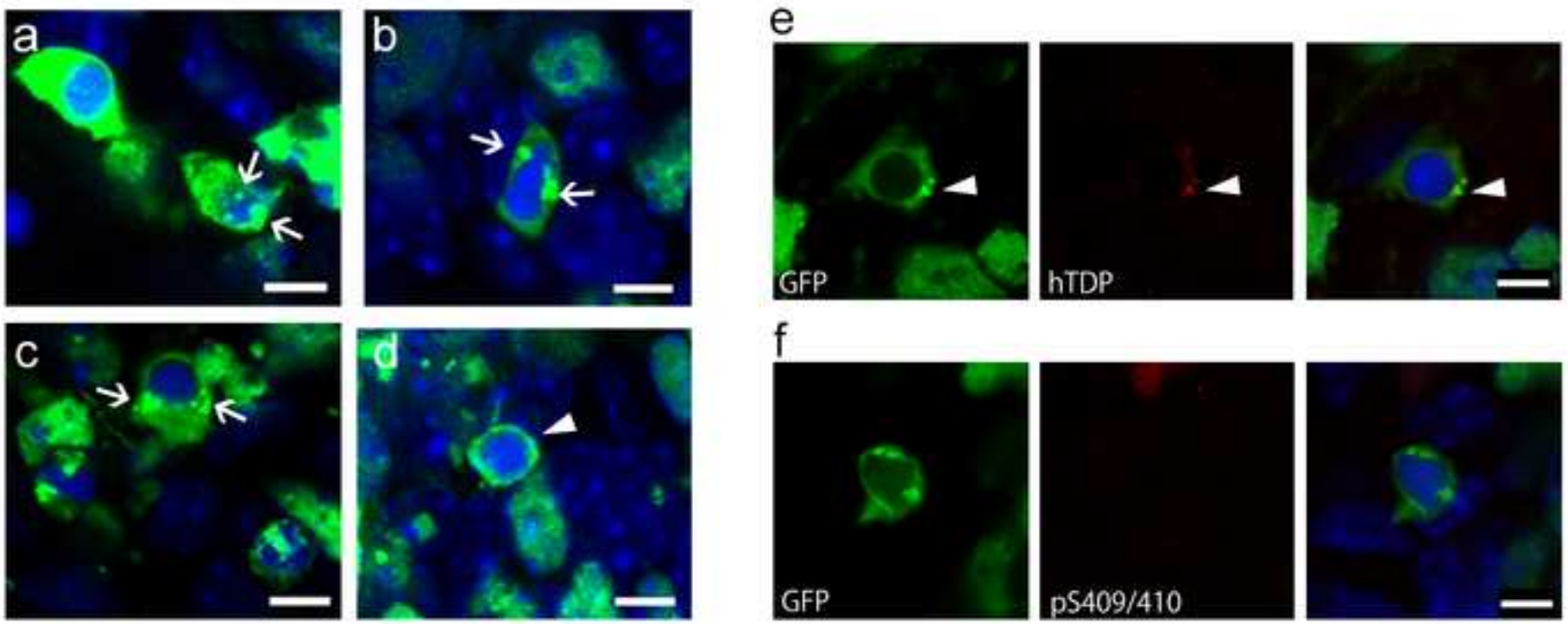

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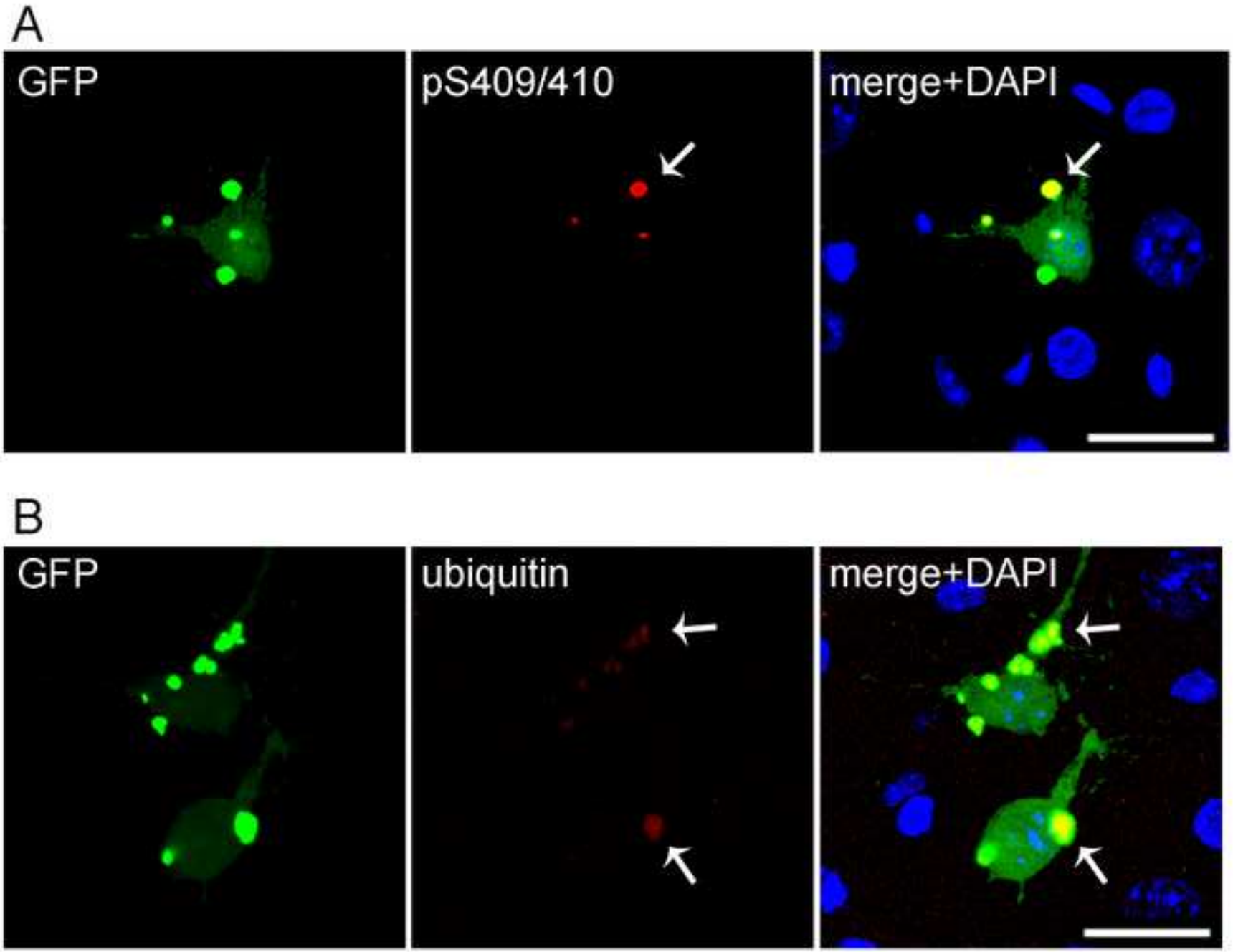

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