博士論文

Role of arginine methylation in the intercellular transmission of FUS (アルギニンメチル化が FUS の細胞間伝播に与える影響の解析)

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§ 1 Introduction

1.1 Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lober Degeneration (FTLD)

Amyotrophic Lateral Sclerosis (ALS)

ALS is a fatal disease caused by the progressive degeneration of upper and lower motor neurons, leading to muscle weakness and atrophy throughout the body. Patients usually die in respiration failure finally. The rate of disease's onset is the highest between 60-70 years old. In Japan, patients with ALS is 7～11 /100,000 people and 1.1～2.5 /100,000 people/year is determined as ALS. The incidence of ALS is 1.3～1.4 times higher in male than in female. Now, the cause of ALS remains unclear and there is no treatment method based on the underlying mechanism (amyotrophic lateral sclerosis clinical practice guidelines 2013). Treatment drugs against the symptom is Riluzole (brand name Rilutek), an inhibiter of glutamate toxicity, and recently Edaravone (brand name Radicut), a radical scavenger. Both of them are expected as protective effects to neurons.

The majority of ALS is sporadic ALS (SALS), but 5 ～10 % of all ALS patients is familial ALS (FALS). In 1992, the superoxide dismutase 1 gene (*SOD1*) was identified as the causative gene of FALS (Rosen *et al*., 1992; Rosen *et al*., 1993). More than 170 mutations in *SOD1* gene have been reported from FALS families to this date, and the most frequent mutations accounting for 20 % of all FALS. Furthermore, in 2008, mutations in the *TARDBP* gene encoding TDP-43 (TAR-DNA binding protein of 43kDa) were reported by FALS and SALS. (Gitcho *et al*., 2008; Kabashi *et al*., 2008; Sreedharan *et al*., 2008; Yokoseki *et al*., 2008). Also, TDP-43 is identified as a major component of ubiquitin-positive inclusions of SALS or FALS except for SOD1-linked FALS (Arai *et al*., 2006; Neumann *et al*., 2006). TDP-43 Pathology is found in more than 95 % of ALS patients, including sporadic cases (Ling, *et al*, 2013).

In 2009, FUS / TLS (fused in sarcoma / translated in liposarcoma) gene was identified as a new etiological gene for FALS (Kwiatowski *et al*., 2009; Vance *et al*., 2009). Because both FUS and TDP-43 proteins have multiple RNA recognition sequences, they are predicted to be involved in the metabolism of DNA/RNA. In terms of structure and function of the similarity, some RNA metabolism abnormality can be involved in ALS onset. (Lagier-Tourenne and Cleveland, 2009). The physiological functions of FUS are described in detail in 1.3. The human *FUS* gene is located at 16p11 on the short

arm of chromosome 16 and was involved in a chromosomal translocation t (12:16) (q13: p11) in mucinous liposarcoma. It was identified as a gene that forms a fusion gene with the transcription factor CHOP on chromosome 16. (Crozat *et al*., 1993). *FUS* gene also in acute myeloid leukemia patients form the fusion gene to (Pangopoulos *et al*.,1994; Panagopoulos *et al.*,1995) chromosomal reciprocal translocation t (16:21) (p11: q22) with ERG has also been reported. 50 or more FALS mutations in FUS have been reported. These are 4 % of FALS. (Da Cruz and Cleveland, 2011; Lanson and Pandey, 2012; ALSoD (http://alsod.iop.kcl.ac.uk/home.aspx)). In Japan, R521C mutation (Yamamoto-Watanabe *et al.*, 2010; Suzuki *et al.*, 2010; Tateishi *et al.*, 2010), P525L mutation (Mochizuki *et al.*, 2012), K510M mutation (Mochizuki *et al.*, 2014) or G504WfsX515 frameshift mutation (Hirayanagi *et al.*, 2016) was reported. Further, H517D mutation, R521H mutation (Nakamura et al., 2016) even in SALS patients have been reported by using the analysis of next-generation sequencing in Japan. The average age of onset of FALS patients associated with FUS mutations reported so far is about 44 years, which is earlier than that of SALS (ALSoD). However, it has been reported that the age of onset, rate of progression, and pathophysiology differ depending on the type of mutation (Mackenzie *et al.*, 2011a).

Frontotemporal lobar degeneration

Frontotemporal lobar degeneration (FTLD) accounts for about 10% of dementia. It develops between the ages of 45 and 65 and causes progressive impairment in behavior, personality, language, and memory retention (Neary *et al.*, 1998, McKhann *et al.*, 2001). In 1892, Arnold Pick who is a psychiatrist in the Czech, reported the overlapping memory mistake (Reduplicative Paramnesia) and argyrophilic spherical structures in the brains of patients (Pick 's Body). In 1926, this disease is determined as Pick 's disease of the non-Alzheimer's type. In 1994 Lund-Manchester study suggested several, clinical character including Pick's disease that exhibit abnormal behavior and personality changes caused by frontal-temporal lobe atrophy are renamed as frontal temporal lobe dementia (Frontotemporal Dementia, FTD) and in 1996, frontotemporal lobar degeneration (FTLD) was proposed as the name of anatomical diagnosis of frontotemporal lobar degeneration. Clinical findings of FTLD are consisted of behavioral variant FTD (bvFTD), progressive non-fluent aphasia (PNFA) and, semantic dementia (SD). As pathology, FTD type (frontal lobe is degenerated), Pick type (frontal and temporal lobe is denatured), and MND (the motor neuron degeneration) were classified.

In the FTLD patient brains, inclusion bodies in the affected neurons are appeared as a pathological feature. As a component of the inclusion bodies, tau, TDP-43, and FUS were identified (Mackenzie *et.al*., 2010; Arai *et.al*., 2006; Neumann *et.al.*, 2009). Depending on the component of inclusion bodies, currently FTLD is pathologically classified into the following types. ① FTLD-Tau (tau-positive inclusions are appeared), ② FTLD-TDP (TDP-43-positive inclusions are appeared), ③ FTLD-FUS (FUS-positive inclusions are appeared), or ④ FTLD-UPS (ubiquitin-positive inclusion bodies, which are negative to tau, TDP-43 and FUS, are appeared). (Mackenzie *et al.*, 2010). Pick's bodies in Pick's disease are positive for tau (Murayama *et.al.*, 1990), so Pick's disease is classified as FTLD-tau. The FTLD-FUS includes neuronal intermediate filament inclusion disease (NIFID), and basophilic inclusion body disease (BIBD) (Neumann *et al.*, 2009, Mackenzie *et al.*, 2011b).

Currently, the diseases whose are characterized by the accumulation of FUS, such as ALS, and FTLD-FUS are collectively known as FUS-opathy, or FUS proteinopathy. In the patients with FUS proteinopathy, FUS may have a crucial role to cause neurodegeneration through some common mechanism. Since patients with FUS mutations mainly exhibit the phenotype of ALS and rarely exhibit FTLD, it is also possible that neurodegeneration may be occurred by a mechanism different from that of wild-type FUS and FALS mutant FUS. The detail mechanism of neurodegeneration considered in FUS proteinopathy are given in the following section.

1.2 FUS

FUS consists of 526 amino acids and is a nuclear ribonucleoprotein which consists of three proteins belong to FET family (FUS, EWS, TAF15). FUS has multiple functional domains, from amino-terminal Gln - Gly - Ser -Tyr (QGSY) rich regions, Gly (G) rich region, RNA recognition motif (RRM), Zn finger region, nuclear localization signal (NLS). Because it includes RRM, it involves in the processing of some DNA / RNA. Further, the area in amino terminal from QGSY-rich region toward G rich region called as a low-complexity domain (LC domain), because its sequence types are biased markedly. Several studies revealed that LC domain is necessary and sufficient for the self-assembly, accumulation in the cells called as stress granules, liquid droplet formation, and fibril

formation with cross- beta structures (Kato *et al.*, 2012, Han *et al.*, 2012, Murakami *et al.*, 2015, Murray *et al*, 2017, Hughes *et al.*, 2018). Moreover, also in our laboratory, using *Drosophila* FUS proteinopathy model, self-assembly of LC domain of FUS through is essential for neurotoxicity. (Matsumoto *et al.*, 2018).

Carboxy-terminus region of FUS has a Pro-Tyr type NLS (PY-NLS), whch is characteristic to FET family. PY-NLS is reported to interact with transportin1 / 2 and responsible for the nuclear translocation of FUS (Dormann *et al.*, 2010). Interestingly, this area is a hotspot of fALS with 50 or more mutations. FUS shuttle between the nucleus and cytoplasm in the cell but is mainly localized in the nucleus (Zinszner *et al.*, 1997, Anderson *et al*., 2008). And these mutations is known to lose its interaction with Transportin 1/2 and FUS shifts the cytoplasm (Dormann *et al.*, 2010). According to these facts, it is possible that the disorder of FUS translocation into the nucleus may be involved in the development of ALS.

FUS can bind to RNA through RRM and Zn finger domain (Lerga *et al.*, 2001, Iko *et al.*, 2004, Kino *et al.*, 2011) and is thought to be involved in several DNA/RNA processing including an expression regulation, RNA transcription, splicing, transport, and degradation (Crozat *et al.*, 1993; Zinszner *et al.*, 1997; Yang *et al*., 1998; Baechtold *et. al.*, 1999; Uranishi *et al*., 2001; Kanai *et al*., 2004; Fujii and Takumi, 2005; Wang *et al*., 2008; Tan and Manley, 2010). In the CLIP-seq analysis using human or mouse brains, 5,000 or more RNAs were identified to interact with FUS protein (Lagier-Tourenne *et al.*, 2012). Because the most of them are intron regions, especially FUS can play an important role in the RNA splicing (Ishigaki *et al.*, 2012, Rogeli et al., 2012). Also, FUS has been reported to act in the biosynthesis of snRNP (small nuclear RNP), which is constituted to the spliceosome, with SMN (Survival of motor Neurons) localized with Cajal bodies (GEMS) (Yang *et al.*, 1998; Meissner *et al.*, 2003; Kameoka S *et al.*, 2004). Furthermore, it also has been reported that FUS is localized in the granular structure in the nucleus and is involved in the sequestration of RNA to the granular structures (Naganuma *et al.*, 2012; Nishimoto *et al.*, 2013). From these findings, it is possible that FUS binds to RNA and is particularly involved in its splicing. *In vitro*, FUS has been shown to bind to GGUG sequences (Lerga *et al.*, 2001). In addition, GUGGU is found in about 60 % of the sequences to which FUS binds in human and mouse tissues (Lagier-Tourenne *et al.*, 2012). Further, FUS was considered to give some feedback effect on its own expression. (Zho *et al.*, 2013).

In addition, FUS can be involved in the transport and local translation of mRNA in the neuron. The motor proteins such as Myo5A, Myo6 and KIF5B, can bind to FUS. (Yoshimura *et al.*, 2006, Takarada *et al.*, 2009). FUS can stabilize actin, reconstitute dendrites and involve in the translation in mRNA in dendrites (Yasudas *et al*., 2013; Fujii *et al.*, 2005). So far, FUS-deficient mice were reported to be lethal in the perinatal period (Hicks *et al.*, 2000, Kuroda *et al.*, 2000). Hippocampal primary cultured neurons from FUS knockout mice exhibited the reduction of NdL-1 in dendrites (Fujii *et al.*, 2005). These findings suggest that FUS may be involved in the RNA transport and processing in neurons.

1.3 FUS proteinopathy

As described in 1.2, diseases associated with FUS pathology such as ALS and FTLD-FUS are generally called FUS proteinopathy, and it is considered that neuronal death may be caused by some common mechanism. Neurodegeneration mechanism by wild-type FUS or FALS with mutant FUS have been considered mainly by following two theories (Kim *et al*., 2020). ① FUS can loss a normal function and it can cause a neuronal death (Loss-of-function hypothesis). ② FUS can get some toxicity and it can cause a neuronal death (Gain-of-toxic-function hypothesis). Therefore, both of these theories are summarized below.

loss of function hypothesis

As described in 1.2, it is known that FUS deficient mice exhibit lethality in the inbred background (Hicks *et al.*, 2000, Kuroda *et al.*, 2000). This finding suggests that loss of FUS function may have a serious impact on cell survival. So far, physiological FUS is known to be mainly localized in the nucleus, on the other hand, in FUS proteinopathy patients, FUS forms intracellular cytoplasmic inclusion bodies in neurons at the lesion site. This finding suggests in neuron, FUS -positive cytoplasmic inclusions can reduce the FUS function in the nucleus. In primary neurons derived from FUS-deficient mice, RNA transport abnormality at spines in dendrites occurred (Fujii and Takumi, 2005). CNS-specific FUS knockdown in the mouse brains altered the splicing of a variety of genes including tau genes and CamK2α (Fujioka *et al.*, 2013, Ishigaki *et al.*, 2017). These suggest that mislocalization or reduction of FUS in the nuclear may reduce neuronal function and cause neuronal

death.

In zebrafish model, Zebrafish homologs *fus* KD has shown motor dysfunction, decrease in motor neuron branches and neuromuscular junction of synaptic activity abnormal phenotype. (Kabashi *et al*, 2011, Armstrong and Drapeau, 2013). Further, in *Drosophila* models, knockdown of *caz*, *Drosophila* homologue of FUS has shown motor dysfunction and life shortening and motor neuron degeneration (Xia *et al.*, 2012, Sasayama *et al*, 2012). These results support the loss-of-function hypothesis that FUS causes impairment or death of motor neuron.

However, it has been also reported that FUS deficient mice in the outbred background grew into adulthood and exhibited vacuolation, hyperactivity and reduction in anxiety-like behavior, but no overt ALS-like phenotype (Kino *et al*., 2015). In humans, malignant liposarcoma patients with the translocation of FUS gene do not develop the ALS or FTLD-like phenotypes. These results may not support the loss-of-function hypothesis.

Gain of toxic function hypothesis

In the FUS proteinopathy patients, FUS-positive cytoplasmic inclusion bodies are formed in the neurons at the lesion site. So there was a possibility that the inclusion body may have some neurotoxicity, or FUS acquires some toxicity during the process of inclusion body formation. Familial ALS linked to R521C FUS overexpressed rats in their whole body or central nervous specifically indicated progressive motor neuron death or the paralysis of motor neuron, but it is reported wild-type FUS did not express any such phenotypes (Huang *et al.*, 2011, Huang *et al.*, 2012). Central neurons specific R521C mutant FUS overexpressing rat showed ubiquitin positive cytoplasmic inclusions in the neurons (Huang *et al.*, 2012). Moreover, even in a mouse model, overexpression of wild-type or fALS mutant FUS showed shedding of motor neuron, motor dysfunction and life shortening like ALS phenotype. Overexpression of FALS mutant FUS compared to the wild-type FUS is reported to show more ALS-like phenotype (Verbeek *et al.*, 2012, Mitchell *et al.*, 2013, Shelkovnikova *et al.*, 2013, Qiu *et al.*, 2014, Sephton *et al.*, 2014). In addition, knock-in mice in which FUS was localized in the cytoplasm by deleting the carboxy end of NLS region were also reported to exhibit progressive motor neuron death (Skekic-Zahirovic *et al.*, 2016, Devoy *et al.*, 2017). These results are particularly fALS mutant FUS or abnormal localizion of FUS in the cytoplasm can acquire neurotoxicity and cause neuronal death.

In *Drosophila* model, overexpression of wild-type or FALS mutant FUS in compound eyes, or in motor neurons, exhibited the degeneration in compound eyes or in motor dysfunction. Phenotypic and life shortening are observed, especially in FALS variants. (Lanson *et al.*, 2011). In nematode model, overexpressed FALS mutant FUS to motor neurons showed motor neuron failure and shortened life (Murakami *et al.*, 2012). These results suggest FUS can acquire some neuronal toxicity and impair neuronal function and support gain-of-toxic-function hypothesis.

1.4 cell-to-cell transmission of pathogenic proteins in neurodegenerative diseases

In a variety of neurodegenerative diseases characterized by the intracellular accumulation of aggregated proteins such as tau in Alzheimer's disease and α-synuclein in Parkinson's disease, the pathogenic protein is known to be expanded along the neuronal circuit with the progress of the disease. (Braak and Braak, 1991, Jucker and Walker, 2013). Recent years, aggregated proteins such as Tau and α-synuclein were considered to be released from neurons into extracellular space, incorporated into neuronal cells, and formed new aggregates (Holmes *et al.*, 2014). The phenomenon is called as a cell-to-cell transmission and may explain the neural circuit-dependent progression of lesions in neurodegenerative diseases. Thus, the molecular mechanism of the “cell-to-cell transmission” involved in the formation of inclusion bodies, and the neurodegeneration is attracting a huge attention.

Prion disease was the first to reveal the phenomenon of abnormally structured protein transmission. Prion disease is collectively called as pathogenic prion proteins neurodegenerative diseases such as Creutzfeldt-Jakob Disease (CJD), and Gerstmann-Straussler-Scheinkerdisease (GSS). Due to neuronal cell death in prion disease brain, spongiform encephalopathy pathological changes are occurred. In part of the prion disease patients’ brains, amyloid accumulated Kuru spots occured (Aguzzi and Polymenidou, 2004). Prion disease has sporadic cases and autosomal dominant inheritance hereditary onset cases. The gene about Familial CJD and GSS in prion protein (PrP) encoding 40 of point mutations has been identified (Hsiao *et al.*, 1989, Prusiner, 1998). These prion diseases PrP acquires protease resistance and changes to scrapie prion (PrP^{SC}) with β-sheet structure. Furthermore, once PrP^{SC} is formed, PrP^{SC} as a template, normal PrP^{C} is changed into PrP^{SC} structure, disease pathology is believed to be progressed (Pan KM *et al.*, 1993; Caughey BW *et al.*, 1991; Safer *et al.*, 1993). The acquired prion diseases is Kuru disease, which is endemic in Papua New Guinea or

iatrogenic CJD and new CJD etc. Prion diseases was caused by eating or by inoculation, for instance, PrP^{SC} enters into the body, normal PrP^{C} is changed into PrP^{SC} by structural changes. From these findings, PrP features in prion diseases are determined as ① self-templating of abnormal structures, and ② intercellular or propagation of abnormal structure between individuals (transcellular spreading). The following details are the findings on the lesions and etiologic proteins of each neurodegenerative disease, especially on the transmission phenomenon.

Amyloid β peptide (Aβ)

Aβ is a major component of senile plaque amyloid in Alzheimer's disease. In Alzheimer's disease brain, senile plaques are known to start accumulating from the neocortex to hippocampus with the progress of the disease and to expand the lesion site (Braak and Braak, 1991). On the other hand, *in vitro* experiment, Aβ fibrillizatiion is known to be dependent on aggregating nucleus as a template (Jarrett *et al.*, 1993), and further, when brain lysates of Alzheimer's disease patients are innoculated into amyloid precursor protein transgenic mouse, which is a pathological model mouse of Alzheimer's disease, they showed certainly accumulation of Aβ (Kane *et al.*, 2000; Meyer- Luehmann *et al.*, 2006). In addition, senile plaque-like Aβ accumulation was observed in the brains of iatrogenic CJD patients (Jaunmuktane *et al.*, 2015, Ritche *et al*., 2017). From these findings, in the Alzheimer's disease brain, there is possibility that some Aβ can become an aggregating seed and it can contribute to the pathological expansion.

Tau

Neurofibrillary tangles in Alzheimer's disease, tau-positive inclusions in FTLD -Tau, Pick sphere in Pick disease, highly phosphorylated tau in neurons of various neurodegenerative diseases are known to be accumulated. Tau accumulation is thought to be closely associated with neurodegeneration and cognitive dysfunction. So, diseases in which tau accumulates are collectively called tauopathy. In the Alzheimer's disease brain, neurofibrillary tangles in first appeare in entorhinal cortex, with the progress of the subsequent disease, the hippocampus, spread to neocortex (Braak and Braak, 1991, 1995). So far, it has been reported that intracerebral injection of inclusion-bearing brain homogenates into transgenic mouse overexpressing FTDP-17 mutant tau, or wild-type mouse led to formation of tau pathology (Clavaguera *et al.*, 2009; Guo *et al*., 2016). It became clear that tau could propagate. Tau is a cytoplasmic protein, but Tau is detected in cerebrospinal fluid (CSF) and also in

interstitial fluid (Arai *et al*., 1995, Yamada *et al*.2011), possibly tau released out some mechanisms. What route tau is released is not consensus yet. The pathways via exosomes (Saman *et al.*, 2012), ectosomes (Dujardin *et al.*, 2014), microvesicles (Simon *et al.*, 2012), synaptic vesicles (Pooler *et al.*, 2013), etc. has been reported.

α - synuclein

α - synuclein is a major component of Lewy body in Parkinson's disease or Lewy body disease (Baba *et al*., 1998). So far in Parkinson's disease patients, α - synuclein is accumulated initially in the brain stem neurons and intestines, then retrogradely expanded to midbrain substantia nigra, and spread into a neocortex (Klingelhoefer and Rechmann, 2015, Braak *et al.*, 2003). It has been reported that Lewy bodies appear in the transplanted fetal midbrain cells, suggesting that α- synuclein can have propagated between neurons (Kordower *et al.*, 2008, Li *et al.*, 2008). Furthermore, when recombinant human α- synuclein fibers were injected into α- synuclein transgenic mouse brains or wild-type mouse brains, they exhibited α- synuclein aggregates (Luc *et al.*, 2012; Masuda-Suzukake *et al.*, 2013). These findings strongly suggest that α- synuclein, which has acquired some cohesiveness, can propagate between neurons and enlarge the lesion.

TDP-43

TDP-43 is similar to FUS in terms of nuclear ribonucleoprotein involved in RNA metabolism. TDP-43 mainly accumulates to form cytoplasmic inclusions in neurons of the SALS and FALS, further FTLD-TDP43 (Arai *et al.*, 2006, Neumann *et al.*, 2006). Accumulated TDP-43 is known to be phosphorylated at 409/410 (Hasegawa *et al.*, 2008), and it became clear that the phosphorylated TDP-43 starts its accumulation from the motor cortex, then the brain stem, and spread the lesions to the spinal cord (Brettschneider *et al.*, 2013). Furthermore, intracerebral injections of brain extracts from FTLD-TDP patients into TDP-43 transgenic mice or wild-type mice led to the induction of TDP-43 pathology (Porta *et al.*, 2018). This suggests that TDP-43, which has acquired some cohesiveness, may expand the lesion by intercellular transmission. How TDP-43, which is mainly present in the nucleus, is released into the cytoplasm is not yet clear. It is likely has been suggested exosomes (Nonaka *et al.*, 2013), microvesicles (Feiler *et al.*, 2015) and tunneling nanotubes (Ding *et al.*, 2015) relate to the propagation.

FUS

There was no report that FUS can be transmitted intercellularly. It is only reported that FUS pathology spreads in brains of FUS-proteinopathy patients as their clinical state. For instance, FUS is accumulated more in frontal or temporal cortex than in other area of the brains of FTLD-FUS patients. Patients with basophilic inclusion body diseases including ALS have also FUS accumulation but it is more in motor cortex or medulla oblongata (Lee *et al.*, 2016).

1.5 Protein arginine methylation

Methylation modification of biomolecules is widely involved in various biological phenomena. It is inferred in the 1960s that proteins are methylated, but in the 1980s arginine residues and lysine residues were revealed to be the target of methylation. However, the actual condition of the enzyme was unknown for a long time (Paik *et al.*,2007). In 1996 fermentation methylate arginine in *Saccharomyces cerevisiae* by arginine methyltransferase 1 (RMT1) (Gary *et al*., 1996). It turns out that its homolog is also present in mammals (Lin *et al*., 1996). Finally, the body of arginine methylase is solved. The methyl group of the arginine residue in the protein is given by protein arginine methyltransferases (PRMTs) from S-adenosylmethionine (AdoMet or SAM) as a methyl group donor. It is a highly conserved protein and has four characteristics motifs (motif I, post I, post Ⅱ, post Ⅲ), and a THW loop. Motif I, post I, and the THW loop forms a binding pocket with the SAM (Zhang *et al*., 2003). PRMTs are classified into several different types. PRMT1, 2, 3, 4, 6, or 8 induces monomethylated arginine and asymmetric dimethylarginine (ADMA) in proteins. On the other hand, PRMT5, or 9 catalyzes monomethylation and symmetric dimethylation (SDMA) in proteins (Miranda *et al*., 2004). It was reported in the 1990s that arginine methylation of proteins occurs in the glycine-arginine-rich region (often a repeating sequence of RG, RGG, RGR) commonly found in RNA-binding proteins called the GAR domain (Najhauer *et.al.*,1993). In cells, PRMT1 is considered to be the most major arginine methylase (Tang *et al*., 2000). PRMT family, including PRMT1, often methylates the GAR domain. However, it has been clarified that many proteins that do not have a GAR domain, such as histones, are also methylated. A method for screening proteins that are arginine-methylated by PRMT1 was developed and showed that not only the GAR domain but also the RXR motif can be a target for methylation (Wada et al., 2002).

FUS contains three RGG repeat domains with RGG/RG motifs that are thought to modify with ADMA by PRMTs. It has been reported that antibodies against unmethylated arginine FUS (UMA-FUS), monomethylated arginine FUS (MMA-FUS), and asymmetric demethylated arginin FUS (ADMA-FUS) were produced. Brain sections of FTLD-FUS patients were stained with these antibodies and found that UMA-FUS- or MMA-FUS- positive inclusions exclusively appeared in the neurons (Suarez-Calvet *et al*., 2016). Moreover, AdOx (adenosine-2',3'-dialdehyde), inhibitor of methyltransferase, can promote the production of liquid-droplet of FUS (Qamer *et al.* 2018). These findings suggest that protein arginine methylation may regulate the formation of FUS inclusions in the brains of FUS proteinopathies.

1.6 Purpose of this study

I hypothesized that protein arginine methylation of FUS has a crucial role in the neuron-to-neuron transmission of FUS protein, leading to the formation of FUS-positive inclusions. To this end, I tried to clarify next 3 points in this study.

① Demonstrate the cell-to-cell transmission of FUS

② Elucidate the effects of protein arginine methylation of FUS in the cell-to-cell transmission of FUS

③ Elucidate the effects of protein arginine methylation of FUS in the oligomerization of FUS

§2 Method Details

2.1 Cell Culture

2.1.1. Cell culture

HEK293 cells (obtained from ATCC) were incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and Penicillin/Streptomycin (PS, Thermo Fisher Scientific) at 37℃, 5% CO_2. Generated each split-luciferase-FUS stably expressing HEK293 cells were incubated in DMEM with 10% FBS and 500 ng/mL Hygromycin B (Wako) at 37℃, 5% CO_2.

2.1.2. cDNA

For the generation of cDNAs expressing split-NanoLuc luciferase (LgBiT:17.6kDa, SmBiT: 11 amino acids, Promega) tagged human FUS proteins (LgBiT FUS wt/pcDNA3.1(+), LgBiT FUS P525L/pcDNA3.1(+), LgBiT FUS allS/pcDNA3.1(+), LgBiT FUS RA213-359/pcDNA3.1(+), LgBiT FUS RA370-491/pcDNA3.1(+), LgBiT FUS RA495-526/pcDNA3.1(+), LgBiT FUS RA495-503/pcDNA3.1(+), LgBiT FUS RA514-518/pcDNA3.1(+), LgBiT FUS RA521-524/pcDNA3.1(+), SmBiT FUS wt/pcDNA3.1(+), SmBiT FUS P525L/pcDNA3.1(+), SmBiT allS FUS/pcDNA3.1(+), SmBiT FUS RA213-359/pcDNA3.1(+), SmBiT FUS RA370-491/pcDNA3.1(+), SmBiT FUS RA495-526/pcDNA3.1(+), SmBiT FUS RA495-503/pcDNA3.1(+), SmBiT FUS RA514-518/pcDNA3.1(+) and SmBiT FUS RA521-524/pcDNA3.1(+)), FUS wt, FUS P525L, or allS FUS cDNA was amplified from VENUS-FUS wt, P525L or allS-FUS/pcDNA 3.1 (+) (Matsumoto et.al., 2018) by the PCR using oligonucleotides pairs (FUS wt, P525L: 5'-AAAAACTCGAGCGGCGGTATGGCCTC-3', allS FUS: 5'-AAAAACTCGAGCGGTATGCCTCAAACG) and (5'-AAAAATCTAGATTAATACAGCCTCTCTCCCTG-3'). *XhoI* and *XbaI* restriction site fused FUS RA213-359, RA370-491, RA495-526, RA495-503, RA514-518 or RA521-524 cDNA was also synthesized by Eurofin genomics. Their sequence were shown in following pages. These cDNAs were inserted between *XhoI* and *XbaI* sites of LgBiT vector or SmBiT vector (pBiT1.1-N[TK/LgBiT], pBiT2.1[TK/SmBiT], Promega). Then, these cDNAs encoding LgBiT-FUS and SmBT FUS were cut with both *HindIII* and *XbaI* and substituted between *XhoI* and *XbaI* sites of Venus-FUS/pcDNA3.1(+). For the generation of CRY2-FUS, CRY2olig-mCherry cDNA was purchased from Addgene (60032). Oligonucleotides encoding CRY2olig-mCherry protein was amplified from CRY2olig-mCherry cDNA by PCR using primer pairs (5'-AAAAAGGTACCACCATGAAGATGGACAAAAAGAC-3') and (5'-AAAAAGGTACCGGCTTGTACAGCTGCTCGTCC-3') and inserted at KpnI site of FUS/pcDNA 3.1 (+).

2.1.3 Bimolecular-luciferase complementation (BiLC) assay

cDNAs were transiently transfected into HEK293 cells using FuGENE6 transfection reagent (Promega) by the manufacture's protocol. In brief, $1.0x10^5$ cells/mL were seeded on 12-well, 24-well (IWAKI) or 96-well white plates for detecting luminsescence (Sumitomo bakelite). 6 µL of FuGENE6 is diluted into 92 µL of DMEM and incubated for 5 min. 200 ng of firefly luciferase (pGL4.54 [luc2/TK] (Promega)) was also added to the reagent. After 10 min incubation, 1 µg of LgBiT-FUS and 1 µg of SmBiT-FUS cDNAs were mixed with the cocktail, incubated for 30 minutes and 10 µL of the solution was added to each well. NanoLuc luminescence was measured at 24 hours after the transfection. After the measurement, ONE-Glo EX Reagent (Promega) was added to the medium and firefly luciferase was measured after another hour incubation.

2.1.4 Generation of LgBiT-FUS or SmBiT-FUS stably expressing HEK293 cells

LgBiT-FUS wt/pcDNA3.1(+), LgBiT-FUS P525L/pcDNA3.1(+), LgBiT-FUS RA213-359/pcDNA3.1(+), LgBiT-FUS RA370-491/pcDNA3.1(+), LgBiT-FUS RA495-503/pcDNA3.1(+), LgBiT-FUS RA513-518/pcDNA3.1(+), LgBiT-FUS RA521-524/pcDNA3.1(+), SmBiT-FUS wt/pcDNA3.1(+), SmBiT-FUS P525L/pcDNA3.1(+), SmBiT-FUS RA213-359/pcDNA3.1(+), SmBiT-FUS RA370-491/pcDNA3.1(+), SmBiT-FUS RA495-503/pcDNA3.1(+), SmBiT-FUS RA514-518/pcDNA3.1(+) or SmBiT-FUS RA521-524/pcDNA3.1(+) were transfected into HEK293 cells with FuGENE6. After 1-day incubation, cells were seeded on 10 cm dish. Culture media were changed with DMEM (+10% FBS and 500 ng/mL Hygromycin B) every 5 days. After 10 days, transfected cells were re-seeded on 96-well plate with sequential dilution and monoclonal stably expressing cells were obtained. Protein expression levels were checked by the western blotting.

2.1.5 Co-culture LgBiT-FUS, SmBiT-FUS stable cells (FUS transmission sensor cells)

$1.0x10^5$ cells/mL of monoclonal stably expressing cells of LgBiT-FUS or SmBiT-FUS were seeded together on 96-well white plates and incubated for a day. Their conditioned media were changed and 25 µL of substrate solution (N2014, Promega) was added to them. After 20 minutes incubation, the luminescence was measured by GloMax ® Navigator with Dual Injectors (GM2010, Promega).

2.1.6. FUS Uptake assay using SmBiT-FUS stably expressing cells

$1.0x10^5$ cells of Sm-FUS staby expressing cells were seeded on 96-well white plate. After 1-day incubation, culture medium was changed with that of Lg-FUS stably expressing cells. They were incubated for another day, and measured luminescence in their supernatant or cells.

2.1.7 Pharmacological studies using FUS transmission sensor cells

FUS transmission sensor cells were seeded at $1.0x10^5$ cells/mL on 96-well white plate or 12-well plate.

24 hours later, cells were treated with 0-30 μM of adenosine-2',3'-dialdehyde (AdOx) diluted in dimethyl sulfoxide (DMSO). Final concentration of DMSO was 0.1% of each culture medium. After 1-day incubation, luminescence in the cells were measured. For the analyses of arginine methylation of FUS, cells were collected and analyzed the level of unmethylated arginine FUS (UMA-FUS), monomethylated arginine FUS (MMA-FUS), or demethylated arginine FUS (ADMA-FUS) by the immunoblotting.

2.1.8 Transfection of PRMT1

cDNA of pcDNA3-HA-human PRMT1 variant 1 was given from Dr. Fukamizu. 1 μg of cDNA was mixed with 3 μL of FuGENE6 and 96 μL of DMEM. After 20 minutes incubation, cDNA/FuGENE6 cocktail was added to FUS transmission sensor cells on 96-well plate or 12-well plate.

2.1.9 TCA precipitation and immunoprecipitation for the protein concentration

HEK293 cells or LgBiT-FUS stably expressing cells seeded on 15-cm dish were incubated for 3 days at 100% confluency. Culture media were collected and centrifuged at 2,000 xg for 10 minutes and the supernatant was collected.

For Trichrolo Acetic Acid (TCA Wako) precipitation, the supernatant was mixed with 20% of TCA, incubated on ice for 30 minutes, and centrifuged at 15,000 xg for 10 minutes. The pellet was washed with 5 mL acetone and centrifuged at 15,000 xg for 10 minutes again. Finally, the pellet were re-solubilized in a 2xSDS sample buffer and boiled at 95 ℃ for 10 minutes.

For immunoprecipitation, the supernatant was mixed with magnetic beads (Pierce ™Protein A/G Magnetic Agarose beads, pre-washed with PBS twice) and incubated with 10 μg/mL of FUS antibody (A300-293A) at room temperature for 1 hour. The magnetic beads were separated from the solution by magnetic field, washed with PBS twice, and eluted in a 2xSDS sample buffer.

2.1.10 Immunocytochemistry

Cells cultured on coverslips were fixed with 4 % parafolmaldehyde (PFA, TAAB Laboratories) in PBS (PFA/PBS) at room temperature for 30 minutes, washed with TBS (50 mM Tris, 150 mM NaCl, pH=7.6) and blocked with blocking buffer (10 % calf serum, 0.1% Triton X-100(Wako) and 0.1 % NaN_2 in PBS) for 30 min. 1st antibodies were reacted on them at 4 ℃ overnight or at room temperature for 3 hours, and washed with TBS for 5 min 3 times. Fluorescent tagged 2nd antibodies were reacted on them at room temperature for 2 hours, washed with TBS for 5 min 3 times, and mounted with PermaFluor mounting (Thermo Fisher Scientific). The fluorescent images were observed on a confocal laser scanning microscope (FV3000, Olympus).

2.1.11 Immunoblotting

Cultured cells were harvested with sample buffer (80 mM Tris-HCL, 2 % SDS (Nacalai tesque), 15 % glycerol (Kanto Chemical) pH=6.8, 100 μL) and sonicated on ice. (Sonifier 250/BRANSON). The supernatant was collected after centrifugation at 20,000 xg for 15 min at 4℃. The protein quantitation was performed with BCA protein assay kit (Takara Bio). Samples were boiled at 95 ℃ for 10 minutes with 1% (V/V) of and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The samples were electrophoresed in 10 %, 12.5 %, or 15 % of acrylamide gels with molecular marker (Precision Plus Protein Standard, Bio-Rad). Gels were transferred to polyvinylidene fluoride (PVDF) membrane (Merck Milllipore). Membranes were blocked with TS-Tween (TBS including 0.1 % Tween 20 (Kanto Chemical) and 5 % skim milk (DIFCO)) for 30 minutes, and incubated with 1st antibody in TS-Tween at room temperature for 3 hours or at 4 ℃ over night. Then, membranes were washed with TS-Tween for 5 minutes 3 times and incubated with 2nd antibody at room temperature for 1 hour. After wash with TS-Tween for 5 minutes 6 times, membranes were developed using immunostar (Wako) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized by LAS-4000 mini (GE healthcare). The intensity of the bands was analyzed by Image Gage Software (GE healthcare).

2.1.12 Proximity Ligation Assay

To detect the amino-terminus - amino-terminus interaction (cis-type interaction) of FUS proteins, CL0190 (mouse, monoclonal, abcam) and JJ09-31 (rabbit, monoclonal, Thermo Fisher Scientific) were used. To detect trans the amino-terminus - carboxy-terminus interaction (trans-type interaction) of FUS proteins, CL0190 (mouse, monoclonal, abcam) and BLR023E (rabbit, monoclonal, abcam) were used. After blocking, fixed cells on cover slips were incubated with 2 antibodies at 37 ℃ for 3 hours, washed with PBS for 5 minutes 3 times. Next, cells were incubated with two 2nd antibodies conjugated with oligonucleotide (Duolink, PLA Anti-Mouse PLUS, PLA Anti-Rabbit MINUS, Sigma Aldrich) at 37 ℃ for 30 minutes and washed with wash buffer A for 5 minutes 3 times. The cells were incubated with 40 μL of ligase diluted in ligase buffer for 60 minutes, washed with wash buffer A for 5 minutes 3 times. The cells were incubated with 40 μL of polymerase diluted in polymerization buffer for 100 minutes, and washed with wash buffer B for 5minutes twice. Cells were incubated with DRAQ5 (1:3000) diluted in wash buffer B for 30 minutes, washed with wash buffer B for 5 minutes twice and mounted on slide glasses. The fluorescent images were observed on a confocal laser scanning microscope.

2.1.13 CRY2-FUS experiments

The CRY2-mCherry-FUS plasmid was transfected to HEK293 cells by FuGENE6 according to the manufacture's protocol. The cells were incubated at 37 ℃ without any light for a day and stimulated by LED blue light in a CO_2 incubator. The distance between LED and the cells were 5 cm. After 1-

day incubation, cells were collected in a dark room for further immunocytochemical experiments. No stimulated cells were incubated covered with aluminum foil to avoid the light.

2.1.14. Primary cultured neurons

Primary cultured neurons were taken from fatal F344 inbred rats at 15 days and collected in Dulbecco's PBS (DPBS). 12-well plates or coverslips were coated by 1 mg/mL of poly-D-Lysin (PDL) for over-night. PDL stock (1 mg/mL) was diluted in desterilized water and filtrated through 0.22 μm once and washed with PBS twice. A pregnant female rat was sacrificed and opened the belly and taken the entire uterine bag containing embryos. Embryos were put on a 10 cm dish containing cold PBS on ice. Heads were cut by forceps and put into a new 35 mm dish containing cold PBS on ice. Skin and skull were discarded and blood vessels were got rid of the cortices. Cortical layers were taken and put into cold PBS. Then, PBS was removed and Neurobasal (Thermo Fisher Scientific) +FBS+PS+Glutamax (1/100) was added. Cortices were dissociated mechanically by pipetting 20 times with P1000 and then 20 times with P200, diluted to Neurobasal+FBS+PS+Glutamax (1/100) and filtrated through cell strainer (100-μm pore). After cell counts, 0.4 x10^6 cells/500μL was seeded on PDL-coated coverslips in a 24-well plate or 0.8x10^6 cells/1.0mL was in a 12-well plate. On the next day (DIV1), half of the media was removed and replaced it with Neurobasal (PS+) +4% B27+ 1% Glutamax (1/100) so that the final concentration would be 2% B27 and 1% Glutamax. On the DIV5, half of the media was removed and replaced it with Neurobasal (PS+) +4% B27 +1% Glutamax (1/100) +2 μM AraC. 50 % of the media was removed and replaced with Neurobasal (PS+) +4% B27 +1% Glutamax (1/100) without AraC every 3 or 4 days after DIV5. AAV9-FUS was infected on DIV4 and AdOx (0, 10 μM) was treated on DIV13. Neurons were collected on DIV14 for western blot or immunocytochemistry.

2.2 Adeno Associated Virus (AAV9)-FUS

2.2.1 Generation of AAV9-FUS

cDNAs encoding Nls-dTomato, P2A-3xFLAG tag, and one of human FUS wt, FUSRA495-503, FUSP525L, and PRMT1 were inserted between *XbaI and HindIII* restriction sites of *pAAV-hSynI-GCaMP6s-P2A-nls-dTomato* cDNA (Addgene 51084). *pAAV-hSynI-nls-dTomato-P2A-3xFLAG-FUSwt (AAV9-FUSwt)*, *pAAV-hSynI-nls-dTomato-P2A-3xFLAG-FUSRA495-503 (AAV9-FUSRA495-503), pAAV-hSynI-nls-dTomato-P2A-3xFLAG-FUSP525L (AAV9-FUSP525L), pAAV-hSynI-PRMT1 (AAV9-PRMT1) or pAAV-hSynI-nls-dTomato (AAV9-dTomato)* were packed into Adeno Associated Virus vector serotype9 (AAV9), purified and titered by the PENN Vector Core at University of Pennsylvania.

2.2.2 Immunocytochemistry of primary cultured neuron

AAV9-FUS infected Primary cultured neurons at DIV14 were washed with DPBS and fixed with PFA/PBS for 30 minutes. Neurons were washed with TBS and blocked with PBS including 10 % calf serum, 0.1 % Triton X-100 and 0.1 % NaN_3 for 30 minutes, and incubated with 1st antibody overnight at 4℃ or for 3 hours at room temperature. Neurons were washed with TBS for 5 minutes 3 times, and incubated with 2nd antibody with fluorescent tag and DRAQ5 for 2 hours at room temperature. After washing with TBS for 5 minutes 3 times, neurons were mounted on slide glasses by PermaFluor mouting. The fluorescent images were observed on a confocal laser scanning microscope.

2.2.3 Infection of AAV9-FUS

$0.5x10^{11}$ genome copies/50μL of AAV9-FUS was injected into temporal vein of postnatal C57BL6/J mice. Rota-rod test was performed at 1, 3, 6 months and immunohistochemical analyses were also performed at 1, 3, 6, 16 months. All mice were incubated at 22-23 ℃ and all the mouse experiments was permitted by the animal committee of the graduate school of medicine, the University of Tokyo (医-P18-068), and performed under the rule for animal experiments of the University of Tokyo.

2.2.4 Rota-rod test

On the day before the behavioral test, mice have gotten used to a machine for 2 minutes. On the test day, mice were trained on the roller at 4 rpm for 2 minutes, and tested three times including 2 resting time over 2 minutes respectively. The time for which a mouse ran on the roller accelerating at 4-40 rpm during 300 seconds was measured. All tests have been performed in a randomized, blind setting.

2.2.5 Brain sections and immunohistochemistry

Mouse brains were fixed with PBS including 4 % paraformaldehyde for 24 hours. After fixation, brans were washed with PBS 3 times and cut in 7 parts every 2-3 mm from anterior to posterior. Brain sections were dehydrated by 70%, 90%, 99% ethanol for 2 hours sequentially, and 99 % ethanol overnight, then, soaked in xylene for 2 hours 3 times, and soaked in melted paraffin at 67 ℃ overnight. The paraffin-embedded brains were sliced in 4 μm by microtome (HM400, MICROM) and sticked onto MAS coated slide glasses (Matsunami).

For immunohistochmiestry, paraffin sections were deparaffinized in xylene for 5 minutes 3 times. They were hydrophilized by 99 %, 99 %, 80 %, 70 % ethanol for 1 minute sequentially and flowing water for 5 minutes respectively. Sections were microwaved in citrate buffer (1.8 mM anhydrous citric acid (Wako), 8.2 mM tri-Sodium citrate anhydrous (Wako), pH=6.0) for 18 minutes, cooled down at 4 ℃ and washed with flowing water. Sections were blocked with 10 % calf serum /PBS for 30 minutes and incubated with 1st antibodies at 4 ℃ over-night. Sections were washed with TBS for 5 minutes 3 times, incubated with 2nd antibodies at room temperature for 2 hours. After washing with TBS for 5 minutes 3 times again and embedded with PermaFluor mounting. The fluorescent images

were observed on a confocal laser scanning microscope.

2.2.6 hematoxylin/eosin staining

For hematoxylin/eosin (H.E.) staining, paraffin-embedded brain sections deparaffinized in xylene for 5 minutes 3 times. They were hydrophilized by 99 %, 99 %, 80 %, 70 % ethanol for 1 minute sequentially and flowing water for 5 minutes respectively. Then, sections were stained with hematoxylin solution (1 g/L hematoxylin (Wako), 50 g/L potassium ammonium sulfate (Wako), 0.2 g/L sodium periodate (Kanto Chemical), 20 % glycerol (Wako)) for 10 minutes, washed with flowing water for 10 minutes and stained with 0.2 % eosin solution (1 % eosin Y diluted with desterilized water) for 5 minutes. H.E. stained sections were dehydrated by 70 %, 80 %, 90 %, 99 %, 99 % Ethanol for 1 minutes sequentially, soaked with xylene for 2 minutes 3 times and mounted by HSR solution (Sysmex) .

2.3 *Drosophila melanogaster*

2.3.1 Generation of transgenic flies

GMR-GAL4 lines (Ellis MC *et.al.*, 1993) and balancers' lines were given from Department of Genetics, the Graduate School of Pharmaceutical Sciences, the University of Tokyo. UAS-LacZ line (Stock No.1776) was purchased from Bloomington Drosophila Stock Center. Flies were incubated at 25 ℃. Baits were given from Department of Genetics, the Graduate School of Pharmaceutical Sciences, the University of Tokyo. FUS transgenic flies (w:WT#2/CyO:GMR/MKRS.Sb, Matsumoto *et al*., 2018) and PRMT1 transgenic flies (w:+:PRMT1/MKRS.Sb) were generated by BestGene co. w:LacZ/CyO:GMR/MKRS.Sb and w:WT#2/CyO:GMR/PRMT1 flies were generated by the mating.

2.3.2 Brain sections and immunohiistochemistry

The heads of female flies were cut under CO_2 anesthesia and their beaks were removed. Fly heads were soaked with 70% ethanol for 1 minutes. After washing with PBS twice, heads were fixed with 4 % paraformaldehyde (PFA/PBS) for 2 hours. After washing with PBS again, heads were dehydrated with 70, 90, 99 % ethanol for 10 minutes and 99 % ethanol over-night, and penetrated with 35 % butanol (Wako)/65 % ethanol, 55 % butanol/45 % ethanol, 75 % butanol/25 % ethanol, 99 % butanol for 10 minutes and 99 % butanol for 1 hour. Then, they were incubated in melted paraffin at 67 ℃ and embedded in a metal tray with plastic ring. These paraffin blocks were sliced in 4 μm by a microtome, and sticked on MAS coated slide glasses.

Paraffin-embedded sections were deparaffinized in xylene for 5 minutes 3 times, and hydrophilized by 99 %, 99 %, 80 %, 70 % ethanol for 1 minute sequentially and flowing water for 5 minutes respectively. Sections were microwaved in citrate acid buffer for 18 minutes, cooled down at 4 ℃

and washed with flowing water. After soaking in TBS, sections were blocked with 10 % calf serum /PBS for 30 minutes and incubated with 1st antibodies at 4 ℃ over-night. After washing with TBS for 5 minutes 3 times, sections were incubated with 2nd antibodies at room temperature for 2 hours. After washing with TBS for 5 minutes 3 times again, sections were embedded with PermaFluor Mounting. The fluorescent images were observed on a confocal laser scanning microscope.

2.3.3 Immunoblotting

The 10 heads of male flies were cut under CO_2 and collected in 1.5 mL eppendorf tubes. 20 μL/1 head of SDS sample buffer (80mM Tris-HCl, 2 %SDS (Nacalai tesque), 15 % glycerol (Kanto Chemical), pH6.8) with 1% 2-melcaptoethanol (Wako) was added and homogenized with homogenizer. These samples were centrifuged at 4 ℃, at 21,800 xg for 10 minutes and the supernatant was collected and boiled at 95 ℃ for 10 minutes. 10 μL of each sample (equal to a half head of flies) was analyzed by SDS-PAGE.

2.4 Antibody list

2.4.1 1st antibody

anti-FLAG (Sigma-Aldrich:M2: 1:2,000)

anti-FUS（Bethyl Laboratories；A300-293A：1：2,000）

anti-FUS (Sigma-Aldrich: JJ09-31, 1:50)

anti-FUS (abcam: CL0190, 1:250)

anti-FUS (Sigma-Aldrich: BLR023E, 1:100)

anti-RFP（MBL;PM005:1:1,000）

anti-UMA-FUS (from Dr. Dorothee Dormann, 1:10)

anti-MMA-FUS (from Dr. Dorothee Dormann, 1:10)

anti-ADMA-FUS (abcam, 9G6, 1:1000)

anti-PRMT1 (abcam, ab73246, 1:1000)

anti-α-tubulin anttibody（Sigma-Aldrich; DM1A；1：2,500）

2.4.2 2nd antibody

Alexa（488）labeled rabbit IgG antibody（Thermo Fisher Scientific；1：1,000）

Alexa（488）labeled mouseIgG antibody（Thermo Fisher Scientific；1：1,000）

Alexa（488）labeled rat IgG antibody （Thermo Fisher Scientific；1：1,000）

Alexa（546）labeled mouse IgG antibody（Thermo Fisher Scientific ；1：1,000）

Alexa（546）labeled rabbit IgG antibody（Thermo Fisher Scientific ；1：1,000）

HRP labeled mouse IgG antibody（Jackson Immunoresearch；1：10,000）

HRP labeled rabbit IgG antibody（Jackson Immunoresearch；1：10,000）
HRP labeled rat IgG antibody（Jackson Immunoresearch；1：10,000）

2.5 Statistics

In this research, data were calculated by Excel（Microsoft）and tested statistically by Prism 6（Graph Pad. When the two groups were compared, unpaired Student' t test was used. When the 3 or more groups were compared, one-way ANOVA with Tukey-Kramer test or Dunnett test was used. When p-value was less than 0.05, significant difference was determined.

§ 3 Result

3.1 Visualization of interneuronal transmission of FUS *in vivo*

3.1.1. **Establishment of the model to visualize interneuronal transmission of FUS *in vivo***

AAV9-FUS (adeno-associated virus serotype 9) vector construct expresses fused protein, which sandwiches in P2A between dTomato and human FUS under human synapsin I promoter. Infected neurons express dTomato and FUS equally by self-cleavage of P2A (donor neurons). If FUS transmits interneuronally, it is expected that there are neurons expressing only FUS without dTomato (recipient neurons) (Fig. 1).

3.1.2. **Analysis of interneuronal transmission of FUS in mice using AAV9-FUS**

In order to investigate the intercellular transmission of FUS in the central nervous system, AAV9-FUS was introduced from the temporal vein of 1-day-old C57BL / 6J wild-type mice, and brain tissue was removed 1 month later. Immunohistochemical analysis was performed. Since the blood-brain barrier of newborn mice is immature, it is expected that the AAV9-FUS virus can infect cells of the central nervous system by this introduction method. As a result, neurons co-expressing dTomato and FUS were observed in the cerebral cortex at 1 month after infection (Fig. 2). Notably, in neurons close to some neurons that co-express FUS with dTomato, that express FUS in the cytoplasm but are negative for dTomato were observed (Fig. 2). This suggested that FUS can transmit between neurons.

3.1.3 **Analysis of the expression of UMA-FUS in AAV9-FUS mice**

Immunofluorescent analyses were performed at the brains of AAV9-FUS injected mice by anti-UMA-FUS, anti-FLAG, and DRAQ5 antibodies. UMA-FUS was localized in nucleus and cytoplasm of donor neurons, and cytoplasm of recipient neurons (Fig. 3). Non-infectious neurons were negative for UMA-FUS (Fig. 3). These data suggested that unmethylated FUS can be involved in the neuron-to-neuron transmission of FUS.

3.2. Investigation of the roles of UMA-FUS in the cell-to-cell transmission of FUS

3.2.1 **Establishment of the FUS transmission assay using a bimolecular luminescence complementation assay (BiLC) technique**

In order to investigate whether FUS transmits between cultured cells, I decided to

apply the method of split-luciferase complementation assay, which emits luminescence when FUS associates with each other. Therefore, I first examined self-assembly of FUS. NanoLuc is a marine luciferase derived from deep-sea luminescent shrimp (*Oplophorus gracilirostris*) and has a smaller molecular weight than the same marine luciferase, *Renilla* luciferase (NanoLuc: 19 kDa, Renilla luciferase: 36 kDa), and is more than 100 times stronger than *Renilla* luciferase (Hall *et al.*, 2012). First, cDNAs were prepared by connecting the amino-terminal fragment of NanoLuc (LgBit: 17.6 kDa) or the carboxy-terminal fragment (SmBit: 11 amino acids) to the amino-terminus of human FUS (LgBiT-FUS, SmBiT-FUS), respectively. cDNAs were transiently transfected in Human Embryonic Kidney 293 (HEK 293) cells. If FUS self-polymerizes and NanoLuc is reconstituted, it is expected to emit luminescence. As a result, it was found that HEK293 cells co-expressing LgBiT-FUS and SmBiT-FUS exhibited luminescence, and no luminescence was observed when only LgBiT-FUS or SmBiT-FUS was expressed (Fig. 4-a). In HEK293 cells co-expressing Lg-FUS and Sm-Halo fused with Halo-Tag, which is thought not to bind to FUS as a control, the degree of luminescence was weak (Fig. 4-a). These data suggested that FUS undergoes self-assembly.

3.2.2 **Establishment of the intercellular transmission model of FUS using BiLC technique**

Since it became possible to measure the self-assembly of FUS using the BiLC, HEK293 cells stably expressing LgBiT-FUS or SmBiT-FUS were generated (FUS sensor cells), and these sensor cells were co-cultured to quantify the intercellular transmission of FUS. (Fig. 4-b). LgBiT-FUS WT and SmBiT-FUS WT cells were co-cultured, and the luminescence was measured after 1, 2, 4, 6, 8, and 10 days. As a result, it was confirmed that the luminescence increased chronologically (Fig. 5). On the other hand, no increase in luminescence was measured in Sm-FUS WT monocultured cells. These results suggest that FUS can transmit intercellularly.

3.2.3 **Investigation of FUS release in the culture media**

To investigate whether FUS is released into culture media in FUS sensor cells, LgBiT-WT stable cells were incubated for 3 days and its culture media was collected. The culture media were concentrated by the TCA precipitation and analyzed by the immunoblotting. As a result, both endogenous FUS migrated at ~70 kDa and LgBit-FUS migrated at ~100 kDa were detected in the culture media (Fig. 6). This suggested that FUS was released into culture media.

3.2.4. **Investigation of roles of arginine methylation of FUS in its cell-to-cell transmission**

Next, to examine the relationship between hypomethylation of FUS and cell-to-cell transmission of FUS, FUS sensor cells were treated with 0, 1, 3, 10, 30 μM of adenosine-2',3'-dialdehyde (AdOx), which is an inhibitor for methyl transferases. By immunoblot analyses with anti-UMA-

FUS, anti-ADMA-FUS, anti-total-FUS, or anti-α-tubulin antibody, AdOx treatment caused an increase of UMA-FUS and decrease of ADMA-FUS in FUS sensor cells in a dose dependent manner, whereas the amount of total FUS was not changed (Fig. 7). Moreover, it is revealed that the level of the luminescence from FUS sensor cells were significantly increased along with the increase in the concentration of AdOx (Fig.8). This data suggested that UMA-FUS was involved in the cell-to-cell transmission of FUS.

3.2.5. **The role of arginine methylation by PRMT1 in the cell-to-cell transmission of FUS**

To further investigate whether PRMT1 methylates FUS, leading the suppression of the cell-to-cell transmission of FUS, I transfected human PRMT1 cDNA in the AdOx-treated FUS sensor cells. After 24 hours from transfection of human PRMT1 cDNA in FUS sensor cells, cells were treated with 0, 1, 3, 10, 30 μM of AdOx and incubated for another 24 hours. In the immunoblot analyses with anti-PRMT1, anti-UMA-FUS and anti-total FUS, the levels of UMA-FUS after the treatment of AdOx were decreased (Fig.9-a). Moreover, I found that overexpression of PRMT1 significantly decreased the levels of luminescence in FUS sensor cells (Fig.9-b). These data suggested that overexpression of PRMT1 methylates FUS proteins and decreases the cell-to-cell transmission of FUS.

3.2.6 **Analyses of the relationship between the level of UMA-FUS and self-assembly of FUS**

To investigate whether UMA-FUS is involved in the self-assembly of FUS, both LgBiT-FUS and SmBiT-FUS cDNAs were transiently transfected in HEK 293 cells. Transfected cells were treated with 0, 1, 3, 10, 30 μM of AdOx for 1 day and their luminescence were measured. I found that there treatment of AdOx did not change the luminescence in the cells (Fig.10), suggesting that UMA-FUS may not be involved in the self-assembly of FUS.

3.2.7 **Search for arginine residues in FUS responsible for its transmission**

Human FUS protein possesses 37 arginine residues. To determine the responsible arginine residues in FUS protein for the involvement in the cell-to-cell transmission, we generated 6 mutant FUS cDNAs in which arginine residues were replaced to alanine residues (Fig. 11). In detail, Arg213, Arg216, Arg218, Arg234, Arg242, Arg244, Arg248, Arg251, Arg259, Arg269, Arg274, and Arg328 were substituted into Ala in FUS RA213-359, Arg371, Arg372, Arg377, Arg383, Arg386, Arg388, Arg394, Arg407, Arg422, Arg441, Arg472, Arg473, Arg476, Arg481, Arg485, Arg487, and Arg491 were substituted into Ala in FUS RA370-491, Arg495, Arg498, Arg503, Arg514, Arg518, Arg521, Arg522, and Arg524 were substituted into Ala in FUS RA495-524, Arg495, Arg498 and Arg503 were substituted into Ala in FUS RA495-503, Arg514 and Arg518 were substituted into Ala in FUS RA514-518, Arg521, Arg522, and Arg524 were

substituted into Ala in FUS RA521-524.

First, I investigated whether those RA FUS mutants underwent the self-assembly by BiLC assay, and found that RA213-359 and RA495-503 mutations did not change the level of self-assembly of FUS, however, RA370-491 and RA514-518 mutations significantly reduced the level of luminescence, and RA495-526 and RA521-524 mutations significantly increased the level of luminescence (Fig. 13, S1). Because RA213-359 and RA495-503 mutations did not change the level of self-assembly of FUS, I further analyzed these 2 RA FUS mutants. Next, I measured the level of cell-to-cell transmission of mutant FUS proteins by FUS sensor cells, and found that RA213-359 and RA495-503 mutations dramatically reduced the level of cell-to-cell transmission with or without AdOx treatment (Fig. 12, S2). Because RA213-359 mutations disrupt the RNA-recognition motif of FUS, FUS RA495-503 mutant was chosen as a cell-to-cell transmission incapable mutant. In immunoblot analyses, AdOx treatment caused a significant increase in the level of UMA-FUS in FUS wt sensor cells in a dose dependent manner, whereas no UMA-FUS was detected in FUS RA495-503 sensor cells (Fig. 11). This suggested that the epitope of anti-UMA-FUS antibody may be in Arg495, 498 and 503. Together, Arg495, Arg498, Arg503 may be important in the cell-to-cell transmission of FUS.

3.2.8. **Investigation of effects of RA495-503 mutations in the interneuronal transmission of FUS in rat primary cultured neurons**

To investgate whether Arg495, 498, 503 were involved in the neuron-to-neuron transmission, AAV9-FUS RA495-503 was generated and interneuronal transmission of FUS wt or FUS RA495-503 in rat primary cultured neurons was observed. Rat primary cultured neurons taken from E15 embryo F344 inbred Rat was seeded on coverslips and infected with $1.0x10^9$ GC/mL of AAV9-FUS WT or RA495-503 at DIV5, In immunocytochemical analyses with anti-FLAG antibody, recipient neurons (FLAG(+), dTomato (-)) were observed in FUS WT, whereas they were not observed in FUS R495-503 (Fig. 14). The number of recipient neurons were 7.0 per 100 FLAG positive neurons in AAV9-FUS WT, whereas 0.0 per 100 FLAG positive neurons in AAV9-FUS RA495-503 (average of n=3). These data suggested that RA495-503 mutant FUS was incapable for the interneuronal transmission.

3.3 **Oligomerization and transmission of FUS**

3.3.1 **Two-oligomers hypothesis of FUS**

Next, I focused on the oligomerization of FUS proteiin. In BiLC assay in this study, cis-type oligomers based on a π-ring conjugated system of tyrosine on the amino-terminal side

were measured (Fig. 15) and the level of cis-type self-assembly of FUS was not affected by the AdOx treatment. In recent years, it has been reported FUS also forms trans-type oligomers based on the cation-π interaction with the π-electron conjugated system of tyrosine residues on the amino-terminal side to arginine residues in the carboxy-terminal RGG region (Fig. 15). It is not clear whether UMA-FUS affect the level of trans-type oligomers of FUS. To this end, I visualized the trans-type FUS oligomers using a Proximity ligation assay (PLA) method, which can visualize the proximity of two molecules.

3.3.2 **Visualization of trans-type oligomers by Proximity Ligation Assay**

In the PLA method primary antibodies from different animal species and oligonucleotide-labeled secondary antibodies were used. If the two antigens are close to each other within 40 nm, the oligonucleotides are ligated together, amplified by the polymerase and visualized by the fluorescent PLA probe (Fig. 17). In the PLA method, trans-type FUS oligomers were visualize mainly in the nucleus of HEK293 cells, on the other hand, knockdown of FUS gene significantly reduced the number of PLA dots. Single antibody did not exhibit any PLA dots in HEK293 cells. Thus, it was considered that the trans-type FUS oligomers in which the amine terminal and the carboxy end are close to each other can be visualized by the PLA method (Fig.17). Interestingly, treatment of 10 μM of AdOx significantly increased the number of PLA dots (Fig.18), suggesting that AdOx increases the level of trans-type interaction in HEK293 cells. Furthermore, the number of PLA dots in FUS RA495-503 stably expressing cells were significantly lesser than that in FUS wt stably expressing cells (Fig.19). This data suggested that the RA495-503 mutation may inhibit the formation of trans-type oligomers.

3.4. Investigation of the effects of familial ALS mutation of FUS in the oligomerization and transmission of FUS

3.4.1 **P525L mutation increases the oligomerlization of FUS and reduces the level of cell-to-cell transmission of FUS**

To investigate the roles of familial ALS-linked mutations of FUS in its oligomerization and transmission, I examined whether P525L mutation affect the self-assembly of FUS by BiLC assay, cell-to-cell transmission of FUS by FUS transmission sensor cells. I found that P525L mutations significantly increased the level of luminescence, and that P525L mutation dramatically reduced the level of cell-to-cell transmission with or without AdOx treatment (Fig.20). In addition, in the PLA method, the number of PLA dots in FUS P525L stably expressing cells were significantly lesser than that in FUS wt stably expressing cells (Fig. 20). These data suggested that the P525L mutation may inhibit the formation of trans-type FUS oligomers, causing the suppression of the cell-to-cell transmission of FUS.

3.5 **Investigation of the roles of FUS oligomers in the arginine unmethylation of FUS**

3.5.1 **Oligomerization of CRY2-FUS induces UMA-FUS**

To investigate whether oligomerization of FUS can trigger hypomethylation of FUS, I introduced CRY2olig system. CRY2olig sequence was developed from *Arabidopsis* photoreceptor cryptochrome 2 as an optogenetic module, allowing the rapid and reversible protein oligomerization under the blue light stimulation. I generated cDNA encoding CRY2olig-mCherry tagged FUS protein (CRY2-FUS) and transfected it to HEK293 cells (Fig. 21). In the stimulation of blue light for 24 hours, CRY2-FUS was accumulated in cytoplasm where UMA-FUS was positive, whereas there was no UMA-FUS-positive structures without any light stimulation (Fig.22). These data suggest that the oligomerization by FUS in cytoplasm may induce the arginine unmethylation of FUS.

3.6 **Investigation of effects of arginine methylation in the neuronal toxicity induced by FUS**

3.6.1 **Overexpression of PRMT1 and FUS in the retinal neurons of *Drosophila Melanogaster***

In this study, I examined the effects of arginine methylation of FUS in the cell-to-cell transmission of FUS, however it is not clear how arginine methylation of FUS affects the neuronal toxicity induced by FUS. To this end, I utilized FUS transgenic (tg) flies which exhibited progressive neuronal death in the retina (Matsumoto et al., 2018). I generated double transgenic flies overexpressing both FUS and PRMT1 in retinal neurons under GMR driver (Fig.S3) and analyzed them by histochemistry and immunohistochemistry. H.E. staining revealed that 5-day-old FUS tg flies exhibited severe retinal degeneration, vacuolization, thinning of the thickness of the retina, and overexpression o PRMT1 in FUS tg flies also exhibited severe retinal degeneration (Fig. S4). There are no significant difference in the retinal thickness between FUS single tg flies and FUS and PRMT1 double tg flies (Fig.S4).

Finally, the level of UMA-FUS and ADMA-FUS in the heads of FUS single tg flies and FUS and PRMT1 double tg flies were analyzed by immunoblotting. In FUS and PRMT1 double tg flies the level of ADMA-FUS or total FUS was increased were increased, suggesting that PRMT1 may stabilize the FUS protein through the arginine dimethylation of FUS in the heads of flies (FigS5).

§ 4 summary and discussion

The facts revealed in our study is summarized below.

- FUS was transmitted between neurons in the AAV9-FUS mouse brains
- In the recipient neurons of the AAV9-FUS mouse brain, transmitted FUS was exclusively localized in the cytoplasm.
- In the recipient neurons of the AAV9-FUS mouse brain, UMA-FUS was also localized in the cytoplasm.
- In FUS transmission sensor cells, FUS was transmitted between cells
- In FUS transmission sensor cells, the intercellular transmission of FUS was significantly increased by the treatment of AdOx.
- Treatment of AdOx did not affect the self-assembly of cis-type FUS oligomers.
- In FUS transmission sensor cells, arginine residues 495, 498, 503 was identified to be involved in the intercellular transmission of FUS.
- RA495-503 mutations suppressed interneuronal transmission of FUS in rat primary cultured neurons.
- FALS-linked P525L mutation increased the self-assembly of cis-type FUS oligomers.
- Using the PLA method, trans-type FUS oligomers can be visualized in the nucleus of HEK293 cells.
- AdOx treatment significantly increased the number of trans-type FUS oligomers, whereas RA495-503 mutation significantly decreased the number of trans-type FUS oligomers.
- UMA-FUS can be increased by the oligomerization of FUS.

・ Overexpression of PRMT1 did not change the neuronal toxicity induced by FUS in the retina of *Drosophila melanogaster*

4.1. **transmission of FUS**

There were no reports about transmission of FUS in the patient with FUS proteinopathy until now. In this study, I developed two experimental systems, FUS transmission sensor cells and AAV9-FUS infected mouse model, to investigate the intercellular transmission of FUS. In both systems, the cell-to-cell transmission of FUS was occurred. In the brain of AAV9-FUS mice, it was clarified that FUS transmitted from neurons to adjacent neurons, on the other hand, there was no evidence that FUS transmitted along perforant pathway from entorhinal cortex to hippocampal dentate gyrus (Watanabe, Master's thesis, 2018). These results suggest that FUS may be transmitted between cell bodies rather than through synaptic pathways. So far, among the proteins related to the pathology of ALS and FTLD, there is a report that TDP-43 has been released from axon terminals by experiments in which primary cultured neurons were separated into cell bodies and axon terminals using a microfluidic device culture system. (Feiler *et al.*, 2015), suggesting the involvement of synaptic transmission pathways. TDP-43 and FUS have strikingly structural and functional similarities, implicating the existence of a common mechanism underlying TDP-43 and FUS proteinopathies. However, FUS may be transmitted between neurons through different manner from TDP-43. To elucidate it, further studies of FUS transmission using a microfluidic device are needed. It is also necessary to elucidate the cellular and molecular mechanisms which involve interneuronal transmission of FUS in future.

It is known that TDP-43 in which Ser409/410 is phosphorylated TDP-43 is specifically accumulated in cytoplasmic inclusion bodies, and using anti-TDP-43 Ser409/410 phosphorylation-specific antibody, the analysis of TDP-43 spreading in the brains or spinal cords has been performed (Brettschneider *et al*, 2013). FUS is known to form cytoplasmic inclusion bodies in ALS or FTLD patients (Kwiatokowski *et al.*, 2009, Mackenzie *et al.*, 2010). It has been also reported that the FUS pathology spreads to the adjacent area as the pathological condition exacerbated (Lee *et al.* 2016). I think that it is possible to analyze the time-dependent spread of FUS pathology in the cerebrum of FTLD patients using anti-UMA-FUS specific antibody as well.

4.2 **demethylation and methylation of FUS**

In our study, I used AAV expression system to visualize the interneuronal transmission

of FUS to clarify whether FUS transmits between neurons and UMA-FUS exists both in donor and recipient neurons. As a result, I found UMA-FUS was exclusively localized in cytoplasm of recipient neurons. Despite FUS protein is mainly localized in the nucleus, FUS-positive cytoplasmic inclusions are pathological hallmark of FUS proteinopathies. The similarity of FUS localization between AAV9-FUS infected mice and FUS proteinopathies is very interesting. In this study, I found AdOx treatment increased the level of UMA-FUS, trans-type FUS oligomers and cell-to-cell transmission of FUS. It may be possible that transmitted trans-type UMA-FUS oligomers in the recipient neurons suppressed its binding with transportin 1/2 to enter into nucleus, leading the cytoplasmic accumulation of UMA-FUS.

PRMT1 is thought to contribute to as much as 85% of all cellular PRMT activity in mammalian cells (Tang *et al*., 2000), and PRMT1 is considered as a candidate to methylate FUS as I have shown in the result part. PRMT1 is belong to the type I PRMTs which catalyze monomethylated arginine and asymmetric dimethylarginine (ADMA), on the other hand, type II PRMTs catalyze monomethylated arginine and symmetric dimethylarginine (SDMA). I confirmed the existence of ADMA-FUS in HEK293 cells by anti-AMDA-FUS specific antibody, this also supports that PRMT1 methylates FUS protein. To further confirm which type I PRMTs is responsible for the methylation of FUS in the neurons, I need knockdown/knockout experiments of PRMTs using primary cultured neurons.

It is unclear why UMA-FUS specifically deposits in the neurons of FTLD-FUS patients. One possibility is the dysfunction of PRMT1 or other PRMTs to induce UMA-FUS. Arginine methylation is mainly observed on several RNA-binding proteins and histones, and involved in the regulation of DNA/RNA processing or epigenetic regulation (Nicholson *et al*., 2009). There is a report that mutation in *PRMT1* gene was not observed in FTLD-FUS patients (Ravenscroft *et al.*, 2013). And there is no report about PRMT1 expression in the brains of FTLD. Another possibility is the accumulation of FUS proteins. In my CRY2-FUS experiments, it is revealed that oligomerization can be a trigger of increasing UMA-FUS. It is still unknown which is happened the first, unmethylation or oligomerization. It is interesting what the first trigger of unmethylation of FUS is.

4.3 **molecular species of FUS for the interneuronal transmission**

In this study, I found that AdOx treatment significantly increased the level of UMA-FUS, the formation of trans-type FUS oligomers, and the cell-to-cell transmission. In addition, I found that the RA495-503 mutation of FUS significantly decreased the level of trans-type oligomer formation of FUS, and reduced the cell-to-cell or interneuronal transmission. These data strongly suggest that UMA-FUS and/or trans-type FUS oligomers may be the responsible

molecular species for the cell-to-cell transmission of FUS.

Here, endogenous FUS or LgBiT-FUS was detected in the culture media of HEK293 cells or LgBiT-FUS stable cells. These data suggest that FUS is released. Because the bands corresponding to the endogenous FUS or LgBiT-FUS in culture media migrated at the similar size as a full-length endogenous FUS or LgBiT-FUS, respectively, in cell lysate in SDS-PAGE, it seemed full-length FUS may be released. In the FUS transmisson sensor cells, I measured the luminescence emitted from reconstituted NanoLuc by the self-assembly of FUS, so it is expected that FUS formed dimers or multimers in the cells to which it was transmitted or in the culture supernatant. To further elucidate the detailed molecular species of FUS for the cell-to-cell transmission, I would like to conduct biochemical experiments using a gel filtration column to examine what size FUS is released and incorporated.

In the primary cultured neuron infected AAV9-FUS, the transmitted FUS was mainly localized in the nucleus, on the other hand, in the recipient neurons in the AAV9-FUS-infected mice, the transmitted FUS was observed to be localized in the cytoplasm. To understand the difference of the subcellular localization of FUS between these models, I need further studies to determine the molecule species of FUS for the interneuronal transmission in both models. This will give us a hint in that the formation of FUS positive cytoplasmic inclusions in the brains of FUS proteinopathies.

4.4 **two type oligomers of FUS**

Recently, it has been reported that the cations of arginine residues at the carboxy-terminal RGG domain interacts with the aromatic rings of tyrosine residue at the amino terminal LC domain in a cation-π interaction, resulting in liquid-liquid phase separation of FUS (Qamar *et al*., 2018). In this study, PLA analyses showed the existence of trans-type FUS oligomers in HEK293 cells presumably by the interaction between amino terminal LC domain and carboxy-terminal RGG domain. Upon my findings that the increases of the levels of UMA-FUS and trans-type FUS oligomers enhanced the cell-to-cell transmission of FUS, these results suggest that the intermolecular binding of FUS by cation-π interaction between carboxy-terminal unmethylated arginine residues such as Arg495, Arg498, and Arg503 and amino-terminal tyrosine residues in LC domain, may be involved in the liquid-liquid phase separation and intercellular transmission of FUS.

Previously, my research group identified that the self-assembly of FUS through the LC domain is necessary for the neurodegeneration induced by FUS in the retina of *Drosophila melanogaster* (Matsumoto, Watanabe *et al*., 2018). *In vitro* biochemical studies revealed that LC domain of FUS is sufficient for the fibril formation (Kato *et al*., 2012; Han *et al*., 2012; Hughes

et al., 2018). In this study, using BiLC technique that can measure the cis-type FUS oligomers by the interaction through its LC domain, I found that fALS-linked P525L mutation significantly increased the formation of cis-type FUS oligomers independent from AdOx-mediated arginine unmethylation, whereas P525L mutation significantly decreased the cell-to-cell transmission. I also found that overexpression of PRMT1 in the retina of FUS transgenic flies did not change the neurotoxicity. These data suggest that cis-type FUS oligomers through its LC-domain may be involved in the fibril formation and neurotoxicity independent from unmethylation of arginine residues in RGG domain and that trans-type FUS oligomers may not be involved in the neurotoxicity. In the future, using FUS transmission sensor cells and PLA method, I will investigate the relationship between trans-type FUS oligomers and cis-type FUS oligomers in more detail. I would like to elucidate the relationship between interneuronal transmission and neuronal damage occurred with FUS by using the AAV9-FUS experimental system to clarify the role of FUS interneuronal transmission in pathological progression.

4.5 **Involvement of Arg495, Arg498, and Arg503 of FUS in the cell-to-cell transmission**

In this study, it was found that the substitution of Arg495, Arg498, and Arg503 to Ala of FUS significantly reduced the intercellular or interneuronal transmission. Arginine residue has positive charge on nitrogen of its side chain, and the positive charge is hidden by methyl groups. When arginine is replaced to alanine, the positive charge is diminished. I also found that the RA495-503 mutation significantly decreased in the level of trans-type oligomer formation of FUS, suggesting that these Arg495, 498, and 503 have an important role in the interaction with tyrosine residues of amino-terminus of FUS to form the trans-type FUS oligomers.

In the arginine mutation analyses, I found that FUS RA213-359 mutant also significantly decreased the cell-to-cell transmission of FUS without any changes in the ability of self-assembly as well as FUS RA495-503 mutant. Because the 213-359 region of FUS is important in the RNA-binding of FUS, I have ruled out this mutant in this study, I will further analyses of the formation of trans-type FUS oligomers and interneuronal transmission of FUS RA213-359 mutant.

I also found that FUS RA370-491 or RA514-518 mutant significantly decreased the level of self-assembly of FUS, and RA495-526 or RA521-524 significantly increased the level of self-assembly of FUS. This may suggest that these arginine residues are involved in the self-assembly of FUS, especially cis-type oligomer. It is also possible that these mutation may affect the subcellular localization of FUS in cytoplasm from in nucleus.

4.6 **difficulties in this study**

We have established the quantitative assay for oligomerization or transmission of FUS by BiLC technique. However, only cis-type oligomerization was measured by this assay, because split-luciferases (LgBiT- or SmBiT-) were fused to N-terminus of FUS. C-terminal end of FUS has nuclear localization signal, thus the fusion of split-luciferase tag to C-terminus of FUS may impede the subcellular localization of FUS into cytoplasm. I will analyze the formation of cis-type FUS oligomers by PLA method as well as trans-type FUS oligomers I have shown in study.

At this time, PLA method was utilized to detect trans-type FUS oligomers. Considering the results based on the principle of this method, fluorescent dots were observed when two molecules came close within 40 nm. Thus, it is possible to think that PLA dots in HEK293 cells using anti-N-terminal region of FUS and C-terminal region of FUS antibodies come from not only intermolecular interaction of FUS, but also innermolecular interactions of FUS. I will further analyze whether FUS forms dimers or multimers using biochemical techniques.

In the CRY2-FUS model, I have observed the UMA-FUS accumulation in HEK293 cells under blue light stimulation, however, it cannot be denied that CRY2-mcherry tag will affect some characteristics of FUS due to its fusion to N-terminal of FUS. Also, in this system, it is considered that cis-type oligomer was only produced by light stimulation. Thus, it is still unknown whether trans-type oligomers induce UMA-FUS. I will further investigate whether RA495-503 mutant affects the UMA-FUS accumulation using CRY2-FUS system.

4.7 **Strategy for therapeutic method based on cell-to-cell transmission of FUS**

In this study I have developed AAV9-FUS system to visualize the interneuronal transmission of FUS, and FUS transmission sensor cells to quantify the intercellular transmission of FUS. These methods revealed the increase of UMA-FUS can enhance the trans-type oligomerization of FUS and promote cell-to-cell transmission, and are useful for the drug discovery for FUS proteinopathies. Now, I am considering there can be 3 targets against progression of FUS pathology below.

1. Promoting the methylation of FUS
2. Inhibition of the formation of trans-type FUS oligomers
3. Decomposition of UMA-FUS in recipient neurons

Needless to say, it is still controversial for UMA-FUS to cause neurotoxicity. To investigate the effect of UMA-FUS in the neurotoxicity, *Drosophila* model expressing PRMT1 and FUS was utilized at this time. As a result, overexpression of PRMT1 did not rescue the

neurotoxicity caused by FUS, whereas the level of UMA-FUS was decreased. To further analyze the effects of UMA-FUS in the transmission and neurotoxicity of FUS, I am currently studying them using AAV9-FUS infected mouse model.

4.8 **Conclusion**

In this study, AAV9-FUS infected mice exhibited the cytoplasmic localization of UMA-FUS in the recipient neurons, indicating that the involvement of arginine unmethylation of FUS in the interneuronal transmission of FUS. Using FUS transmission sensor cells and PLA method, I found that unmethylation of arginine residues of the RGG region of FUS, especially Arg495, 498, and 50,3 induced the interaction with LC domain of FUS, inducing the cell-to-cell transmission of FUS. Furthermore, I also found that oligomerization of FUS increased the level of UMA-FUS. This vicious cycle may be involved in the development of pathology in FUS proteinopathies.

§ 5 Figures

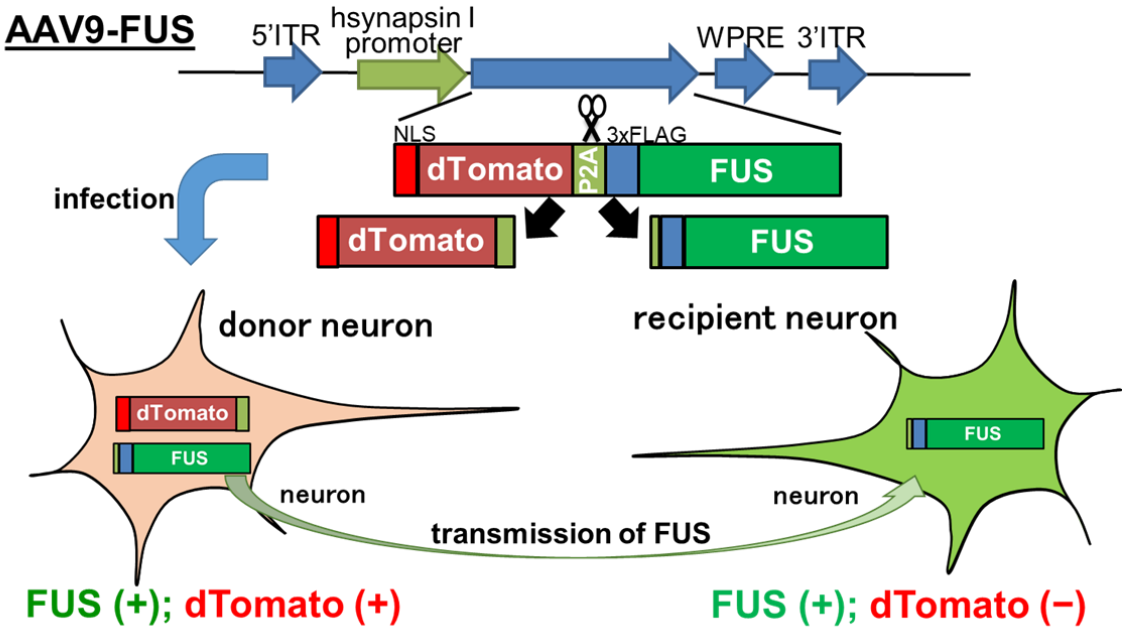


Fig.1：**Establishment of the model to visualize interneuronal transmission of FUS *in vivo***

AAV9-FUS (adeno-associated virus serotype 9) vector construct expresses fused protein, which sandwiches in P2A between dTomato and human FUS under human synapsin I promoter. Infected neurons express dTomato and FUS equally by self-cleavage of P2A (donor neurons). If FUS transmits interneuronally, it is expected that there are neurons expressing only FUS without dTomato (recipient neurons).

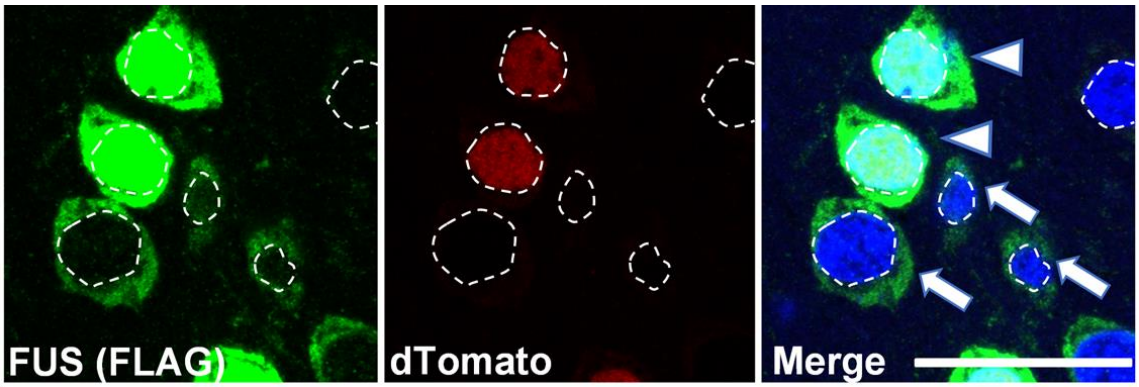


Fig.2 AAV9-FUS mice showed transmission of FUS from donor neuron to recipient neuron. FUS in the recipient neurons was exclusively localized in cytoplasm

Immunofluorescence analyses of the brains of AAV9-FUS injected mice by anti-FLAG (FUS), anti-RFP (dTomato), and DRAQ5. In donor neurons, both FUS and dTomato were positive (arrowheads). Notably, FUS-positive, dTomato-negative neurons were observed beside the donor neurons (arrows). Nuclei are indicated in white dotted lines. Scale bar is 25 μm.

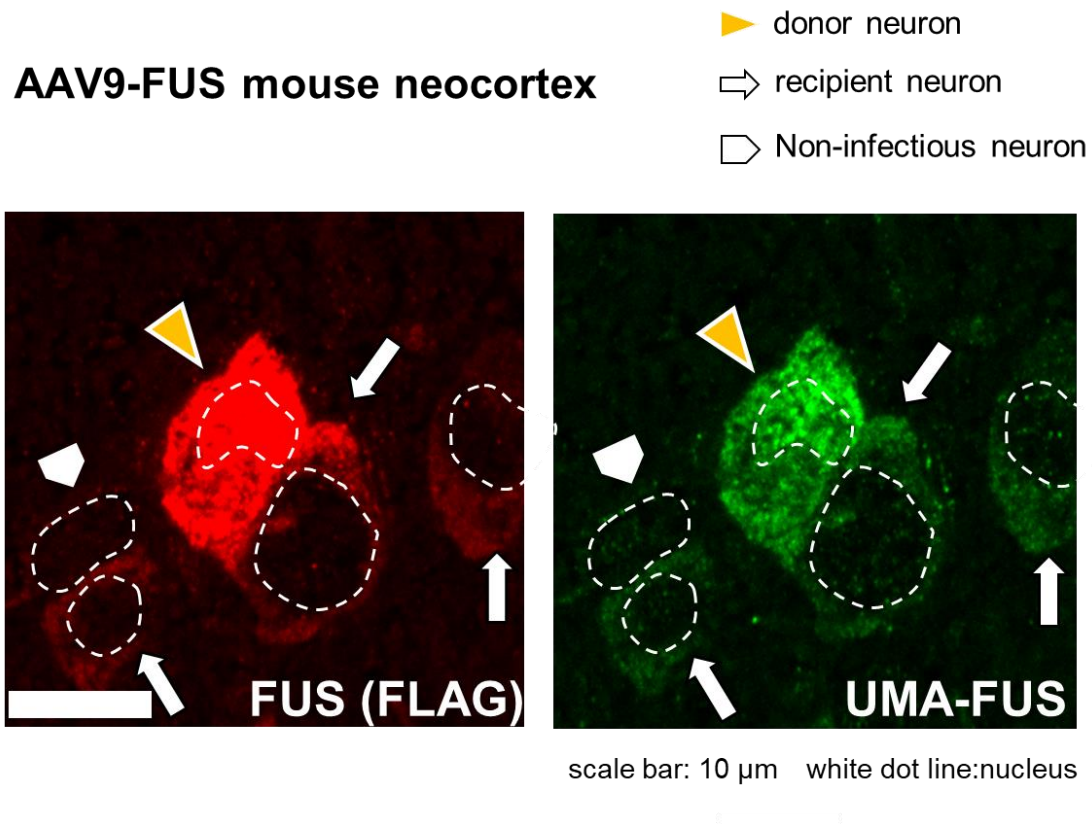


Fig.3. Unmethylated FUS was detected in nuclei and cytoplasm of donor neurons, and cytoplasm of recipient neurons

Immunofluorescent analyses were performed at the brains of AAV9-FUS injected mice by anti-UMA FUS, anti-FLAG, and DRAQ5 antibodies. Both donor (arrowheads) and recipient (arrows) neurons are positive for UMA-FUS. Non-infectious neurons (pentagons) were negative for UMA-FUS. Nuclei are indicated in white dotted lines. Scale bar is 25 μm.

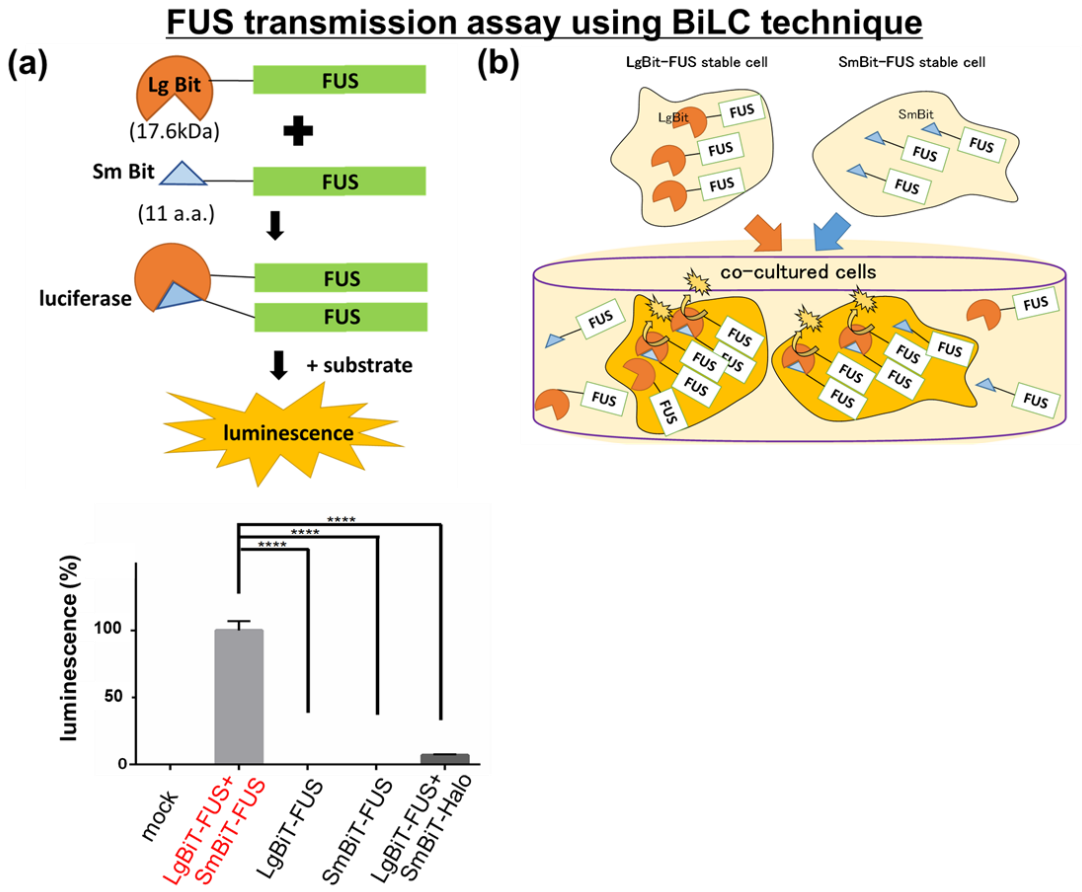


Fig.4. Establishment of the FUS transmission assay using a bimolecular luminescence complementation assay (BiLC) technique

(a) If FUS self-polymerizes and NanoLuc is reconstituted, it is expected to emit luminescence. HEK293 cells co-expressing LgBiT-FUS and SmBiT-FUS exhibited luminescence, and no luminescence was observed when only LgBiT-FUS or SmBiT-FUS was expressed (100% at Lg-FUS+Sm-FUS, N=3, One-way Anova (****) $p<0.01$) **(b)** Establishment of the intercellular transmission model of FUS using BiLC technique. If FUS is transmitted between cells stably expressing LgBiT-FUS or SmBiT-FUS (FUS sensor cells) , and these sensor cells were co-cultured to quantify the intercellular transmission of FUS.

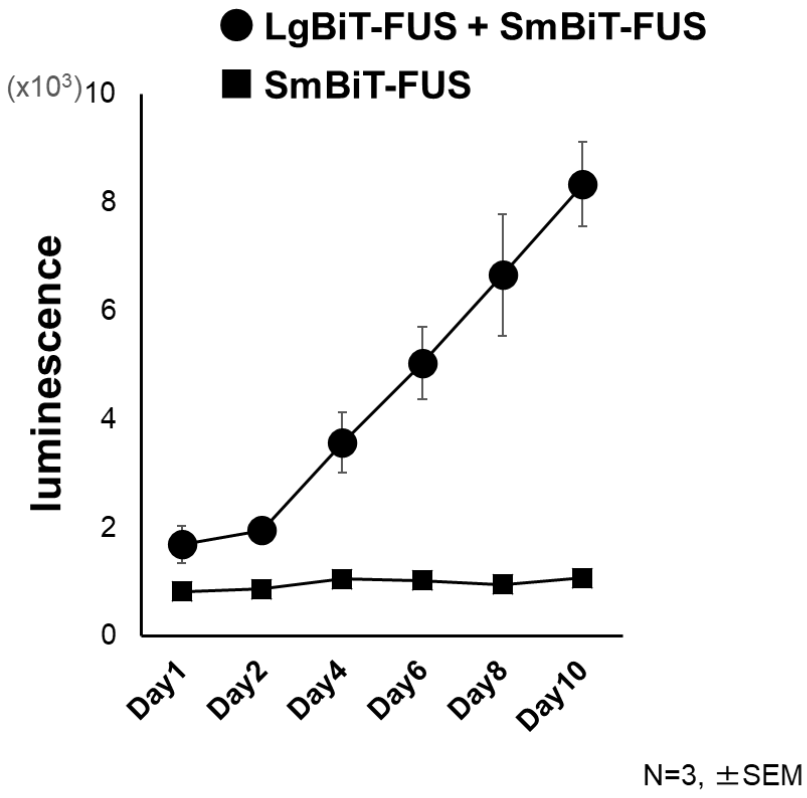


Fig.5: Luminescence in FUS sensor cells

LgBiT-FUS WT and SmBiT-FUS WT cells were co-cultured, and the luminescence was measured after 1, 2, 4, 6, 8, and 10 days. As a result, it was confirmed that the luminescence increased chronologically. On the other hand, no increase in luminescence was measured in Sm-FUS WT monocultured cells.(N=3,±SEM)

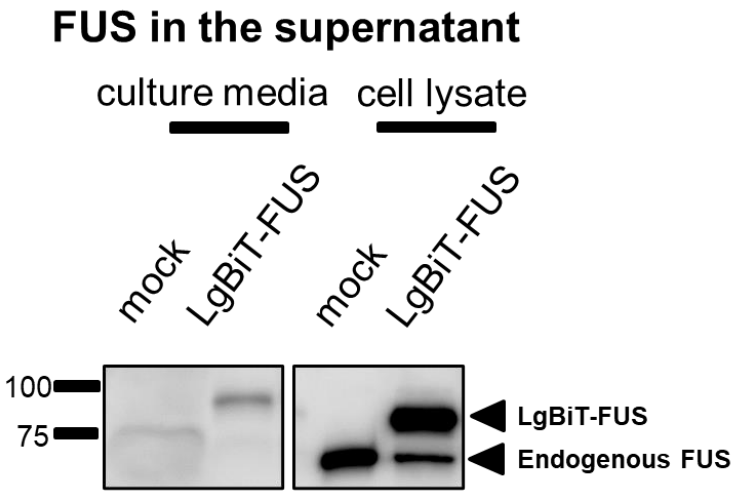


Fig.6: Investigation of FUS release in the culture media

LgBiT-WT stable cells were incubated for 3 days and its culture media was collected. The culture media were concentrated by the TCA precipitation and analyzed by the immunoblotting. Both endogenous FUS migrated at ~70 kDa and LgBit-FUS migrated at ~100 kDa were detected in the culture media.

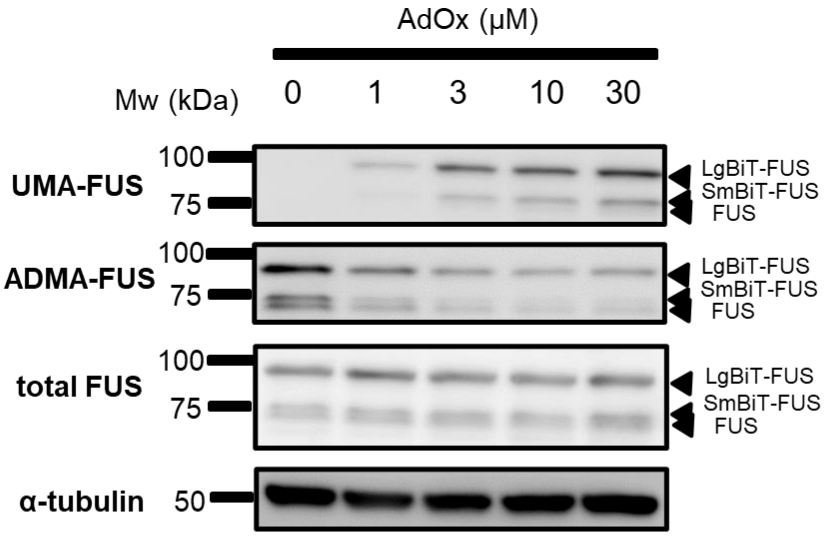


Fig.7. Investigation of arginine methylation of FUS in its cell-to-cell transmission

FUS sensor cells were treated with 0, 1, 3, 10, 30 μM of adenosine-2',3'-dialdehyde (AdOx), which is an inhibitor for methyl transferases. By immunoblot analyses with anti-UMA-FUS, anti-ADMA-FUS, anti-total-FUS, or anti-α-tubulin antibody, AdOx treatment caused an increase of UMA-FUS and decrease of ADMA-FUS in FUS sensor cells in a dose dependent manner, whereas the amount of total FUS was not changed.

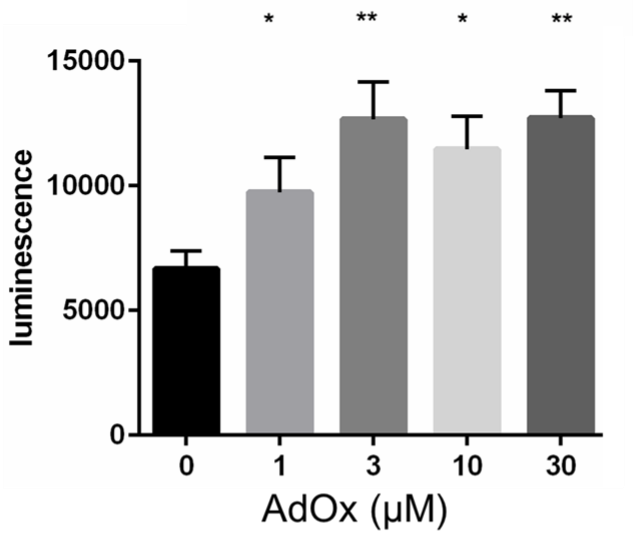


Fig.8 UMA-FUS was involved in the cell-to-cell transmission of FUS.

The level of the luminescence from FUS sensor cells were significantly increased along with the increase in the concentration of AdOx. N=3, ±SEM. One-way ANOVA post hoc Dunnett test, (**) $p<0.01$, (*) $p<0.05$

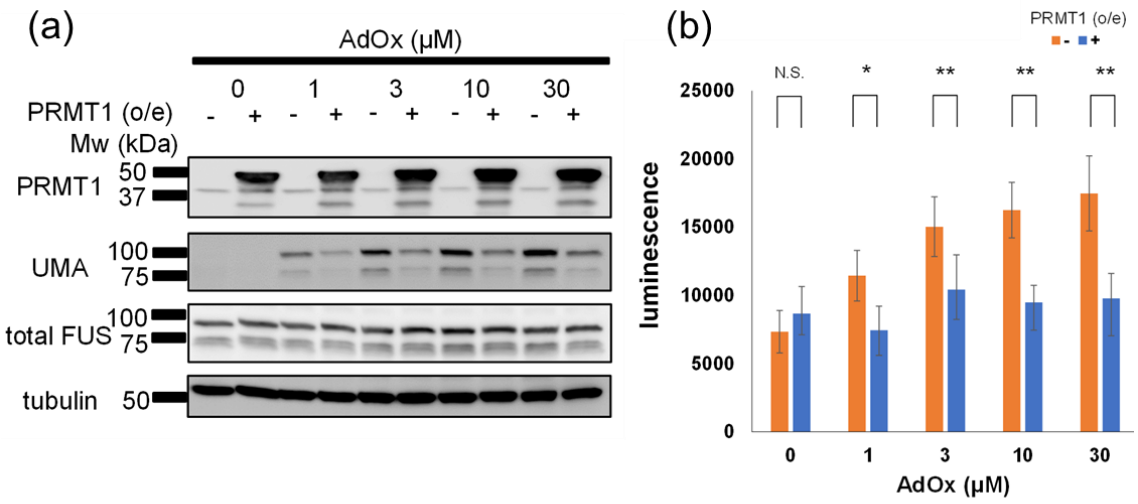


Fig.9 The role of arginine methylation by PRMT1 in the cell-to-cell transmission of FUS

(a) After 24 hours from transfection of human PRMT1 cDNA in FUS sensor cells, the cells were treated with 0, 1, 3, 10, 30 mM of AdOx and incubated for another 24 hours. In the immunoblot analyses with anti-PRMT1, anti-UMA-FUS and anti-total FUS, the levels of UMA-FUS after the treatment of AdOx were decreased.

(b) Overexpression of PRMT1 significantly decreased the levels of luminescence in FUS sensor cells. N=5, unpaired t-test, (**) $p<0.01$, (*) $p<0.05$.

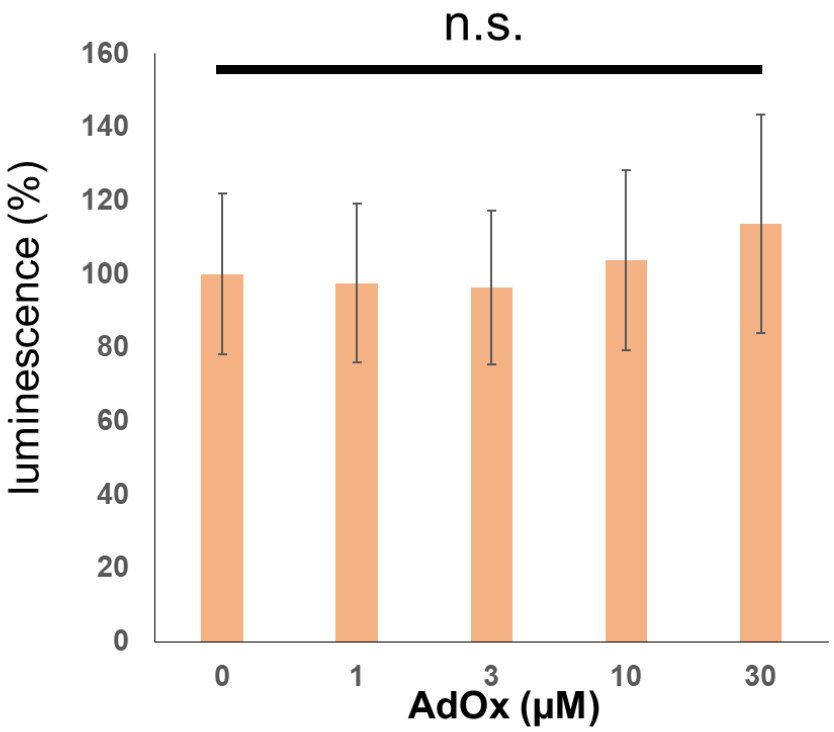


Fig.10 Analyses of the relationship between the level of UMA-FUS and self-assembly of FUS

Both LgBiT-FUS and SmBiT-FUS cDNAs were transiently transfected in HEK 293 cells. Transfected cells were treated with 0, 1, 3, 10, 30 μM of AdOx for 1 day and their luminescence were measured. There treatment of AdOx did not change the luminescence in the cells. N=5, One-way ANOVA post hoc Dunnett test, (**) $p<0.01$, (*) $p<0.05$.

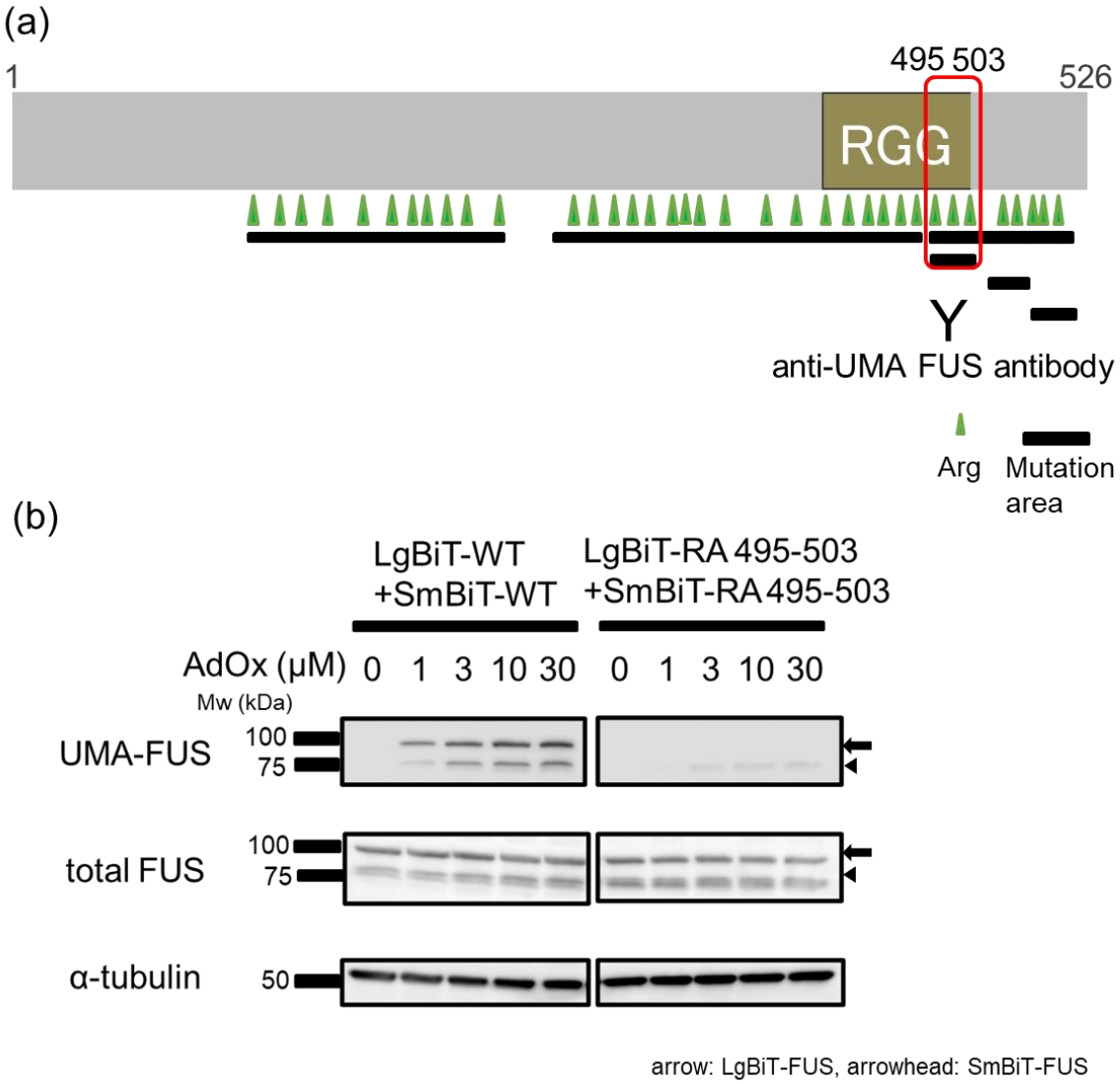


Fig.11 Search for arginine residues in FUS responsible for its transmission

(a) 6 mutant FUS cDNAs in which arginine residues were replaced to alanine residues. Arg495, Arg498 and Arg503 is surrounded with a rectangular.
(b) UMA-FUS and total FUS was detected with immunoblot. AdOx treatment caused a significant increase in the level of UMA-FUS in FUS wt sensor cells in a dose dependent manner, whereas no UMA-FUS was detected in FUS RA495-503 sensor cells.

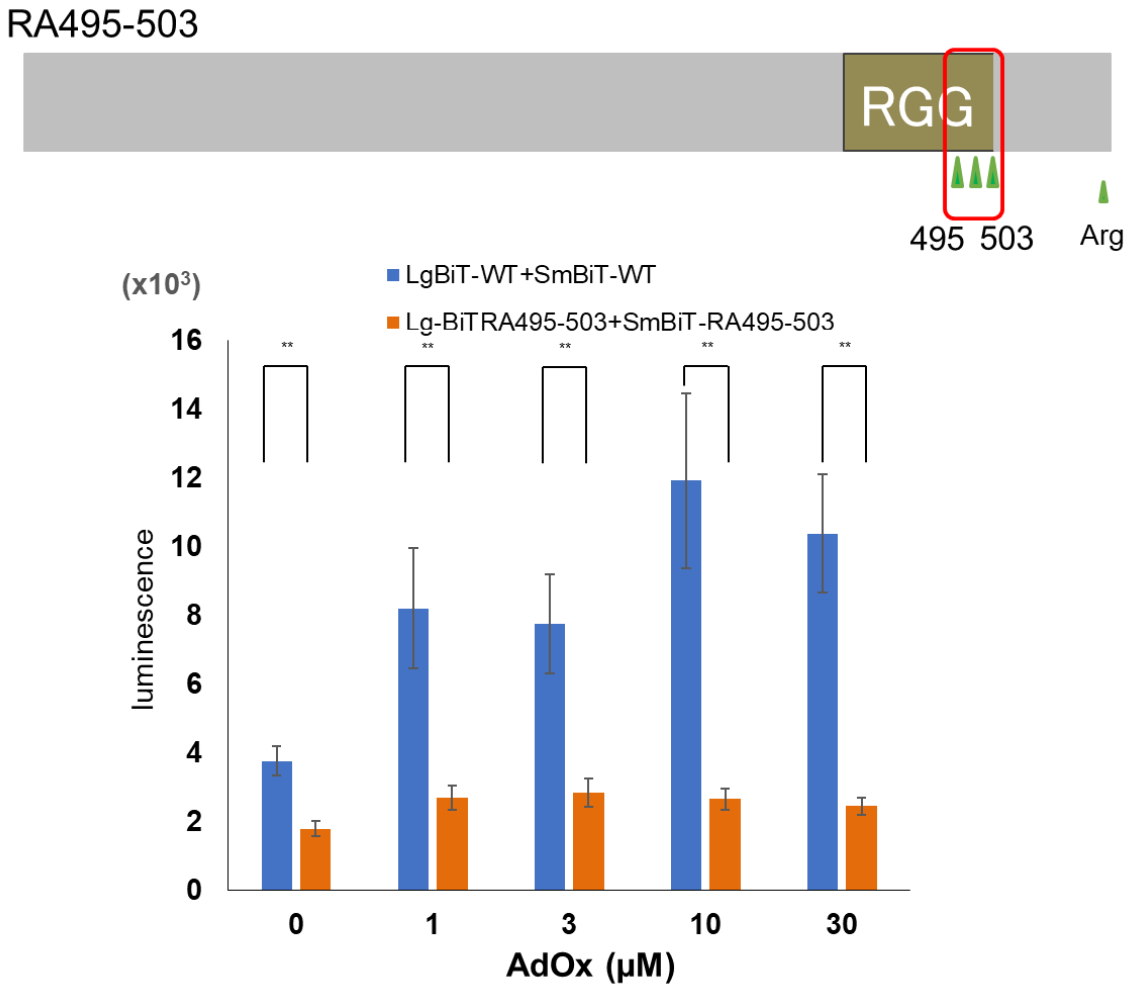


Fig.12 transmission of FUS with arginine at 495, 498 and 503 changed to alanin

Luminescence of sensor cells (LgBiT-WT+SmBiT-WT/LgBiT-RA495-503+SmBiT-RA495-503) treated with AdOx. RA495-503 mutations dramatically reduced the level of cell-to-cell transmission with or without AdOx treatment. N=5, unpaired t-test, (**) $p<0.01$

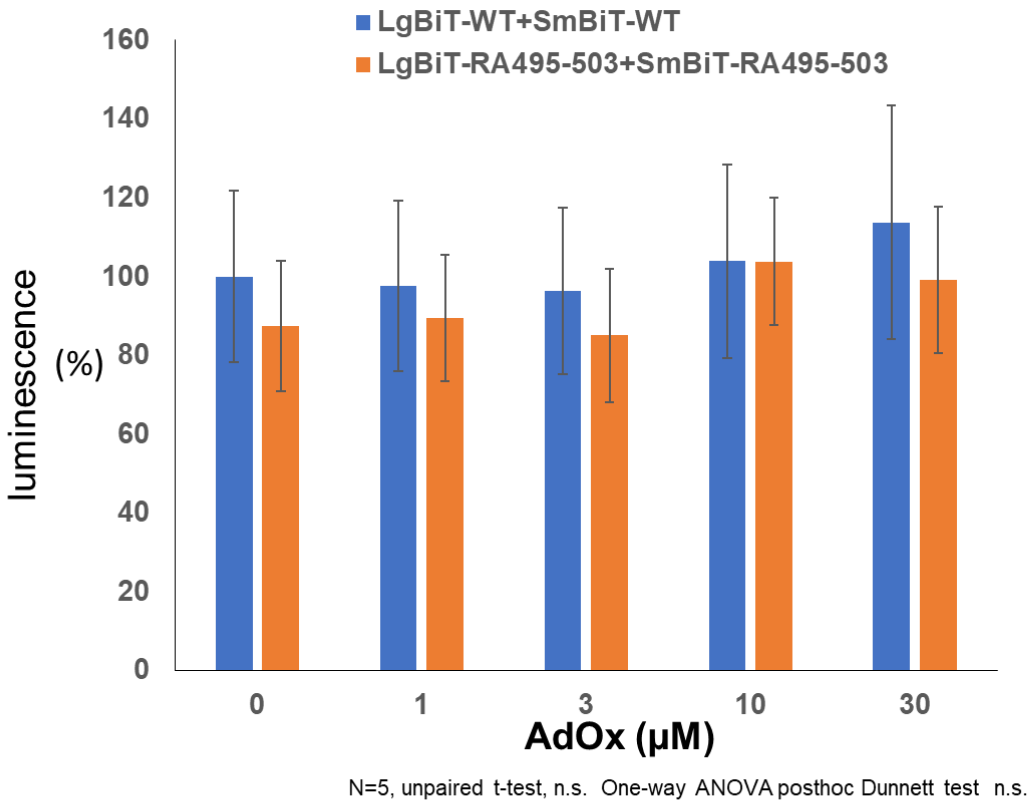
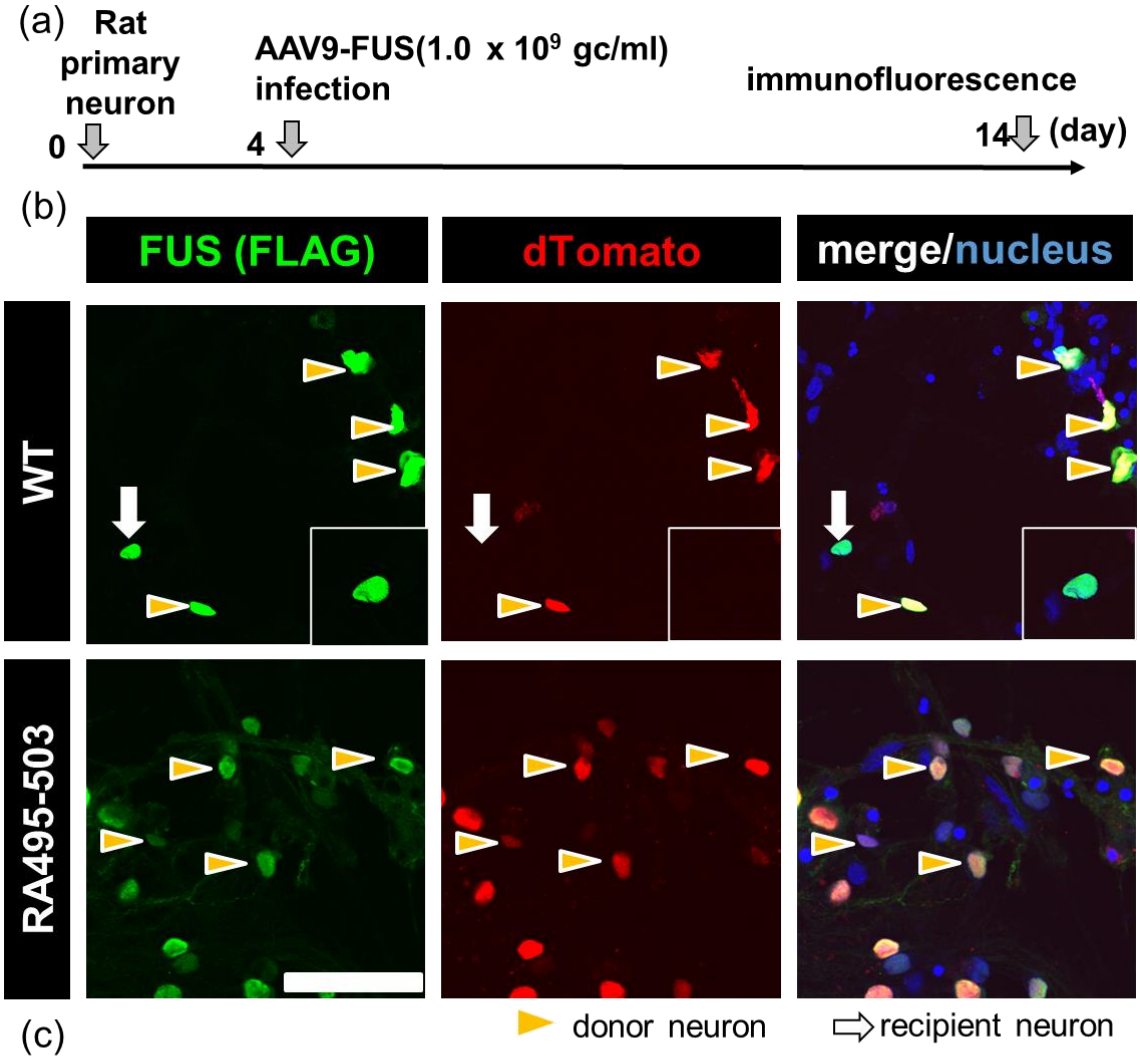


Fig.13 self-assembly of FUS with arginine at 495, 498 and 503 changed to alanin

Luminescence of HEK293 cells co-expressed with LgBiT-WT+SmBiT-WT/LgBiT-RA495-503+SmBiT-RA495-503 treated with AdOx. RA495-503 mutations did not change the level of self-assembly of FUS. N=5, unpaired t-test, n.s. One-way ANOVA posthoc Dunnett test n.s. Standardized by firefly luciferase and 100% at AdOx 0 μM.



(c)

	dTomato (-) cells/ FLAG(+) cells
WT	7.0/100
RA495-503	0.0/100

Fig.14 Investigation of effects of RA495-503 mutations in the interneuronal transmission of FUS in rat primary cultured neurons

(a) Timeline

(b) In immunocytochemical analyses with anti-FLAG antibody, recipient neurons (FLAG(+), dTomato (-)) were observed in FUS WT, whereas they were not observed in FUS R495-503.

(c) The number of recipient neurons (average of n=3)

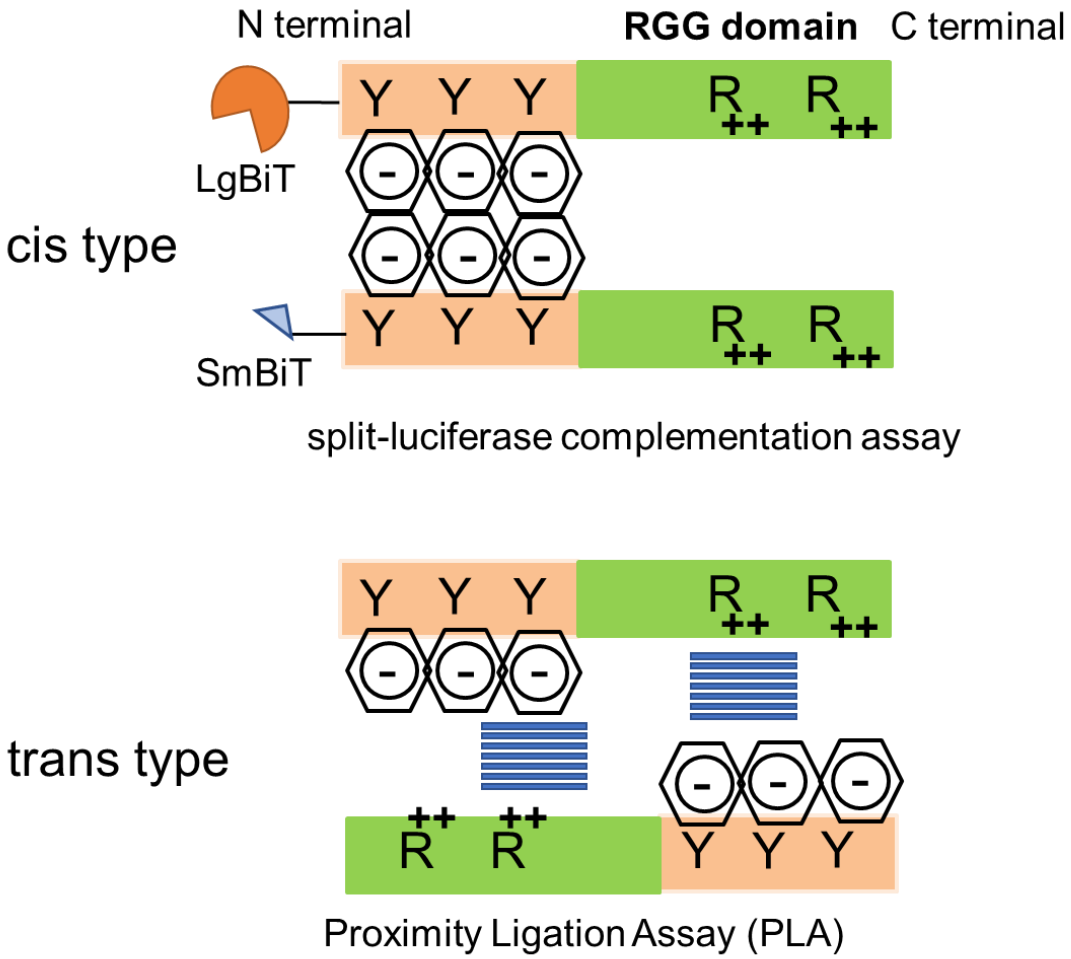


Fig.15 Two-oligomers hypothesis of FUS

In BiLC assay in this study, cis-type oligomers based on a π-ring conjugated system of tyrosine on the amino-terminal side were measured, and the level of cis-type self-assembly of FUS was not affected by the AdOx treatment. In recent years, it has been reported FUS also forms trans-type oligomers based on the cation-π interaction with the π-electron conjugated system of tyrosine residues on the amino-terminal side to arginine residues in the carboxy-terminal RGG region. It is not clear whether UMA-FUS affect the level of trans-type oligomers of FUS. I visualized the trans-type FUS oligomers using a Proximity ligation assay (PLA) method, which can visualize the proximity of two molecules.

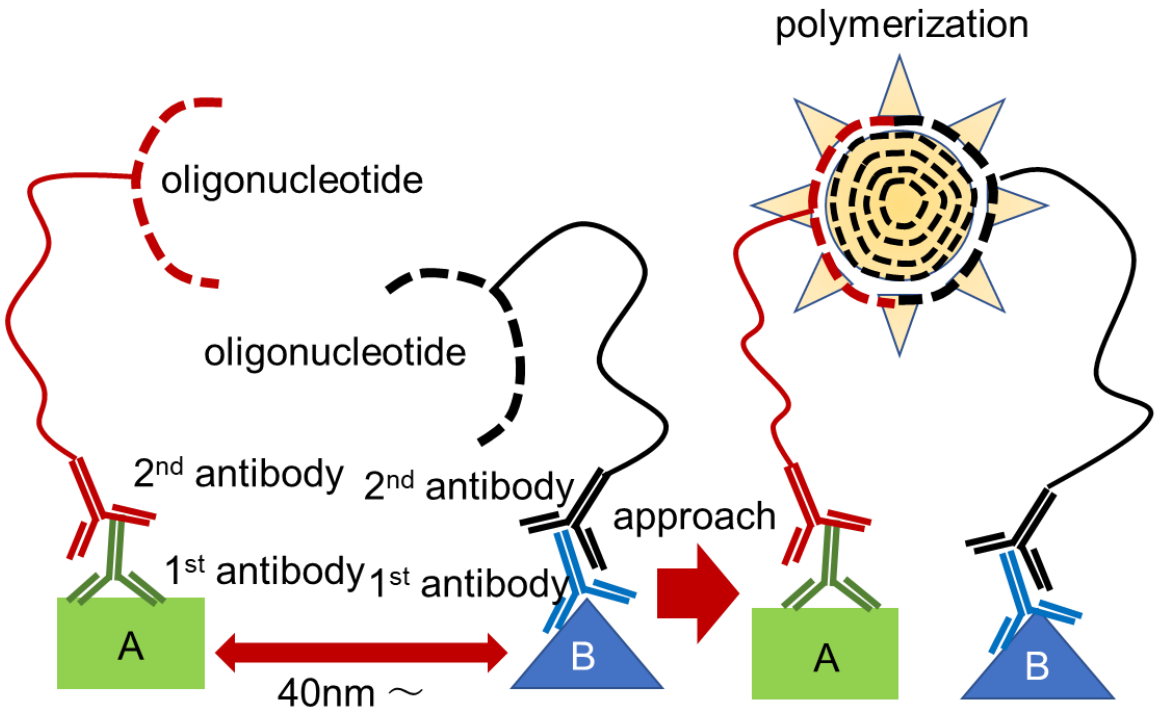
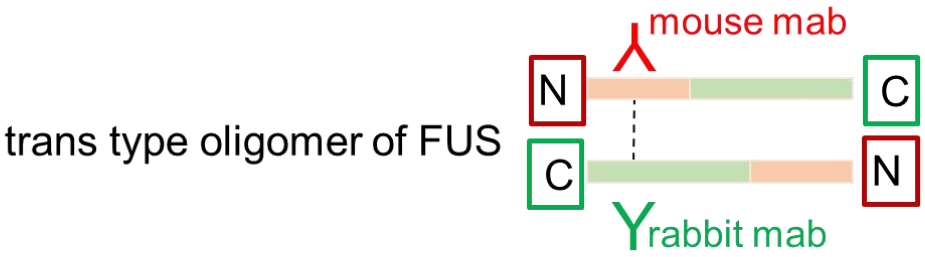


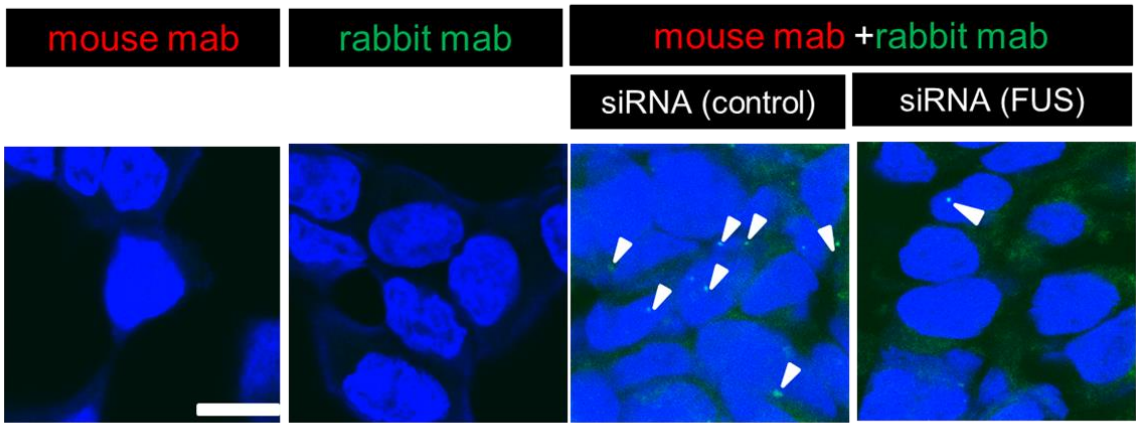
Fig.16 Visualization of trans-type oligomers by Proximity Ligation Assay

In the PLA method primary antibodies from different animal species and oligonucleotide-labeled secondary antibodies were used. If the two antigens are close to each other within 40 nm, the oligonucleotides are ligated together, amplified by the polymerase and visualized by the fluorescent PLA probe.

(a)



(b)



Scale: 10μm, white arrowheads:fluorescent dots

(c)

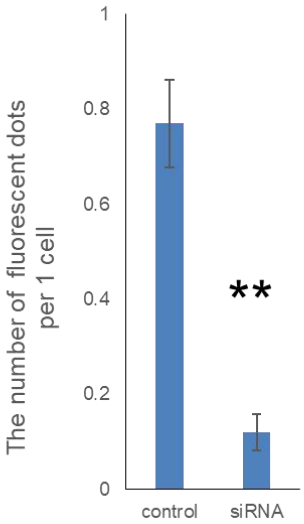


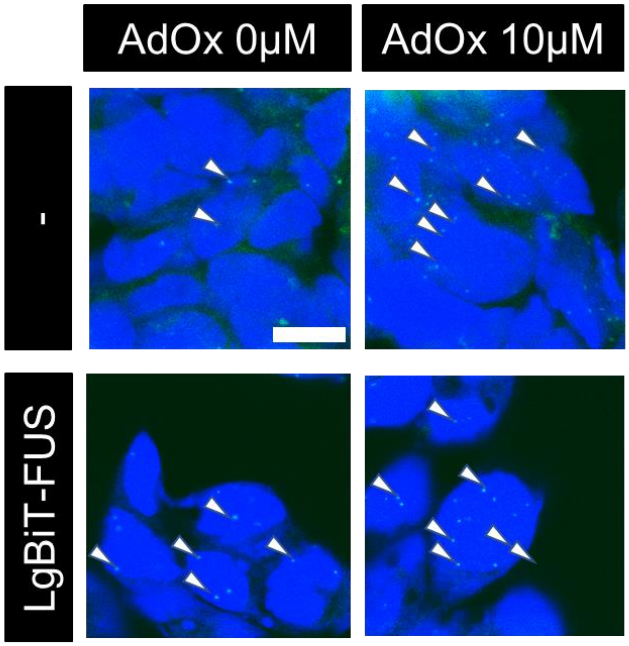
Fig.17 Trans type oligomer was detected by Proximity Ligation Assay

(a) 2 kinds of antibodies generated from different animals (mouse or rabbit) were used for detecting trans type oligomer of FUS

(b) Single antibody did not exhibit any PLA dots in HEK293 cells. Trans-type FUS oligomers were visualize mainly in the nucleus of HEK293 cells, on the other hand, knockdown of FUS gene significantly reduced the number of PLA dots.

(c) Quantification of fluorescent dots in siRNA knockdown (100 cells, unpaired t-test, (**) $p<0.01$)

(a)



Scale: 10μm, white arrowheads:fluorescent dots

(b)

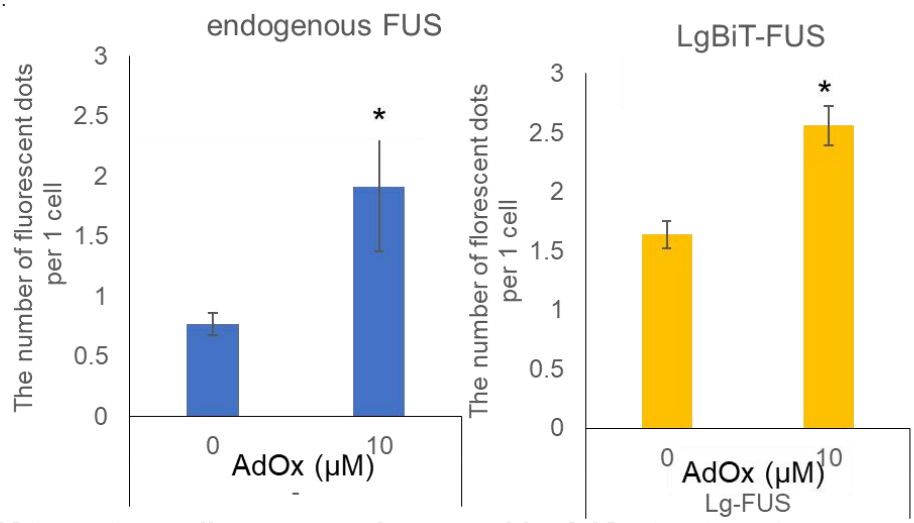


Fig.18 trans type oligomer was increased by AdOx treatment

(a) Fluorescent dots were shown by PLA in control or LgBiT-FUS stably expressing HEK293 cells with or without AdOx treatment (0 μM, 10μM).
(b) Quantification of fluorescent dots in the case of (a). 100 cells, unpaired t-test, (**) $p<0.01$

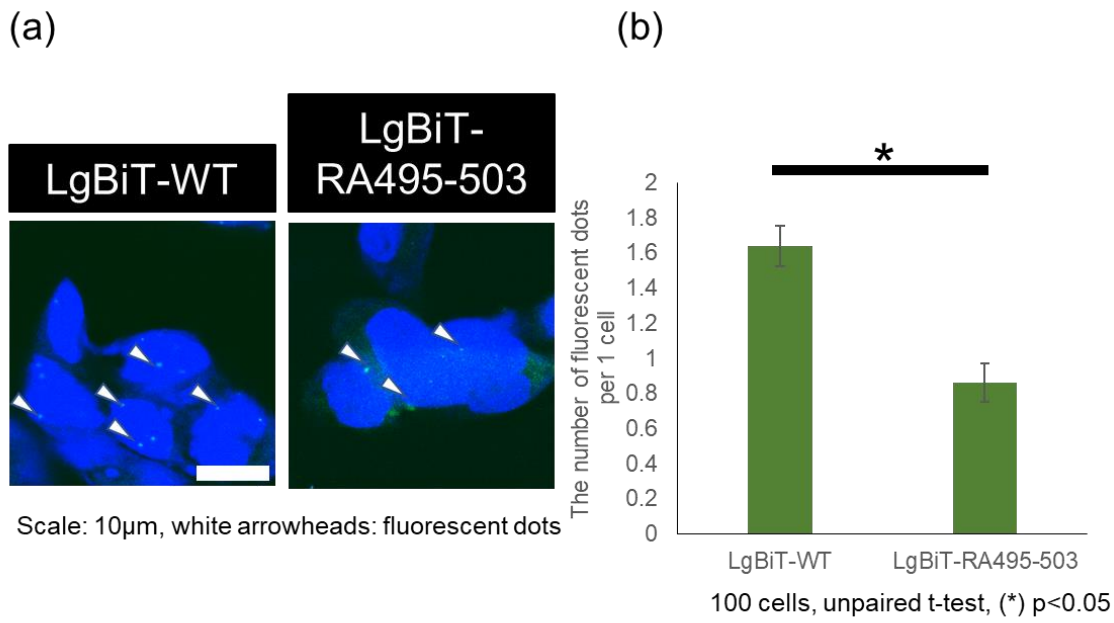


Fig.19 trans type oligomer was decreased by LgBiT-RA495-503 FUS stably expressing cells

(a) Fluorescent dots were shown by PLA in LgBiT-FUS WT or LgBiT-FUS RA495-503 stably expressing HEK293 cells.
(b) Quantification of fluorescent dots in the case of (a) 100 cells, unpaired t-test, (**) p<0.01

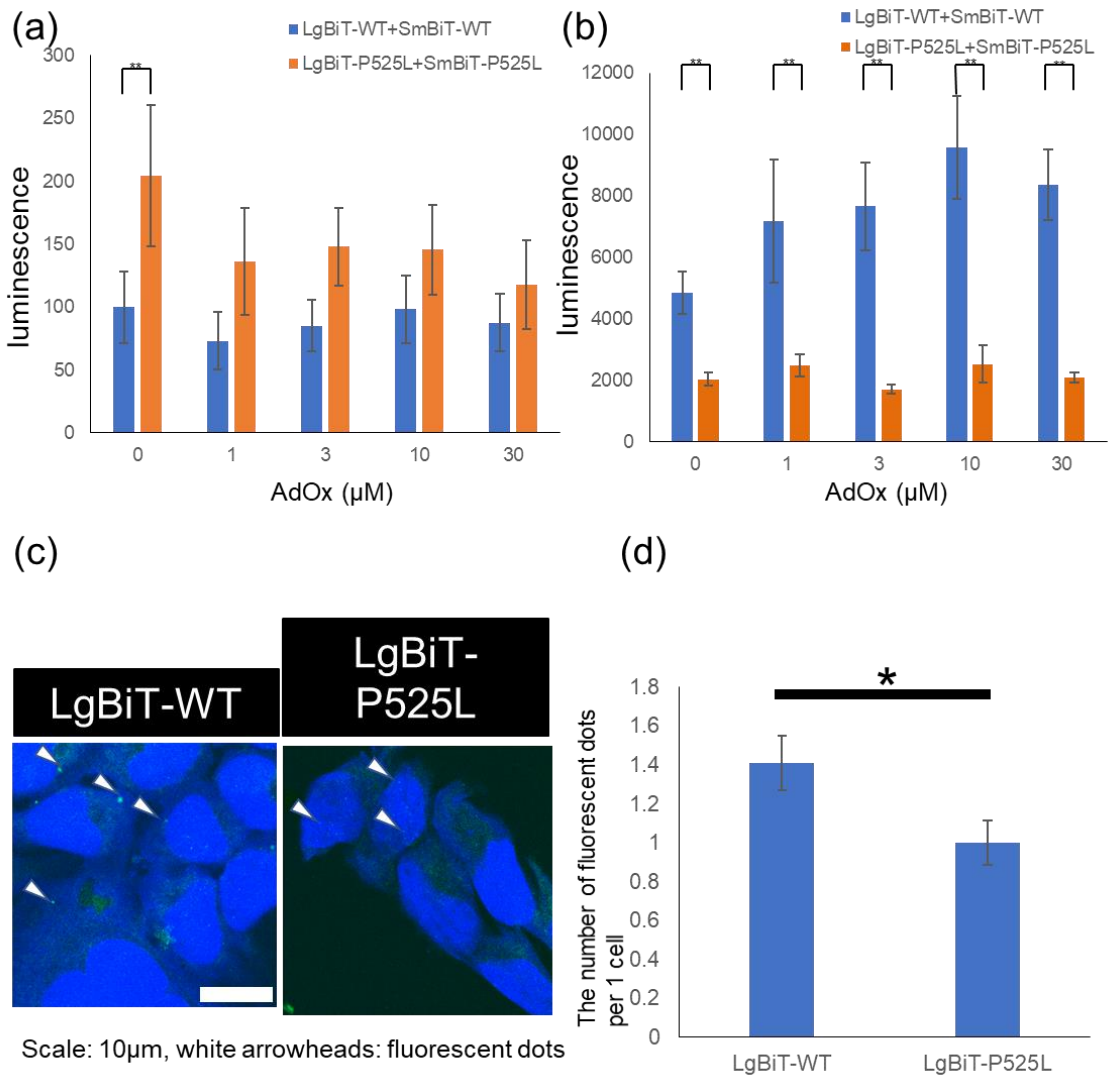


Fig.20 P525L mutation increases the oligomerlization of FUS and reduces the level of cell-to-cell transmission of FUS

(a) luminescence of HEK293 cells co-expressed with LgBiT-WT+SmBiT-WT/LgBiT-P525L+SmBiT-P525L treated with AdOx, unpaired t-test, (**) $p<0.01$ 100 cells

(b) luminescence of sensor cells (LgBiT-WT+SmBiT-WT/LgBiT-P525L+SmBiT-P525L) treated with AdOx, unpaired t-test, (**) $p<0.01$) 100 cells

(c) Fluorescent dots were shown by PLA in LgBiT-FUS WT or LgBiT-FUS P525L stably expressing HEK293 cells.

(d) Quantification of fluorescent dots in the case of (a), unpaired t-test, (*) $p<0.05$

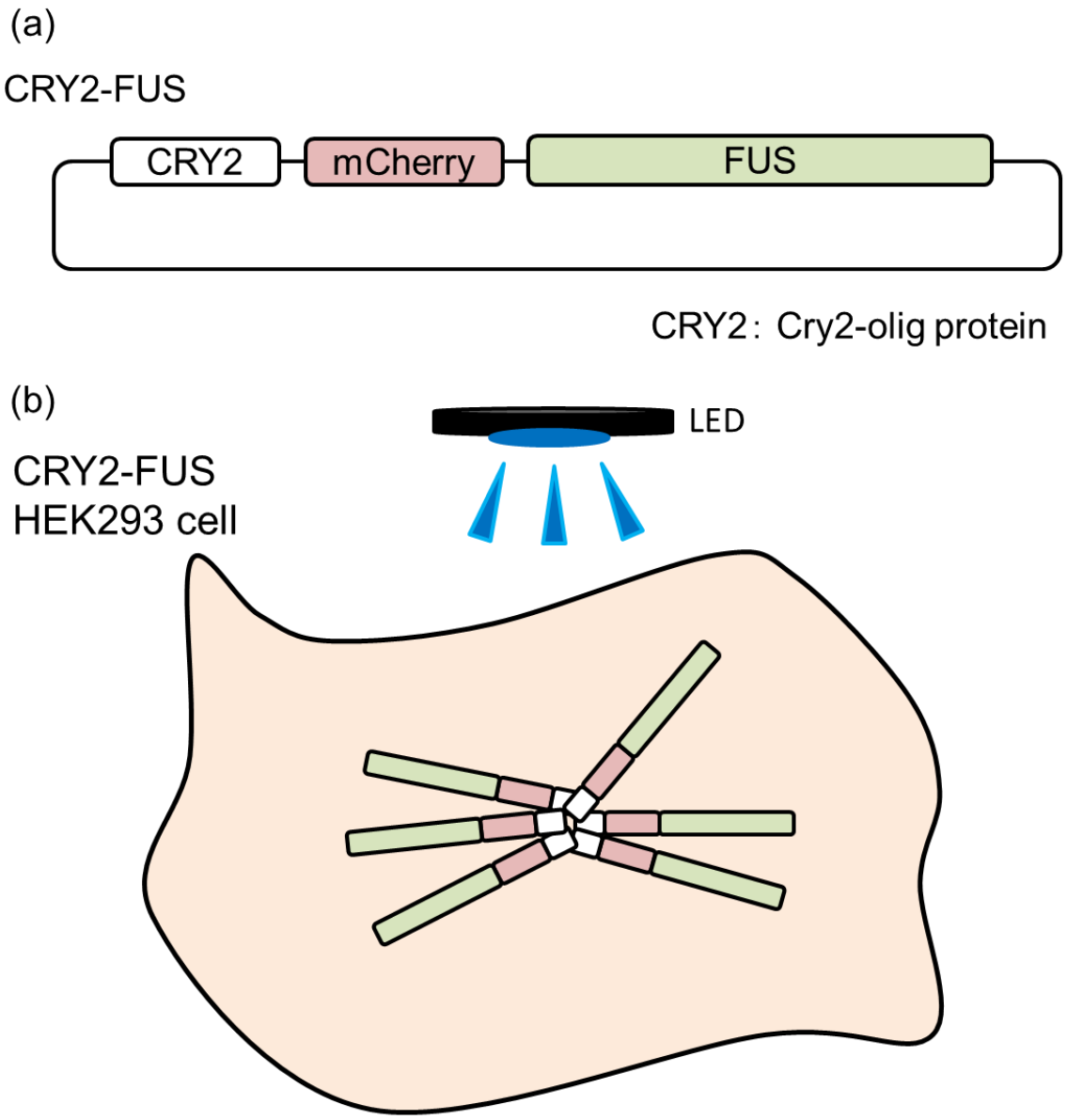


Fig.21 Oligomerization of CRY2-FUS under blue light stimulation

(a) CRY2-mCherry FUS construct was generated
(b) The scheme of gathering FUS by CRY2-FUS under blue light LED.

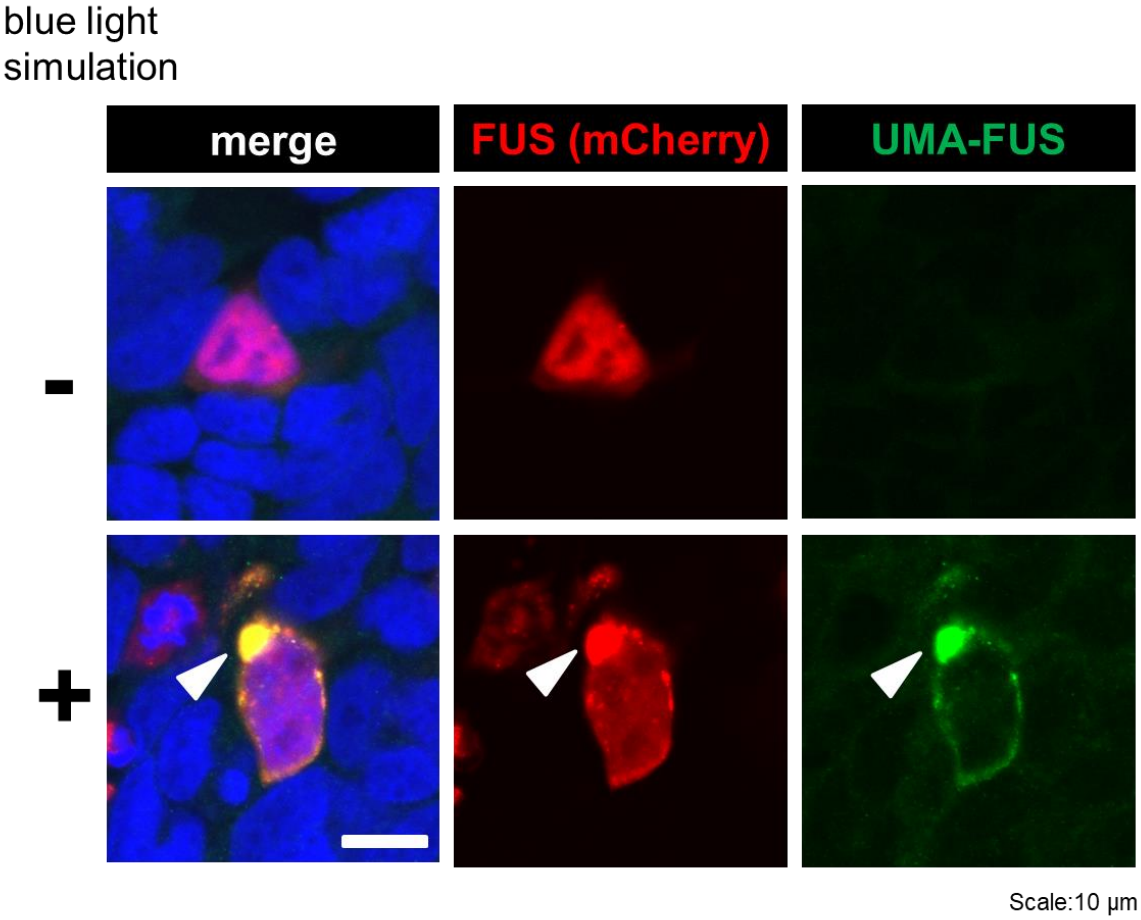


Fig.22 Oligomerization of CRY2-FUS induces UMA-FUS

CRY2-FUS was transfected to HEK293 cells transiently and the cells were incubated for 24 hours without light stimulation. In the stimulation of blue light for 24 hours, CRY2-FUS was accumulated in cytoplasm where UMA-FUS was positive, whereas there was no UMA-FUS-positive structures without any light stimulation

supplemental figures

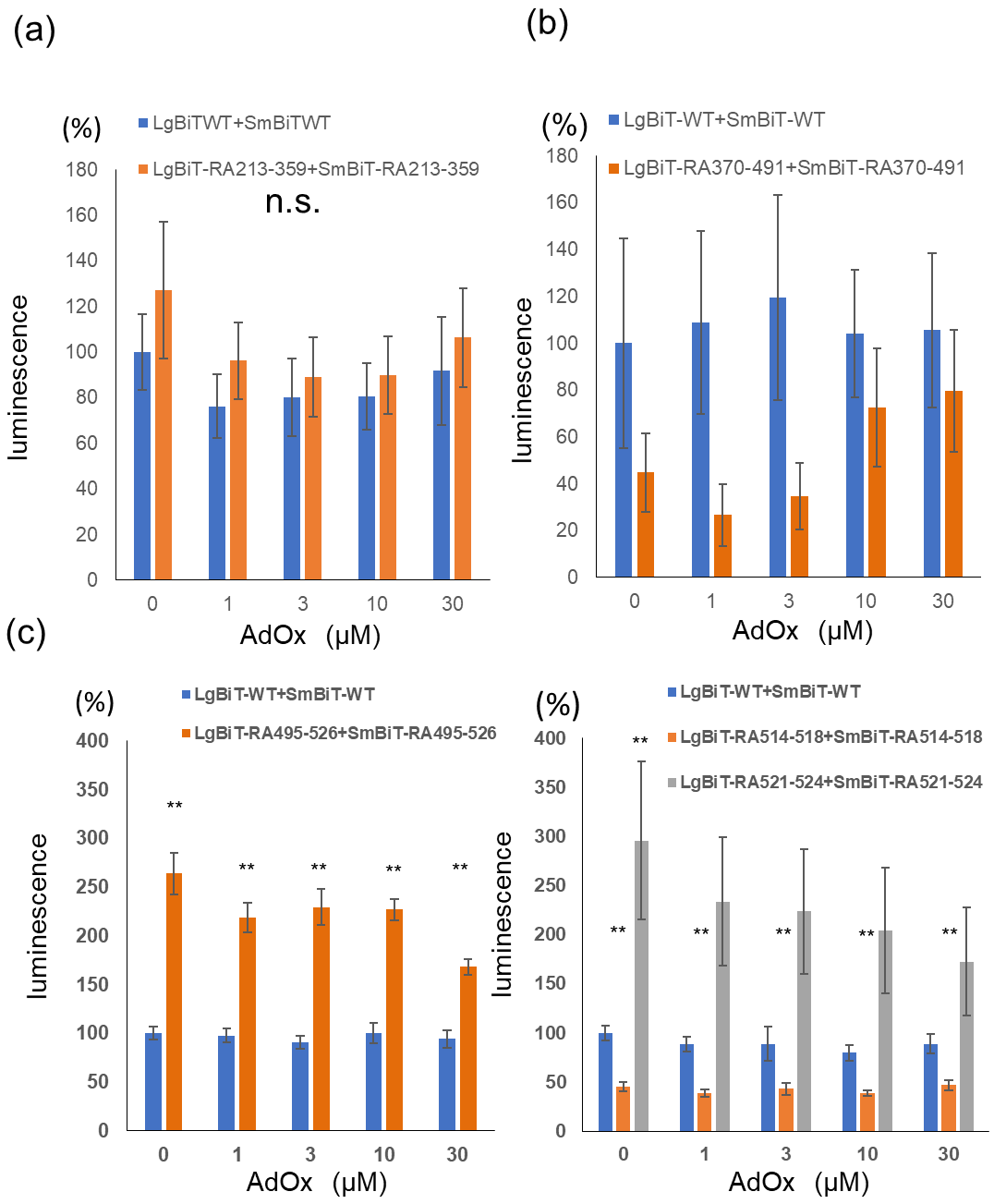


Fig.S 1 Self-assembly of RA213-359 FUS

Luciferase assay of HEK293 cells co-expressed with LgBiT-WT+SmBiT-WT/LgBiT-RA213-359+SmBiT-RA213-359/LgBiT-RA370-491+SmBiT-RA370-491/LgBiT-RA495-524+SmBiT-RA495-524/LgBiT-RA514-518+SmBiT-RA514-518/LgBiT-RA521-524+SmBiT-RA521-524 treated with AdOx. Standardized by firefly luciferase and 100% at AdOx 0 μM of WT, One-way ANOVA posthoc Dunnett test n.s.

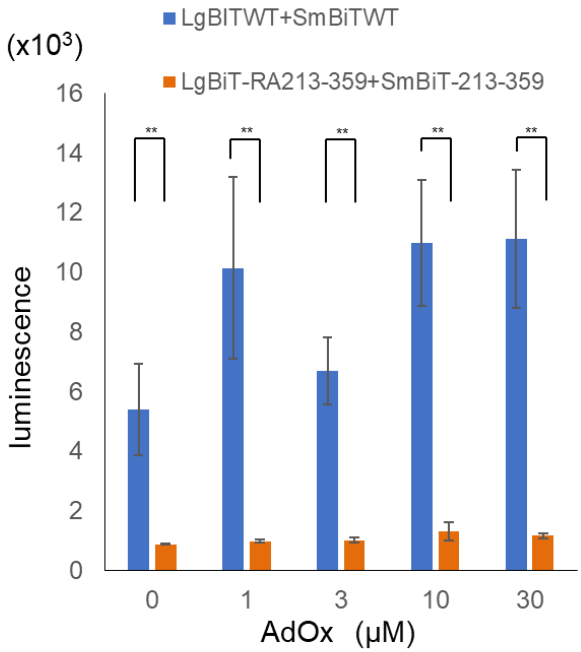


Fig.S 2 transmission of RA213-359 FUS

Luminescence of sensor cells (LgBiT-WT+SmBiT-WT/LgBiT-RA213-359+SmBiT-RA213-359) treated with AdOx. RA213-359 mutations dramatically reduced the level of cell-to-cell transmission with or without AdOx treatment. (N=5, unpaired t-test)

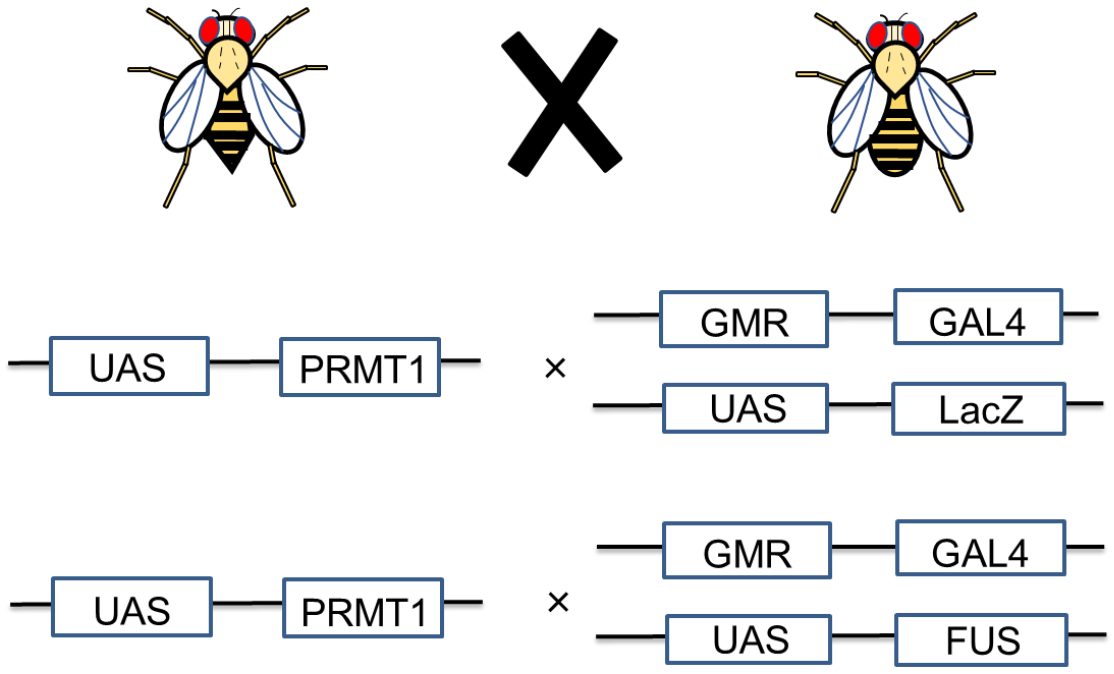


Fig.S3 Overexpression of PRMT1 and FUS in the retinal neurons of *Drosophila Melanogaster*

Double transgenic flies overexpressing both FUS and PRMT1 in retinal neurons under GMR driver were generated.

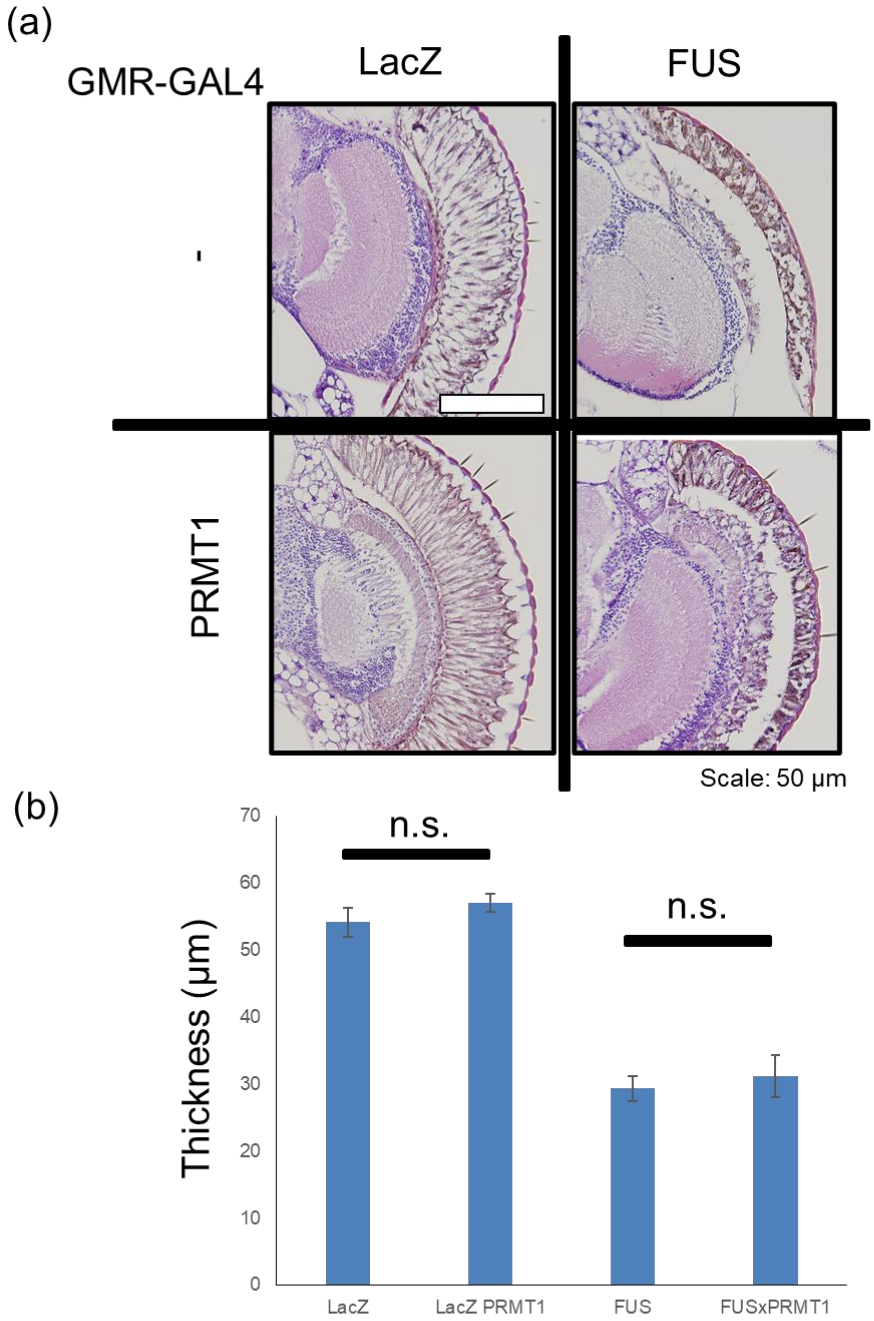


Fig.S 4 immunohistochemistry of flies' compound eyes
(a) Flies' heads were stained by hematoxylin-eosin (H.E.)
(b) Quantification of thickness of letina. n=10 (n=9, FUSxPRMT1) one-way ANOVA, post-hoc Tukey's test

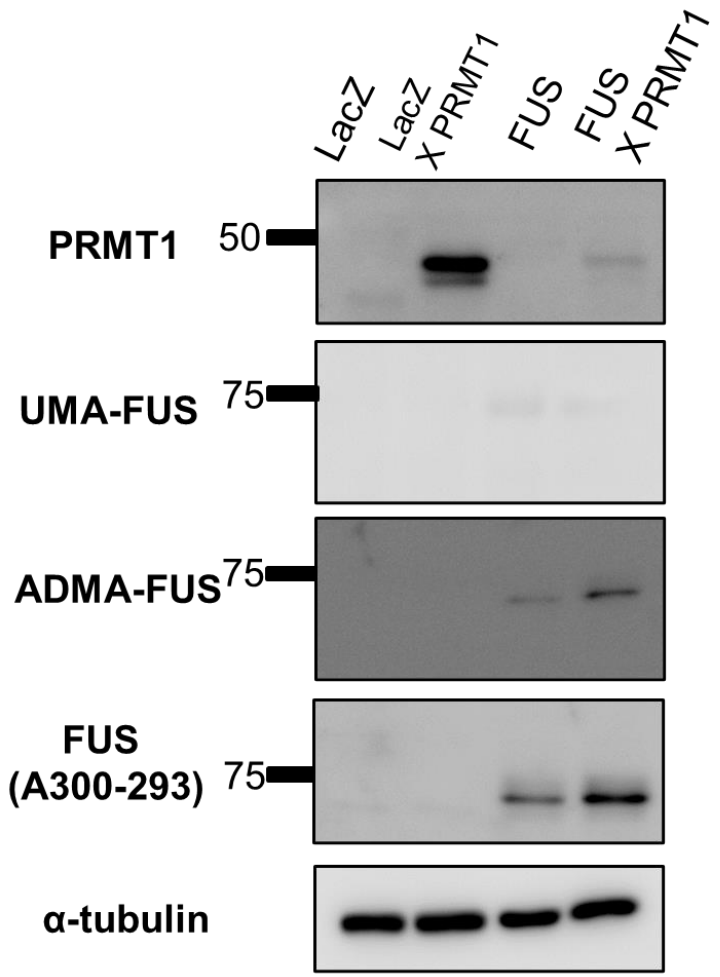


Fig.S5 Checked expression of flies expressing both PRMT1 and FUS in compound eyes

The level of UMA-FUS and ADMA-FUS in the heads of FUS single tg flies and FUS and PRMT1 double tg flies were analyzed by immunoblotting. In FUS and PRMT1 double tg flies the level of ADMA-FUS or total FUS was increased.

XhoI -FUS forward primer	AAAAACTCGAGCGGTATGGCCTC
FUS -Xba1 reverse primer	AAAAATCTAGATTAATACGGCCTCTCCCTG
P525L-Xba1 reverse primer	AAAAATCTAGATTAATACAGCCTCTCCCTG

XhoI allS primer 1	AAAAACTCGAGCGGTATGGCCTCAAACGATTCTAC
Xho-allS primer 2	AAAAACTCGAGCGGTATGGCCTCAAACG
XhoI -FUS forward primer	AAAAACTCGAGCGGTATGGCCTCAAACGATTATACC
XhoI -FUS forward primer2	AAAAACTCGAGCGGTATGGCCTCAAACG
KpnI-CRY2-mCherry	AAAAAAGGTACCACCATGAAGATGGAC
CRY2-mCherry-KpnI	AAAAAAGGTACCGCCTTGTACAGCTCGTCC

Fig.S 6 primer information for LgbiT-/SmBiT-FUS (WT, P525L. allS) or CRY2-FUS

GGATCCATGGCCTCAAACGATTATACCCAACAAGCAACCCAAAGCTATGGGGCCTACCCCA
CCCAGCCCGGGCAGGGCTATTCCCAGCAGAGCAGTCAGCCCTACGGACAGCAGAGTTAC
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GCCAGAGCCAGAACACAGGCTATGGAACTCAGTCAACTCCCCAGGGATATGGCTCGACT
GGCGGCTATGGCAGTAGCCAGAGCTCCCAATCGTCTTACGGGCAGCAGTCCTCCTACCCT
GGCTATGGCCAGCAGCCAGCTCCCAGCAGCACCTCGGGAAGTTACGGTAGCAGTTCTCA
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GACAGCAGCAAAGCTATGGACAGCAGCAAAGCTATAATCCCCCTCAGGGCTATGGACAGC
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GGCCAAGATCAATCCTCCATGAGTAGTGGTGGTGGCAGTGGTGGCGGTTATGGCAATCAA
GACCAGAGTGGTGGAGGTGGCAGCGGTGGCTATGGACAGCAGGACCGTGGAGGCCGC
GGCAGGGGTGGCAGTGGTGGCGGCGGCGGCGGCGGCGGTGGTGGTTACAACCGCAGC
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GCGGAAGTGACCGTGGTGGCTTCAATAAATTTGGTGGCCCTCGGGACCAAGGATCACGT
CATGACTCCGAACAGGATAATTCAGACAACAACACCATCTTTGTGCAAGGCCTGGGTGAG
AATGTTACAATTGAGTCTGTGGCTGATTACTTCAAGCAGATTGGTATTATTAAGACAAACAA
GAAAACGGGACAGCCCATGATTAATTTGTACACAGACAGGGAAACTGGCAAGCTGAAGGG
AGAGGCAACGGTCTCTTTTGATGACCCACCTTCAGCTAAAGCAGCTATTGACTGGTTTGAT
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ATCGGGGTGGTGGCAATGGTCGTGGAGGCCGAGGGCGAGGAGGACCCATGGGCCGTG
GAGGCTATGGAGGTGGTGGCAGTGGTGGTGGTGGCCGAGGAGGATTTCCCAGTGGAGG
TGGTGGCGGTGGAGGACAGCAGCGAGCTGGTGACTGGAAGTGTCCTAATCCCACCTGTG
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CCCAGGAGGGGGACCAGGTGGCTCTCACATGGGGGGTAACTACGGGGATGATCGTCGT
GGTGGCAGAGGAGGCTATGATCGAGGCGGCTACCGGGGCCGCGGCGGGGACCGTGGA
GGCTTCGCAGGGGGCGCAGGTGGTGGGGACGCAGGTGGCTTTGGCCCTGGCAAGATG
GATTCCAGGGGTGAGCACAGACAGGATCGCAGGGAGAGGCTGTATTAATAAGCTT

Fig.S 7 FUS sequence for generating AAV9-FUS (RA495-503, P525L)
This sequence was inserted between BamHI and HindIII of AAV9-FUS WT.
orange: BamHI, light blue: HindIII, green: FUS
blue paint: RA495-503, purple: P525L

GGATCCCCGCCATGGCGGCAGCCGAGGCCGCGAACTGCATCATGGAGAATTTTGTAGCCACC
TTGGCTAATGGGATGAGCCTCCAGCCGCCTCTTGAAGAAGTGTCCTGTGGCCAGGCGGAAAG
CAGTGAGAAGCCCAACGCTGAGGACATGACATCCAAAGATTACTACTTTGACTCCTACGCACA
CTTTGGCATCCACGAGGAGATGCTGAAGGACGAGGTGCGCACCCTCACTTACCGCAACTCCA
TGTTTCATAACCGGCACCTCTTCAAGGACAAGGTGGTGCTGGACGTCGGCTCGGGCACCGGC
ATCCTCTGCATGTTTGCTGCCAAGGCCGGGGCCCGCAAGGTCATCGGGATCGAGTGTTCCAG
TATCTCTGATTATGCGGTGAAGATCGTCAAAGCCAACAAGTTAGACCACGTGGTGACCATCATC
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ACCATCGACCTGGACTTCAAGGGCCAGCTGTGCGAGCTGTCCTGCTCCACCGACTACCGGAT
GCGCTGAGAATTCTGCAGATCAAGCTT

Fig.S 8 PRMT1 sequence for generating AAV9-PRMT1

This sequence was inserted between BamHI and HindIII of AAV9-FUS WT.
orange: BamHI, light blue: HindIII, blue: PRMT1

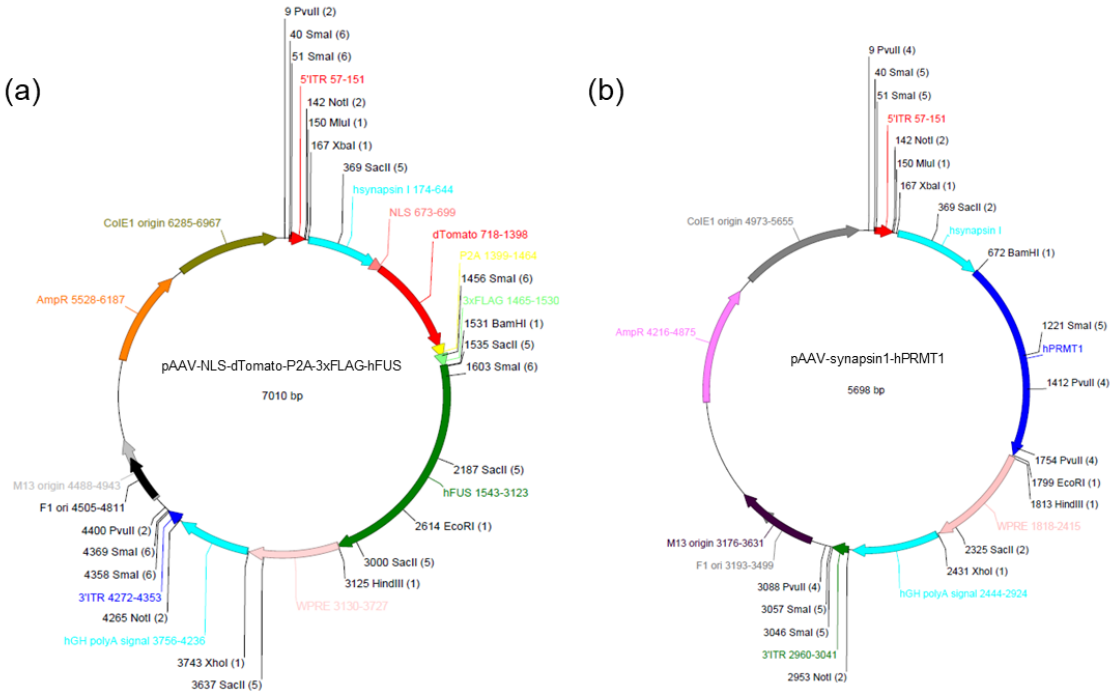

Fig.S 9 sequential image of AAV9-FUS or PRMT1

(a) AAV9-FUS WT, RA495-503, P525L

(b) AAV9-PRMT1

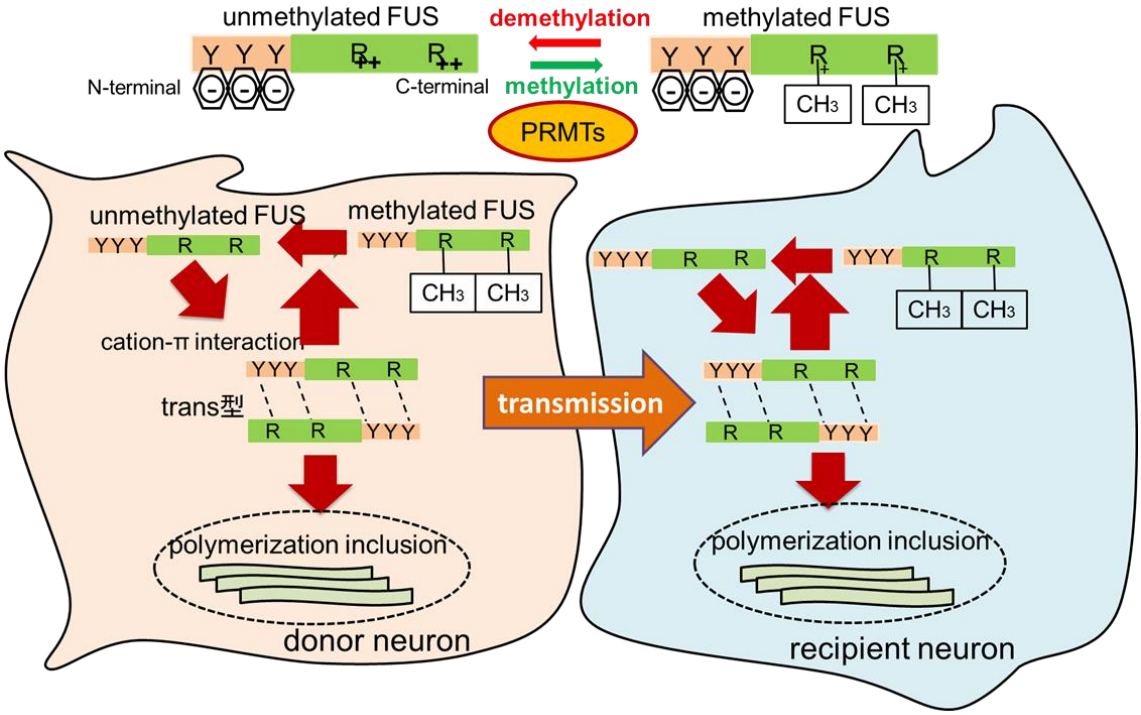

Fig.S 10 Mechanism of FUS transmission

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