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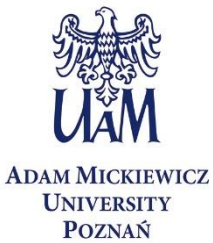
Zależna od FUS obróbka snoRNA do sdRNA i regulacja modyfikacji potranskrypcyjnych rybosomowego RNA - powiązania ze stwardnieniem zanikowym bocznym (ALS)

Kishor Gawade

Promotor: Prof. UAM, dr hab. Katarzyna Dorota Raczyńska



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Adam Mickiewicz University in Poznan

Faculty of Biology

Institute of Molecular Biology and Biotechnology (IBMiB)

Department of Gene Expression

Laboratory of RNA Processing

Doctoral Thesis

FUS-dependent processing of snoRNAs into sdRNAs and regulation of ribosomal RNA modifications: implications in Amyotrophic Lateral Sclerosis (ALS)

Kishor Gawade

Supervisor:

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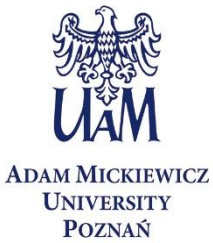

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Dedicated to my parents and siblings

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List of publications included in the thesis:

1. **Kishor Gawade**, Patrycja Plewka, Sophia J. Häfner, Anders H. Lund, Virginie Marchand, Yuri Motorin, Michal W. Szczesniak & Katarzyna D. Raczynska, FUS regulates a subset of snoRNA expression and modulates the level of rRNA modifications. Sci Rep 13, 2974 (2023). https://doi.org/10.1038/s41598-023-30068-2

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MNiSW points: 140

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2. **Kishor Gawade**, Katarzyna D. Raczynska, Imprinted small nucleolar RNAs: Missing link in development and disease? WIREs RNA, 2023. DOI: http://doi.org/10.1002/wrna.1818

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3. Cichocka M, Karlik A, Plewka P, **Gawade K**, Stępień A, Świergiel P, Gadgil A, Raczyńska KD. The Novel Role of hnRNP UL1 in Human Cell Nucleoli. *Int J Biol Sci* 2022; 18(13):4809-4823. doi:10.7150/ijbs.75084. https://www.ijbs.com/v18p4809.htm

IMPACT FACTOR: 9.2

MNiSW points: 100

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Abbreviations

ALS	Amyotrophic lateral sclerosis
AS	Angelmann syndrome
ASD	Autism spectrum disorder
ASE	Anti-sense element
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
dCAMP	N6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt
DCS	Decoding center
DKC1	Dyskerin Pseudouridine Synthase 1
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
eGFP	Enhanced green fluorescent protein
ESCs	Embryonic stem cells
FBL	Fibrillarin
FBS	Fetal bovine serum
FUS	Fused in sarcoma
FUS P525L	Proline at position 525 substituted by leucine
FUS R495X	Arginine at position 495 changes into a premature stop codon
FUS R521C	Arginine at position 521 substituted by cysteine
FUS R521L	Arginine at position 521 substituted by leucine
GAR1	Nucleolar Protein Family A, Member 1
GAS5	Growth arrest-specific 5
GDNF	Glial cell-derived neurotrophic factor
HEK293T	Immortalized human embryonic kidney cells
iPSCs	Induced pluripotent stem cells
IRES	Internal ribosome entry site
KOS14	Kagami-Ogata Syndrome
LSU	Large subunit rRNA (28S, 5.8S and 5S rRNAs)

MAP2	Microtubule-associated protein 2
MNs	Motor neurons
mRNAs	Messenger RNAs
NGS	Next-generation sequencing
NHP2	Nucleolar Protein Family A, Member 2
NLS	Nuclear localization signal
NOP10	Nucleolar Protein 10
NOP56	Nucleolar Protein 56
NOP58	Nucleolar Protein 58
P53	Tumor Protein P53
PBS	Phosphate buffer saline
PCA	Principal component analysis
PFA	Paraformaldehyde
piRNAs	Piwi-interacting RNAs
PLO	Poly-L-ornithine
PMA	Purmorphamine
PTC	Peptidyl transferase center
PWS	Prader-Willi syndrome
RA	Retinoic acid
rRNAs	Ribosomal RNAs
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
scaRNAs	Small Cajal body-specific RNAs
sdRNAs	SnoRNA-derived RNAs
SH-SY5Y	Derived from the SK-N-SH neuroblastoma cell line
smNPCs	Small molecule-derived neural progenitor cells
SNHG1	Small Nucleolar RNA Host Gene 1
SNORA	Small nucleolar RNAs, H/ACA box
SNORD	Small nucleolar RNAs, C/D box
snoRNAs	Small nucleolar RNAs
snoRNP	Small nucleolar ribonucleoproteins
snRNAs	Small nuclear RNAs

SSU	Small subunit rRNA (18S rRNA)
TGF-β3	Transforming growth factor - β3
tRNAs	Transfer RNAs
TS14	Temple syndrome
WT	Wild-type

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STRESZCZENIE

FUS jest białkiem wiążącym DNA/RNA, zaangażowanym w wiele etapów metabolizmu RNA. Mutacje w obrębie sygnału lokalizacji jądrowej (NLS, ang. nuclear localization signal) białka powodują błędną lokalizację FUS w cytoplazmie i w konsekwencji tworzenie agregatów cytoplazmatycznych, co jest powiązane z chorobą neurodegeneracyjną stwardnienie zanikowe boczne, ALS (ang. amyotrophic lateral sclerosis). Małe jąderkowe RNA (snoRNA) to rodzina małych niekodujących RNA, które są zaangażowane w 2'-O-metylację (2'-O-Me) i pseudourydylację rybosomowego RNA (rRNA) i małych jądrowych RNA (snRNA). Te modyfikacje epitranskryptomiczne zapewniają stabilność i zachowanie wiernej struktury rybosomów. Co ciekawe, wbrew wcześniejszemu przekonaniu, około dwie trzecie miejsc w rRNA jest zmodyfikowanych częściowo; zapewnia to dodatkowy poziom generowania heterogeniczności rybosomów. Oprócz funkcji w nadawaniu modyfikacji rRNA i U snRNA, snoRNA klasy C/D i H/ACA mogą być procesowane do mniejszych, stabilnych fragmentów, zwanych sdRNA (ang. snoRNA-derived RNAs, RNA pochodzące ze snoRNA). Cząsteczki sdRNA mogą działać jako mikroRNA i regulować ekspresję genów na poziomie transkrypcji i translacji. Co istotne, rola FUS w biogenezie mikroRNA jest znana i dobrze udokumentowana, nie ma natomiast danych na temat udziału białka FUS w regulacji ekspresji snoRNA i ich dalszej obróbce do sdRNA.

W niniejszej pracy, z zastosowaniem technik wysokoprzepustowego sekwencjonowania RNA, wykazano, że FUS reguluje poziom snoRNA w komórkach linii ludzkiej neuroblastomy SH-SY5Y. Następnie, ponieważ snoRNA biorą udział w potranskrypcyjnych modyfikacjach rRNA i snRNA, wykorzystano ilościowe techniki oparte na sekwencjonowaniu nowej generacji (NGS, ang. next generation sequencing) typu RiboMeth-seq i HydraPsiSeq, do mapowania zmian w poziomach 2'-O-Me i pseudourydyny, w komórkach typu dzikiego i komórkach pozbawionych białka FUS. W wielu miejscach 2’-O-Me w rybosomowych RNA, które były zmodyfikowane częściowo, obserwowano wzrost poziomu modyfikacji w komórkach pozbawionych FUS. Równocześnie podwyższonej ekspresji ulegała też grupa snoRNA klasy C/D, biorąca udział we wprowadzaniu tych modyfikacji. Ponadto, zaobserwowano drobne zmiany w poziomie pseudourydylacji w komórkach pozbawionych FUS, które również wykazywały tendencję wzrostową, podobnie jak zmiany w ekspresji odpowiedzialnych za te modyfikacje snoRNA klasy H/ACA. W kolejnych analizach, w których wykorzystano komórki SH-SY5Y niosące mutację FUS R495X związaną z ALS, prowadzącą do syntezy białka pozbawionego sygnału NLS, również obserwowano znaczące zmiany w poziomach snoRNA oraz 2’-O-Me i pseudourydyny, w porównaniu z kontrolą typu dzikiego. W badaniach wykorzystano również fibroblasty pochodzące od pacjentów z ALS z mutacjami FUS oraz, jako kontrole, fibroblasty pochodzące od dopasowanych wiekiem i płcią osób zdrowych. Zgodnie z oczekiwaniami, w fibroblastach pochodzących od osób z „silną” mutacją FUS P525L, zaobserwowano największą liczbę znacząco zmienionych miejsc 2’-O-Me, podczas gdy w fibroblastach pochodzących od osób z „łagodnymi” mutacjami FUS R521C i R521L, zmienionych miejsc było mniej. Wyniki te uzupełniono danymi dotyczącymi 2’-O-Me z izogenicznej pary indukowanych pluripotencjalnych komórek macierzystych z mutacją FUS P525L, różnicowanych następnie do neuronalnych komórek progenitorowych i neuronów ruchowych. Co ciekawe, większość miejsc ze zmienionym profilem 2’-O-Me i pseudourydylacji położona jest w zewnętrznych partiach rybosomu 80S, sugerując, że te częściowo zmodyfikowane miejsca, w zależności od poziomu ich modyfikacji, mogą wpływać na oddziaływania z białkami rybosomalnymi i z innymi czynnikami.

Jak wspomniano wyżej, analiza danych pochodzących z sekwencjonowania małych cząsteczek RNA wykazała, że wiele cząsteczek snoRNA ulega zróżnicowanej ekspresji w komórkach SH-SY5Y z wyciszeniem białka FUS. Ponadto, zidentyfikowano liczną grupę sdRNA powstających ze snoRNA klasy C/D i H/ACA. Wiele sdRNA pochodzących ze snoRNA klasy C/D zawierało zakonserwowane motywy „C” lub „D”. Co więcej, z jednego snoRNA mogły powstawać różne sdRNA, wykazujące różne poziomy

ekspresji. Profil sdRNA był inny w przypadku proliferujących i zróżnicowanych komórek SH-SY5Y, co sugeruje, że zewnętrzne sygnały, takie jak traktowanie kwasem retinowym, mogą również wpływać na produkcję sdRNA ze snoRNA. Wyniki te wskazują, że białko FUS wpływa na ekspresję snoRNA i modyfikację rybosomalnego RNA. Co więcej, snoRNA są procesowane do sdRNA w sposób zależny od FUS. Jednakże, funkcja tych sdRNA pozostaje wciąż niezbadana. Konieczne są dalsze badania funkcjonalne, aby określić wpływ poszczególnych miejsc modyfikacji rRNA na translację i wpływ mutacji FUS związanej z ALS na ten proces.

Słowa kluczowe – snoRNA, ALS, FUS, 2'-O-Me, pseudourydyna, sdRNA.

ABSTRACT

FUS is a DNA/RNA binding protein involved in many aspects of RNA metabolism. Moreover, mutations within the nuclear localization signal (NLS) of FUS result in the mislocalization of this protein into the cytoplasm, resulting in the formation of cytoplasmic aggregates, and it is associated with amyotrophic lateral sclerosis, a neurodegenerative disease. Small nucleolar RNAs (snoRNAs) are a family of small non-coding RNAs that guide site-specific 2'-O-methylation (2'-O-Me) and pseudouridylation of ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs). These epitranscriptomic modifications provide stability and maintain the structural fidelity of the ribosomes. Additionally, contrary to the previous belief, about two-thirds of these sites on the rRNA are fractionally modified; this provides another layer of generating ribosomal heterogeneity. Not limited to only guiding rRNA and snRNA modifications, both C/D and H/ACA box types of snoRNAs can be processed into smaller, stable fragments called sdRNAs (snoRNA-derived RNAs). These sdRNAs may function as microRNAs and regulate gene expression at transcriptional and translational levels. Moreover, the role of FUS in the biogenesis of microRNAs is known and well documented, but its role in regulating snoRNA expression and processing into sdRNAs is not explored.

In this work, using high-throughput sequencing, it was identified that FUS regulates snoRNAs in SH-SY5Y (neuroblastoma) cells. Since snoRNAs are involved in guiding rRNA and snRNA modifications, quantitative, next-generation sequencing (NGS)-based techniques, RiboMeth-seq and HydraPsiSeq were used to map changes in 2'-O-Me and pseudouridine levels in wild-type and FUS-depleted cells (FUS KO). Many fractionally modified 2'-O-Me sites on ribosomal RNAs showed a higher proportion of modification in FUS-depleted cells, and a subset of guide C/D box snoRNAs were also upregulated. Furthermore, pseudouridine changes in the FUS-depleted cells were subtle, but an overall increase in the modification of rRNAs was noticeable, along with changes in guide H/ACA box snoRNAs. Next, SH-SY5Y cells carrying ALS-associated *FUS* R495X mutation that lack an NLS also displayed significant changes in snoRNAs and 2'-O-Me and pseudouridine levels compared to wild-type control. In addition, ALS-patient-derived fibroblasts with FUS mutations and age-sex-matched controls were used to explore if 2'-O-Me changes are also observed in ALS patients with FUS mutations. As expected, fibroblasts carrying 'strong' FUS P525L mutation displayed the highest number of significantly changed 2'-O-Me sites, whereas 'mild' FUS mutations R521C and R521L displayed fewer sites. These results were complemented by 2'-O-Me data from an isogenic pair of induced pluripotent stem cells, neural progenitor cells and motor neurons carrying FUS P525L mutation. Interestingly, most of the 2'-O-Me and pseudouridine sites mapped to the outer periphery of the 80S ribosome, suggesting that depending on their modification levels, these fractionally modified sites may regulate the binding of ribosomal proteins or other factors.

As mentioned above, small RNA sequencing data showed that some snoRNAs were differentially expressed in SH-SY5Y FUS KO cells and, that many sdRNAs are generated from C/D and H/ACA box snoRNAs. In the case of the C/D box snoRNAs, these sdRNAs showed conserved box C or box D motifs. Moreover, a single snoRNA produced multiple sdRNAs with varying levels of expression. The sdRNA profile was different for proliferating and differentiated SH-SY5Y cells, suggesting that external cues such as retinoic acid treatment can also influence the processing of snoRNAs into sdRNAs. These results indicate that FUS influences snoRNA expression and ribosomal RNA modification. Secondly, some snoRNAs are processed into sdRNAs in a FUS-dependent manner. However, the function of these sdRNAs remains to be explored. Functional studies are necessary to explore the effects of individual rRNA modification sites on translation and how ALS-associated FUS mutation influences this process.

Keywords – snoRNA, ALS, FUS, 2'-O-Me, pseudouridine, sdRNAs.

1. Background and forming the hypothesis

FUS protein is involved in DNA and RNA binding and regulates downstream gene expression. Moreover, FUS involvement in microRNA biogenesis and processing has already been reported by previous studies[1,2]. Additionally, RNA immunoprecipitation data from our laboratory and others suggested that this protein binds to small non-coding RNAs, including small nucleolar RNAs (snoRNAs)[1,3], which are involved in guiding modifications of ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs). Therefore, the obvious question was identifying which snoRNAs are differentially expressed in FUS-depleted cells and analyzing the effect on ribosomal RNA modifications. Secondly, snoRNAs are known to be processed into smaller fragments called snoRNA-derived RNAs (sdRNAs). These sdRNAs can regulate gene expression and function like microRNAs[4]; hence, part of the work was to identify if snoRNAs are processed into sdRNAs in a FUS-dependent manner.

FUS mutations are associated with familial amyotrophic lateral sclerosis (ALS)[5,6]. Depending on the site of amino acid substitution, these mutations could be related to late-onset or rapidly progressive forms of the disease. Hence, the second part of the thesis focused on identifying changes in the snoRNA expression and its downstream effects in cells with ALS-FUS mutations. These cells were either already established cell lines, like neuroblastoma SH-SY5Y cells with FUS deletion and mutation, ALS-patient-derived fibroblasts and age-sex-matched controls and induced pluripotent stem cells reprogrammed from fibroblast with FUS P525L mutation and isogenic wild-type control, further differentiated into neuronal progenitor cells (NPCs) and motor neurons.

The following questions were explored in this doctoral thesis:

1. Is there a significant difference in the expression of snoRNAs between wild-type and FUS knockout cells in both proliferating and differentiated cell states?

2. Do differentially expressed snoRNAs have a significant downstream effect on ribosomal RNA modifications, specifically 2'-O-ribose methylation (2'-O-Me) and pseudouridylation, as measured by high-throughput sequencing techniques RiboMeth-seq and HydraPsiSeq?

3. Are there quantifiable changes in 2'-O-Me in ALS patient-derived fibroblasts compared to age-sex-matched control samples?

4. Can quantifiable changes in 2'-O-Me be observed in iPSCs, smNPCs, and motor neurons carrying the FUS P525L mutation compared to isogenic wild-type control samples?

5. Are snoRNAs processed into sdRNAs (snoRNA-derived RNAs) in a FUS-dependent manner in SH-SY5Y cells under proliferating and retinoic acid-induced differentiated conditions?

2. Introduction

FUS (Fused in sarcoma) is an RNA-binding protein belonging to the FET family that also includes EWSR1 (Ewing sarcoma breakpoint region 1) and TAF15 (TATA-box binding protein associated factor 15)[7]. FUS binds to pre-mRNA introns and facilitates alternative splicing[8]. FUS is also known to be involved in microRNA biogenesis and processing; this highlights its role in regulating small non-coding RNAs[1,2]. Our work earlier showed that FUS is also involved in replication-dependent histone gene expression in complexes with U7 snRNP[9,10]. FUS binds to a plethora of RNAs that may or may not contain the consensus FUS binding sequences GUGGGU motif[11]. Not limited to the consensus binding motif, FUS binds to RNAs lacking this sequence or a defined secondary structure, suggesting a more global RNA binding ability[12]. It also binds single and double-stranded DNA, facilitating genome maintenance while participating in DNA repair[8].

Amyotrophic lateral sclerosis is a neurodegenerative disease that can be affected without any known cause, called sporadic ALS, while mutations within genes like *C9orf72*, *SOD1*, *TDP-43*, *FUS* and others can cause 'familial ALS'[5,6]. ALS primarily causes progressive loss of upper and lower motor neurons, resulting in progressive paralysis and death with no direct treatments or medicines available[13]. Even though many genes have been linked to this disease, only a fraction of the cases can be attributed to a familial form of ALS with parental inheritance; most cases are sporadic and hence no known causal relationship exists[13]. Another RNA-binding protein linked to ALS, TDP-43, shares many structural and functional similarities with FUS[14]. Hence, aberrant RNA processing and metabolism have been proposed as significant pathways affected in ALS associated with *TDP-43* and *FUS* mutations[11,14]. Even though involved in mRNA nucleocytoplasmic shuttling, FUS protein is mainly localized in the nucleus. In contrast, mutant FUS is frequently mislocalized to the cytoplasm and is known to form cytoplasmic aggregates that include other RNA-binding proteins, RNP complexes and mRNAs[15,16]. The ALS progression is not uniform among *FUS* mutations; mutations like FUS P525L, where amino acid proline is substituted by leucine (P to L at 525 position within NLS), and FUS R495X, where whole NLS signal is missing due to introduction of a premature stop codon, are associated with juvenile-onset and rapidly progressing ALS where clinical symptoms are visible at a very young age as opposed to other, more common, slow-progressing mutations like FUS R521C (arginine to cysteine at position 521 within NLS)[17].

Small nucleolar RNAs are mainly located in the nucleolus and are primarily involved in guiding ribosomal or small nuclear RNA modifications. Moreover, recent studies suggest they can also guide modifications on mRNA and tRNAs[18–20]. These small RNAs are mainly located within introns of protein-coding or non-coding genes, while some snoRNAs are transcribed as independent transcription units[21]. snoRNAs can be mainly divided into two subtypes based on the conserved motifs within sequences;

C/D box snoRNAs consist of two conserved sequence motifs, C box (RUGAUGA) and D box (CUGA) located at 5' end and 3' end, respectively. Apart from these conserved motifs, less conserved C' and D' boxes also exist, mainly localized to the middle of the sequence[22,23]. The C/D box snoRNAs form ribonucleoprotein complexes with NOP56, NOP58, 15.5K and methyltransferase fibrillarin (FBL). C/D box snoRNP complex recognizes the target through a specific sequence called an anti-sense element (ASE). The methyltransferase FBL then carries out 2'-O-methylation of the ribose on 5th nucleotide upstream of the D or D' box[22] (Figure 1A). H/ACA type of snoRNAs contains a box H (ANANNA, N is any nucleotide) and box ACA (ACA trinucleotide) motifs. H/ACA snoRNAs have a more complex secondary structure with hairpin-hinge-hairpin-tail where box H is present in the hinge region and box ACA is located within the 3' end of the tail. A 9-13 nucleotide region within the internal loops recognizes the target sequence by base complementarity[24]. H/ACA box snoRNP includes NHP2, GAR1, NOP10 and pseudouridine synthase involved in target pseudouridylation, dyskerin (DKC1)[24] (Figure 1B). A third type of snoRNAs are small Cajal body RNAs (scaRNAs) localized to Cajal bodies; they consist of a Cajal-body-specific motif UGAG[25]. scaRNAs are mainly involved in guiding 2'-O-methylation and pseudouridylation of snRNAs[25].

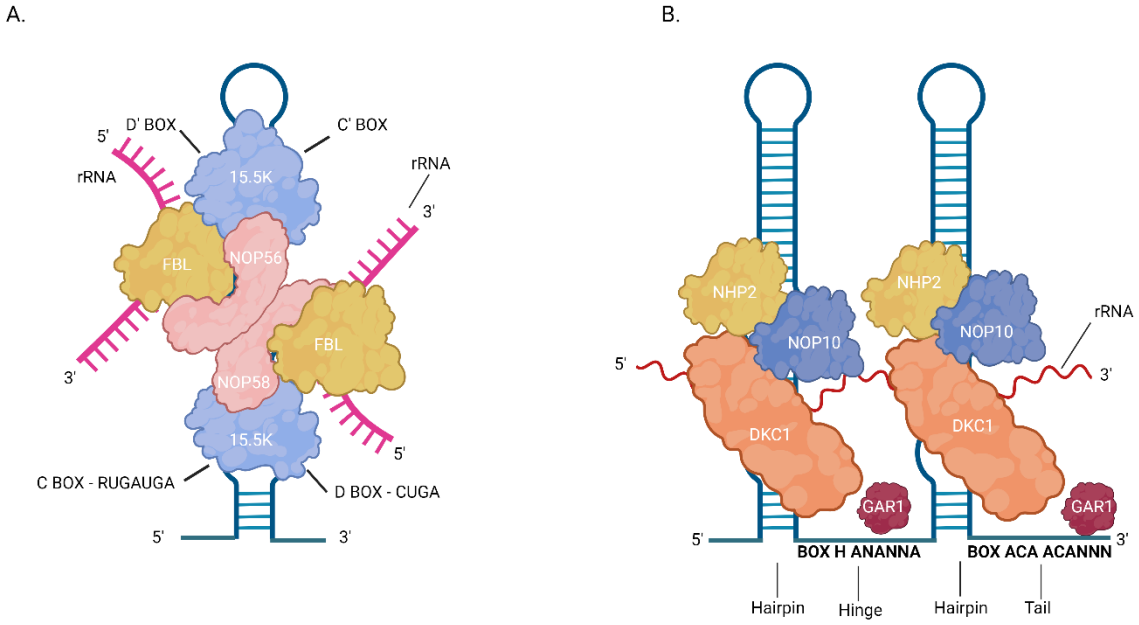


Figure 1: C/D box and H/ACA box snoRNP complexes: **A.** FBL, NOP56, NOP58 and 15.5kD proteins assemble on a C/D box snoRNA to form a functional snoRNP. Fibrillarin catalyzes 2'-O-Me on target rRNA while other proteins provide necessary structural and functional stability. **B.** DKC1, NHP2, NOP10 and GAR1 form the box H/ACA snoRNP assembly. DKC1 facilitates pseudouridylation of the target RNA while other proteins are necessary for stability and catalytic activity of DKC1. (Prepared using https://biorender.com/).

Interestingly, not limited to RNA modifications, snoRNA of both C/D and H/ACA classes are known to be involved in many other cellular processes, including alternative splicing, post-transcriptional regulation, pre-rRNA processing and tumorigenesis[21,22,26]. Even though many C/D box snoRNAs have been known to guide 2'-O-methylation at a specific site in rRNA, about 50% of these snoRNAs are considered 'orphan.' Moreover, most of these 'orphan' C/D box snoRNAs are concentrated in two major snoRNA clusters, *SNORD113-SNORD114* on chromosome 14 (14q32.2) and *SNORD115-SNORD116* on chromosome 15 (15q11-q13). The 14q32.2 and 15q11-q13, also known as *DLK1-DIO3* and *SNURF-SNRPN*, respectively, are known as 'imprinted regions' where parent-of-origin-specific imprinting control centers control the allelic expression[27,28]. Deletions, epimutations and uniparental disomy within the *SNORD115-SNORD116* cluster are known to be involved in neurodevelopmental disorders, Prader-Willi syndrome and Angelmann syndrome. Notably, similar genetic defects within the *SNORD113-SNORD114* cluster are associated with Kagami-Ogata syndrome and Temple syndrome[27–29].

Non-coding RNAs like tRNAs, rRNAs, and snoRNAs are known to undergo processing and produce shorter RNA fragments that may act as microRNAs and regulate post-transcriptional and post-translational gene expression[26,30,31]. The stable shorter fragments of 20-35 nucleotides produced from snoRNAs are known as sdRNAs (snoRNA-derived RNAs), this size may vary depending on the class of parent snoRNA[30]. Some of these sdRNAs depend on Drosha/DGCR8 mediated processing, as observed in the case of scaRNA15 (ACA45)[32]. Interestingly, 'orphan' C/D box snoRNA, HBII-52 (SNORD115) can be processed into smaller fragments and these sdRNAs are involved in alternative splicing of *DPM2*, *TAF1*, *RALGPS1*, *PBRM1* and *CRHR1* pre-mRNAs[33]. In another work, sdRNAs processed from SNORD114-1, another orphan C/D box snoRNA, are associated with endothelial cell abundance and tumor vascularisation[34]. In recent years, sdRNAs have been associated with cancers as having both tumorigenic and anti-tumorigenic functions[26,30]. For example, the level of sdRNA-93 derived from SNORD93 correlates with breast cancer invasiveness; this sdRNA regulates the expression of Pipox, a sarcosine metabolism-associated protein that determines the molecular subtype of breast cancer[35]. Another study implicated a feedback loop between anti-tumorigenic P53, snoRNA host gene SNHG1, and sno-MiR-28 derived from SNORD28[36].

As described earlier, the C/D box and H/ACA class of snoRNAs are involved in site-specific guiding of 2'-O-Me and pseudouridylation on rRNAs, respectively. Due to recent advances in detecting RNA modifications by next-generation sequencing (NGS)-based techniques, it is possible to map 2'-O-Me and pseudouridine levels in rRNAs and snRNAs quantitatively[37,38]. Most known modified sites are present in the functional regions of the ribosome, like peptidyl transferase center (PTC), decoding center (DCS) and tRNA-interacting sites[38,39]. These sites are primarily fully modified in any given

physiological condition. In contrast, 2'-O-Me and pseudouridine sites on ribosomal periphery are substoichiometric, and the proportion of modification can be regulated depending on the internal or external environmental processes[39,40]. These substoichiometric sites, therefore; provide another layer for generating heterogeneous ribosomes that could be involved in the translation of specific sets of mRNAs. Indeed, recent studies implicate specific 2'-O-Me sites that regulate the translation of a specific set of mRNAs, and this adds to the growing evidence for ribosomal heterogeneity[41–43].

3. Summary of the doctoral work

The following questions were answered in this section:

1. Is there a significant difference in the expression of snoRNAs between wild-type and FUS knockout cells in both proliferating and differentiated cell states?

2. Do differentially expressed snoRNAs have a significant downstream effect on ribosomal RNA modifications, specifically 2'-O-ribose methylation and pseudouridylation, as measured by high-throughput sequencing techniques RiboMeth-seq and HydraPsiSeq?

Kishor Gawade, Patrycja Plewka, Sophia J. Häfner, Anders H. Lund, Virginie Marchand, Yuri Motorin, Michal W. Szczesniak & Katarzyna D. Raczynska, **FUS regulates a subset of snoRNA expression and modulates the level of rRNA modifications.** Sci Rep 13, 2974 (2023). https://doi.org/10.1038/s41598-023-30068-2

We wanted to check the effect of FUS protein on snoRNA expression, and for this purpose, I developed HEK293T cells with FUS knockout (FUS KO). A colleague from our group developed SH-SY5Y cells with ALS-associated FUS mutation R495X that lack a nuclear localization signal. Neuroblastoma SH-SY5Y cells with homozygous FUS knockout were a kind gift from Dr. Marc-David Ruepp from King's College, London, UK. SH-SY5Y cells were treated with retinoic acid (RA) for ten days in DMEM +10% FBS to generate neuron-like cells to mimic the effect of FUS mutations in neurons[9].

As the first part of our work, we performed high-throughput small RNA sequencing on wild-type (WT) and FUS knockout SH-SY5Y cells in proliferating and differentiated conditions (RA-treated). After processing the raw sequencing files and quality control, we performed differential gene expression analysis (DEG). Surprisingly, many small non-coding RNAs were differentially expressed between WT and FUS KO cells, in both proliferating and differentiated conditions. In proliferating and differentiated SH-SY5Y FUS knockout cells, orphan C/D box snoRNAs from the known imprinted clusters, *SNORD113-SNORD114* and *SNORD115-SNORD116*, were mainly downregulated. Some of these 'orphan' imprinted snoRNAs have regulatory functions, discussed in our review, forming the second published part of this doctoral thesis[29]. HEK293T FUS KO and SH-SY5Y FUS R495X cell lines were generated much later in work and not sent for small RNA sequencing. It would have been interesting to see in the future, if FUS mutation and depletion can produce similar changes in small RNA transcriptomes in cells of different

origins. Secondly, using an isogenic line after restoring the wild-type FUS gene in mutant cells could have been a better control, and we addressed this in the next part of this study (unpublished work).

C/D box snoRNAs guide 2'-O-methylation, while H/ACA box snoRNAs guide pseudouridylation on mostly ribosomal and small nuclear RNAs. Our previous results from small RNA sequencing data drove us to look for the downstream effects of differentially expressed snoRNAs. For this purpose, we utilized the RiboMeth sequencing technique to map 2'-O-Me on ribosomal RNAs quantitively. I used RNA from SH-SY5Y WT and FUS KO cells in proliferating and differentiated conditions, SH-SY5Y FUS R495X in differentiated conditions and HEK293T cells with FUS knockout and WT control. The RiboMeth sequencing library preparation and data analysis were performed at the University of Copenhagen, Denmark, according to published protocols[37]. We considered 2'-O-Me positions as affected by FUS when the modification levels at particular positions differed by at least 0.05 (5%). Using this criterion, we had an interesting observation where substoichiometric 2'-O-Me sites were highly modified in FUS KO or FUS R495X cells compared to WT controls. This effect aligns with our small RNA sequencing and RT-qPCR analysis, where some of the corresponding guide C/D box snoRNA levels were upregulated in FUS KO and FUS R495X cells. Fractionally modified positions, like 18S-Um354 and 18S-Cm1272, showed higher modification in HEK293T FUS KO, SH-SY5Y FUS KO and FUS R495X cells, while 18S-Gm436 exhibited higher 2'-O-Me levels specifically in neuroblastoma cells with FUS mutation and depletion. Interestingly, most positions differentially modified in FUS KO and FUS R495X cells belong to 'fractionally modified' sites. The proportion of modification of these sites might be an essential contributor to generating ribosome heterogeneity. Moreover, we used the Snoscan prediction server to identify probable 2'-O-Me positions within ribosomal RNAs[44]. Of the putative sites, SNORD44 expression and the level of 2'-O-Me at 18S-Cm1000 in SH-SY5Y FUS KO proliferating cells were downregulated. A similar expression pattern was observed for other 'putative' sites, like 28S-Cm2075, which was changed in all the cells. However, it is necessary to confirm these 'putative' sites using deletion/reconstitution of corresponding snoRNAs, mass spectrometry, and other high-throughput sequencing techniques like direct RNA Nanopore sequencing.

Furthermore H/ACA box snoRNAs that guide pseudouridine synthase, DKC1 to introduce site-specific pseudouridine on rRNAs and snRNAs were differentially expressed in our small RNA sequencing data. To explore downstream effect of differentially expressed H/ACA box snoRNAs on pseudouridine levels we used HydraPsiSeq[38]. This high-throughput sequencing technique quantitatively maps pseudouridine levels at specific sites on ribosomal RNAs[38]. As previously known, most of the pseudouridine sites were fully modified. The pattern of changes of the fractionally modified sites was similar as observed in the case of 2'-O-Me, where modification was higher in FUS KO and FUS R495X cells. Interestingly, most of the significantly changed sites were present on 28S

rRNA. Upregulated expression of SNORA44 was associated with higher pseudouridine at the 18S-Psi897 site, and higher expression of SNORA47, SNORA37, SNORA43 and SNORA33 was associated with higher pseudouridylation at corresponding sites 28S-Psi1779, 28S-Psi4673, 28S-Psi4973 and 28S-Psi5001, respectively. Similar to our observations for 2'-O-Me, only a subset of H/ACA box snoRNA expression correlates with pseudouridine levels. This suggests that apart from H/ACA box snoRNA levels, different mechanisms that regulate pseudouridine levels may exist. Not only limited to rRNAs, we also observed some changes in the pseudouridine levels of snRNAs where this modification was increased at specific sites on U2, U5A, and U6 snRNAs.

To shed some light on the mechanisms involved in the FUS-mediated changes in snoRNA levels, we checked the expression of snoRNA host genes; these host genes were primarily upregulated in HEK293T FUS KO cells. Furthermore, RNA immunoprecipitation showed that FUS binds to both snoRNAs and snoRNA host gene transcripts, which made it impossible to distinguish whether FUS binds directly to mature snoRNAs or solely to their host gene transcripts. The snoRNP complex proteins FBL, NOP56, and DKC1 were not significantly changed in FUS KO or FUS mutant cells, which suggests that these proteins are probably not responsible for the observed changes in rRNA modifications. Furthermore, to mark where the significantly changed modified sites are present on the 80S ribosome, we performed structural analysis using Pymol 2.0 software and a published 80S ribosome structure[45]. This analysis suggested that most sites are present on the surface of the 80S ribosome rather than at critically essential positions like DCS, E-site, and PTC. Some 2'-O-Me sites, like 18S-Um354 or 18S-Cm1440, map to the periphery where they may interact with ribosomal proteins and hence are regulated according to environmental stimuli. Another fractionally modified site, 18S-Cm1272, is present near DCS, and interestingly, the guide C/D box SNORD66 is also significantly changed in FUS-depleted and mutant cells. To elucidate whether changes in rRNA modifications affect global translation, we performed a SUnSET (Surface Sensing of Translation) assay that involves treating the cells with puromycin followed by western blotting. This assay revealed a slight reduction in global translation in HEK293T FUS KO cells, while no changes were observed in SH-SY5Y FUS KO and FUS R495X cells. The latest studies suggested that changes in 2'-O-Me at specific sites may result in changes in the translational efficiency of a particular set of mRNAs involved in dedicated pathways[41,46].

4. Summary of the doctoral work:

Kishor Gawade, Katarzyna D. Raczynska, Imprinted small nucleolar RNAs: Missing link in development and disease? WIREs RNA, 2023. http://doi.org/10.1002/wrna.1818

As mentioned earlier, FUS depletion resulted in differential expression of snoRNAs, and the majority of these snoRNAs belong to two 'orphan' C/D box snoRNA clusters present within 14q32.2 (*DLK1-DIO3*) and 15q11-q13 (*SNURF-SNRPN*) imprinted domains. Imprinting is a type of epigenetic regulation where the expression of an allele is parent-of-origin specific. Moreover, recent work has assigned some functions to these imprinted 'orphan' snoRNAs.

Prader-Willi/Angelmann syndrome (PWS/AS) is caused by uniparental disomy, deletions or epimutations within the 15q11-q13 imprinted region. The role of deletions within the *SNORD115-SNORD116* cluster in developing clinical phenotypes in PWS is well-studied[27]. Moreover, Kagami-Ogata syndrome (KOS14) and Temple syndrome (TS14) are imprinting disorders caused by uniparental disomy, deletions or epimutations within the 14q32.2 imprinted region[28]. Interestingly, this region harbors *SNORD113-SNORD114* imprinted C/D box snoRNA cluster, and the role of deletions within this cluster has not been directly linked to KOS14. In this review, we have highlighted that the *SNORD113-SNORD114* cluster is responsible for developing at least some clinical phenotypes in KOS14/TS14[29]. KOS14 phenotype involves abnormal thoracic and cardiovascular development. We highly reviewed studies highlighting how individual snoRNAs in the *SNORD113-SNORD114* cluster may contribute to cardiovascular development and disease[19,20].

The most striking involvement of these snoRNAs is observed in autism spectrum disorder (ASD). Intellectual disability and ASD have high co-occurrence; importantly, KOS14 and TS14 individuals display intellectual disability and developmental delay phenotypes. *SNORD113-SNORD114* cluster is involved in the splicing of ASD-relevant pre-mRNAs, and changes in the expression of this cluster in KOS14/TS14 may contribute to aberrant splicing and ASD phenotypic development[47]. Not restricted to only these conditions, snoRNAs from this cluster have been implicated in neurodegenerative disorders, placental development, and cancers. Additionally, we have focused on piwi-interacting RNAs (piRNAs) from this cluster involved in developing neurodegenerative diseases and cancers. Our review provides insights into the *SNORD113-SNORD114* cluster in development and disease and opens up new frontiers that can be delved into.

5. FUS mutation-dependent changes in ribosomal RNA modifications in ALS patient-derived fibroblasts, iPSCs, smNPCs, and motor neurons (unpublished results part 1)

Following questions were answered in this section:

3. Are there quantifiable changes in 2'-O-Me in ALS patient-derived fibroblasts compared to age-sex-matched control samples?

4. Can quantifiable changes in 2'-O-Me be observed in iPSCs, smNPCs, and motor neurons carrying the FUS P525L mutation compared to isogenic wild-type control samples?

5.1. Materials and Methods: iPSCs, smNPCs, ALS patient-derived fibroblasts and controls were received from our collaborators at the Medical University of Rostock, Germany. The isogenic pair of iPSCs, WT and FUS P525L, contain an eGFP tag attached to the FUS protein.

Table 1: Material needed for Proliferation and differentiation of smNPCs into motor neurons.

Supplement name	Catalogue No.	Weight/Vol.	Manufacturer
L-Ascorbic acid	A4544-25G	25 gm	Sigma
Chiron99021	13122	5 mg	Cayman chemicals
PMA	10009634	5 mg	Cayman chemicals
BDNF	B3795-5UG	5 µg	Sigma
GDNF	SRP3309-10UG	10 µg	Sigma
dCAMP	D0627-100MG	100 mg	Sigma
TGF- β3	100-36E	10 µg	Peprotech
RA (Tretinoin)	1674004	30mg	USP
Neurocult SM1- without RA	05731	10 mL	StemCell Tech.
Poly-L-ornithine solution (PLO) (0.01%)	A-004-M	100 ml	Sigma
Mouse laminin I	3400-010-02	1 mg	R&D systems
DMEM/F-12, GlutaMAX™ supplement	10565018	500 ml/bottle	Gibco
Neurobasal™ medium	21103049	500 ml/bottle	Gibco
Accutase	25-058-CI058CI	100 ml/bottle	Corning

BDNF - brain-derived neurotrophic factor, dCAMP - N6,2'-O-dibutyryladenosine 3',5'-cyclic monophosphate sodium salt, GDNF - glial cell-derived neurotrophic factor, PMA – Purmorphamine, RA - retinoic acid, TGF-β3 – transforming growth factor - β3.

Fibroblast Culture: ALS-Fibroblasts were cultured according to the protocol published in Scientific Reports that forms the main part of this thesis[3].

smNPC culture and differentiation: smNPCs were cultured and differentiated into motor neurons as previously described by our collaborators[48]. To have more mature neurons, differentiation was continued in maturation media for four weeks instead of the three weeks recommended[48]. Detailed protocol for expansion and differentiation of smNPCs is explained in Figure 2, and stock concentrations of all the chemicals and factors used are listed in Table 2.

Table 2: Dilution and stock concentrations of chemicals/factors.

Name of the chemical	Procedure for stock preparation	Working stock concentration	storage
L-Ascorbic acid	528.36 mg diluted in 10 ml sterile H_2O, sterile filter.	0.3 M	-20^{0}C
Chiron99021	5 mg in 3.58 ml DMSO	3 mM	-20^{0}C
PMA	5 mg in 5 ml DMSO	1 mg/ml	-20^{0}C
BDNF	5 µg in 5 ml sterile 1X PBS +1% BSA.	1 ug/ml	-20^{0}C
GDNF	10 µg in 1ml	10 ug/ml	-20^{0}C
dCAMP	24.57 mg in sterile 1 ml H_2O	50 mM	-20^{0}C
RA	30 mg RA + 6 ml DMSO = 16.64 mM RA 1 ml RA (16.64 mM) + 7.32 ml DMSO	2 mM	-80^{0}C
TGF- β3	10 µg in 5 ml citric acid monohydrate (5 mM) 5 mM citric acid – 10.507 mg in 10 ml sterile H_2O	2 ug/ml	-20^{0}C

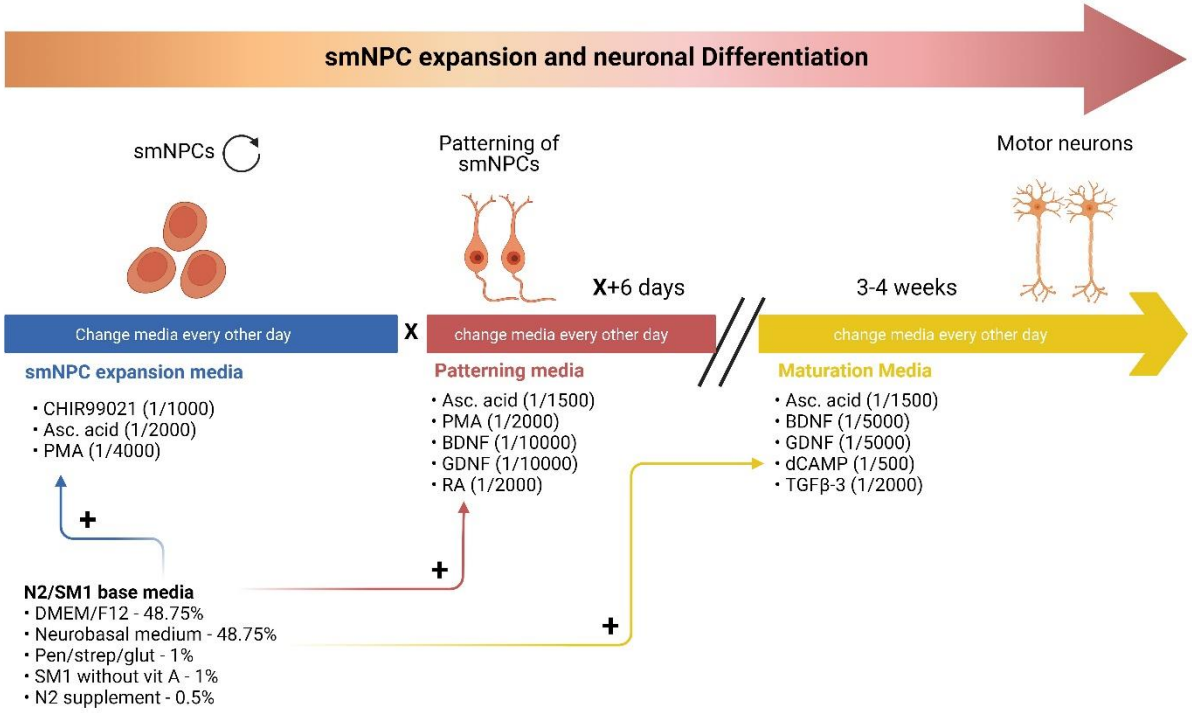


Figure 2: Protocol for expansion and differentiation of smNPCs. N2/SM1 base media forms the basis of all the media used for expansion and differentiation; the volume of each component needed to prepare N2/SM1 media is mentioned as a percentage of the total volume. The circular arrow near smNPCs represents self-renewal potential. The final dilution for each component used are mentioned in the brackets. 'X' is the starting point of the patterning media and is continued for 6 days. (Generated using biorender.com).

Before starting a revival or culture of smNPCs, cell culture plates/flasks were coated with matrigel diluted 1:100 in 1x PBS. Cells were split at a 1:10 ratio using accutase. To start the differentiation, smNPC expansion media was replaced with patterning media (Figure 2). It was changed every other day for 6 days. For motor neurons, tissue culture plates were coated with 15% PLO (0.01%) diluted in 1X PBS and incubated at 37 °C overnight. The next day, PLO was removed, and the plate was washed with 1x PBS two times. Next, laminin was diluted at 1:100 in 1x PBS, and the plates were coated and incubated overnight at 37 °C. The following day, plates were washed two times with 1x PBS before transferring 'patterned' smNPCs into PLO/laminin-coated plates. Patterned smNPCs were seeded at an appropriate cell quantity (5,00,000/well of a 6-well plate), and the medium was changed to maturation media (Figure 2).

Immunofluorescence: Fibroblasts, smNPCs, and neurons were grown on chambered coverslips (Ibidi). The differentiation of smNPCs into neurons was carried out, as mentioned earlier in the chambered coverslips. smNPC-derived neurons were fixed after four weeks in the maturation media. All the cells were fixed with 4% PFA and permeabilized in 1x PBS pH 7.0 + 0.5% Triton X-100 (PBS-T). For staining, cells were washed twice with 70% ethanol for 2 min and three times with PBS for 5 min. Next, the cells were incubated with a blocking solution (1% BSA in PBS) for 30 minutes at room temperature. Primary antibodies used included anti-FUS antibody (Santa Cruz, SC 47711, 1:500), anti-MAP2 (Abcam, ab5392, 1:500), anti-β-Tubulin III (Sigma, T8578, 1:500), and anti-FBL (Santa Cruz, H140, 1:500) for 1 hour at room temperature. After washing the cells with blocking solution, staining was performed using the following secondary antibodies, Alexa Fluor 555 antibody (Thermo Fisher Scientific A21422), Alexa Fluor 488 (Invitrogen, A32723 or A315532), Alexa fluor 647 (Jackson ImmunoResearch, Code: 703-605-155) in blocking solution for 45 min RT. The image acquisition was performed using a confocal scanning microscope (Nikon A1Rsi) using a 100×/1.4 or 63×/1.4 oil-immersion objective.

RNA extraction and RNA quantitation was performed as described in my previous work[3].

Data availability: The RiboMeth sequencing data used for this analysis will be deposited in NCBI GEO once the results are published in a peer-reviewed journal.

5.2. Results

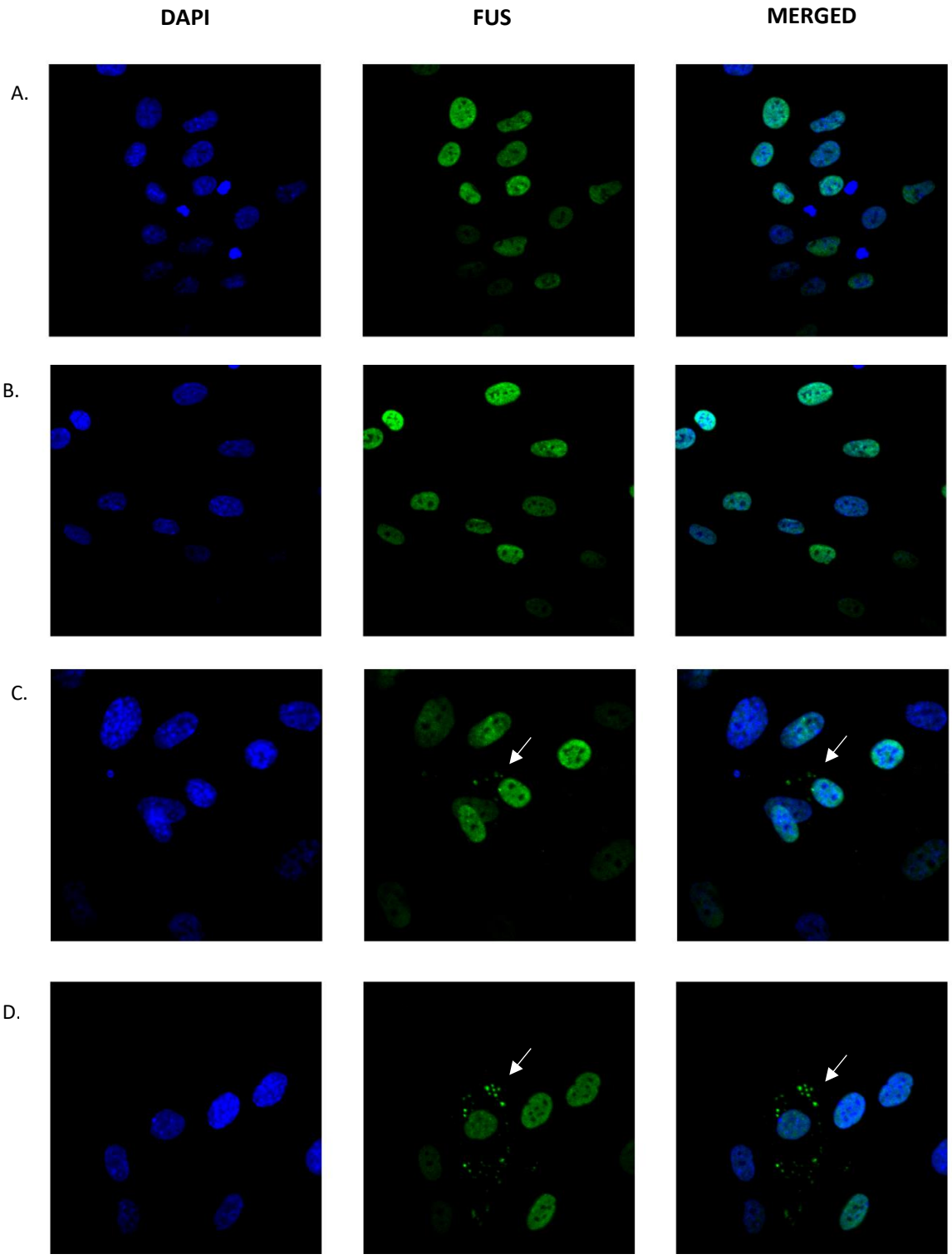
In our previous work, we identified a substantial number of differentially expressed snoRNAs and changes in the ribosomal RNA modifications. To complement our previous work, I needed to determine if I could observe any changes in the ribosomal RNA modifications in ALS patient-derived cells. For this purpose, we collaborated with Prof. Andreas Hermann's laboratory at the Medical University of Rostock, Germany. iPSC culture and RNA extraction were performed by our collaborators, and I received the extracted RNA for RiboMeth sequencing analysis. We received three lines of FUS-mutant ALS patient-derived fibroblasts and age-sex-matched control fibroblasts. The clinical phenotypes of these FUS-ALS patients are presented in Table 3.

Table 3: Clinical details of the healthy individuals and ALS patients.

Fibroblast line name	Age at biopsies	Sex	FUS mutation	Clinical symptoms
AK	48	W	Control 1	NA
KA	28	W	Control 2	NA
WK	34	W	FUS P525L	Not available
KG	58	W	FUS R521C	ALS, arms and bulbar
ML	65	W	FUS R521L	ALS, flail arm, DD spinal, disease duration three years

NA – not applicable, W - woman

A wild-type FUS protein is mainly localized to the nucleus. In contrast, ALS-associated FUS mutant proteins are mislocalized to the cytoplasm. Mislocalized FUS proteins form cytoplasmic aggregates, which is a hallmark of ALS[49,50]. Motor neurons are the primary cell type affected in ALS, and previous studies have shown that mutant FUS mislocalized to the cytoplasm in these cells[49,51]. It was necessary to check if this hallmark of mutant FUS is also present in other cell types, such as patient-derived fibroblasts. I prepared the slides with all the ALS-patient fibroblast lines to check for FUS protein mislocalization. As observed in Figure 3, some mutant FUS protein mislocalization is visible in WK FUS P525L and KG FUS R521C fibroblast cells. In contrast, ML R521L line, as well as control fibroblast cells showed no evidence of FUS mislocalization.



A. AK_CNTRL1, **B.** ML_FUS_R521L, **C.** KG_FUS_R521C, **D.** WK_FUS_P525L.

Figure 3: Localization of FUS protein in ALS patient-derived and control fibroblasts. ALS-FUS fibroblasts were stained with nuclear stain DAPI (blue) and anti-FUS antibodies + fluorescent secondary antibody (green). White arrows represent mutant FUS mislocalized to the cytoplasm in KG_FUS_R521C and WK_FUS_P525L fibroblasts, while no FUS mislocalization was observed in ML_FUS_R521L or AK control line.

Three biological replicates from each line (except the KA line, for which only two biological replicates were used) were sent for RiboMeth-seq analysis to identify 2'-O-Me changes in these ALS fibroblasts. Interestingly, we did observe changes in the 2'-O-Me levels of 18S and 28S rRNA, but the differences were subtle and not as prominent as in SH-SY5Y cells with R495X mutation[3]. The heatmap presents all the 2'-O-Me sites within rRNAs (Figure 4). Even though a few sites showed some changes in the 2'-O-Me, most were fully modified. In the case of 18S-Cm1440, there is a complete absence of modification, which is interesting as this site is modified in the cells of neuronal origin[3].

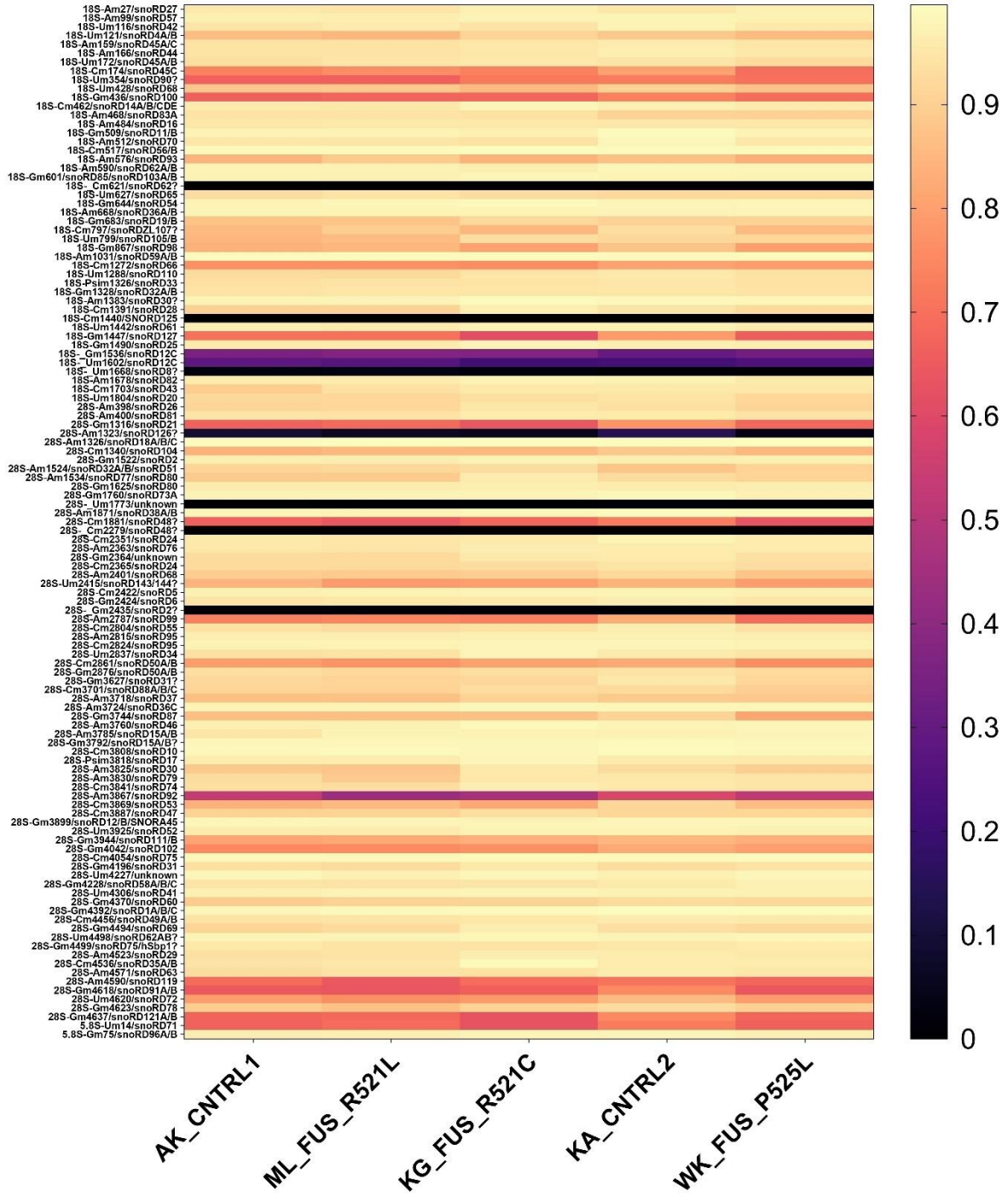


Figure 4: All the 2'-O-Me sites on rRNAs in ALS patient-derived fibroblasts compared to age-sex-matched control. Respective rRNA, 2'-O-Me site, and probable guide C/D box snoRNA associated with the site are mentioned for each row, while column names represent the name of the fibroblast cell line. AK_CNTRL1 is a control line for ML_FUS_R521L and KG_FUS_R521C; KA_CNTRL2 is a control line for WK_FUS_P525L. The legend describes the colour associated with the fraction of 2'-O-Me at a given site on the heatmap. The black coloured rows represent a complete lack of 2'-O-Me at that site.

Further, I set the criteria of a significantly changed 2'-O-Me position on ribosomal RNA as follows:

1. Significance based on paired-T-test is less than 0.05;

2. The difference between the level of 2'-O-Me in the control fibroblast vs. FUS mutant line is more than 5%.

I identified some significantly changed 2'-O-Me positions on 5.8S, 18S, and 28S rRNA with the above criteria. They mainly correspond to substoichiometric modifications (Figure 4, Table 4). As represented in Figure 5, only two positions were significantly altered between control and FUS R521L fibroblasts, while five positions were significantly changed in FUS R521C fibroblasts. Interestingly, both these mutations are considered slow progressing, and ALS development is also observed at a significantly later age[17] (Table 3). Further, WK (FUS P525L) line analysis revealed twelve significantly changed 2'-O-Me sites on rRNA; these sites were spread on 18S, 28S, and one on 5.8S rRNA (Figure 5, Table 4). Unlike our previous results (Gawade et al., 2023), these sites showed lower 2'-O-Me levels than the age-sex-matched control fibroblast cells (Table 4).

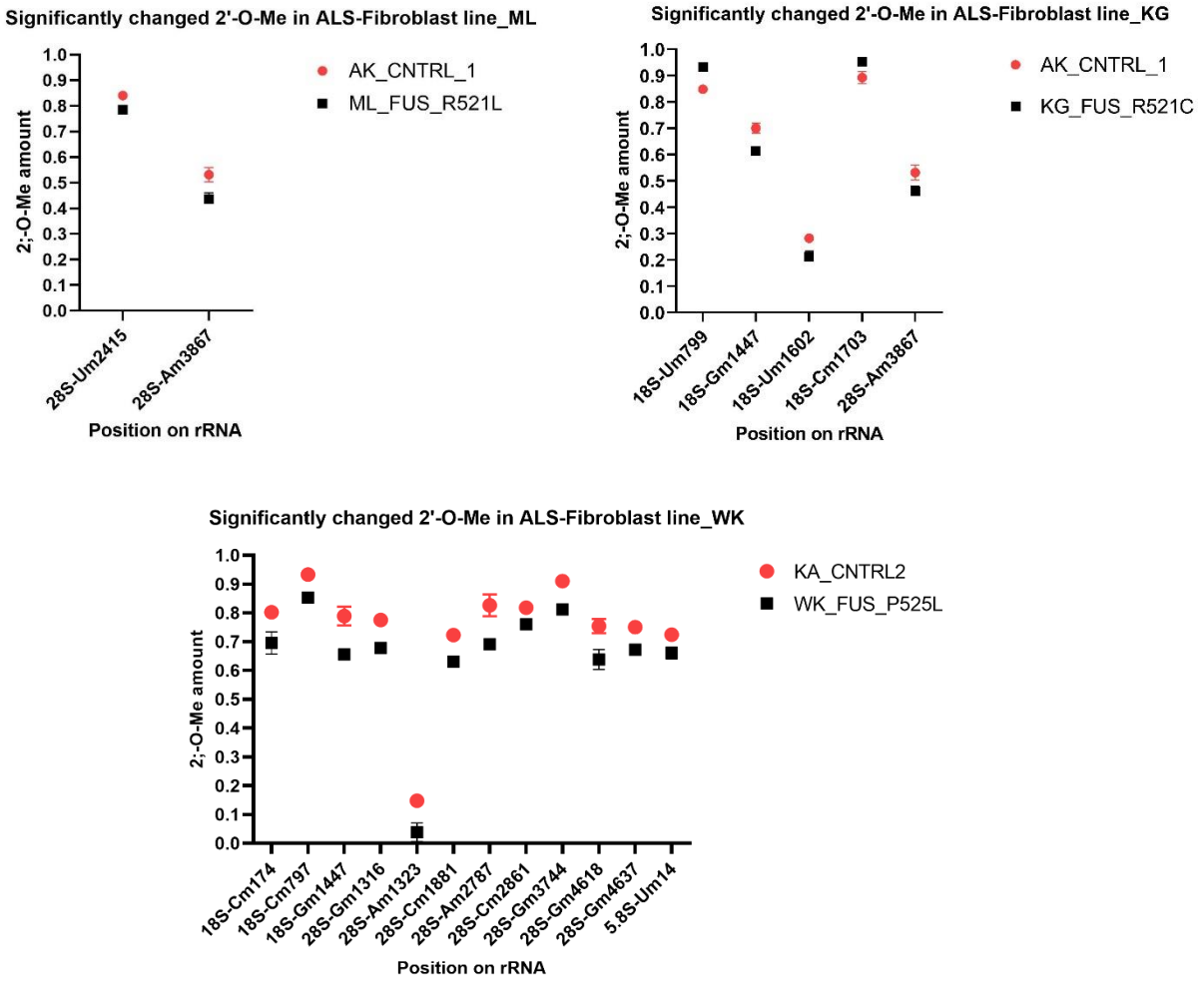


Figure 5: Significantly changed 2'-O-Me positions in ALS patient-derived fibroblasts compared to age-sex-matched control. AK_CNTRL_1 is a control fibroblast line for ML (FUS R521L) and KG (FUS R521C) fibroblast lines, while KA_CNTRL2 is a control fibroblast line for WK (FUS P525L) line. As observed in the scatter plot, the FUS P525L mutation, which is known to be associated with rapidly progressing juvenile ALS, had more significantly changed 2'-O-Me positions compared to the 'mild' FUS mutations, FUS R521L and R521C. The X-axis represents the position on the rRNA; the Y-axis represents a fraction

of 2’-O-Me at the corresponding position on the X-axis. *Error bars of standard deviation (std dev) are invisible when it is not very high.

Table 4: Significantly changed 2’-O-Me sites in ALS patient-derived fibroblasts compared to age-sex-matched controls.

Position on the rRNA	Average 2’-O-Me in AK_control_1 fibroblast	Average 2’-O-Me in ML_FUS R521L fibroblasts	T-test	C/D box snoRNA guiding 2’-O-Me
28S-Um2415	0.840798	0.785525	0.000167	SNORD143/144?
28S-Am3867	0.531408	0.436163	0.0119	SNORD92
Position on the rRNA	Average 2’-O-Me in AK_control_1 fibroblast	Average 2’-O-Me in KG_FUS R521C fibroblasts	T-test	C/D box snoRNA guiding 2’-O-Me
18S-Um799	0.848161	0.931478	0.000839	SNORD105A/B
18S-Gm1447	0.699905	0.612757	0.002335	SNORD127
18S-Um1602	0.282279	0.212276	0.009922	SNORD12C
18S-Cm1703	0.892297	0.953662	0.009663	SNORD43
28S-Am3867	0.531408	0.460888	0.02424	SNORD92
Position on the rRNA	Average 2’-O-Me in KA_control_2 fibroblasts	Average 2’-O-Me in WK_FUS P525L fibroblasts	T-test	C/D box SNORNA guiding 2’-O-Me
18S-Cm174	0.801677	0.695011	0.034788	SNORD45C
18S-Cm797	0.932719	0.852959	0.012533	SNORDZL107?
18S-Gm1447	0.789028	0.655305	0.005255	SNORD127
28S-Gm1316	0.775028	0.677927	0.006839	SNORD21
28S-Am1323	0.147724	0.037994	0.022885	SNORD126?
28S-Cm1881	0.723092	0.630537	0.011072	SNORD48?
28S-Am2787	0.82614	0.690805	0.006951	SNORD99
28S-Cm2861	0.818043	0.760173	0.043494	SNORD50A/B
28S-Gm3744	0.910089	0.81169	0.003031	SNORD87
28S-Gm4618	0.754127	0.637811	0.028536	SNORD91A/B
28S-Gm4637	0.750106	0.671579	0.005731	SNORD121A/B
5.8S-Um14	0.724112	0.661156	0.035578	SNORD71

Furthermore, because the analysis was performed on three different FUS mutations, the next thing was to check if these mutations indeed share any significantly changed 2’-O-Me sites. A common 2’-O-Me site between multiple mutations may highlight its importance in ALS pathogenesis by generating ribosomal heterogeneity, which might help in the differential translation of mRNAs belonging to specific pathways. While there was no detection of a joint in significantly changed 2’-O-Me sites between all the three investigated FUS mutations, site 28S_Am3867 was shared between FUS R521L and FUS R521C mutations. At the same time, 18S-Gm1447 was common between FUS R521C

and FUS P525L lines (Figure 6). Interestingly, the 2'-O-Me levels decreased compared to the control at both sites (Table 4). Moreover, both sites are fractionally modified, suggesting a regulatory role for these sites. One striking observation is that FUS P525L, associated with rapidly progressing juvenile ALS, presents more significantly changed 2'-O-Me sites than other 'mild' FUS mutations (Table 4, Figure 5).

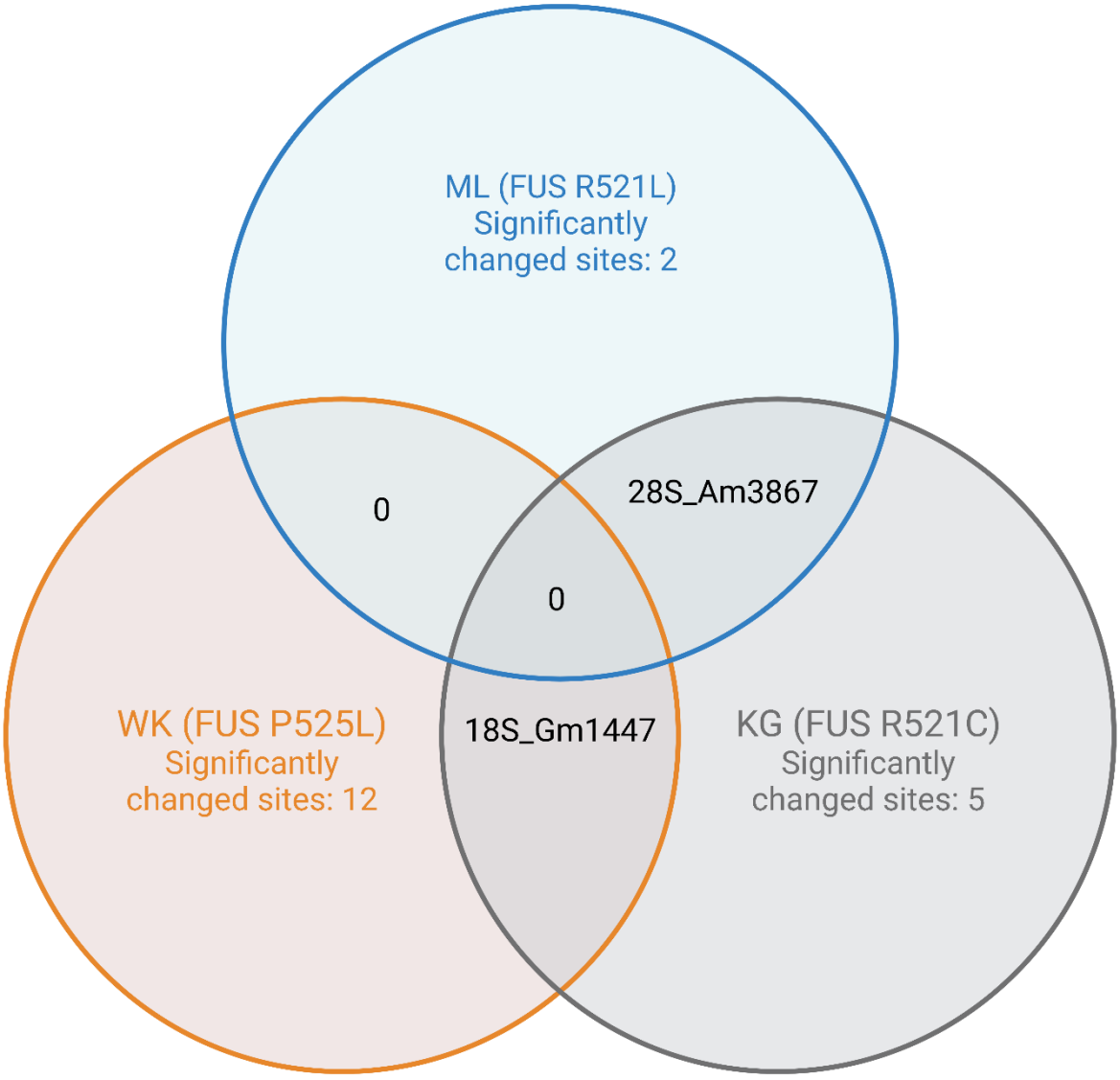


Figure 6: Venn diagram displays significantly changed 2'-O-Me sites in the three ALS patient fibroblast lines with FUS mutations. 28S_Am3867 is a common site between ML_FUS_R521C and KG_FUS_R521C lines, while 18S_Gm1447 site is common between WK_FUS_P525L and KG_FUS_R521C lines.

As fibroblast studies involved a comparison between ALS patient fibroblasts and controls, it was difficult to rule out the possibility of differences between 2'-O-Me profiles at the individual level. Different individuals may display a unique 2'-O-Me profile; a larger sample size is required to eliminate

this bias. Further, we performed only three FUS mutants and the corresponding age-sex-matched control comparison; hence, our smaller sample size may not represent the global 2'-O-Me scenarios in ALS patients. Motor neurons are the primary cell type that undergoes progressive and age-dependent loss in ALS, and changes in 2'-O-Me profiles in fibroblasts may not correctly capture the situation in ALS disease. Therefore, to address these concerns, we expanded our analysis to induced pluripotent stem cells generated from ALS patient fibroblasts and other cells that can be differentiated from them. Induced pluripotent stem cells provided a unique opportunity to examine the changes in 2'-O-Me profiles during differentiation into neuronal progenitor cells and motor neurons.

Our collaborators at the Medical University of Rostock, Germany, developed an isogenic iPSC line from KG_FUS_R521C fibroblasts[48]. Fibroblasts derived from ALS patients with a R521C (mild) mutation in the FUS gene were changed to wild-type and then to FUS P525L, a strong mutation associated with juvenile ALS. An eGFP tag was added to the isogenic WT and FUS P525L lines to better visualize FUS localization in live cells[48]. Due to the change of the original patient mutation R521C to P525L, we can observe a more severe phenotype. The isogenic iPSC lines provided a unique opportunity to determine the changes in 2'-O-Me when these cells are differentiated. Not only iPSCs but stable and self-renewable smNPCs were generated from the isogenic iPSC lines. These smNPCs were further differentiated into motor neurons, the primary cell type lost during the progression of ALS. This differentiation protocol is already standardized and published[48]. Hence, we only performed staining with neuron markers, MAP2 and β-Tubulin III, to confirm the successful differentiation of smNPCs into neurons (Figure 7). Three biological replicates from isogenic WT and FUS P525L iPSCs, smNPCs, and motor neurons differentiated from smNPCs were sent for RiboMeth sequencing to our collaborators at the University of Lorraine, France.

The changes in the 2'-O-Me sites on rRNA in iPSCs, smNPCs, and motor neurons are evidenced by the heatmap in Figure 8. Like earlier fibroblast data, the changes are concentrated amongst fractionally modified 2'-O-Me sites. Interestingly, a trend in the increase in 2'-O-Me levels upon differentiation of iPSCs into smNPCs and smNPCs to motor neurons is observable for fractionally modified sites. This trend suggests that fractional 2'-O-Me sites are increasingly modified as iPSCs are differentiated into different cell types with limited differentiation potential and higher dedicated functionality, like motor neurons. Intriguingly, most fractionally modified sites in iPSCs and smNPCs are completely modified in terminally differentiated motor neurons (Figure 8).

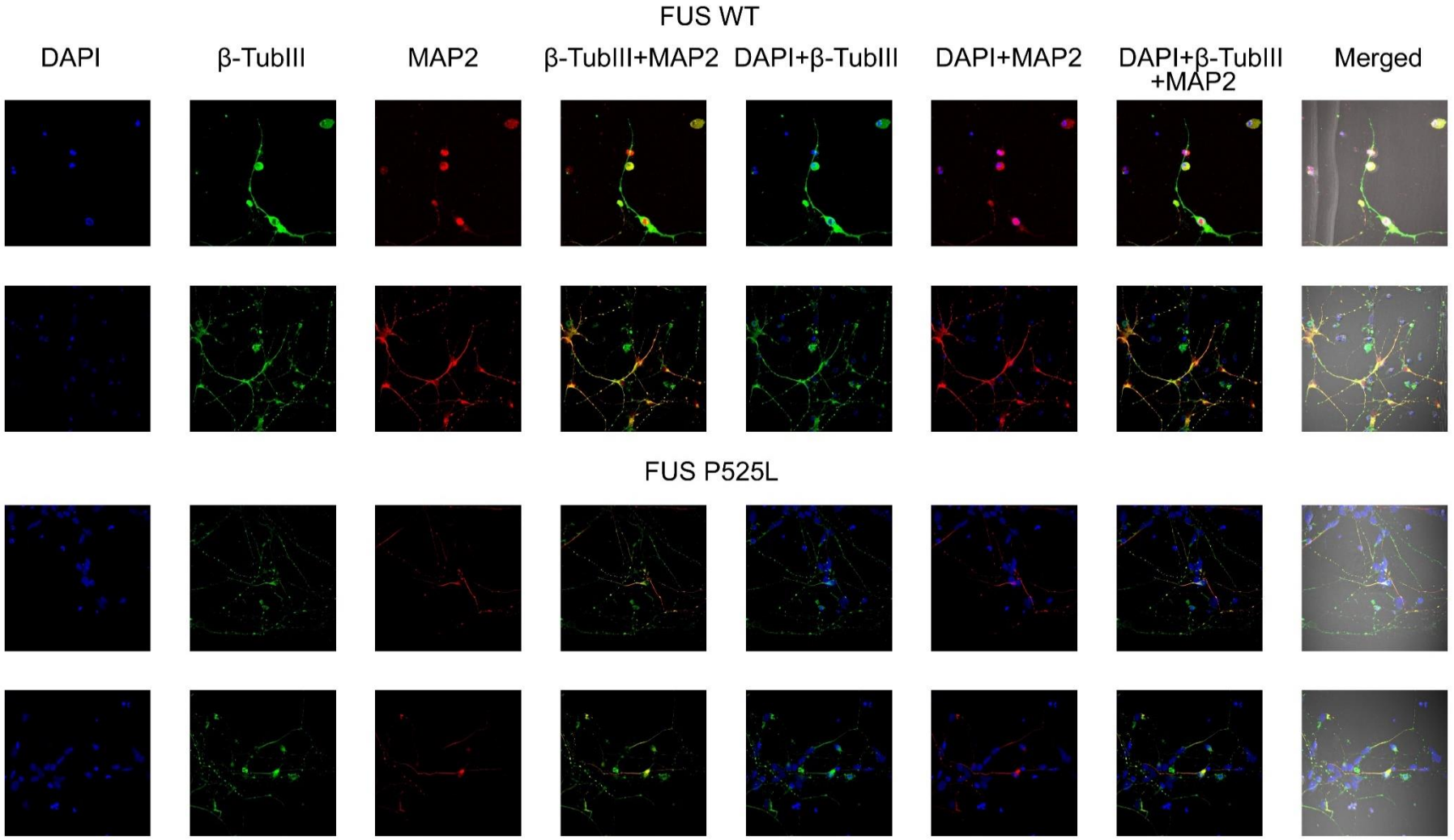


Figure 7: Confirmation of smNPC differentiation into neurons. smNPCs differentiated for three weeks were stained with MAP2 and β-tubulin III (β-TubIII) antibodies followed by fluorescent-tagged secondary antibodies. Fluorescence signals reveal that smNPCs were successfully differentiated into neurons. Both WT and FUS P525L MN expressed MAP2 and β-TubIII, as evidenced by the red and green fluorescence.

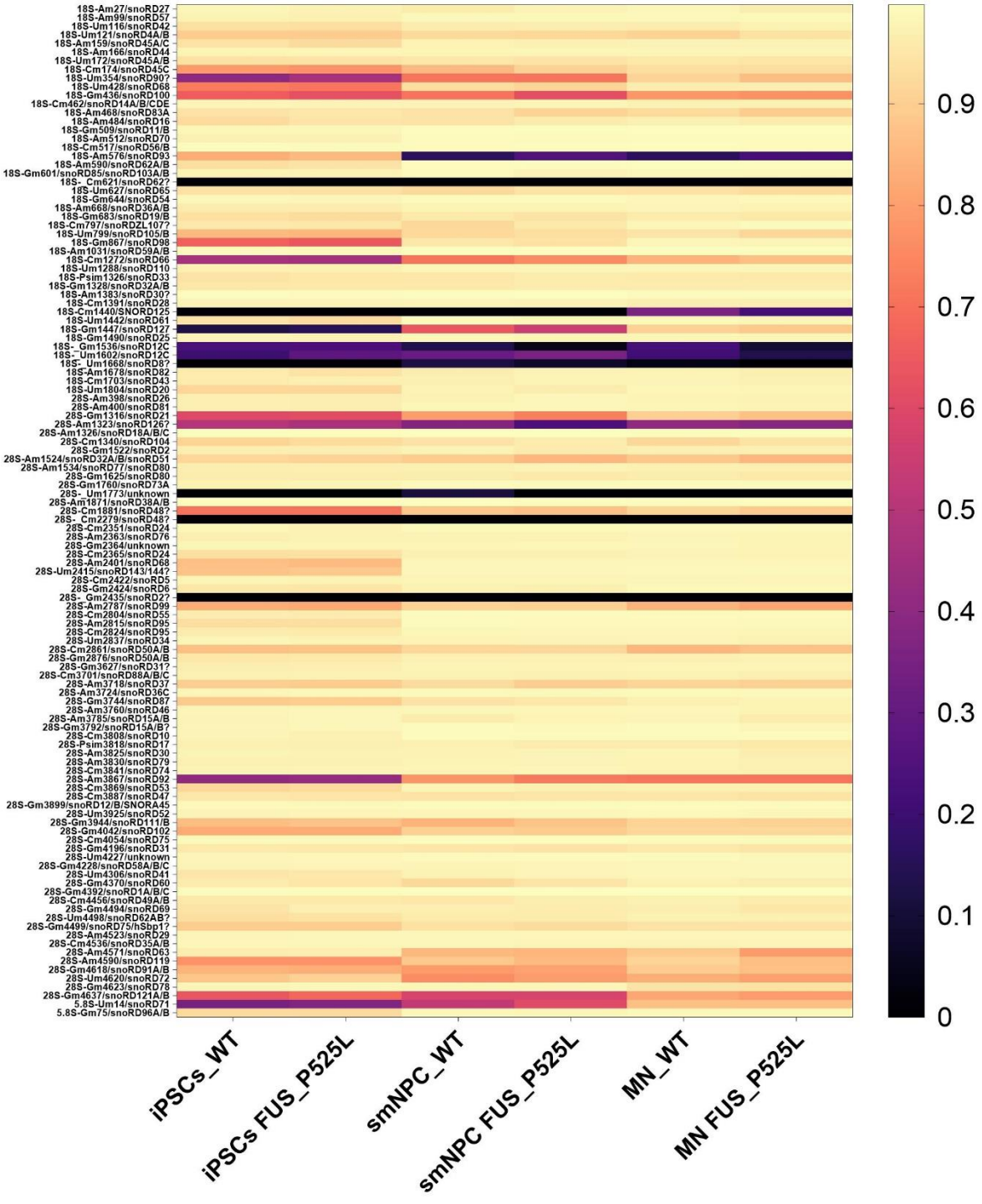


Figure 8: All 2'-O-Me sites on rRNAs in FUS P525L iPSCs, smNPCs, and motor neurons, compared to respective isogenic WT controls. Respective rRNA, 2'-O-Me site, and probable guide C/D box snoRNA associated with the site are mentioned for each row, while column names represent the name of the cell line. The legend describes the color related to the fraction of 2'-O-Me at a given site on the heatmap. The black-colored rows represent a complete lack of 2'-O-Me at that site.

As opposed to previously observed changes in the WK_FUS_P525L fibroblasts, in iPSCs, smNPCs, and motor neurons differentiated from smNPCs, there were fewer significantly changed 2'-O-Me sites (Figure 9, Table 5). In the case of iPSCs, only one 2'-O-Me site 18S-Um1602 was significantly changed, where the fraction of modification was higher in FUS P525L cells compared to the WT control. Moreover, in smNPCs, only 18S-Gm436 and 5.8S-Um14 sites were significantly changed, where the prior site showed a lower fraction of modification, and the latter displayed higher modification levels in cells with FUS P525L mutation (Figure 9, Table 5). Motor neurons are the primary cell type progressively lost in ALS, so the expectation of 2'-O-Me changes in these cells was higher. Surprisingly, only 28S-Am4571 was significantly changed, where motor neurons carrying FUS P525L mutation showed lower levels of 2'-O-Me at this site (Figure 9, Table 5). More sites in ALS patient fibroblasts with P525L mutation displayed significant changes in the 2'-O-Me levels compared to the control. However, this was not the case for motor neurons carrying the same FUS mutation. The differences may arise due to differences in cell types, and each cell type might display a type-specific methylome [3,52,53]. Similar observations were made during our previous work, where HEK293T cells and SH-SY5Y cells in proliferating and differentiated conditions displayed unique 2'-O-Me methylome on ribosomal RNAs[3].

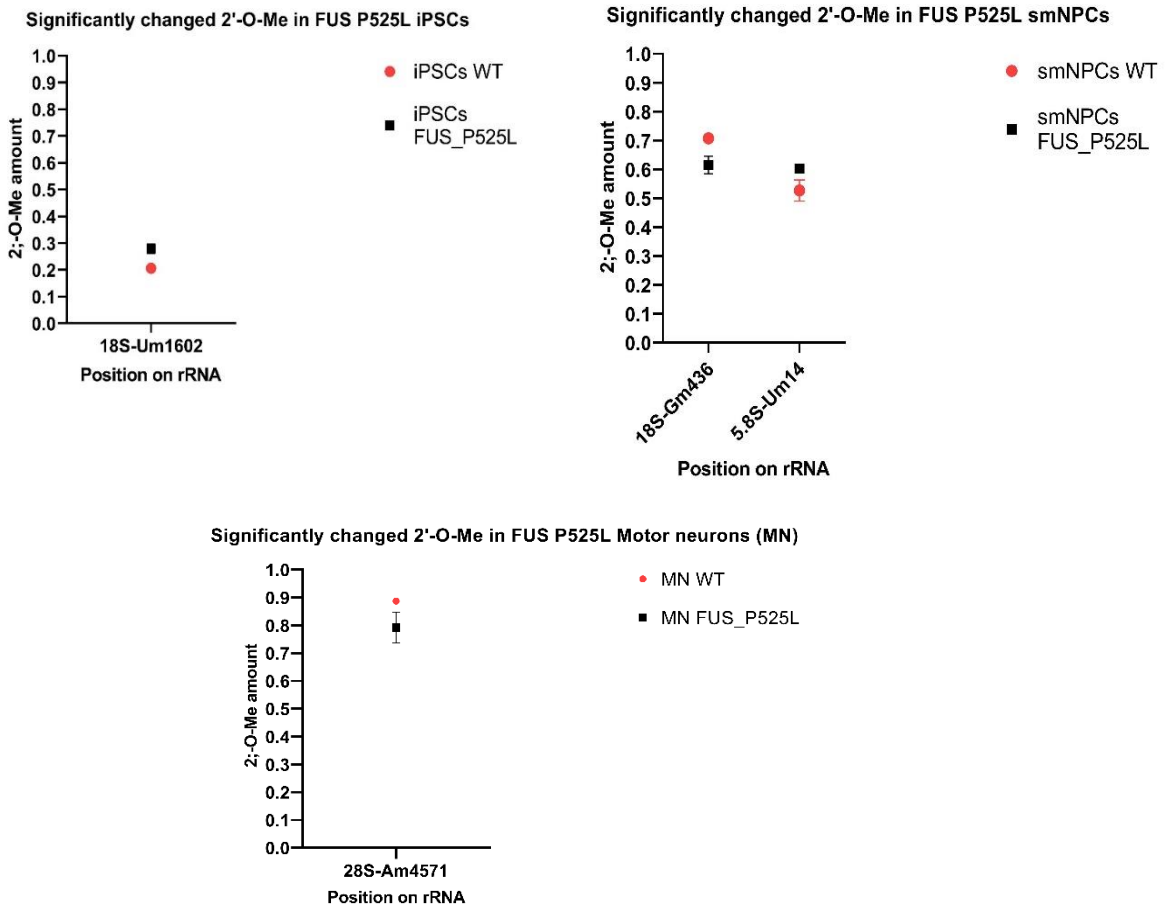


Figure 9: Significantly changed 2'-O-Me positions in WT and FUS P525L iPSCs, smNPCs, and motor neurons. In iPSCs, 18S-Um1602 is the only significantly changed position between WT and FUS P525L

conditions. Similarly, 28S-Am4571 is the only site with significant changes in motor neurons. In the case of smNPCs, 2'-O-Me at 18S-Gm436 is lower, in contrast the same is increased at the 5.8S-Um14 site. The X-axis represents the position on the rRNA; the Y-axis represents a fraction of 2'-O-Me at the corresponding position on the X-axis. *Error bars of standard deviation (std dev) are invisible when it is not very high.

Table 5: Significantly changed 2'-O-Me positions in WT and FUS P525L cells.

Position on the rRNA	Average 2'-O-Me in iPSCs WT	Average 2'-O-Me in iPSCs FUS P525L	T-test	C/D box snoRNA guiding 2'-O-Me
18S-Um1602	0.205993	0.279417	0.004648	SNORD12C
Position on the rRNA	Average 2'-O-Me in NPCs WT	Average 2'-O-Me in smNPCs FUS P525L	T-test	C/D box snoRNA guiding 2'-O-Me
18S-Gm436	0.707508	0.615392	0.006956	SNORD100
5.8S-Um14	0.527367	0.602625	0.023916	SNORD71
Position on the rRNA	Average 2'-O-Me in motor neurons WT	Average 2'-O-Me in motor neurons FUS P525L	T-test	C/D box snoRNA guiding 2'-O-Me
28S-Am4571	0.887572	0.792136	0.044375	SNORD63

All the 2'-O-Me profiles from ALS and control fibroblasts, iPSCs, smNPCs, and motor neurons were plotted on a PCA plot to understand whether the investigated cell types had significantly different methylome (Figure 10). As expected, fibroblasts, iPSCs, smNPCs, and motor neurons formed unique clusters on the PCA; additionally, smNPCs and motor neuron clusters were closer yet separate, suggesting different neuronal cells might display a unique 2'-O-Me profile (Figure 10). Similar changes in 2'-O-Me profiles were recently reported during murine embryonic stem cell differentiation into neuronal cell types[52]. Interestingly, the PCA plot did not show a separation of control and FUS-mutant cells in the observed clusters, suggesting a limited number of 2'-O-Me sites may respond to changes such as FUS mutations. Moreover, only a few sites changed significantly in iPSCs, smNPCs, and motor neurons, hinting that the same FUS mutation (FUS P525L) may regulate different 2'-O-Me sites depending on the context, such as differentiation and, of course, cell type.

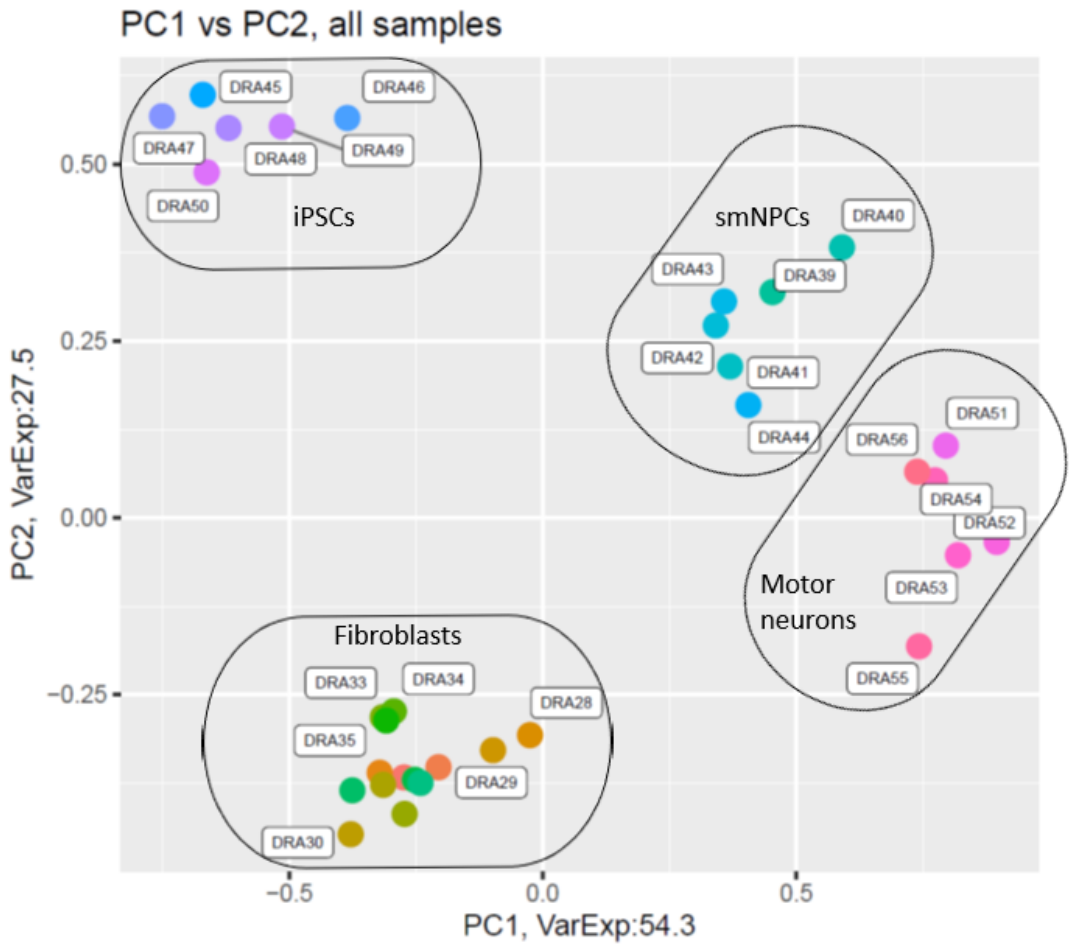


Figure 10: Principal component analysis of all the samples analysed through RiboMeth-seq for 2'-O-Me. PCA plot shows that all the samples from each cell type cluster together and that each cell type displays enough differences in 2'-O-Me profiles to form a unique cluster. Sample names are as follows:

DRA25: AK control 1	DRA30: ML FUS R521L 1	DRA36: WK FUS P525L 1	DRA45: iPSCs WT 1	DRA39: smNPC WT 1	DRA51: MN WT 1
DRA26: AK control 2	DRA31: ML FUS R521L 2	DRA37: WK FUS P525L 3	DRA46: iPSCs WT 2	DRA40: smNPC WT 2	DRA52: MN WT 2
DRA27: AK control 3	DRA32: ML FUS R521L 3	DRA38: WK FUS P525L 3	DRA47: iPSCs WT 3	DRA41: smNPC WT 3	DRA53: MN WT 3
DRA28: KA control 1	DRA33: KG FUS R521C 1		DRA48: iPSCs FUS P525L 1	DRA42: smNPC FUS P525L 1	DRA54: MN FUS P525L 1
DRA29: KA control 2	DRA34: KG FUS R521C 1		DRA49: iPSCs FUS P525L 2	DRA43: smNPC FUS P525L 2	DRA55: MN FUS P525L 2
	DRA35: KG FUS R521C 3		DRA50: iPSCs FUS P525L 3	DRA44: smNPC FUS P525L 3	DRA56: MN FUS P525L 3
Fibroblasts			iPSCs	smNPCs	Motor neurons

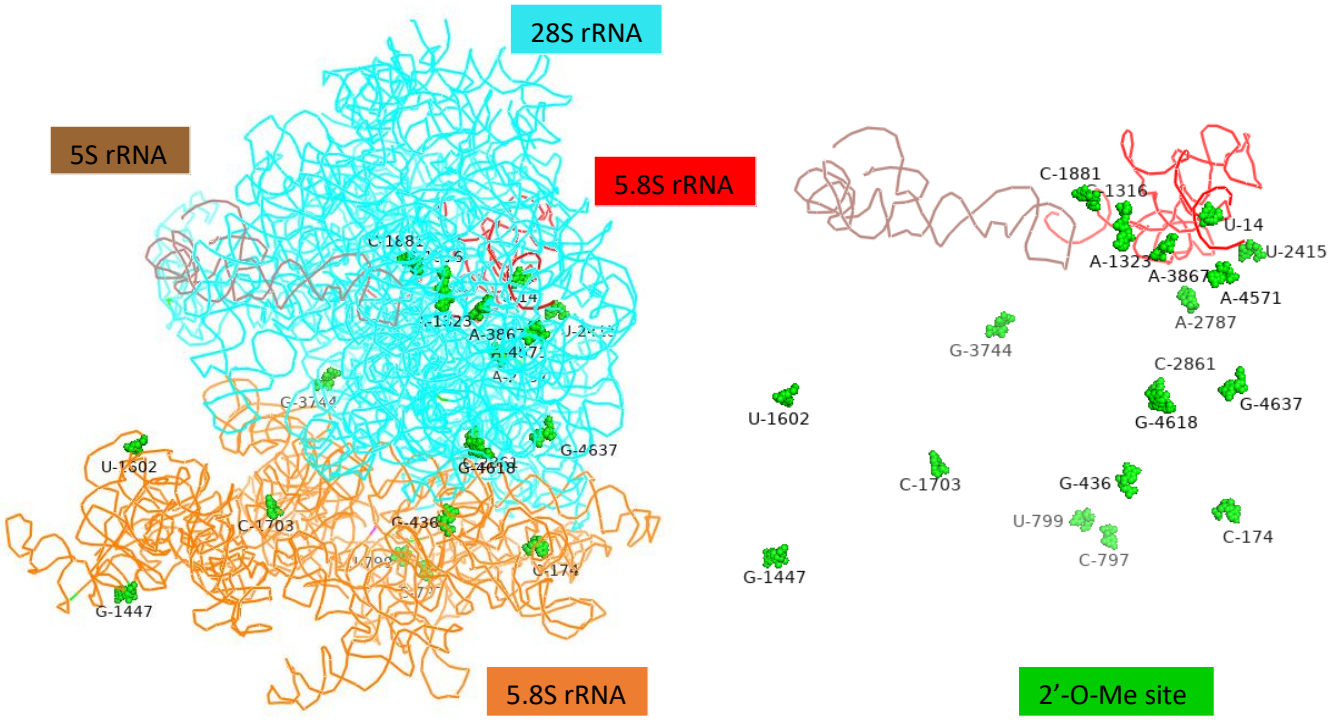


Figure 11: Visualization of the 2'-O-Me sites with significant differences in all the cells investigated on the LSU (28S, 5.8S, and 5S rRNA) and SSU (18S rRNA). The color box and name correspond to the unique color of the ribbon representing rRNAs, and green color spheres represent significantly changed 2'-O-Me sites in all the investigated cells. (80S ribosome structure from PDB – 4UG0 file). (Created using Pymol 2.0).

Next, in order to elucidate the location of significantly changed 2'-O-Me sites on the 80S ribosome, I imported PDB file 4UG0[45] in Pymol 2.0; the complex structure was converted to ribbon structure with dedicated colors for each subunit of rRNA (Figure 11). Individual 2'-O-Me sites were denoted as green spheres, as observed in Figure 11; these sites are not concentrated in one region but located on different parts of the rRNA subunits. Interestingly, 18S-Gm436, 28S-Am1323, 28S-Cm1881, 28S-Cm2861, 28S-Am3867 and 5.8S-Um14 sites which were significantly changed in our earlier study; some of these sites are also altered in the ALS fibroblasts and ALS iPSCs-derived cells; suggesting these sites may be involved in ALS pathogenesis by affecting ribosomal heterogeneity and translation. Furthermore, one significantly changed site, 5.8S-Um14, showed 6% lower 2'-O-Me levels in WK-FUS-P525L ALS-patient fibroblasts cells compared to control. Recently, it was discovered that in SNORD71 knockout cells, which is a guide RNA for 5.8S-Um14, internal ribosome entry site (IRES)-mediated translation was significantly increased, and it also enhanced the translation of COL1A1 (collagen type I alpha 1 chain) and contributed to the pathology of osteoarthritis[43]. In the same fibroblast cells, 18S-Cm174 levels were significantly lower compared to age-sex-matched control; this site has been recently implicated in the MYC-dependent translation of specific mRNAs[41]. 18S-Gm1447 and 28S-

Gm4618 modification levels vary among breast cancer types and tumor grades[54]. These studies suggest that some of the significantly changed 2'-O-Me sites observed in this study are known to influence specific translational changes. While for other significantly changed 2'-O-Me sites, no associated known functions exist. Another peculiarity within these sites is their 2'-O-Me status under physiological conditions, where most sites are hypomethylated (< 0.65). These substoichiometric sites can provide another layer of ribosomal heterogeneity, as suggested by previous studies[41,46,54,55]. Moreover, as many of these sites are positioned on the surface of the ribosome and not within any functional centers, this suggests their possible role in interaction with specific ribosomal proteins.

5.3. Discussion

As discussed in our previous study, about 66% of all the 2'-O-Me sites on rRNAs are fully methylated at given physiological conditions; similarly, as observed in Figure 4 and Figure 8, in fibroblasts as well as in iPSC-derived cells, most of the 2'-O-Me sites are fully methylated. The methylome of ALS patient-derived fibroblasts suggests that the known aggressive FUS mutations, like FUS P525L, are responsible for more significant changes in the 2'-O-Me at specific sites as compared to 'milder' FUS R521C and R521L mutations. A comprehensive ALS cohort study associated with FUS mutations revealed that the age of onset and clinical phenotypes among FUS-ALS patients vary depending upon the severity of the FUS mutation[17]. While difficult to directly correlate, some of these changes in site-specific 2'-O-Me of rRNA could be associated with the mislocalization of mutant FUS to the cytoplasm, as observed in Figure 3. Of the significantly changed 2'-O-Me sites, most of these sites show a lower level of modification compared to the control; this is in stark contrast to our earlier work that utilized FUS deletion and FUS R495X mutation in SH-SY5Y neuroblastoma cells[3]. This difference could be attributed to different cell types used; earlier studies and current work also highlight unique methylome for each cell type investigated[3] (Figure 10).

Recently, it was reported that 2'-O-Me profiles have lineage-specific patterns[52], and here we also observed similar changes when iPSCs were differentiated into smNPCs and smNPCs were further differentiated into motor neurons (Figure 8). Some substoichiometric 2'-O-Me sites are gradually modified to the saturated levels as the iPSCs are differentiated into other cell types. These sites include 18S-Um354, 18S-Cm1272 and 18S-Gm1447 on SSU and, 28S-Gm1316, 28S-Am3867, 28S-Gm4637, 5.8S-Um14 on LSU (Figure 8). Cell-type-specific 2'-O-Me profiles have been reported recently for cells derived from hematopoietic stem cells such as B and T lymphocytes, monocytes and granulocytes[53]. Interestingly, a prior study suggests that an increase in 2'-O-Me at 18S-Um354 may hamper ribosomal efficiency and decrease cell growth[39]. Moreover, 18S-Gm1447, modified by SNORD127 C/D box snoRNA, has been associated with elevated levels of amino acids in leukemic stem cells (LSCs)[53]. Furthermore, substoichiometric 2'-O-Me sites that respond to FBL levels were linked to the translation of amino acid transporter proteins, where upregulation of FBL increased, and knockdown decreased the level of these proteins[53]. Additionally, individual 2'-O-Me sites have been shown to influence cell fate decisions, as observed in the case of 28S-Um3904, for which 2'-O-Me levels drop specifically during embryonic stem cells (ESCs) to early NPC differentiation and affect WNT signaling pathway[46].

We observed only one, 28S-Am4571, significantly changed 2'-O-Me sites in motor neurons. Motor neurons are the primary cell type that is progressively lost in ALS, the changes in many 2'-O-Me sites were expected, as observed in ALS-patient fibroblasts that carry the same FUS P525L mutation (Table 4, Table 5). This lack of significant changes in the motor neurons could be attributed to

reprogramming, as it involves global changes in the age-associated epigenetic markers[56]. The cells generated by differentiating iPSCs into desired cell types lack the age-associated changes and, age-wise, are more similar to fetal cells, limiting disease modeling of ALS where age is one of the contributing factors[57]. In order to replicate age-associated changes, small-molecule-based direct conversion of patient cells into desired cell type provides a better alternative[56–58]. I am establishing a similar protocol in our laboratory, using small molecules and lentiviruses encoding relevant neuronal factors to convert ALS patient-derived fibroblasts into motor neurons. This approach will help us explore, in the future, how FUS mutations and age-associated changes contribute to 2'-O-Me changes in rRNAs and whether these changes contribute to broader translational dysregulation in ALS. I have not checked the expression pattern of the C/D box snoRNAs that guide the significantly changed 2'-O-Me sites in ALS patient-derived fibroblasts, iPSCs, smNPCs, and motor neurons; it will be done in the future using quantitative RT-qPCR and next-generation sequencing.

6. FUS-dependent processing of snoRNAs into snoRNA-derived RNAs (sdRNAs) (unpublished results part 2)

The following question was answered in this section:

5. Are snoRNAs processed into sdRNAs (snoRNA-derived RNAs) in a FUS-dependent manner in SH-SY5Y cells under proliferating and retinoic acid-induced differentiated conditions?

6.1. Bioinformatics analysis:

The quality control of raw small RNA sequencing files was done as previously published by our group[3]. The Cutadapt tool[59] (https://cutadapt.readthedocs.io/en/stable/guide.html) was used for trimming the adapter sequence, and separate files were generated for reads between 18-35 bp (basepairs) and reads above 35 bp[31,60]. Too short reads below 18 bp and reads that did not contain an adapter sequence were also discarded.

The 18-35 bp reads containing fastq files were collapsed and converted to fasta files using fastx_toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). A snoRNA index[61] (from the snoRNA database) was generated using the bowtie tool[62] (bowtie-build). The alignment was performed using the bowtie tool, and no mismatches were allowed to align the possible sdRNA with the host snoRNA completely. The Python scripts used for the analysis were received from Prof. UAM, Dr. hab. Michal Szczesniak (Department of Computational Biology, UAM).

The usage of each script is as follows:

Get_snoRNA_sRNAs.py ------> Creates a file (snoRNA_sRNA.txt) with unique snoRNA fragment sequences from bowtie-aligned output (.out) files.

sRNA_expression.py ------> This script generated two files with raw and RPM (reads per million) normalized data for the snoRNA fragments.

prepare_for_deseq_mirna.py ------> It extracts and organizes data generated during previous steps. I provided a text file containing details of the samples and defined if the sample is part of the control or treatment. The output contains two files: a text file containing one read per line and read counts from each replicate in columns; the second file contains the R-language script used to perform differential gene expression analysis using the DESeq2 tool.

The raw read text file containing sdRNAs was then used in DESeq2 analysis[63]. The DESeq normalization method was applied to get differentially expressed sdRNAs with a p-adjusted value of less than 0.05.

The normalized reads were also used to generate PCA plots and heatmaps using the ggplot2 library (https://ggplot2.tidyverse.org/), while the volcano plot was created using the EnhancedVolcano[64] library (https://bioconductor.org/packages/devel/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html).

annotate_deseq2.py ------> This Python script annotates the differentially expressed snoRNA fragments. The input1 is a fasta file consisting of all snoRNA sequences, and input2 is a file with differentially expressed sdRNAs. This script annotated sequences in input2 using sequences from input1 and created an annotated file. The flowchart explains the steps involved in the bioinformatics analysis (Figure 12).

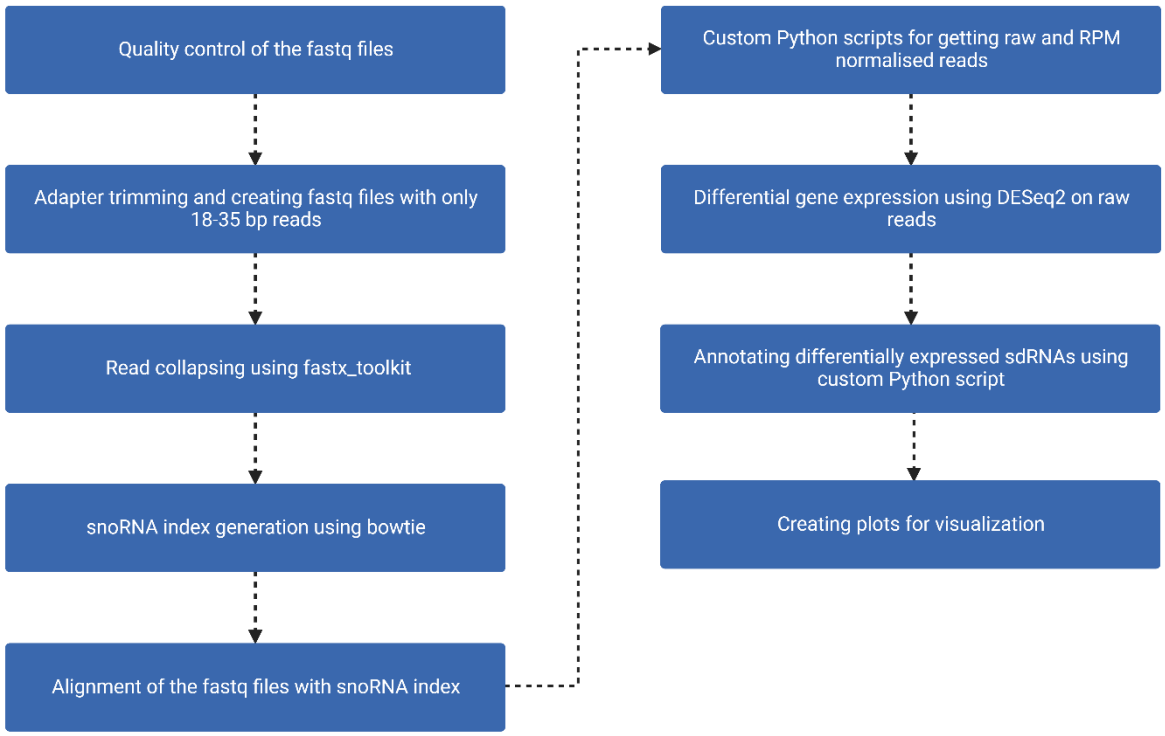


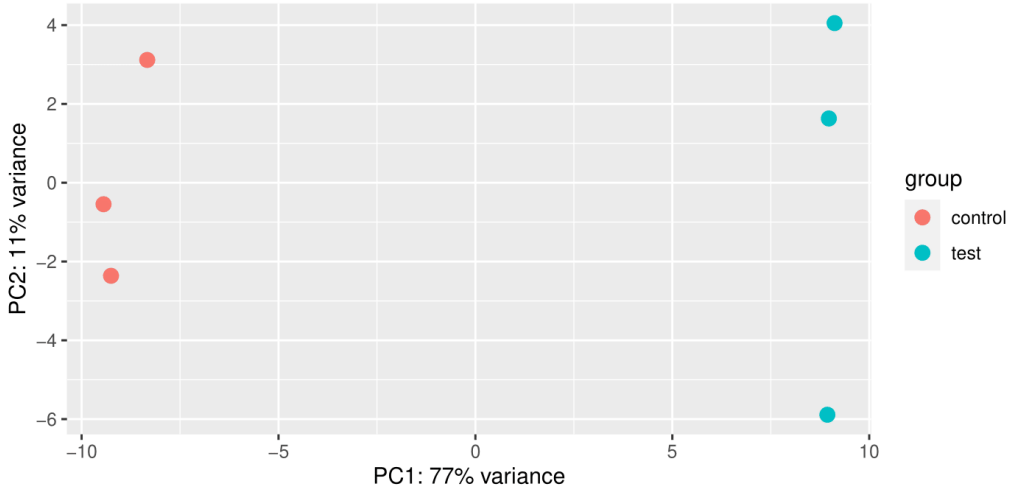
Figure 12: Flowchart describing small RNA sequencing data analysis steps to identify differentially expressed sdRNAs.

Data availability: The small RNA sequencing data used for this analysis is deposited in NCBI GEO under accession number GSE202531. The Python scripts used for the analysis will be deposited in relevant depositories once the results are published in a peer-reviewed journal.

6.2. Results

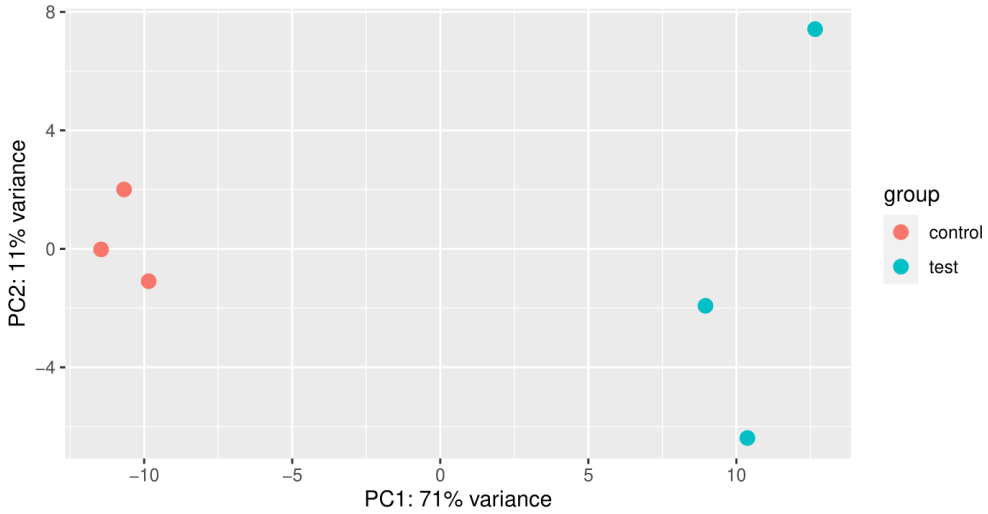
Our previous FUS immunoprecipitation study highlighted that many snoRNAs are enriched in the FUS fraction compared to IgG control. As previously explained, snoRNAs can be processed into smaller fragments called sdRNAs that regulate gene expression. With this possibility, I wanted to explore if FUS protein can influence the processing of the snoRNAs into sdRNAs and what happens to the snoRNA processing when FUS protein is depleted (in FUS knockout cell lines). For this purpose, I used the same small RNA sequencing data from SH-SY5Y neuroblastoma cells, described in our earlier work[3]. The small RNA sequencing data also contained longer reads that are not generally considered 'sdRNAs' (> 35 bp); I restricted the analysis to reads between 18 – 35 bp.

The principal component analysis (PCA) plots were generated using the ggplot2 library. Figures 13A and 13B suggest, that FUS KO proliferating and differentiated neuroblastoma cells clustered separately from the wild-type control. There is a high variation in the PC1 component between WT and FUS KO cells. These high variations for both proliferating and differentiated FUS-depleted samples suggest that sdRNA profiles for these cells are significantly different than control cells.



A. PCA plot: SH-SY5Y proliferating cells

Control – SH-SY5Y WT cells, test – SH-SY5Y FUS KO cells.



B. PCA plot: SH-SY5Y differentiated cells

Control – SH-SY5Y WT cells, test – SH-SY5Y FUS KO cells.

Figure 13: Principle component analysis of the SH-SY5Y cells in proliferating (A) or differentiated conditions (B). SH-SY5Y FUS KO cells, in both proliferating and differentiated conditions, have a significantly different cluster with high PC1 variance.

Next, I wanted to check if the sdRNA profile varies drastically between wild-type and FUS knockout cells and which sdRNAs are differentially expressed. Heatmaps allowed us to visualize the overall expression pattern between samples and conditions, so I plotted the DESeq normalized sdRNA reads on a heatmap. As Figures 14A and 14B suggest, the sdRNA profile for individual sdRNAs varies significantly between wild-type and FUS knockout conditions. Moreover, SH-SY5Y cells in proliferating and differentiated conditions display unique sdRNA profiles. This suggests that retinoic acid (RA)-based differentiation of neuroblastoma cells can influence the processing of sdRNAs (Figure 14B).

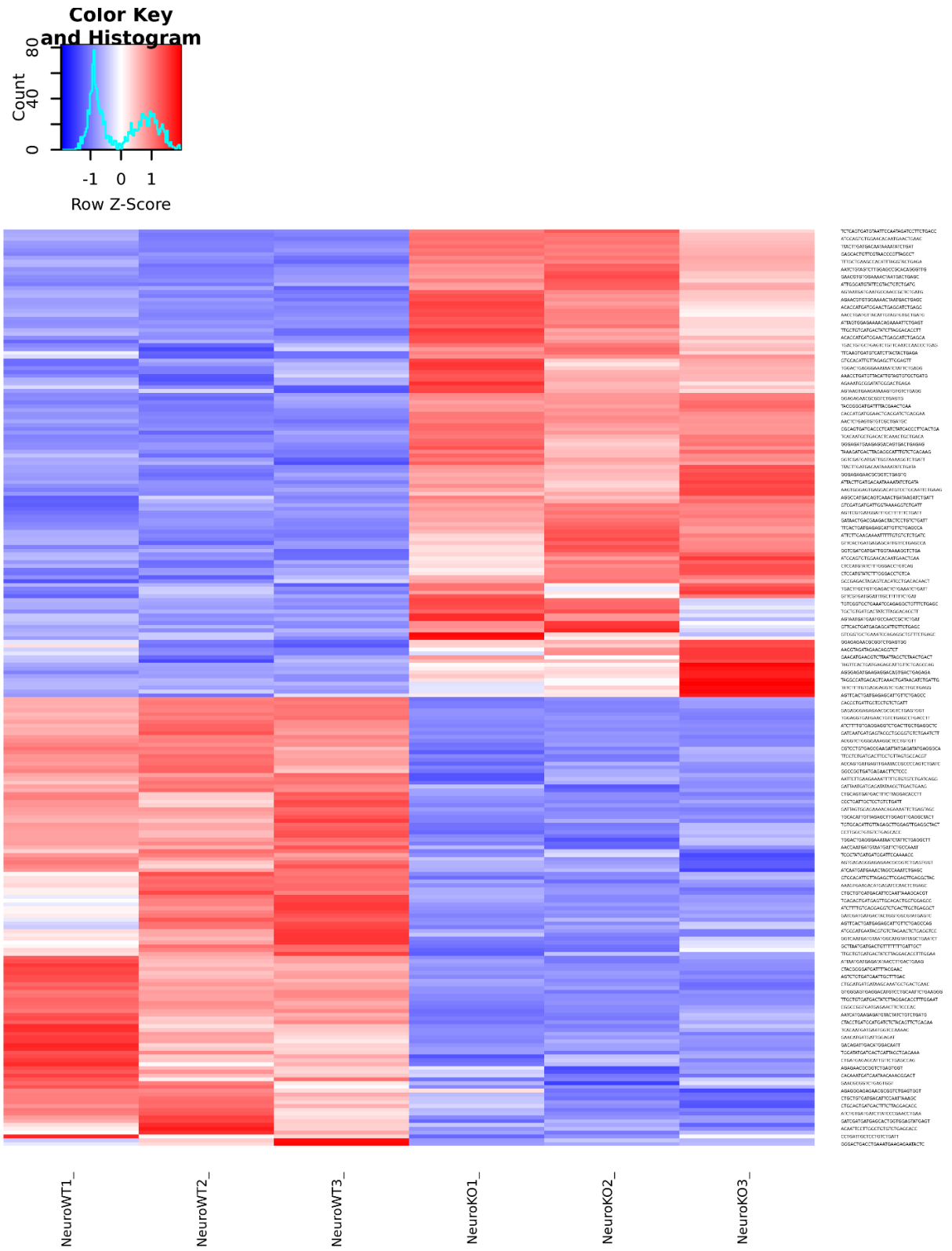


Figure 14A: Heatmap depicting differentially expressed sdRNAs in SH-SY5Y proliferating cells. The row name represents a sequence of the individual sdRNA. Column names represent biological replicates from each wild-type and FUS knockout cells. Red color represents upregulated and blue represents downregulated sdRNAs.

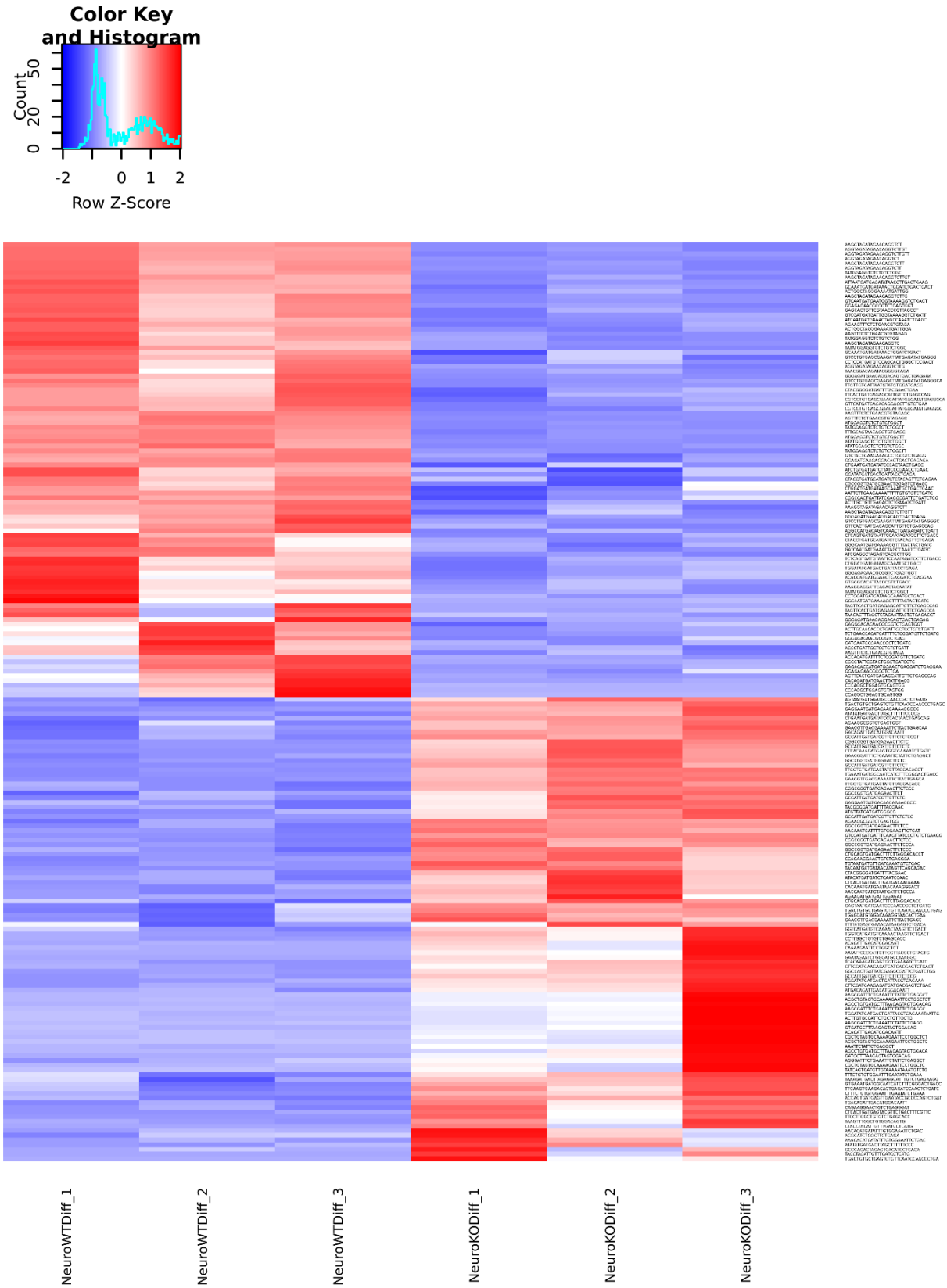


Figure 14B: Heatmap depicting differentially expressed sdRNAs in SH-SY5Y differentiated cells. The row name represents a sequence of the individual sdRNA. Column names represent biological replicates from each wild-type and FUS knockout cells. Red color represents upregulated and blue represents downregulated sdRNAs.

I plotted the normalized data on a volcano plot to visualize statistical differences and log2FC (log2 fold change) variation between samples. Figures 15A and 15B depict sdRNAs up and downregulated in proliferating and differentiated FUS KO neuroblastoma cells. Interestingly, in proliferating cells, many significantly changed sdRNAs are processed from the C/D box type of parent snoRNAs.

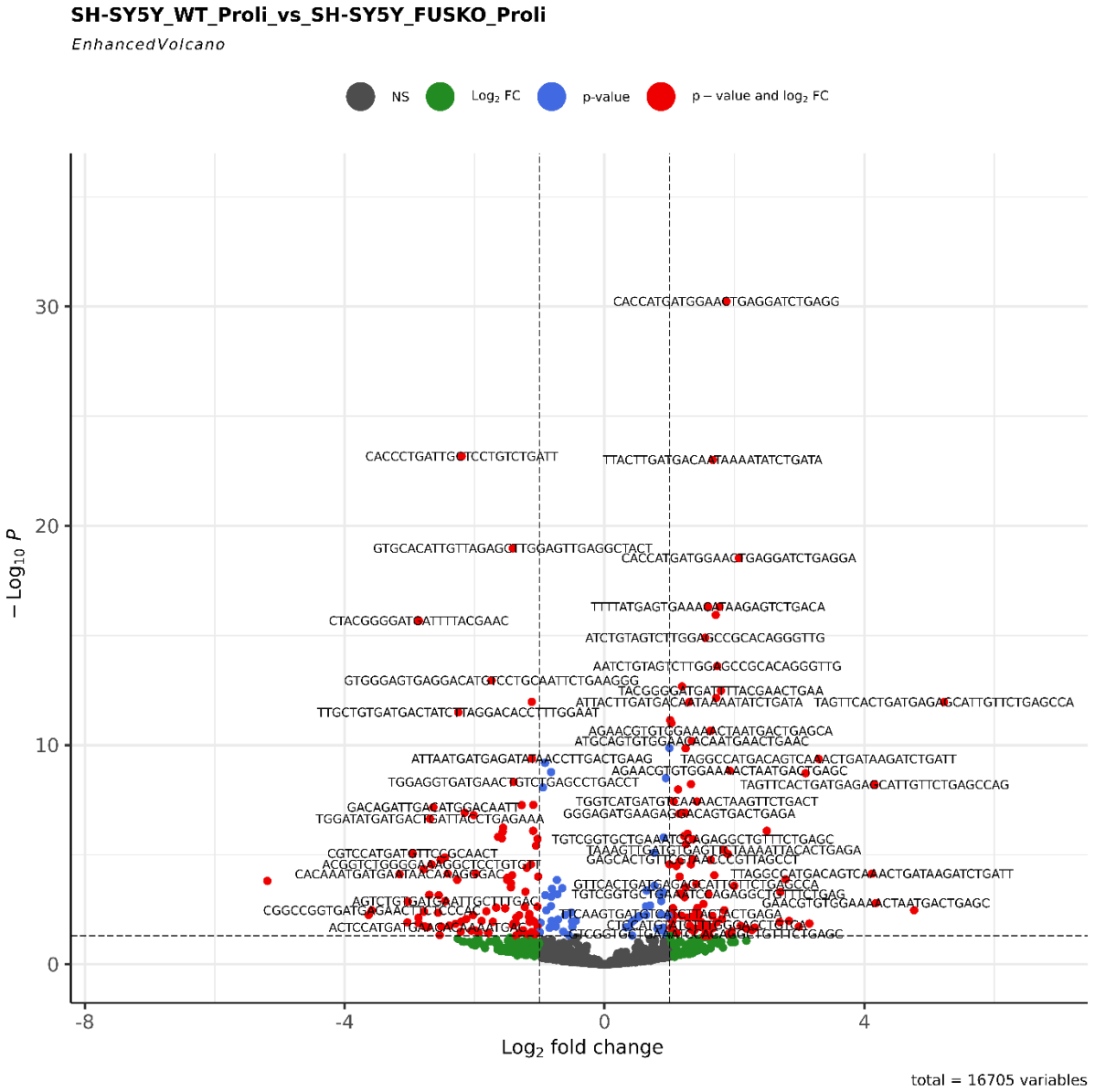


Figure 15A: Volcano plot representing differentially expressed sdRNAs in SH-SY5Y proliferating cells. The plot was generated using the Enhanced Volcano R library. X-axis = Log2 fold change of sdRNAs, Y-axis = p-adjusted value. Each red dot represents a significantly changed sdRNA. NS -non-significant.

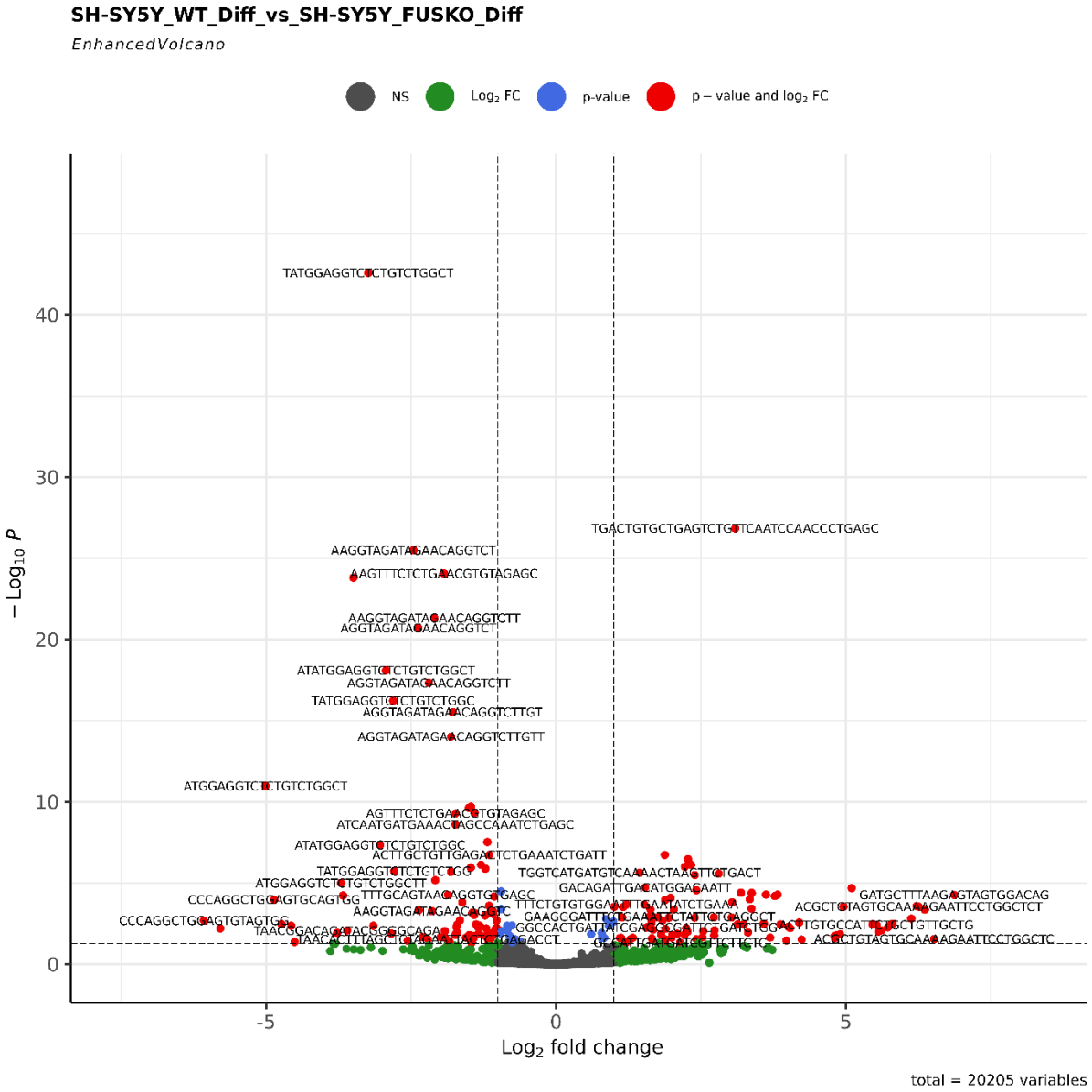


Figure 15B: Volcano plot representing differentially expressed sdRNAs in SH-SY5Y differentiated cells. The plot was generated using the Enhanced Volcano R library. X-axis = Log2 fold change of sdRNAs, Y-axis = p-adjusted value. Each red dot represents a significantly changed sdRNA. NS -non-significant.

Many C/D box snoRNAs can indeed produce multiple sdRNAs that are very similar to each other; for example, SNORD66 in FUS KO SH-SY5Y proliferating cells is processed into multiple sdRNAs with varying levels of expression (Figure 14A, 15A and Table 6). The sdRNAs processed from C/D box snoRNAs also harbor conserved D-box motif (CUGA), or in some cases box C (RUGAUGA) is conserved (Figure 15A, table 6). Interestingly, some of these sdRNA66s (name derived from SNORD66) display higher expression than other processed from the same parent snoRNA (Table 6). The long non-coding RNA GAS5-hosted SNORD74 and SNORD81 produce highly expressed sdRNAs which are upregulated in SH-SY5Y FUS KO proliferating cells; in contrast sdRNAs derived from SNORD44, which is also hosted within GAS5 intron, are downregulated in these cells (Table 6). In SH-SY5Y FUS KO differentiated cells,

the top 25 differentially expressed sdRNAs are primarily derived from scaRNA3 and scaRNA15 (small Cajal body RNAs). As seen in the case of SNORD66-derived sdRNAs, scaRNA3 and scaRNA15 also produce multiple sdRNAs with variable expression patterns. Moreover, these sdRNAs differ from each other by one or two nucleotides. Nevertheless, their expression levels change drastically (Figure 14B, 15B and Table 7).

Table 6: Top 25 differentially expressed sdRNAs in SH-SY5Y proliferating cells

sdRNA	baseMean	log2FC	padj
CACCATGATGGAACTGAGGATCTGAGG_SNORD66	948.077318	1.87	5.79E-31
CACCCTGATTGCTCCTGTCTGATT_SNORD118	258.526399	-2.19	6.71E-24
TTACTTGATGACAATAAAATATCTGATA_SNORD81	1148.51091	1.66	9.73E-24
GTGCACATTGTTAGAGCTTGGAGTTGAGGCTACT_SNORA62	1251.29645	-1.41	1.05E-19
CACCATGATGGAACTGAGGATCTGAGGA_SNORD66	162.298041	2.06	2.95E-19
TTTTATGAGTGAAACATAAGAGTCTGACA_SNORD101	415.300666	1.59	4.92E-17
TCACAATGCTGACACTCAAACTGCTGACA_SNORD71	208.978061	1.77	4.92E-17
ACACCATGATGGAACTGAGGATCTGAGG_SNORD66	529.546262	1.71	1.16E-16
CTACGGGGATGATTTTACGAAC_SNORD26	83.5773014	-2.85	2.11E-16
ATCTGTAGTCTTGGAGCCGCACAGGGTTG_SCARNA13	317.096916	1.55	1.27E-15
AATCTGTAGTCTTGGAGCCGCACAGGGTTG_SCARNA13	176.956752	1.73	2.51E-14
GTGGGAGTGAGGACATGTCCTGCAATTCTGAAGGG_SNORD96A	158.883823	-1.73	1.12E-13
GTTCGTGATGGATTTGCTTTTTTCTGATT_SNORD51	486.687155	1.19	2.10E-13
TACGGGGATGATTTTACGAACTGAA_SNORD26	145.03425	1.79	3.31E-13
ACACCATGATGGAACTGAGGATCTGAGGA_SNORD66	157.421514	1.71	7.01E-13
CTGGATGATGATAAGCAAATGCTGACTGAAC_SNORD44	717.272542	-1.11	1.07E-12
ATTACTTGATGACAATAAAATATCTGATA_SNORD81	639.445498	1.30	1.09E-12
TAGTTCACTGATGAGAGCATTGTTCTGAGCCA_SNORD103	85.0443351	5.22	1.09E-12
TTGCTGTGATGACTATCTTAGGACACCTTTGGAAT_SNORD58C	78.8605856	-2.25	3.20E-12
AATTCTTGAAGAAAATTTTTGTGTGTCTGATC_SNORD111B	662.128879	1.01	7.26E-12
AGTAATGATGAATGCCAACCGCTCTGATG_SNORD74	1584.20343	1.02	9.95E-12
AGAACGTGTGGAAAACTAATGACTGAGCA_SNORD63	166.685165	1.63	2.22E-11

ATGCAGTGTGGAACACAATGAACTGAAC_SNORD98	338.105181	1.34	6.50E-11
GGAGGTGATGAACTGTCTGAGCCTGACC_SNORD57	948.466999	0.99	1.36E-10
CACCATGATGGAACTGAGGATCTGAGGAA_SNORD66	230.785922	1.24	1.41E-10

Log2FC – log2 fold change, padj – p-adjusted value.

Table 7: Top 25 differentially expressed sdRNAs in SH-SY5Y differentiated cells

sdRNA	baseMean	log2FC	padj
TATGGAGGTCTCTGTCTGGCT_SCARNA3	433.787638	-3.23	2.59E-43
TGACTGTGCTGAGTCTGTTCAATCCAACCCTGAGC_SNORD69	367.945103	3.09	1.41E-27
AAGGTAGATAGAACAGGTCT_SCARNA15	866.675117	-2.45	3.10E-26
AAGTTTCTCTGAACGTGTAGAGC_SNORD3A|U3	1424.12362	-1.92	8.55E-25
TATGGAGGTCTCTGTCTGGCTT_SCARNA3	121.242994	-3.49	1.55E-24
AAGGTAGATAGAACAGGTCTT_SCARNA15	1078.50175	-2.09	4.72E-22
AGGTAGATAGAACAGGTCT_SCARNA15	240.782877	-2.37	1.94E-21
ATATGGAGGTCTCTGTCTGGCT_SCARNA3	105.316827	-2.93	7.86E-19
AGGTAGATAGAACAGGTCTT_SCARNA15	293.365339	-2.19	4.63E-18
TATGGAGGTCTCTGTCTGGC_SCARNA3	106.586878	-2.80	5.79E-17
AGGTAGATAGAACAGGTCTTGT_SCARNA15	595.21579	-1.78	2.93E-16
AGGTAGATAGAACAGGTCTTGTT_SCARNA15	329.608159	-1.80	9.69E-15
ATGGAGGTCTCTGTCTGGCT_SCARNA3	39.4761889	-5.00	1.03E-11
AAGGTAGATAGAACAGGTCTTGT_SCARNA15	709.036495	-1.46	2.01E-10
ATTAATGATGAGATATAACCTTGACTGAAG_SNORD119	404.267298	-1.50	2.35E-10
GGGAGATGAAGAGGACAGTGACTGAGAGA_SNORD62A	522.909404	-1.40	4.70E-10
AGTTTCTCTGAACGTGTAGAGC_SNORD3	157.870601	-1.73	5.08E-10
ATCAATGATGAAACTAGCCAAATCTGAGC_SCARNA9	219.796535	-1.73	2.43E-09
AAGGTAGATAGAACAGGTCTTG_SCARNA15	1322.11392	-1.18	2.95E-08
ATATGGAGGTCTCTGTCTGGC_SCARNA3	35.8925821	-3.03	4.45E-08
ACTTGCTGTTGAGACTCTGAAATCTGATT_SNORD30	1423.42418	-1.14	1.80E-07
GGCCGGTGATGAGAACTTCTC_SNORD33	82.124033	1.87	1.83E-07
GGCCGGTGATGAGAACTTCTCC_SNORD33	59.1033968	2.27	3.30E-07
CGGCCGGTGATGAGAACTTCTC_SNORD33	47.1093728	2.33	7.52E-07
GTCGATGATGATTGGTAAAAGGTCTGATT_SCARNA5	440.543258	-1.29	7.52E-07

Log2FC – log2 fold change, padj – p-adjusted value.

6.3 Discussion

Many non-coding RNAs, including rRNAs, tRNAs, snoRNAs and scaRNAs, are known to be processed into stable smaller fragments. These stable small RNA fragments may act as miRNAs, regulate gene expression, or influence alternative pre-mRNA splicing. One example is HBII-52 (SNORD115), which is processed into smaller fragments, and these stable snoRNA fragments are involved in alternative splicing of *DPM2*, *TAF*1, *RALGPS1*, *PBRM1* and *CRHR1* pre-mRNAs[33]. Some studies indicate that the snoRNA fragments work as piwi-interacting RNAs and perform epigenetic regulation of its targets[65].

I identified a unique pattern of processing of snoRNAs where conserved motifs such as C-box and D-box are present in the processed snoRNA fragments (sdRNAs); this feature of sdRNAs has been reported previously[60]. Moreover, the sdRNA profiles of SH-SY5Y cells differ significantly between proliferating and differentiated cells, indicating that cell state also contributes to snoRNA processing (Figure 15A, 15B). While many C/D box sdRNAs are differentially expressed in proliferating cells, differentiated cells displayed more sdRNAs derived from scaRNAs (Figure 15B, Table 7). As we have previously reported, the snoRNA expression pattern between proliferating and differentiated cells also varies significantly, and this could be one of the contributing factors for significantly different sdRNA profiles for these cells[3]. Interestingly, the processing of scaRNAs ACA45 (scaRNA15) has been reported to be independent of DROSHA/DGCR8 microprocessor complex but dependent on Dicer[32]. Furthermore, the sdRNAs derived from scaRNA15 regulated the expression of CDC2L6 (cyclin-dependent kinase 19, CDK19) by targeting its 3'-UTR (untranslated region)[32]. Moreover, we observed differentially expressed sdRNAs from SNORD44, SNORD74 and SNORD81 in FUS KO proliferating cells; interestingly, sdRNAs derived from the 5' end of SNORD44 are known to be upregulated in malignant prostate cancer tissues[66]. Apart from the usual function of guiding RNA modifications, some C/D box snoRNAs like U3 (SNORD3) and U8 (SNORD118) are involved in pre-rRNA processing[67]. Our data suggests that U8 snoRNA is processed into stable sdRNAs, downregulated in SH-SY5Y FUS KO proliferating cells; similarly, U3 snoRNA-produced sdRNAs show a similar expression pattern in differentiated cells. Recently, it was shown that U3 snoRNA is exported to the cytoplasm and processed into U3-miRNAs (sdRNAs), and these U3-miRNAs regulate sortin nexin 27 (SNX27) expression[68].

It is established from the current data that many snoRNAs are processed into stable sdRNAs, which are differentially expressed in FUS-depleted cells. Still, I have not worked on identifying a direct link between FUS and snoRNA processing. Given that FUS is involved in the biogenesis of some microRNAs and binds to pri-microRNAs[1], it is reasonable to hypothesize a similar function in snoRNA processing. Detailed functional studies are needed to decipher the role of FUS in the processing of

snoRNAs, especially in those where the processing profile resembles that of microRNA processing. Future studies are also needed to identify possible targets for these sdRNAs using target-prediction tools and functional confirmation of these targets. Moreover, because FUS mutations are associated with amyotrophic lateral sclerosis, it is necessary to address whether mutant-FUS influences the snoRNA processing pattern. This exploratory study only identified differentially expressed sdRNAs in FUS-depleted cells, and future studies from our group will address the functional role of FUS in the biogenesis of these sdRNAs and its consequences.

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AUTHOR STATEMENT

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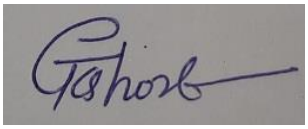
I declare that the research article by Gawade, K., Plewka, P., Häfner, S.J. et al. FUS regulates a subset of snoRNA expression and modulates the level of rRNA modifications. Sci Rep 13, 2974 (2023). https://doi.org/10.1038/s41598-023-30068-2, of which I am the first author, is part of my Ph.D. dissertation. I declare that my part of the article is as mentioned in the 'contributions' section of the published article.

Contributions

K.G., P.P. and K.D.R. contributed to the conception and design of the work. K.G., P.P., S.J.H., V.M., and Y.M. performed the experiments. K.G., P.P., S.J.H., V.M., Y.M. and K.D.R. analyzed and interpreted the data. K.D.R., K.G. and P.P. wrote the manuscript. S.J.H., A.H.L., V.M., Y.M., M.W.S analyzed and revised the manuscript.

Signature: 

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Contributions

K.G., P.P. and K.D.R. contributed to the conception, and design of the work. K.G., P.P., S.J.H., V.M., and Y.M. performed the experiments. K.G., P.P., S.J.H., V.M., Y.M. and K.D.R. analyzed and interpreted the data. K.D.R., K.G. and P.P. wrote the manuscript. S.J.H., A.H.L., V.M., Y.M., M.W.S analyzed and revised the manuscript.

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Contributions

K.G., P.P. and K.D.R. contributed to the conception, and design of the work. K.G., P.P., S.J.H., V.M., and Y.M. performed the experiments. K.G., P.P., S.J.H., V.M., Y.M. and K.D.R. analyzed and interpreted the data. K.D.R., K.G. and P.P. wrote the manuscript. S.J.H., A.H.L., V.M., Y.M., M.W.S analyzed and revised the manuscript.

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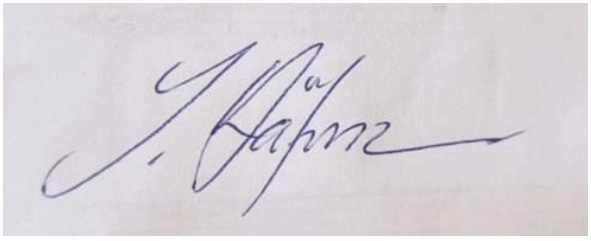

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I declare that the research article by Gawade, K., Plewka, P., Häfner, S.J. et al. FUS regulates a subset of snoRNA expression and modulates the level of rRNA modifications. Sci Rep 13, 2974 (2023). https://doi.org/10.1038/s41598-023-30068-2', of which I am a co-author, is part of a Ph.D. dissertation by Kishor Gawade. I declare that my part of the article is as mentioned in the 'contributions' section of the published article.

Contributions

K.G., P.P. and K.D.R. contributed to the conception, and design of the work. K.G., P.P., S.J.H., V.M., and Y.M. performed the experiments. K.G., P.P., S.J.H., V.M., Y.M. and K.D.R. analyzed and interpreted the data. K.D.R., K.G. and P.P. wrote the manuscript. S.J.H., A.H.L., V.M., Y.M., M.W.S analyzed and revised the manuscript.

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23.08.2023

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COAUTHOR STATEMENT

'FUS regulates a subset of snoRNA expression and modulates the level of rRNA modifications'

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19.09.2023

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Gawade, K., & Raczynska, K. D. (2023)

Imprinted small nucleolar RNAs: Missing link in development and disease?

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AUTHOR STATEMENT

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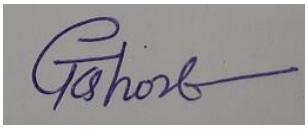
Contributions

Kishor Gawade: Conceptualization (lead); formal analysis (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). Katarzyna D. Raczynska: Conceptualization (equal); formal analysis (equal); funding acquisition (lead); resources (lead); supervision (lead); writing – review and editing (equal).

Signature: 

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