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Doctoral thesis

The effect of ALS-associated FUS mutations on U7 snRNP activity and the expression of core canonical histone genes in neuronal cells

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Dedicated to my wife

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STRESZCZENIE

Stwardnienie zanikowe boczne (ang. ALS, amyotrophic lateral sclerosis) to choroba neurodegeneracyjna, która polega na postępującej utracie górnych i dolnych neuronów ruchowych układu nerwowego. W 90 do 95% przypadków ALS jest klasyfikowany jako sporadyczny ALS (sALS), autosomalna dominująca postać rodzinnego ALS (fALS) stanowi pozostałe 5 do 10% przypadków. Za postać fALS odpowiadają mutacje w różnych genach, między innymi w genie *FUS*. FUS jest białkiem jądrowym podlegającym konstytutywnej ekspresji, zaangażowanym w naprawę DNA i regulację transkrypcji, splicing RNA i eksport RNA do cytoplazmy.

Mutacje FUS związane z ALS (ALS-FUS) występują głównie w zachowawczym regionie końca C białka i prowadzą do nieprawidłowej lokalizacji białka i tworzenia cytoplazmatycznych agregatów. Wcześniej wykazaliśmy, że białko FUS oddziałuje z cząstką U7 snRNP i bierze udział w regulacji transkrypcji i wydajności dojrzewania końca 3' genów histonów zależnych od replikacji (RDH, ang. replication dependant histones).

Bazując na dostępnych danych i naszych wcześniejszych obserwacjach, za cel pracy doktorskiej obrałem analizę wpływu mutacji ALS-FUS na aktywność U7 snRNP oraz efektywność transkrypcji i dojrzewanie pre-mRNA kanonicznych histonów rdzeniowych, jako mechanizmu molekularnego leżącego u podstaw ALS. Wyniki, jakie uzyskałem na modelu komórkowym i pierwotnych neuronów szczurów wskazują, że mutacje ALS-FUS prowadzą do błędnej lokalizacji w agregatach cytoplazmatycznych wraz z białkiem FUS również cząstki U7 snRNP. To cytoplazmatyczne uwięzienie ALS-FUS wraz z U7 snRNP ma następnie znaczący wpływ na aktywność transkrypcyjną i nieprawidłowe dojrzewanie końca 3' pre-mRNA RDH. W proliferujących komórkach neuroblastoma transfekowanych mutantami ALS-FUS zaobserwowałem zahamowanie transkrypcji genów RDH i upośledzenie dojrzewania końca 3' ich pre-mRNA. Jednocześnie, w terminalnie zróżnicowanych komórkach nie zaobserwowałem zaburzenia dojrzewania końca 3' pre-mRNA, ale znaczne obniżenie poziomu transkryptów z uwagi na zahamowaną transkrypcję.

Uzyskane wyniki wskazują, że cząstka U7 snRNP jest jedną z cząstek snRNP, na których aktywność wpływają mutacje ALS-FUS, w efekcie prowadząc do szeregu zaburzeń, od nieefektywnego splicingu po zaburzoną obróbkę transkryptów RDH. Niewątpliwie, istnieje jeszcze więcej parametrów, które nie zostały wciąż zbadane. Niemniej, podsumowując dostępne dane, zwiększenie uszkodzeń DNA i zakłócone dojrzewanie pre-mRNA genów RDH spowodowane mutacjami ALS-FUS może prowadzić do niestabilności genomu i może być mechanizmem molekularnym leżącym u podstaw ALS.

Słowa kluczowe – ALS, FUS, U7 snRNP, histony zależne od replikacji

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder which involves the progressive loss of upper as well as the lower motor neurons of the nervous system. ALS is usually categorized as sporadic ALS (sALS) which constitutes for about 90 to 95% of the cases and autosomal dominant familial ALS (fALS) comprising of the remaining 5 to 10% cases. Different genes involving varied mutations have been discovered to be involved in fALS with mutations in *FUS* gene being one of them. FUS is a ubiquitously expressed predominantly nuclear protein involved in DNA repair and transcription regulation, RNA splicing and export to cytoplasm.

The ALS-linked FUS mutations (ALS-FUS) are mainly observed in the conserved C terminus region and are associated with mislocalization and cytoplasmic inclusion formation. We have previously shown that the FUS protein interacts with U7 snRNP and is involved in the regulation of transcription and 3’ end processing efficiency of the replication-dependent histone (RDH) genes.

Based on the available data and our previous observation, the aim of the thesis was to analyze the effect of ALS-FUS mutations on U7 snRNP activity and the efficiency of transcription and processing of core canonical histone pre-mRNAs as the molecular mechanism underlying ALS. From the data obtained from our experimental results, it shows that ALS-FUS mutations along with itself mislocalize U7 snRNP in the cytoplasmic aggregates of cellular models and rat primary neurons. This cytoplasmic entrapment of ALS-FUS along with U7 snRNP has a significant impact on the transcriptional activity and aberrant 3’ end processing of RDH pre-mRNAs. In proliferating neuroblastoma cells transfected with ALS-FUS mutants we observed inhibition of RDH gene transcription and impairment of the 3’ end pre-mRNA maturation. At the same time, in terminally differentiated cells we observed no obvious impairment in the 3’ end maturation but significant downregulation of transcript level due to inhibited transcription.

The obtained results indicate U7 snRNP is one of the snRNP whose activity is affected by ALS-FUS mutations resulting in a range of complications from inefficient splicing to disturbed RDH transcripts processing. Undoubtedly, there are even more parameters which are yet to be explored. But summarizing the available data, increased DNA damage and disrupted RDH pre-mRNA processing caused due to ALS-FUS mutations can cause genomic instability and may be the molecular mechanisms underlying in ALS.

Keywords – ALS, FUS, U7 snRNP, replication-dependent histones

LIST OF SCIENTIFIC WORKS INCLUDED IN THE DISSERTATION

The results of the experimental works are described in the following paper:

1. **Gadgil A**., Walczak A., Stępień A., Mechtersheimer J., Nishimura A.L., Shaw C.E., Ruepp M.D., Raczyńska K.D. (2021) ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells. *Scientific Reports*, 2021; 11(1):11868, doi:10.1038/s41598-021-91453-3
   PMID: 34088960, PMCID: PMC8178370
   Ministry points (MNiSW) - 100
   Impact factor (2021) – 4,379

In addition, the following review paper concerning U7 snRNA and its role in gene therapy is published.

2. **Gadgil A**., Raczyńska K.D. U7 snRNA: A tool for Gene therapy. *Journal of Gene Medicine* 2021;23(4): e3321, doi:10.1002/jgm.3321
   PMID: 33590603, PMCID: PMC8243935
   Ministry points (MNiSW) - 40
   Impact factor (2021) – 4,565

ABBREVIATIONS

RDH	–	Replication dependent histone
FUS	–	Fused in sarcoma
ALS	–	Amyotrophic lateral sclerosis
ALS-FUS	–	ALS linked FUS mutations
R495X	–	Truncation after arginine amino acid at position 495 in FUS amino acid chain
P525L	–	Proline to Leucine substitution at position 525 in FUS amino acid chain
WT	–	Wild type
FUS KO	–	FUS knockout
U snRNP	–	Uridine rich small nuclear ribonucleoprotein
snRNA	–	Small nuclear ribonucleic acid
fALS	–	Familial amyotrophic lateral sclerosis
sALS	–	Sporadic amyotrophic lateral sclerosis
hiPSC	–	Human induced pluripotent stem cells
SH-SY5Y	–	Neuroblastoma cell line
UTR	–	Untranslated region
CDS	–	Coding DNA sequence
SIC	–	Spreading initiation center
RNAP II	–	RNA polymerase II

Important achievements during PhD studies

1. Received the Włodziemierz Mozolowski award for oral presentation (young scientist under 30 years) at the international conference 'Congress BIO 2018' organized by the Polish Biochemical Society, Gdańsk, Sep 2018.
2. Recipient of the EMBO scientific exchange grant #8906. (Formerly known as EMBO short-term fellowship). During which I visited the University of Eastern Finland for a duration of 90 days. 04 January 2021 to 03 April 2021. The visit proved beneficial in establishing a new collaboration and extend the observations of my PhD studies to prostate cancer research.

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Scientific collaboration

The experiments were performed at the Laboratory of RNA Processing, Department of Gene Expression, Institute of Molecular Biology and Biotechnology, Faculty of Biology, Adam Mickiewicz University, Poznan, Poland, in collaboration with:

1. Group of Dr. Marc-David Ruepp UK Dementia Research Institute Centre at King's College London, Institute of Psychiatry, Psychology and Neuroscience, King's College London, UK.

2. Group of Dr. Leena Latonen at the Institute of Biomedicine, University of Eastern Finland, Kuopio, Finland.

Main thesis including results and summary

Aim of the study

In 2009, mutations in the *FUS* gene were identified in patients with an inherited form of amyotrophic lateral sclerosis. Mislocalization of this FUS protein is a hallmark of ALS wherein ALS-linked mutations in *FUS* genes disrupt nuclear import and trap the protein in cytoplasmic aggregates. Mislocalized FUS mutants sequester RNA-binding proteins and U snRNPs. Our laboratory previously showed that endogenous FUS can interact with U7 snRNP. FUS protein is also involved in the transcription of replication-dependent histone genes and the 3' end processing of their pre-mRNAs along with U7 snRNP.

Thus, the main goal of the project is to analyze the effect of ALS-linked FUS mutations on U7 snRNP activity and the efficiency of transcription and processing of core canonical histone pre-mRNAs as the molecular mechanism underlying ALS.

Experimental workplan included:
1. To check the colocalization of ALS-linked FUS mutants and U7 snRNA.
2. To check the colocalization of ALS-linked FUS mutants and Lsm11 protein.
3. To check the effect of ALS-linked FUS mutations on the RNA polymerase II (RNAP II) occupancy on histone genes.
4. To check the effect of ALS-linked FUS mutations on the transcription of replication-dependent histone genes.
5. To check the effect of ALS-linked FUS mutations on the 3' end processing of replication-dependent histone genes.

Background and introduction

ALS is a progressive neuromuscular disease characterized by the progressive degenerative of upper and lower motor neurons in the brain and spinal cord, respectively. Early symptoms include muscle weakness or stiffness, muscle twitching in arm, leg, muscle cramps, slurred or thick speech and difficulty in projecting the voice, loss of motor control in the arms and legs leading to tripping and falling. As the disease progresses, the affected individuals gradually lose their strength, ability to speak, swallow, and commonly the cause of death being inability to breathe. Diagnosis is usually performed using muscle and imaging tests, like blood/urine tests, muscle/nerve biopsy, nerve conduction study, magnetic resonance imaging (MRI), electromyography. Currently, there is no available treatment or cure to halt or reverse the progression of the disease. Although, aid can be provided to control symptoms, prevent unnecessary complications and make living with the disease easier.

ALS is neurodegenerative disease reported worldwide irrespective of any race or ethnic background. The cause of ALS is still unknown with about 90-95% cases occurring sporadically at random with no clearly associated risk factors and no family history of the disease. However, the remaining 5-10% of the cases are familial wherein a clear genetic history exists [1,2]. More than 25 genes are known to be associated with ALS of which often most mutated are *C9orf72*, *SOD1*, *TARDBP* and *FUS* [3].

Notably, in 2009, mutations in the *FUS* gene were identified in patients with an inherited form of ALS [4,5]. Most of these mutations are missense mutations localized around the conserved C terminus region which contains the nuclear localization signal and lead to almost abolished nuclear import of FUS protein. Moreover, the C terminus contains RNA recognition motif, Arginine-Glycine (RGG) repeats and zinc finger domain involved in RNA processing.

The ALS-FUS mutants thus tend to aggregate in the cytoplasm and a liquid to solid phase transition is accelerated by the disease mutation(s) [6]. When aggregating in the cytoplasm, the ALS-FUS mutants sequester other RNA-binding proteins, FUS binding proteins (hnRNP A1, hnRNP A2, SMN), poly(A) mRNAs and U snRNA/U snRNP ((U1 snRNPs, U2 snRNPs, U1 snRNA, U11 snRNA and U12 snRNA) [19-23].

Our lab previously reported an important observation which provided valuable information for forming the basis of my PhD studies. We observed that the endogenous FUS protein acts as a positive regulator of replication-dependent histone gene transcription and the 3'end processing of their pre-mRNAs during the S phase of the cell cycle. Furthermore, we suggested that FUS might be involved in U7 snRNP-dependent repression of histone gene expression outside of S phase, thereby preventing the synthesis of extra histones that would be harmful to the cells [7]. U7 snRNP is one of the key factors involved in the processing of RDH pre-mRNAs. It consists of the U7 snRNA and five Sm proteins that are shared with spliceosomal snRNPs (SmB/B', SmD3, SmE, SmF and SmG) and two unique Sm-like proteins: Lsm10 and Lsm11 [8,9]. These proteins along with other proteins form a histone cleavage complex, recognize the histone downstream element and perform a single endonucleolytic cleavage to produce mature RDH mRNA. By introducing controlled changes at the histone binding sequence and the Sm motif of the U7 snRNA, it can be utilized as a tool for gene therapy [25].

Our preliminary FISH experiment showed that the ALS-FUS mutants traps U7 snRNA/snRNP in the cytoplasm. Therefore, I hypothesized that a consequence of the loss of the nuclear function of ALS-linked FUS mutants and U7 snRNP mislocalization, is deregulation of RDH gene expression which might be relevant to altered glial cells and motor neurons homeostasis in ALS. In glial cells, which do proliferate, decreased histone gene transcription and decreased efficiency of the 3'end processing of pre-mRNAs might be observed. On the other hand, in terminally differentiated neurons, FUS mutations can lead to destabilized transcription control mechanism that in turn might results in aberrant activation of histone proteins synthesis. Nevertheless, disturbed histone gene expression resulting in genome

instability or toxic effect of excess of histones may be the molecular mechanisms underlying altered glial cells and motor neurons homeostasis in ALS.

The experiments to identify the effect of ALS-linked FUS mutations on U7 snRNP activity and on transcription and the 3'end processing of replication-dependent histone genes were performed during the PhD studies and the observations are published as a research article in the *Scientific reports* in 2021 [24].

After discussing the role of U7 snRNP in diseases we also were interested to elaborate the therapeutic importance of U7 snRNP. The different unique aspects of U7 snRNA, its advantages and limitations are discussed in our review U7 snRNA: A tool for gene therapy.

Moreover, we have also studied the role of FUS and U7 snRNP in prostate cancer and have presented the data in this thesis.

The main thesis and achievements of the work

Gadgil A., Walczak A., Stępień A., Mechtersheimer J., Nishimura A.L., Shaw C.E., Ruepp M.D., Raczyńska K.D. (2021) ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells. *Scientific Reports*, 2021; 11(1):11868, doi:10.1038/s41598-021-91453-3

The experiments were carried out using HeLa and SH-SY5Y FUS knockout cell lines rescued by transient transfection with wild type FUS and with ALS-associated FUS mutants. One plasmid carrying the P525L mutation and the other plasmid with FUS R495X mutation were used for the experiments. Importantly, the SH-SY5Y cell line was used as proliferating cells as well as terminally differentiated neuron-like cells. For differentiation, the SY-SY5Y proliferating cells were treated using retinoic acid for a period of 10 days, confirmed for terminal differentiation, and then transfected. The aim behind this strategy was to explore if the effect of ALS-FUS mutations depends on the proliferation status of the cell.

Further, we also used human induced pluripotent stem cells (hiPSC's) derived from ALS patients harboring FUS R514G mutation to perform all the experiments performed on cell lines. The experiments were focused to observe the effect of ALS-FUS mutations on localization of U7 snRNP and their effect on 3'end processing of replication-dependent histone mRNAs. However, we weren't able to observe any significant effect as in cell lines. This was due to both heterozygous and mild nature of R514G mutation. Therefore, these results were not included in the publication. Unfortunately, due to extreme difficulty of obtaining patient samples we could not proceed further with other planned experiments, including patient samples with R495X or P525L mutations.

Even then keeping up with high standards of quality of data produced, we then performed the localization studies in wild-type rat brain primary neurons expressing the ALS-FUS protein by transient transfection. Here we observed similar and significant observations as in the cell lines, further supporting our hypothesis.

In summary, in the localization studies performed using FUS knockout cells lines of HeLa, SH-SY5Y and samples of rat brain primary neurons, it was evident that ALS-FUS mutants form aggregates and sequester U7 snRNA along with it into the cytoplasm. We were then interested to check if the mislocalization is limited to the U7 snRNA only or it also affects the complete U7 snRNP. To address this question, we analyzed the localization of Lsm11 protein in correlation with ALS-FUS. Based on the results, it was evident that the Lsm11 protein is also mislocalized into the cytoplasm along with mutant ALS-FUS protein. Thus, these consistent observations in multiple cellular models as well as rat primary neurons proved that the ALS-FUS protein mislocalize the whole U7 snRNP complex into the cytoplasm.

With the first two questions being addressed we were then interested to check the effect of ALS-linked FUS mutations on the RNAP2 occupancy on RDH genes, H2AC, H2BJ and H4J. This question was necessary to address, because in our previous publication [7] we reported that decreased levels of total histone transcripts correlate with weaker binding of FUS to histone promoters and diminished levels of RNAP2 on histone genes. It was thus necessary to investigate the effect of ALS-FUS mutations on the activation of RDH gene transcription. We performed ChIP assay to address this question, however, we performed it only for proliferating cells. We also attempted to perform it on differentiated neuron-like cells, but we had difficulties with isolation of the required amount of DNA and thus we could not proceed further. RNAP2 occupancy was analyzed on the 5'UTR, open reading frame (CDS) and the 3'UTR for the three RDH genes by qPCR. The data revealed that RNAP2 occupancy is decreased in cells transfected with ALS-FUS mutants compared to FUS WT in all three regions of the three analyzed histone genes. We thus interpret it that in proliferating cells the ALS-FUS mutations cause inhibition of the transcription of RDH genes due to weak mounting and binding of RNAP2 on histone genes.

After addressing initial three questions, we then moved to our next aim, which was to analyze the effect of ALS-FUS mutations on the transcription efficiency and 3' end processing activity of the RDH pre-mRNAs. Based on the previous publication from our lab in 2015, it was proven that FUS interacts with U7 snRNP and participates in influencing the transcription activity and the 3'end processing of RDH pre-mRNAs [7]. Therefore, knowing that the ALS-FUS mutants mislocalize the whole U7 snRNP complex into the cytoplasm, we expected significant effect on the RDH pre-mRNA 3'end processing as well as pre-mRNAs. We selected the RDH genes based on our previous publication [7]. The replication-independent histone gene H2A.Z which undergoes cleavage and polyadenylation, was used as a reference gene. From these experiments, we observed that compared to the cells transfected with WT FUS, the proliferating SH-SY5Y FUS KO cells transfected with the ALS-FUS mutants showed significantly affected processing efficiency in the majority of the analyzed histones. We interpret that this effect resulted from an elevated level of extended transcripts, with the total level of transcripts significantly downregulated. The lower level of total histone transcripts confirmed our previous observation of decreased transcription efficiency of RDH genes, analyzed by ChIP. Along with FUS KO cell lines, we transiently transfected WT FUS and ALS-FUS in proliferating SH-SY5Y WT and HeLa WT cells, but the effect we observed was

more prominent in the FUS KO cells transfected with the ALS-linked FUS mutants. This difference could be due to high expression of endogenous FUS playing the role of negative autoregulation in wild-type cells[24] combined with low transfection efficiency achieved in neuroblastoma cell.

In real life scenario, ALS affects the motor neurons which are terminally differentiated cells. Thus, to study the real-life situation as precisely as possible we differentiated SH-SY5Y cells into neuron-like cells and confirmed it using qPCR for cellular differentiation markers, expression of MAP2 protein using western blotting, and actin phalloidin staining assessed with immunofluorescence to confirm the morphology. Interestingly, in these neuron-like cells, we observed no change in the 3'end maturation, although we observed significant downregulation of 'processing efficiency' when we compared the ratio of total to polyadenylated transcripts. However, we hypothesized this could be due to inhibited transcription rather than affected the 3'end processing. It is previously shown that terminally differentiated cells synthesize only polyadenylated histone transcripts in the process that is independent of U7 snRNP [10]. Therefore, we conclude that the results which we observed in neuron-like cells are due to the accumulation of mature polyadenylated mRNAs and inhibited transcription instead of the incorrectly processed transcripts.

The main thesis and achievements of the work

Gadgil A., Raczynska K.D. U7 snRNA: A tool for Gene therapy. *Journal of Gene Medicine* 2021;23(4):e3321, doi:10.1002/jgm.3321

With the thesis focused to study the effect of ALS-FUS mutations on U7 snRNP activity, RDH gene transcription and the 3'end processing of RDH pre-mRNAs, we found it interesting is necessary to shed light onto U7 snRNP, and its potential therapeutic importance. The U7 snRNP is not only a unique snRNP in terms of its cellular activity but its capacity to be used in therapy makes it commercially important too. U snRNPs are complexes involved in splicing of pre-mRNAs, but U7 snRNP is an exception since it is not involved in splicing but instead in the 3'end processing of RDH pre-mRNAs.

The U7 snRNP being such a unique molecule has unique uses too. By changing the unique Sm binding sites for Lsm10 and Lsm11, to consensus sequence for spliceosomal U snRNAs, it leads to the formation of modified U7 snRNP which efficiently accumulates in the nucleus and can be involved in pre-mRNA splicing. Modifying the unique binding sites of the U7 snRNA renders U7 snRNP particle nonfunctional in RDH pre-mRNA processing but can be targeted for correcting splicing and is of therapeutic importance in diseases that are an outcome of splicing defects.

In the review we have discussed elaborately about the studies conducted using modified U7 snRNA in treatment of myotonic dystrophy, Duchenne muscular dystrophy (DMD), ALS, β-thalassemia, HIV-1 infection, and spinal muscular atrophy (SMA). Some studies have shown

progress and have reached clinical trial stage; a few are already approved for treatment. We have in detail discussed about the benefits and limitations about using U7 snRNA as a tool for gene therapy. Wherein, we have tried to cover varied points such as reduced risk of immune response against U7 snRNA, ability to provide lifelong therapeutic effect, and the drawbacks in delivery, economic constraints, deciding the timepoint of therapy and the dose.

So overall, in this review, we have discussed about U7 snRNP, its role in RDH gene processing, the scientific background about designing it as a tool for gene therapy, detailed analysis about the diseases targeted and the advantages and limitations, thus trying to make it informative with a wider perspective.

Unpublished data under the project funded by 'EMBO scientific exchange grant, #8906'

Although argued, the role of FUS protein in prostate cancer has been widely reported. It has been shown that FUS and some ribonucleoproteins are found in spreading initiation centers (SIC) - structures typical for early stages of cell spreading and responsible for cellular migration [11]. FUS and other FET family proto-oncoproteins show cell type-specific expression patterns and involvement in cell spreading [12]. These reported findings point out that apart from their housekeeping role, the FUS protein is involved in stress response, translational control, and adhesion, suggesting involvement in human cancer and neurodegeneration. Few publications mention the correlation of ALS and cancer mentioning that prostate cancer survivors have a reduced chance of ALS death [13,14,15]. Based on our observations related with ALS-FUS mutants and the reported publications, I further hypothesized that FUS may be the interlink in these observations.

Our collaborator, Leena Latonen group from the University of Eastern Finland, performed a preliminary study on prostate cancer samples and observed that FUS and histone proteins are significantly increased in castration resistant prostate cancer compared with the primary stage. Additionally, they also observed increased cytoplasmic accumulation of FUS in the cytoplasm of castration resistant prostate cancer cells as compared to primary stage prostate cancer cells.

Association of histone gene expression with cancer is reported, exhibiting upregulation of histone cluster 1 H1A and poor prognosis in individuals with higher expression of histone gene sets in breast cancer [16]. Another evidence for increased histone expression was reported in cervical cancer patients, and histone cluster 1 H2A, H2B, and H4 were found to be of prognostic importance [17]. Moreover, reduced FUS expression is reported to severely impair cellular proliferation and an increase in phosphorylated histone H3, a marker of mitotic arrest [18]. Thus, based on these observations, I hypothesized, that a surge in the number of RDH in prostate cancer may stimulate migration and invasion of prostate cancer cells.

My first question in the project was to check if U7 snRNP is colocalized in the cytoplasm along with FUS and is a part of the SICs. I hypothesized that if U7 snRNP is mislocalized into the cytoplasm, it would result in increase of wrongly processed

(polyadenylated) RDH mRNA transcripts. This would provide the cancer cells essential environment of genomic instability and providing continuous supply of RHD for highly proliferating cancer cells.

To check my hypothesis, I began with analyzing the cells in spreading stage, for which the procedure mentioned in de Hoog et al., 2004 was followed [11]. For staining, the combined immunofluorescence FISH protocol mentioned in our publication Gadgil et al., 2021 [24] was followed. For these experiments, I used PC3 and LNCaP cells which are androgen receptor negative and androgen receptor positive cell lines, respectively. First, I stained PC3 cells for FUS and vinculin (a marker for cell spreading) and observed that FUS and vinculin colocalize in the proposed SICs as shown previously (Fig. 1).

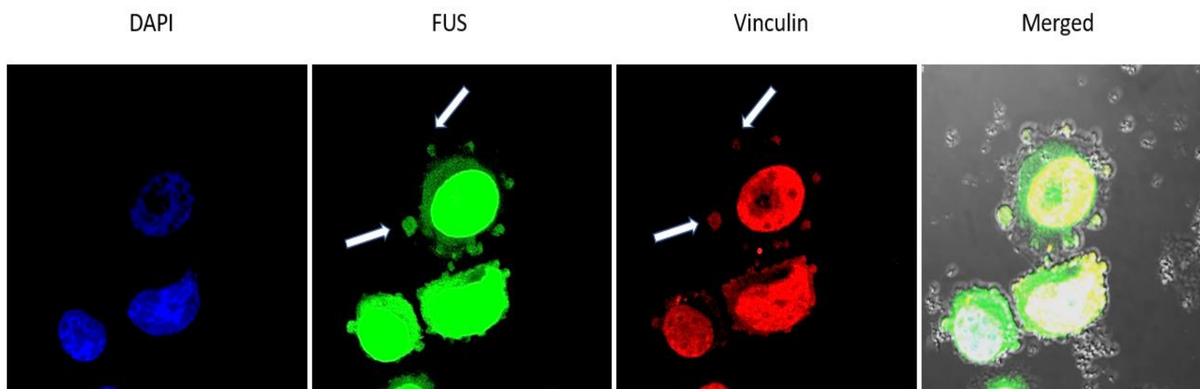

Figure 1 – PC3 cells stained with FUS and vinculin to observe colocalization in SICs.

Further, I stained PC3 cells for U7 snRNA (FISH) and vinculin (IF) and observed that U7 snRNA and vinculin colocalize in the proposed SICs as well. (Fig. 2)

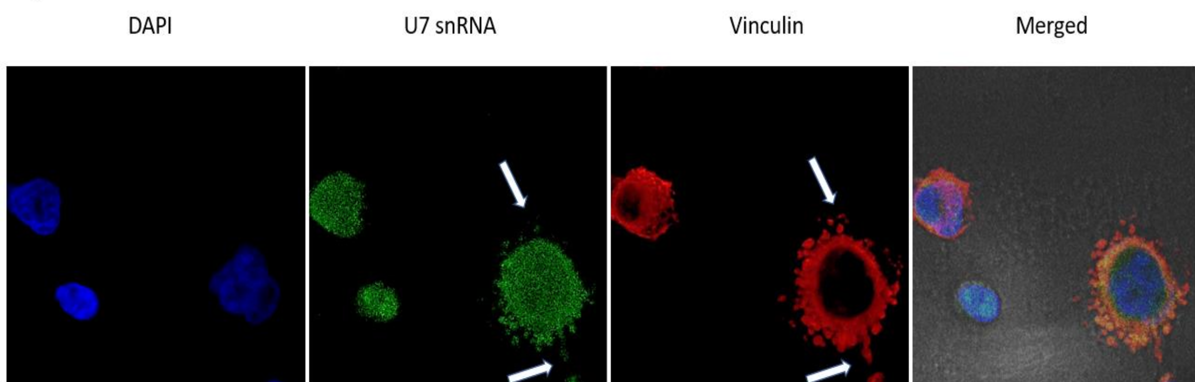

Figure 2 – PC3 cells stained with U7 snRNA and vinculin to observe colocalization in SICs.

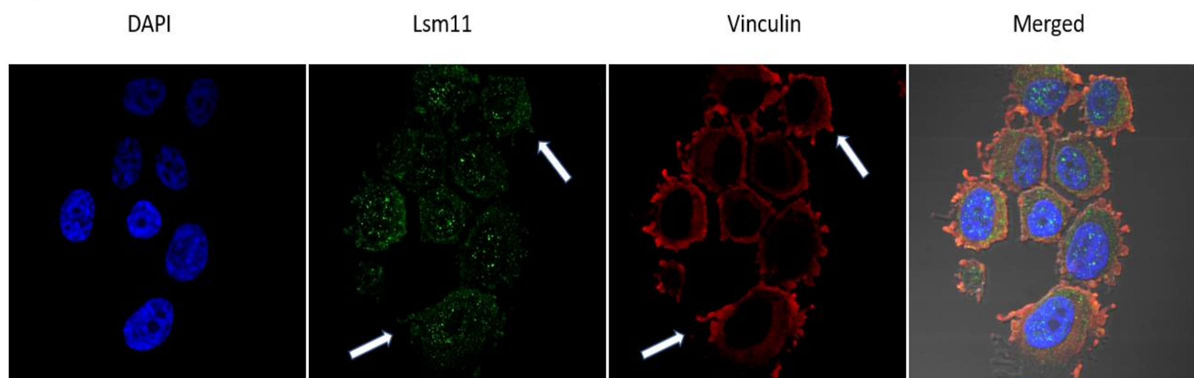

Figure 3 – PC3 cells stained with Lsm11 and vinculin to observe colocalization in SICs.

Importantly, I stained Lsm11 and vinculin to confirm if the whole U7 snRNP is colocalized in SICs. And indeed, I observed that the Lsm11 protein does colocalize with vinculin in the SICs (Fig. 3). It is an important observation showing that the whole U7 snRNP complex colocalizes with vinculin, similarly like FUS, and can be present in the SICs. Due to limitations in using and detecting multiple fluorescence markers, FUS, vinculin, and U7 snRNA together were not analyzed in SICs and needs to be done in future to make any concluding remark in this regard.

My next aim was to analyze the effect of FUS overexpression and knockdown on RDH gene expression. For this, I transfected LNCaP and PC-3 cells with plasmid for overexpression of *FUS* gene and another set of cells transfected with siFUS RNA for FUS knockdown. Cells stressed with sodium arsenite were used as a control for stressed condition. The extracted RNA was used for RT-qPCR experiments using the method as described in our publication Gadgil et al., 2021 [24]. We expected that since U7 snRNP is localized in the SIC it would elevate wrongly 3'end processed RDH pre-mRNA (polyadenylated) transcripts and increase cell proliferation. It would be a deciding factor in increased cell proliferation. Though we could observe some changes in the transcription levels and 3'end processing efficiency of RDH genes, there was no significant changes, and we are not able to have any final conclusion yet.

SUMMARY

The achievement of a doctoral dissertation prepared by me consists of one research publication which elucidates the molecular mechanism behind the pathogenesis of ALS. My PhD topic is focused to study the effect of ALS-FUS mutations on U7 snRNP activity and their effect on transcription of RDH genes and the 3'end processing of these gene transcripts. We found that ALS-FUS mutations do have an impact on U7 snRNP localization as well as their efficiency to process RDH pre-mRNAs. It was also necessary to write in detail about U7 snRNP which we discussed it in our review publication. Further, ALS is also one of the diseases that is under study for U7 snRNA-based therapy and has shown promising results. It was thus important to mention this in the review.

The obtained results published in the research paper encouraged me to write an EMBO scientific exchange grant to study the role of FUS protein and U7 snRNP in prostate cancer cells. Exploring the role of FUS and U7 snRNP in prostate cancer will add to our knowledge of the progression of the disease. Reports are published wherein the association of ALS and cancer is discussed involving the *FUS* gene. Some of these reports mention that prostate cancer survivors have a reduced chance of ALS death. Exploring the role of FUS and U7 snRNP may thus be a step towards understanding prostate cancer, and the association between prostate cancer survivors having a reduced chance of ALS death.

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Publication

ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells

DOI: 10.1038/s41598-021-91453-3

Publication

U7 snRNA: A tool for gene therapy

DOI: 10.1002/jgm.3321

Author statement

AUTHOR STATEMENT

for the research article 'ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells'

I declare that the research article 'Gadgil, A. *et al.* **ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells**. *Sci Rep* 11, 11868 (2021). doi: /10.1038/s41598-021-91453-3', is a part of my PhD dissertation. I, Ankur Gadgil, along with my supervisor Katarzyna Dorota Raczyńska designed the research. I, performed combined FISH and immunofluorescence staining to analyze localization of FUS, Lsm11 and U7 snRNA in cells transfected with plasmids carrying FUS WT and ALS-linked FUS mutations (Fig. 1 A, and Fig. 1 B, and Supplementary Figure, Fig. S2), I analyzed replication-dependent histone gene expression in cells transfected with plasmids carrying FUS WT and ALS-linked FUS mutations (Fig. 2), I assessed the RNA Polymerase II (RNAPII) occupancy on histone genes by chromatin immunoprecipitation assay (Supplementary Figure. S3), and I confirmed the differentiation of SH-SY5Y FUS KO proliferating cells to neuron-like cells using qPCR, immunofluorescence and western blot (Supplementary Figure Fig. S4). I wrote the manuscript, prepared figures, I performed data analyses and I did the editorial work.

Poznan, 15th April 2022

Name: Ankur Gadgil

AUTHOR STATEMENT

for the review article 'U7 snRNA: A tool for gene therapy'

I declare that the review article 'Gadgil A, Raczyńska KD. **U7 snRNA: A tool for gene therapy**. J Gene Med. 2021 Apr;23(4):e3321. doi: 10.1002/jgm.3321', is a part of my PhD dissertation. I, Ankur Gadgil, conceptualized the idea, wrote the manuscript, prepared all the figures Fig.1, Fig.2, Fig.3, Fig.4, and Table 1, and performed all the editorial work.

Poznan, 15th April 2022

Name: Ankur Gadgil

Co-author statement

CO-AUTHOR STATEMENT

for the research article 'ALS-linked FUS mutants affect the localization of U7 snRNP and replication-dependent histone gene expression in human cells'

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ALN performed data analyses and contributed to the writing process.

CES provided material, lab space and contributed to the revision of the manuscript.

MDR designed and constructed the U7 snRNA probe for FISH, was involved in performing combined FISH and immunofluorescence staining and contributed to the revision the manuscript.

KDR designed research, supervised all experiments, analyzed the data, edited, and finalized all the images, shared funding and wrote the manuscript.

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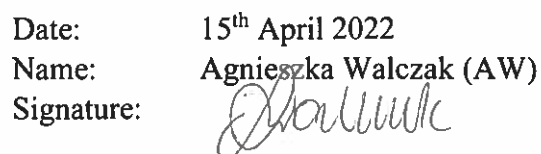

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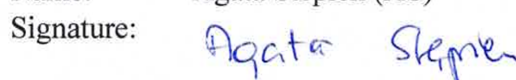

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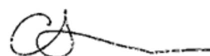

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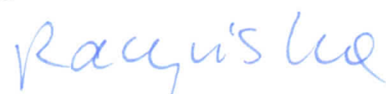

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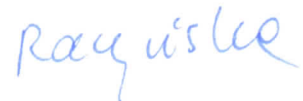
KDR supervised in conceptualizing the idea, finalizing all the figures and the table, writing the manuscript and editing it.

Date: 15th April 2022
Name: Katarzyna Dorota Raczyńska



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