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Versatility of RNA-Binding Proteins in Living Cells

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Abstract: This conference paper aims at demonstration of complexity or multiplicity of RNA-binding protein (RBP) and its related RNA. The data in the manuscript are based upon the third Annual Meeting of the RNA study group, held at the Tokyo office of the Kyoto University on May 23 in 2019. The purpose of the meeting is to present recent progress of the study group and have discussion regarding RNA and related molecules, especially focusing on RBP in this year. We had fourteen sessions and documented ten of them in the manuscript. There were exciting and fruitful debates. Actual presentations are shown as follows. Specific RBP Sfpq and Qk are involved in neuronal development in mouse system. Also in mouse, liquid- and solid-like RNA granule formation plays pivotal roles in neuronal functions. Biochemical experiments demonstrate that affinity profiles categorize RNA-Binding proteins into distinctive groups. This presents basis for biological divergence of RBPs. In the context of pathology, RNA aptamers against prion and Alzheimer's diseases may present therapeutic outcome. Computational analysis is another utility for approaching riddle of RBPs. The study of X-chromosome inactivation is pioneering field of long noncoding RNAs. Redundant triplex interactions between lncRNA and LINE-1s are supposed to be crucial in X-chromosome inactivation. Myogenesis-related lncRNA, *Myoparr* promotes skeletal muscle atrophy caused by denervation through the regulation of

GDF5/*BMP14* expression. In neurodegenerative disorders functional loss of TLS/FUS induces onset of frontotemporal lobar degeneration. Conformational change of TLS is induced with binding of various nucleic acid molecules. TLS is also involved in phase separation into aggregation to pathogen generation of neurodegenerative diseases. The meeting has been successfully prorogued. We utilize the data to boost the activity of the field of RNA and RBP. The achievements of the meeting have confirmed pivotal roles of RBPs in living cells. It should be in complexity. We will be able to analyze intricate phenomena of RBP biological actions and contribute to uncover the veiled rules in divergent biological programs.

Keywords: RNA-Binding Protein, Long Noncoding RNA, Amyotrophic Lateral Sclerosis, TLS FUS, X Chromosome Inactivation, Phase Separation, Neuron, Computer Simulation

1. Introduction

The divergent biological activity of RNA-binding protein (RBP) has been focused owing to its contribution to biological programs including homeostasis, development, and heredity. The intention of this conference paper is to announce activity of the 3rd Annual Meeting of the RNA Study group related to divergent property of RNA-binding protein (RBP). Divergent researchers also contribute to this article. This implies that divergent readers should be attracted to the article.

The Research activity of RBPs has been radically in progress. Actually, it has been shown that more than 1500 proteins function as RBP in the human genome [1]. These RBPs contain established or canonical RNA binding domains, which include the RNA recognition motif, the heterogeneous ribonucleoprotein K homology domain, and C3H1 zinc-finger domain [1] Some of the proteins have previously unidentified or non-canonical RBPs [2]. RBPs are involved in all aspect of RNA metabolism. Recently, it has shown that many RNA processing steps are well coupled with transcription [3]. RBPs are prevalently localized on active chromatin regions. The promoters of active genes are major hot spots for interaction with RBPs. Multiple RBPs are directly involved in transcriptional regulation [3]. It has been reported a novel algorithm called mCross to accurately define RBP specificity by registering protein-RNA crosslink sites utilizing CLIP data [4]. This technique was applied to more than one hundred RBPs and identified a non-canonical binding motif SRSF1, which denotes the protein in regulating phase separation [4]. Now, phase separation is one of the most exciting topics in the RBP field [5-7]. At the conference, we have been exploring these kinds of attractive enigmas of basic sciences.

On Thursday, May 23 in 2019, the third Annual Meeting of the RNA study group was held at the Tokyo office of the Kyoto University, located at the Tokyo station. This gave us a lot of beneficial discussion. We really appreciate every attendee there. We have obtained a plan of the meeting at the chat of the dinner after the session at the 39th annual meeting of the Molecular Biology Society of Japan in Yokohama in December, 2016. Katahira, Yamashita, and Kurokawa voluntarily have started to take charge of the meeting. We decided to have a first meeting at the Research Center for Advanced Science and Technology (RCAST) on Thursday, May 18 in 2017, and also had second meeting on Thursday, May 24 in 2018 there [8]. We did not have any funding on it. This means that it should be a small meeting with less luxurious setting, but have the freedom to take time for discussion. We have prepared for the meeting with groping for a way to make better one. Fortunately, we have attained a great success on it. We have it as a closed format for secured exchange of unpublished data between attendees. Therefore, limited numbers of people were permitted to attend. Then, we had a plenty of time to have productive discussion at the sessions. Official meeting of the big academic society has stringent regulation of the program because of time limitation. Then, we have appreciated the liberty of discussion there.

We have determined to have the date of the fourth meeting on Thursday, May 21 in 2020 somewhere in Japan. This will lead another successful history of the Meeting.

2. Multiplicity in RBPs in Living Cells

Akihide Takeuchi et al. present the data regarding RBP regulation in neuronal development. To decipher physiological roles of mRNA regulation especially in nervous system development, we are analyzing the function of RBPs with multidisciplinary approaches from *in vitro* to *in vivo*. Using transcriptome studies, we identified Sfpq and Q k as highly and selectively expressed RBPs in embryonic mouse brains. Sfpq is expressed in differentiating neurons which are generated from neural stem cells. Using gene targeted mice and genome wide RNA biology approaches, we found that Sfpq critically facilitates transcriptional elongation of long neural genes >100 kbp in length and comprehensively regulates developmentally essential genes as functional clusters called “regulon [9, 10]. Q k is specifically expressed in neural stem cells in embryonic mouse brains and functions as the significant regulator in neurogenesis-to-gliogenesis switching. Q k regulates mRNAs of the certain pathway that is essential for the fate determination of neural stem cells. From series of these studies, we propose the significance of mRNA regulation in neural functions and developmental processes of brain.

Nobuyuki Shiina has shown that RNA granules are membrane-less assemblies of RNA-protein complexes formed by liquid-liquid phase separation. Recently, RNA granules have been shown to contain solid-like cores rather than just liquid droplets [11]. However, it remained unclear how these different substructures are formed and whether different RNA granule scaffold proteins induce the formation

of different substructures. We found that RNA granule scaffold proteins, when expressed separately in cultured fibroblasts, induce the formation of either liquid-type or solid-type granules. When co-expressed in the same cells, the liquid-type and solid-type scaffold proteins were combined into the same granules and formed liquid-type shells and solid-type cores therein, respectively. Being combined with liquid-type scaffold proteins, solid-type scaffold proteins increased their mobility and reduced their dose-dependent translation inhibition in the granules [12]. RNG105 (also known as caprin1) was a liquid-type scaffold protein with remarkably strong activity to change the dynamics and translation inhibition ability of solid-type scaffold proteins. This activity of RNG105 suggested that in RNG105 knockout neurons, RNA granule cores could be very immobile and translation activity in the granules could be decreased. These changes in RNA granule dynamics and translation activity might underlie reduced dendritic localization of mRNAs in RNG105 knockout neurons and severe defects in long-term memory formation in RNG105 conditional knockout mice [13].

Riki Kurokawa have established a novel assay to detect interaction of RBPs with RNA, using biotinylated RNA oligos to capture RBPs with Western blot of specific antibodies against RBPs. The assay detects RNA binding more confidently than the traditional gel shift assay. Starting with completely randomized RNA oligos from 5mer through 12mer length, their binding was examined with HeLa cell nuclear extract (NE). Coomassie brilliant blue-based (CBB) staining did not detect any strong signal. Western blot analysis of typical six RBP antibodies showed four RBPs bound with the random RNA oligos. hnRNPUL2 bound to all from 5mer to 12mer of RNA oligos, while no TLS and hnRNPH1 signal was detected in the random RNA oligo samples. Next, base specificity was examined using sets of oligos of RNA fixed at G, A, U, and C (GAUC RNA oliogs). The RNA oligos fixed at "G" (G RNA oligos) have the most prominent protein bands. A, U, and C of RNA oligos were shown to bind less numbers of protein. Western blot indicated that hnRNPUL2 and hnRNPU bind all four oligos of GAUC at the 10mer length. Contrarily, TLS and hnRNPH1 have no binding with these oligos of GAUC. Then, poly G, A, U and C of RNA at the length of 100mer were tested to see binding profile of RBPs. The CBB staining of the fractions bound with these four polymers of RNA showed that more bands were bound than GAUC RNA oligos. hnRNPU bound well to poly G, A, and, U, but slightly less to poly C. Intriguingly, TLS and hnRNPH1 have binding only to poly G, and also to their common specific sites consisting of GGUG motifs. These data demonstrate that RNA binding is regulated with three factors, length, base composition, and sequence. Furthermore, hnRNPU and hnRNPUL2 have low specificity binding to RNAs, while TLS and hnRNPH1 exert high specific binding. These different propensities in bindings of RBPs are supposed to support specific biological roles in living cells [14].

Mamiko Iida and Masato Katahira identified an RNA aptamer against a prion protein, r (GGAGGAGGAGGA) (R12). We showed that R12 forms a unique quadruplex structure with a tetrad and a hexad by NMR and reduces a level of the abnormal prion protein (PrP), PrP^{Sc}, in the mouse neuronal cells, implying its therapeutic potential as to prion diseases [15, 16]. This time, we developed an RNA aptamer that exhibits much higher anti-prion activity than R12 through the logical design of the RNA sequence on the basis of the R12 sequence. The structure of this aptamer was determined by NMR. The origin of the much higher anti-prion activity was rationally interpreted by this structure. This aptamer has therapeutic potential as to the prion disease. The neurodegenerative disorder of Alzheimer's disease (AD) is associated with the accumulation of misfolded proteins. It was revealed that the amyloid β (Aβ) oligomers bind to prion protein, which acts as a receptor on the cell membrane, resulting in AD [17]. Thus, it is thought that compounds that can disrupt the formation of the prion-Aβ oligomer complex may prevent AD. Fluorescence anisotropy revealed that R12 is capable of binding to PrP, resulting in dissociation of PrP with Aβ. This is the first demonstration of disruption of the PrP-Aβ complex by a nucleic acid [18]. This ability of R12 implied its therapeutic potential as to AD.

Takefumi Yamashita presents a theoretical approach using molecular dynamics (MD) simulations of proteins and RNAs. MD simulations have been applied to many phenomena of biological polymers (e.g. protein-protein interaction) [19-24] as well as chemical reactions [25-27]. In these studies, detection of structural change is essential. Recently, we have proposed that the mathematics-based approach and machine learning-based approach are useful to detect the characteristic structural change efficiently [28, 29]. For example, the persistent homology analysis method, one of the computational mathematics methods, characterizes point clouds. By applying this method to the water molecules of the antigen-antibody complex solution system, we quickly detected water molecules located in the antigen-antibody interface [29], which should largely influence the binding entropy [22]. The persistent homology analysis does not require any thermodynamic knowledge, but can automatically detect characteristic water distributions, which may influence thermodynamic properties.

Yoko Matsuno presents data of redundant triplex interactions between lncRNA and LINE-1s in X-chromosome inactivation [30]. Associations between X-inactive transcript (Xist) -long noncoding RNA (lncRNA) and chromatin are crucial intermolecular interactions in the process of X-chromosome inactivation (XCI). Even with high-resolution analyses of the Xist RNA-binding sites, specific interaction sequences are yet to be identified. Using a combination of bioinformatics, computational simulations, and quartz crystal microbalance measurements *in vitro*, we characterized redundant UC/TC (r-UC/TC) or AG (r-AG) motifs (≥5 nucleotides in length) that are abundantly localized in Xist/XIST/RNA-on-the-silent X (Rsx) RNAs and long interspersed nuclear element 1 (LINE-1) in mouse, human, and opossum genomes. These multiple short motifs in lncRNA and

DNA molecules could be brought together to form Hoogsteen or reverse Hoogsteen triplexes, which co-operatively enhance their associations. In addition, we showed the conservation of r-AG motifs in LINE-1s of any length (100-7,000 bp) or base composition, possibly through convergence in LINE-1 nucleotide variations, which provided a similar motif-length distribution across the three species (Kolmogorov–Smirnov distance <0.100). On the basis of this conserved motif-length distribution pattern, the triplex interactions would preferentially occur via shorter motifs (5-7 nucleotide in length) with their high occupancy rate, whereas longer motifs (≥8 nucleotides in length), although fewer in number, would provide more chances for multiple shorter target motifs to bind. This mode of interaction may impart redundant properties to the association between lncRNAs and LINE-1s in the XCI process.

Keisuke Hitachi et al. have shown that *Myoparr* promotes skeletal muscle atrophy caused by denervation through the regulation of *GDF5*/*BMP14* expression. Hundreds of long non-coding RNAs (lncRNAs) are expressed from the cis-regulatory regions including promoter and enhancer. The promoter region of the *myogenin* gene, coding for a pivotal regulator of myogenesis, produces a lncRNA *Myoparr*. *Myoparr* regulates myogenesis through the activation of neighboring *myogenin* gene expression and skeletal muscle miRNA expressions (*miR-133b/206* and *miR-675*) [31]. Intriguingly, *Myoparr* also promotes skeletal muscle atrophy caused by surgical denervation. However, the downstream genes of *Myoparr* during muscle atrophy remain unclear. We found that *Myoparr* activates the expression of *myogenin* gene, which also promotes neurogenic muscle atrophy by inducing the expression of E3 ubiquitin ligases. In addition, RNA-seq analysis revealed that *Myoparr* decreases the expression of *GDF5/BMP14* gene to repress the activity of BMP signaling [32]. BMP signaling increases the anabolic pathway and antagonizes neurogenic muscle atrophy. Thus, our findings indicate that *Myoparr* promotes neurogenic muscle atrophy by increasing *myogenin* expression and decreasing *GDF5/BMP14* expression. Moreover, our comparative transcriptome analysis between mouse myoblast cell line C2C12 and mouse tibialis anterior muscles showed that the downstream genes of *Myoparr* largely differ between myogenic differentiation and muscle atrophy condition, indicating that the cell status and context affect the downstream genes of lncRNAs, possibly depending on their binding proteins.

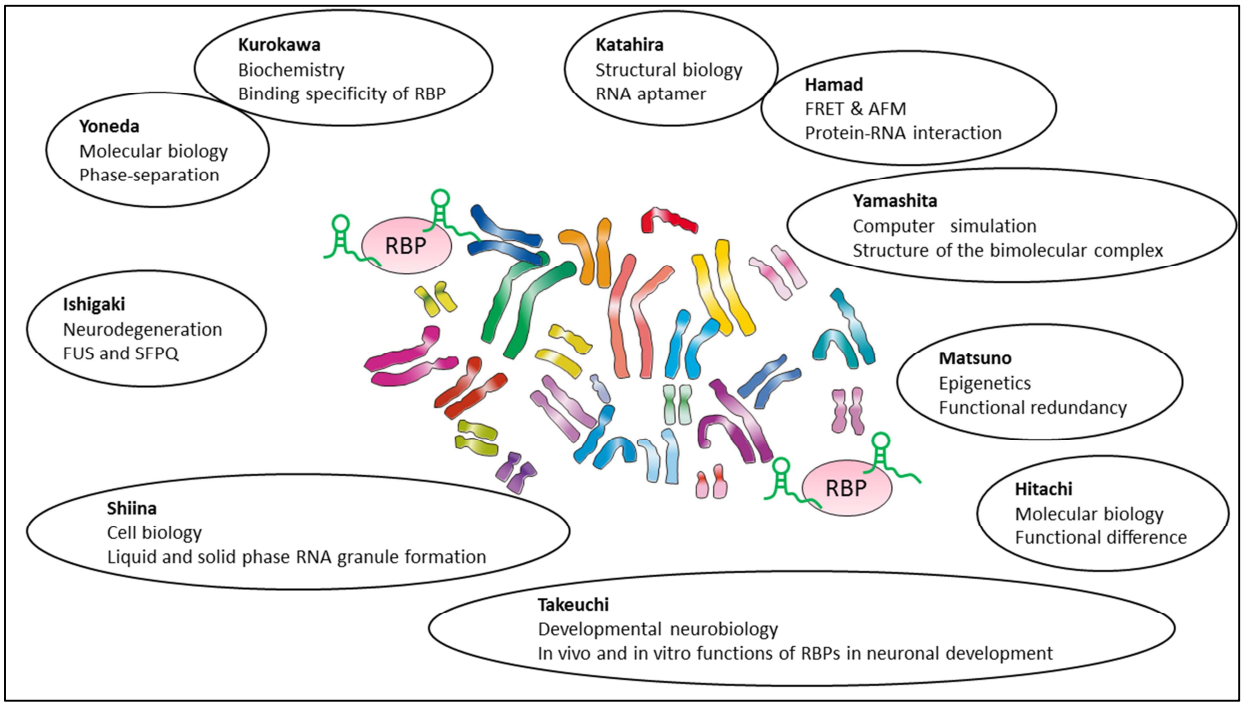

Figure 1. *Summary of the Talks.*

Shinsuke Ishigaki and Gen Sobue show Importance of Functional Loss of FUS in in Neurodegenerative Disorders. Fused in sarcoma (FUS) is an RBP that regulates RNA metabolism including alternative splicing, transcription, and RNA transportation. FUS is genetically and pathologically involved in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). FUS-related ALS/FTLD pathology is characterized by mislocalization of FUS to the cytoplasm with a reduction in nuclear expression in affected neurons, implying that functional loss of FUS could lead to neuronal dysfunction and/or neuronal cell death. We have reported that FUS and SFPQ regulate alternative splicing of *Mapt* gene at exon10 which generates two isoforms of neural microtubule-associated protein tau (Tau) protein. Silencing of FUS or SFPQ in the hippocampus of mice resulted in the increased ratio of 4-repeat tau (4R-tau) / 3-repeat tau (3R-tau) followed by FTLD-like abnormal behaviors, accumulation of phosphorylated tau, and neuronal loss [33]. Additionally, loss of FUS function can affect dendritic spine maturations by destabilizing mRNAs such as

Glutamate receptor 1 (GluA1) [34], and Synaptic Ras GTPase-activating protein 1 (SynGAP1) [35]. Since abnormalities in post-synapse are often observed in neurodegenerative diseases, a link among loss of FUS function, tau isoform alteration, aberrant post-synaptic function, and phenotypic expression, might be a possible sequential biological cascade leading to FTLD.

Nesreen Haamad and Masato Katahira have shown the conformational change of translocated in liposarcoma, TLS, upon binding to various non-coding nucleic acids. Translocated in liposarcoma (TLS), also known as fused in sarcoma (FUS), is a multifunctional RNA/DNA binding protein. TLS mutations are associated with numerous neurodegenerative disorders, such as amyotrophic lateral sclerosis (ALS). TLS plays a series of critical roles in RNA processing, including transcription, splicing, transport, translation, and decay. DNA damage triggers the transcription of a long non-coding RNA (lncRNA) from the 5′ upstream region of cyclin D1 gene (*CCND1*), named promoter-associated non-coding RNA (pncRNA) [36, 37]. Subsequently, TLS is recruited to the damage site. The C-terminal region of TLS interacts with pncRNA, which allows N-terminus TLS to bind CREB-binding protein (CBP) /E1A-binding protein P300 (p300) and to regulate the histone acetyltransferase HAT activity, resulting in transcription repression of *CCND1*. Although it was proposed that the interaction between TLS and pncRNA can convert TLS from closed to open conformation, this structural change has not been experimentally confirmed, so far. This time, we have spectroscopically detected the structural change of TLS upon binding pncRNA. Then, we have further confirmed the structural change on the basis of images of TLS.

Ryoma Yoneda shows phase separation and fibrillization of TLS/FUS-effect of mutation, RNase, and drugs. TLS/FUS is a DNA/RNA binding protein whose mutations cause familial amyotrophic lateral sclerosis (ALS). Severe ALS-related mutations occur in nuclear localization signal at the C-terminal end of TLS/FUS. These mutations cause TLS/FUS aggregation in the cytoplasm, and leads to cytotoxicity. In order to reproduce the TLS/FUS aggregation *in vitro*, we constructed GFP-fusion TLS/FUS with two kinds of ALS-related mutations (H517D and R521G). We first examined the phase separation of wild type (wt) and mutated TLS/FUS in different NaCl concentration, and observed promoted phase separation in low NaCl concentration, but no specific difference between wt and mutated TLS/FUS. RNase treatment also facilitated phase separation of all three TLS/FUS. We finally tested the effect of bisox, a reagent induces TLS aggregation [38], and similar dipeptide named bLH on TLS/FUS fibrilization. As expected, bisox induced fibrilization of all three TLS/FUS examined, and interestingly, bLH inhibited the TLS/FUS aggregation formed by bisox. Further analysis is required to reveal the inhibitory effect of bLH on bisox independent TLS/FUS fibrilization.

3. Conclusion

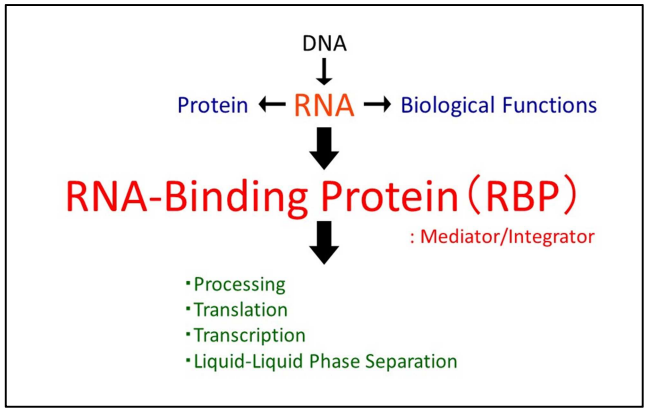

Figure 2. *Conclusion of the Conference RNA-binding protein plays a central role in RNA function in living cells.*

The presentations of the meeting cover variety of biomedical sciences, indicating the significance of RBPs and cognate RNA molecules in each field (Figure 1). The achievements of the meeting are to make sure a point of view of versatility of RBPs in living cells. It should be in complexity or chaos. We will focus on analyzing such intricate phenomena of RBP actions and unraveling the hidden rules. We will have more fruitful activity in the next year's meeting.

RNAs with divergent sequences are transcribed from the human genome. The RNAs have their own intrinsic property, but not so many RNAs are functional themselves. Most of RNAs needs to capture their binding partner for exerting their function. It is RBPs which mediate or integrate divergent sequence information of the RNAs into biological actions in living cells (Figure 2). Therefore, RBPs function as mediator or integrator for divergent RNA sequences. The possible model is presented there (Figure 2). Then, we will extensively examine the model to demonstrate exact molecular mechanism of RBPs in living organisms.

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