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Identification of Long Noncoding RNA Recognized by RNA-Binding Protein TLS/FUS: Purification of RNAs by Affinity Chromatography of GST-TLS

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Abstract: RNA binding protein TLS/FUS binds promoter-associated noncoding RNA-D (pncRNA-D), long noncoding RNA (lncRNA), and inhibits histone acetyltransferase (HAT) activity in cellular nuclei to repress the cyclin-D1 gene transcription. TLS is expressed in neuronal cells and plays pivotal roles in neuronal develop and function. Its precipitation in motor neurons is supposed to cause amyotrophic lateral sclerosis (ALS) one of neurodegenerative diseases. Our preliminary experiments indicate that pncRNA-D should repress the phase separation and the resultant precipitation of TLS in biochemical conditions. This implies that pncRNA-D be a seed for effective drug against ALS. It is conceivable that a pool of lncRNAs bound to TLS should be a competent library screening for an ALS drug. Then, we decided to search for more lncRNAs to regulate phase separation and precipitation. In this manuscript, we develop a simple and swift technology of the affinity purification of unidentified RNAs from HeLa cell total RNA using bacterially expressed glutathione S-transferase-tagged-TLS (GST-TLS). Screening of the GST-TLS bound RNA has been performed with a human lncRNA microarray using a fluorescent dye, the cy3-labeled bound RNA as a probe. 1728 lncRNAs of more than two-fold increase at the fluorescent signals have been identified, compared to those of the input HeLa cell total RNA. The top 25 lncRNAs from the 1728 lncRNAs were expressed at more than 12-fold induction. Tentatively, the top four putative lncRNAs were employed for further analysis. Then, these lncRNAs have been shown to have specific binding to GST-TLS and also cellular TLS. The precipitation based experiment to detect phase separation has shown that these lncRNAs inhibit the phase separation-induced precipitation which is dissolved in 1, 6-hexanediol (1,6-HD). There is no significant sequence homology over these lncRNAs, although a consensus conformation of these lncRNAs is the loops and stems structure based upon the predicted secondary structures of the top 25 lncRNAs selected. These data confirm that the GST-TLS based affinity purification of RNA bound to TLS works well to provide the novel lncRNAs specific to TLS. The method to identify novel lncRNAs recognized by TLS provides a profitable technique for initiating the biology of TLS-bound lncRNAs in cellular programs.

Keywords: TLS/FUS, pncRNA-D, GST-TLS, Long Noncoding RNA, Phase Separation, Intrinsically Disordered Region, IDP

1. Introduction

TLS has been reported to work as a causative gene for amyotrophic lateral sclerosis (ALS) because the mutated TLS is leaked from cell nucleus to cytoplasm area and forms toxic aggregates [1-5]. This aberrant localization of TLS induces its aggregation there in cytosol, leading to the ALS and other neuronal diseases like frontotemporal lobar degeneration (FTLD) [1, 2, 6-8]. Mislocalization of the mutated TLSs is supposed to prompt formation of toxic aggregation of TLS, and also loss of function of TLS, leading to the diseases. It largely remains uncovered how dysregulation of function of TLS should cause the disease. For clear understanding mechanisms of the onset of ALS, more analysis of TLS roles

in neuronal functions should be demanded. Deletion of TLS using gene editing technology with CRISPR/CAS9 in organoids derived from human iPS cells induces proliferation and differentiation of neuronal cells in cortical brain-organoids, but impairs these phenotypes in spinal cord organoids [9]. These reciprocal responses in cortical and spinal cord- organoids, mediated through cellular signaling with neurotrophic factors regulated with TLS. These experiments present direct evidence of the role of TLS in the neuronal development in the human central nervous system (CNS).

TLS knock in mouse strains bearing the ALS-related mutant TLSP525L were generated in order to uncover how the mutated TLS causes neurodegeneration in ALS [10]. These mutated mice are grown to gain toxic aggregations of TLS and lose motor neuron in dose-dependent manner of the TLS mutation. An antisense oligonucleotide against TLS, ION363, proficiently silences TLS and lowers degeneration of motor neurons in the brain and spinal cord. Preliminary examinations of a patient with ALS associated mutant TLSP525L demonstrate that intrathecal injections of ION363 lessen expression of wild type and mutant TLS and reduce the load of the pathological aggregation in the CNS. These sets of experiments using mouse systems and a human clinical subject demonstrate that reduction of the affected TLS with treatment of INO363 should be efficient strategy of therapeutics against ALS caused by TLS mutations [10].

In the past decade, we have experienced a rapid progress in divergent field of RNA studies in biology and medicine, making radical alteration in a view points from Central Dogma which regards RNA as just steps of gene expression [11-20]. Extensive analyses of transcriptomes from the human genome have demonstrated that tremendous numbers of RNA transcribed from the noncoding DNA [21-26]. Mostly, the unappreciated transcripts are found to be long noncoding RNAs (lncRNAs) of which length is more than 200 bases and their biological activity remains largely unidentified [27]. However, lncRNAs need to have RNA-binding proteins (RBPs) for their functions [28-32].

RNA binding is much more enigmatic activity than DNA binding. Some RBPs have high specificity binding to their target RNA, while others have low specificity binding, because RBPs play divergent biological roles in living cells and their specificities might fit each specific biological tasks. Occasionally, the single RBP like TLS has both high and low specificity bindings to RNAs. In this case, differential interaction domains of TLS to RNAs remain unrevealed. Now, we have been analyzing binding regions of TLS to different species of RNA oligonucleotides to obtain molecular mechanism for generating two different specificity domains to distinctive RNA. This helps us to understand biological meaning of multivalent binding sites in a single molecule.

RBPs form ribonucleoprotein (RNP) complexes with other RBPs and regulate gene expressions at various steps like translation and maturations of RNA molecules [33, 34]. RBPs exert their functions by interacting with RNAs through their RNA binding domains [35], including RNA recognition motif (RRM) [36], and DEAD box helicase domain [37]. Recently, more investigating the structures of large RNP complexes like ribosome [38-40] and spliceosome [41, 42] uncovered novel interactions between proteins and RNAs with no canonical RNA binding domains [43, 44]. These findings imply that possibly more unconventional RNA binding site could work in living cells.

It has been shown intrinsically disordered regions (IDRs) in most of RBRs [45-51]. These IDRs have been found to play critical roles in targeting RBPs into cellular structures including RNA granules and paraspeckles [52-54]. Their active and amorphous structures still remain uncovered. Gel-shift binding assay with ^{32}P-RNA probes indicated that RGG domains of TLS, FMRP, and hnRNPU bind specific RNA sequences with moderate affinities [48]. NMR analysis demonstrated that RGG domains significantly increase binding affinity of TLS-RNA complex and promote destabilization of structured RNA conformation, enabling additional binding in a sequence-independent manner [55]. These findings have shown that IDR could function as a interaction surface to RNA, implying that IDR is a novel motif for RNA binding [50, 51]. Recent our experiments indicated that lncRNA pncRNA-D1 represses phase separation and resultant aggregation. This means that lncRNA could be a drug seed for ALS. Searching ALS drug seed requires a good library of TLS-specific lncRNAs. For this purpose, we need to develop systematic method of identifying TLS-specific lncRNAs.

In this manuscript, we set project to develop affinity purification of RNAs recognized by GST-TLS and plan to identify novel TLS-specific lncRNAs based upon detection with the human lncRNA microarray. Series of experiments to test specific binding to TLS and also functional assay to indicate these effects on the precipitation caused by TLS-induced phase separation. These data demonstrate that the GST-TLS affinity purification is effective method to identify biological active lncRNAs to TLS.

2. Materials and Methods

2.1. Antibodies and Reagents

Mouse anti-TLS/FUS antibody (611385, Lot no. 2209827) was purchased from BD Biosciences (New Jersey, USA). Rabbit anti-TLS/FUS antibody (11570-1-AP) was purchased from Protein Tech (Illinois, USA). Rabbit anti-mouse HRP conjugated IgG (P0161, 20017456) was purchased from Dako (Glostrup, Denmark). Goat anti-rabbit HRP-conjugated IgG (7074S, 25) was purchased from Cell Signaling Technology (Massachusetts, USA). HeLa cell nuclear extract (NE) was prepared as previously described protocol [56-59]. RNeasy plus Mini Kit was purchased from Qiagen (Düsseldorf, Germany). 1,6-hexanediol (1,6-HD) was purchased from Sigma-Aldrich Co. (Missouri, USA).

2.2. Affinity Purification of lncRNA with GST-TLS

HeLa cell total RNA fraction was prepared from one 10cm

plate of HeLa cells. The cells with sub-confluent status were harvested. The cell pellet was extracted by an RNA preparation kit (ReliaPrep™ RNA Miniprep Systems) from Promega (Wisconsin, USA). The bacterially expressed GST-TLS (1.14 μg/100 μl) was incubated with one μg of HeLa cell total RNA fraction. The RNA-bound GST-TLS was applied to RNeasy plus Mini Kit (Qiagen) following producer's protocol to obtain 594 ng of the GST-TLS bound RNA.

The GST-TLS bound RNA fraction has been analyzed on the human long noncoding microarray kit from Agilent (California, USA). Parallel samples of the GST-TLS bound RNA were analyzed for agarose gel electrophoresis to inspect the quality of the RNAs. The complemental Cyanine 3-labelled RNA probes were synthesized from the GST-TLS bound RNA fraction and also from the HeLa cell total RNA as a negative control. The cyanine 3-probe of the GST-TLS bound RNA and the probe of the negative control of the HeLa cells were hybridized to the lncRNA microarray which is fixed with human 5139 transcripts with 60 nucleotides consist of some noncoding RNAs, but of coding RNAs. The fluorescent intensity of the hybridized microarray plates were analyzed with the Agilent scanner and presented increments of the fluorescent intensity.

Table 1. *The list of the novel lncRNAs identified. I: Intensity of the TLS RNA signal/Intensity of the HeLa cell RNA signal; II: Chromosome number.*

		ProbeName	GeneName	Ⅰ	Description	Ⅱ
1	lncRNA.1	A_22_P00025249	lnc-CEACAM18-2:1	117.29	DB453801 RIKEN full-length enriched human cDNA library, testis Homo sapiens cDNA	chr19
2	lncRNA.2	A_22_P00012656	lnc-PYCARD-1:1	81.10	LNCipedia lincRNA (lnc-PYCARD-1), lincRNA	chr16
3	lncRNA.3	A_21_P0008265		79.05	long intergenic non-protein coding RNA 567	chr13
4	lncRNA.4	A_19_P00320038	RNA 574	66.39	Homo sapiens long intergenic non-protein coding RNA 574	chr6
5		A_21_P0001336		65.93	leucine rich repeat containing 8 family, member C	chr1
6		A_23_P376088	Lck	54.86	Homo sapiens Lck interacting transmembrane adaptor 1 (LIME1)	chr20
7	lncRNA.5	A_21_P0009846		39.84		chr20
8		A_33_P3382137		38.21		chr20
9		A_24_P187614	LOC100129648	27.80	Homo sapiens cDNA FLJ40318 fis, clone TESTI2030556.	chr22
10	lncRNA.6	A_33_P3357626		25.04		chr1
11		A_33_P3277373	oncostatin M	22.21	Homo sapiens oncostatin M (OSM), mRNA	chr22
12		A_23_P73609	Norrie disease (pseudoglioma)	18.25	Homo sapiens Norrie disease (pseudoglioma) (NDP),	chrX
13		A_33_P3292083	RNA 1301	17.49	long intergenic non-protein coding RNA 1301	chr8
14		A_22_P00014095	lnc-SASS6-1:1	15.30	LNCipedia lincRNA (lnc-SASS6-1), lincRNA	chr1
15		A_33_P3418846		15.27	cancer susceptibility candidate 4 pseudogene 1	chr13
16		A_33_P3416757	prolactin receptor	14.95	Homo sapiens prolactin receptor (PRLR), transcript variant 2, mRNA	chr5
17		A_22_P00009826		14.49	Homo sapiens mRNA; cDNA DKFZp434O1311	chr17
18		A_22_P00006571	lnc-FMN1-3:1	14.47	BX088994 Soares_testis_NHT Homo sapiens cDNA clone IMAGp998G023475	chr15
19		A_21_P0011718		14.00	Homo sapiens small integral membrane protein 17 (SMIM17),	chr19
20		A_22_P00018830	lnc-PANK3-7:1	13.87	DB067147 TESTI4 Homo sapiens cDNA clone TESTI4008690 5',	chr5
21		A_22_P00024191		13.58		chr18
22		A_23_P106887	FUS RNA binding protein	13.51	Homo sapiens FUS RNA binding protein (FUS), transcript variant 1,	chr16
23		A_21_P0009078		12.87		chr16
24		A_33_P3274642	unc-80 homolog (C. elegans)	12.86	Homo sapiens cDNA FLJ14677 fis, clone	chr2
25		A_33_P3326275		12.82	Homo sapiens cDNA: FLJ23120 fis, clone	chr2

The analysis of the GST-TLS-bound RNA hybridized to the plate displayed 1728 more than two-fold increased samples of the fluorescent intensity compared to the HeLa cell RNA hybridized. These analyses also showed 162 samples of more than five-fold increased, 49 samples of more than ten-fold increased, eleven samples of more than twenty-fold increased.

Top four putative lncRNAs were performed for further analysis (Table 1). There is not so rich information from the annotation of these sequences. At least no description for TLS-binding is found. Therefore, these four RNA sequences are supposed to be novel lncRNAs bound to TLS. The biotinylated RNA oligos with 60 nucleotides based on sequence data in the microarray were synthesized and used for RNA binding assay to detect binding to GST-TLS. The intensity of the protein bands occasionally does not represent to the order of the microarray assay. This should be because of difference of assay system.

Next, the RNA binding assays were performed with TLS in HeLa cell NE using Western blot by a TLS antibody. These binding assays confirm that TLS expressed in HeLa cells binds to the novel lncRNAs, indicating that the GST-TLS affinity chromatography works well to identify authentic lncRNAs binding to TLS in living cells.

2.3. RNA Binding Assay

The RNA binding assays were performed previously described [60-62]. Briefly, Dynabeads-M280 (Thermo Fisher)

was washed with PBS containing 0.02% Tween 20. One µmol of biotinylated RNA oligos or RNAs was added to the beads and incubated for 15 min at room temperature with rotation. Subsequently, the beads were incubated with HeLa cell NE for 1 h at 4°C with rotation. Beads were then washed three times in 1 ml of WCE buffer and resuspended in SDS sample buffer and boiled at 100°C for 2 min. Finally, the Dynabeads were removed and supernatants were analyzed by a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) following for stain with the coomassie brilliant blue (CBB) of a SimplyBlue™ SafeStain (Thermo Fisher) or Western blotting analyses. The pncRNA-D1 (32 to 62; 31mer) is shown as (1-1) and the pncRNA-D1 (32 to 44, 13mer) is shown as 5 (1-1). Then, (1-1) and 5 (1-1) are used as an equivalent positive control for RNA binding assay.

2.4. Phase Separation and Precipitation Assay of GST-TLS

Purified GST-TLS with glutathione-agarose beads was employed for development of assays to observe the phase separation mediated precipitation. Firstly, biotinylated isoxazole (BISOX) is added to GST-TLS solution at 50 µM and incubated at 4°C for 60 mins to give a precipitation. Furthermore, procedure without any chemical has been developed. WCE buffer with 1.4 µg of GST-TLS is incubated at 4°C for 60 mins, and centrifuged at 3500 rpm or 5000 rpm for 5 mins to generate precipitation. The precipitation is resuspended with 15% 1,6-HD washing twice. The precipitation is recovered by centrifugation at the same rpm for 5 mins. The precipitation is analyzed by SDS-PAGE following the CBB staining procedure to visualize.

2.5. Protein Analysis

SDS-PAGE was performed with 10% polyacrylamide gels following CBB staining [56, 58, 59]. Western blotting was done with anti-TLS monoclonal antibody with the dilution ratio 1:2000 using standard protocol shown previously.

2.6. RNA Sequences Used in the Experiments

lncRNA1 60 mers:
UUCUCCUCCAAGAACCUUGGCAUCCAGGCGGCCCC
CUAACCUGGCAGCUGCAGGAUGGAU
lncRNA2 60 mers:
UAUAACCACUGUAACUCUGCUGUCCGUAGGGCUG
ACUGCUCUGCUGGGAAUAGCCCUGCC
lncRNA3 60 mers:
UAAACUUUCCUAACCUGGGCUCAACCUUGGUUUCG
UCUCUCAGUCUUAAUUUUGCUUCAG
lncRNA4 60 mers:
GUUGCGUUUUCGUACGGCUGACUAAAGCGGAUAC
CGGUGGCGACUCAUUUCUCGUUUUAU
lncRNA5 60 mers:
UGUCCUCCAGCAGCUCUAGCCUGGAUGCGGUCCCA
GAGAUAAAUCAUAUCUCUUUAAAAA
lncRNA6 60 mers:
GGGGUCAAAUCCAUCCCUAGUCAUGGCCCCCUGGA
GAAGUGGCAAGCCUUGUACUCAUGA
(1-1): pncRNA-D1-32-62 (31 mers):
GUUAAGAGGGUACGGUGGUUUGAUGACACUG
5 (1-1): pncRNA-D-32-44 (13 mers):
GUUAAGAGGGUAC

3. Results

3.1. Affinity Purification of lncRNAs Recognized by GST-TLS

Total RNA fraction was extracted from the HeLa cell culture using RNA extraction kit (Figure 1A). The total RNA obtained was used for affinity chromatography of GST-TLS to get RNA fraction specifically bound to TLS (Figure 1B). GST-TLS contains endogenous bacterial RNA mostly consisting of ribosomal RNA (Lane 1, Figure 1B). To remove the bacterial RNA, treatment of GST-TLS in an Eppendorf tube with 20 units of micrococcal nuclease (MNase) was performed. The resultant sample lost most of signals of bacterial RNAs and should be used for further purification by the QIAGEN kit. After the final purification procedure, more than 500 ng of the TLS-bound RNA fraction was obtained and performed for analysis with the lncRNA microarray.

3.2. RNA Binding Assays to Detect Specific Binding of TLS to the Novel lncRNAs

The analysis of the GST-TLS-bound RNA hybridized to the lncRNA microarray plate displayed 1728 more than two-fold increased samples of the fluorescent intensity compared to the HeLa cell RNA hybridized with the same plate. These analyses also showed 162 more than five-fold increased samples, 49 more than ten-fold increased samples, eleven more than twenty-fold increased samples. The top 25 RNA entries including coding ones are shown (Table 1). Tentatively, the top four putative lncRNAs were performed for further analysis. There is not so abundant information from the annotation of these sequences. At least no description for TLS-binding is found. Therefore, these four RNA sequences are supposed to be novel lncRNAs bound to TLS.

The biotinylated RNA oligos with 60 nucleotides based on sequence data in the microarray were used for RNA binding assay to detect binding to GST-TLS (Figure 2A). The intensity of the protein bands does not represent to the order of the microarray assay. This should be because of difference of assay systems.

Next, the RNA binding assays were performed with TLS in HeLa cell NE using Western blot by a TLS antibody (Figure 2B). These binding assays confirm that TLS expressed in HeLa cells binds to the novel lncRNAs, indicating that the GST-TLS affinity chromatography performs successfully to identify authentic or bona fide lncRNAs bind to TLS in living cells.

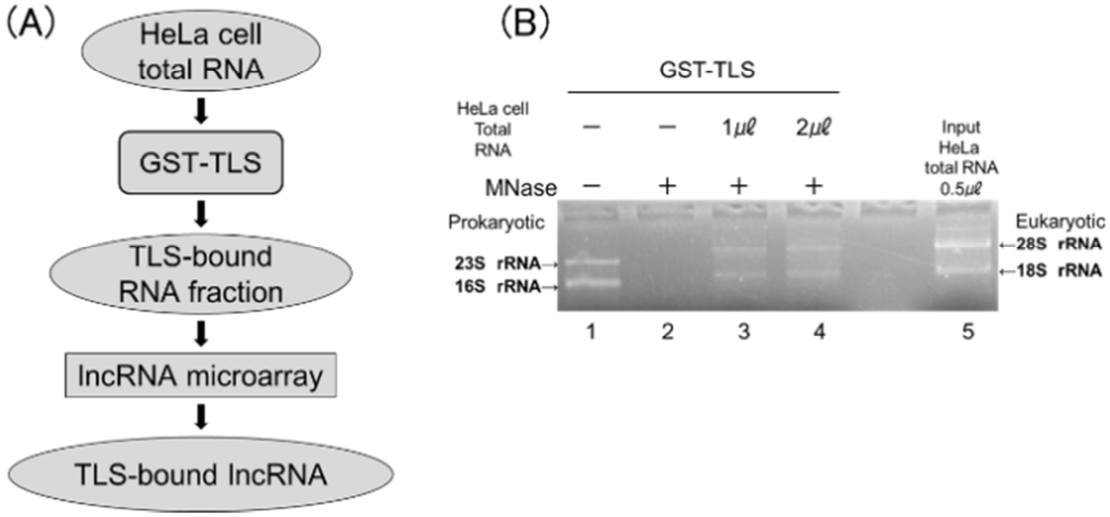

Figure 1. *Isolation of the RNA fraction bound by GST-TLS from HeLa cell total RNA.*

(A) Scheme of the experiment. Detailed procedures are described in text.
(B) Agarose gel electrophoresis of the RNA samples bound by GST-TLS from the HeLa cell total RNA. The samples of each lane are depicted at the top of the gel image. Actual procedures are described in text.

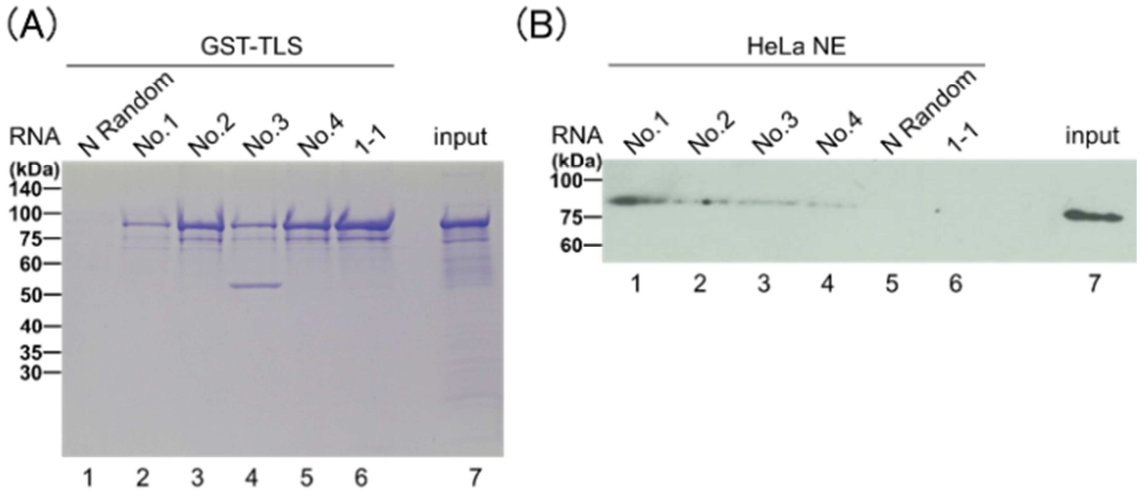

Figure 2. *The biotinylated RNA-binding assays indicate that identified lncRNAs bind to TLS.*

(A) The SDS-PAGE shows that the lncRNAs bind to GST-TLS. The bands are detected with coomassie brilliant blue (CBB)-staining. Each lane is depicted at the top of the gel image. Lanes 2, 3, 4, and 5 of lncRNAs shown. Lane 1 contains negative control of randomized RNA oligos. Lane 6 is from positive control of fragment of (1-1: pncRNA-D (32-62)). The 50% input is shown in all figures except specifying the distinctive % input.
(B) Western blotting analysis shows that biotinylated lncRNAs bind to TLS molecules in the HeLa cell nuclear extract. The samples of each lane are described at the top of the gel image. The negative and positive controls are the same ones shown at (A). Rabbit polyclonal antibody is used for the Western blotting. The 10% input is indicated.

3.3. Specific lncRNAs Have Distinctive Effect on the Precipitation

Our previous data show that the 5'-region of pncRNA-D has inhibitory effect on the phase separation and resultant precipitation detected with NMR or microscopic observation system [63]. The process of the detection required the specialized equipment and also technologies. Then, we decided to develop a simple and swift biochemical method to detect and evaluate the precipitation based on phase separation. Our observation showed that the purified GST-TLS at around 1 μg tends to be precipitated without any chemicals or any centrifugation. Then, we develop a strategy to employ mild centrifugation on the GST-TLS solution to gain the 1,6-HD sensitive precipitation induced by phase separation.

Treatment of the precipitation with 1, 6-HD dissolved it effectively (Figure 3A), suggesting the precipitation of GST-TLS should be induced through the phase separation. A series of spin on the GST-TLS in WCE was examined for generating the precipitations. This procedure provides a condition of centrifugation at 5000 rpm for five minutes should give the best precipitation of GST-TLS (Figure 3B). We have employed this condition to evaluate the precipitation of GST-TLS.

We explored milder reaction condition for detection of effect of the lncRNAs on the GST-TLS and obtained a reaction at 37°C for 60 mins following the 4°C incubation without any centrifugation. The precipitation assay demonstrated all lncRNAs tested have inhibitory effect on the phase separation-based precipitation, although of slight deference in the effect (Figure 4AB). The effect of the 5 (1-1) was less inhibitory on the precipitation than that of lncRNA1 and lncRNA2 (Figure 4A). The experiment to test inhibitory activity of these lncRNAs on the precipitation indicated that slight differences over the five lncRNAs tested (Figure 4B).

These data show that the inhibitory effect of the lncRNAs on the precipitation have distinct specificity. Then, we design to confirm specificity of the functions of these lncRNAs by mapping functional domains of TLS using the GST-TLS fragments shown below.

3.4. Specificity of RNA Binding to TLS Is Generated by Functional Interactions of the TLS Domains

To confirm activity of the lncRNAs, mappings of the TLS binding domains of the lncRNAs were performed (Figure 5A). Previously, we have reported the mapping experiment of the methylated arginine residues at the TLS amino acid sequence using four fragments across the full-length TLS [58]. The full-length GST-TLS has differential binding to these lncRNAs from No. 1 through No. 6 (Figure 5B). LncRNA 2, lncRNA4, and lncRNA5 have relatively stronger bindings than the rest of the lncRNAs. The GST-TLS fragment 1 has almost equivalent and marginal bindings to all of the lncRNAs tested (Figure 5C). There were no significant binding to all lncRNAs tested on the GST-TLS fragment 2 and fragment 3 (Figures 5 D and E). The firm and equivalent bindings of the all lncRNAs were detected on the GST-TLS fragment 4 (Figure 5F). Contrast to the bindings of the GST-TLS fragment 4, the full-length GST-TLS binds to the sets of lncRNAs with differential affinities. Actually, the full-length TLS binds lncRNA 1, lncRNA3, and lncRNA 6 slightly weaker affinity than the rest of the lncRNAs (Figure 5B). The summary of the mapping experiments is shown at table 2.

Table 2. *Summary of the Mapping of the TLS interaction domains with the lncRNAs.*

		lncRNA					
		1	**2**	**3**	**4**	**5**	**5 (1-1)**
GST-TLS	fragment.1	-	-	-/+	-	-/+	-
	fragment.2	-	-	-	-	-	-
	fragment.3	-	-	-	-	-	-
	fragment.4	+	+	-/+	+	+	+
	full length	+	+	+	+	+	+

These data suggest that binding of the fragment 4 might be interfered or inhibited by another fragment of GST-TLS. A putative inhibitory domain should be on the fragment 1, because the fragment 2 and fragment 3 have no binding to any lncRNA tested. Then, we designed an experiment to find out the inhibitory domain in TLS using the fragment 1 and fragment 4. Incubation of the GST-TLS fragment 1 and fragment 4 resulted in less binding to the fragment 4, and appeared some binding to the fragment 1 on the lane of lncRNA3 (Figure 5G), The data indicated that the fragment 1 inhibits the binding of lncRNA3 to the fragment 4 (the lane 3, Figure 5G). The data imply that the fragment 1 could deprive the fragment 4 from lncRNA 3. It shows a putative regulatory region on the GST-TLS fragment 1 against the firm binding of the fragment 4.

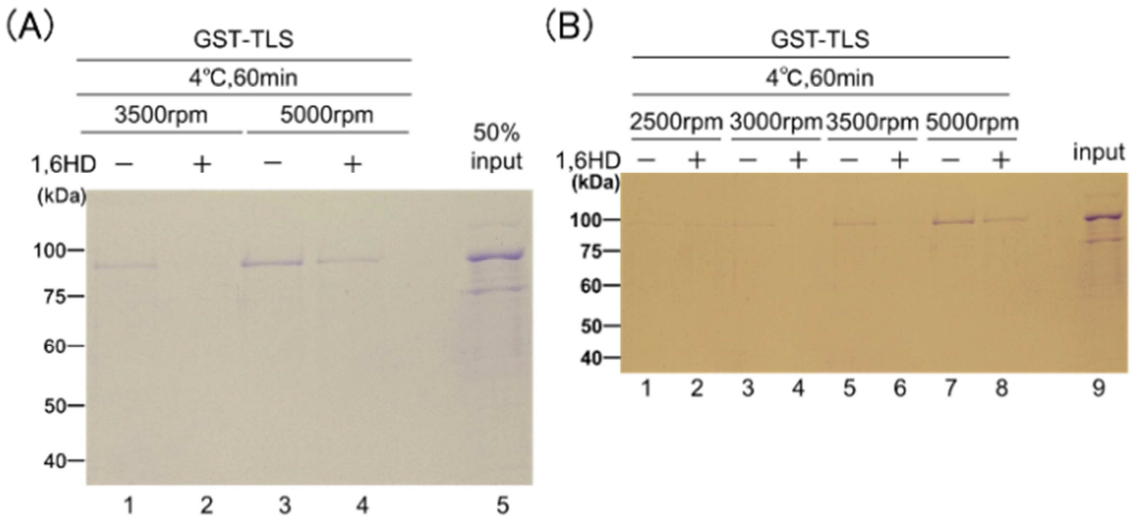

Figure 3. *Dependency of the precipitation on phase separation is examined by treatment with 15% 1,6HD.*

(A) The precipitation of GST-TLS at 3500 rpm and 5000 rpm with treatment by 1,6-HD Precipitated GST-TLS samples at 3500 rpm are washed with 1,6HD (lane 1), or WCE (lane 2) as a negative control. Precipitated GST-TLS samples at 5000 rpm are washed with 1,6HD (lane 3) or WCE (lane 4). The 50% input is depicted at the right edge of the gel.

(B) Extensive conditioning of centrifugation from 2500 rpm through 5000 rpm. The precipitated samples are treated as shown at each lane.

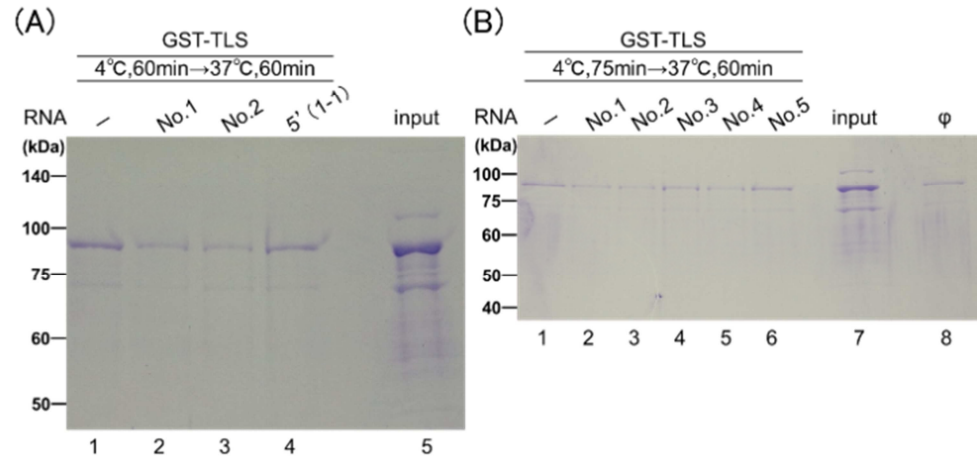

Figure 4. *The lncRNAs inhibit the phase-separation induced precipitation of TLS without centrifugation.*

(A) The effect of lncRNAs is examined for the precipitation of GST-TLS at milder condition, without centrifugation. The tested lncRNAs are shown at the lanes.
(B) The effect of lncRNA 1 through lncRNA 5 on the precipitation of GST-TLS at the milder centrifugation condition shown.

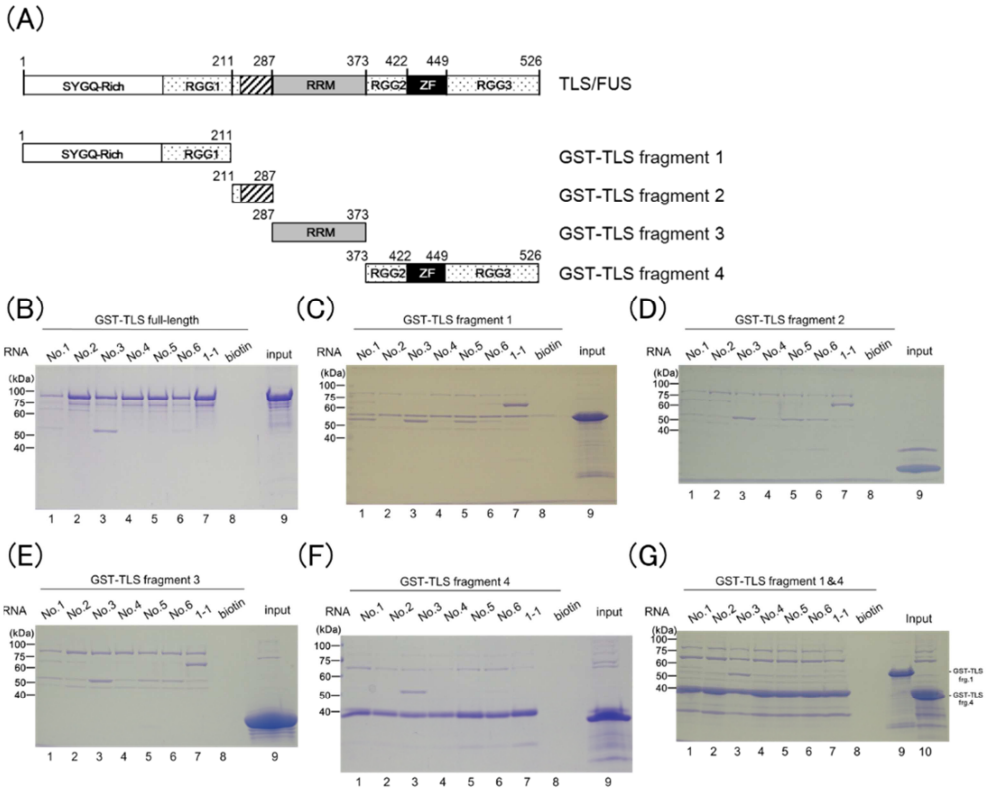

Figure 5. *Mapping of the interaction region on TLS with the lncRNAs.*

(A) The domain structure of TLS. The full-length GST-TLS is divided into the fragments based upon its domain structures.
(B) Mapping of the five lncRNAs to the full-length GST-TLS (1.41 μg) with positive and negative controls. Binding assay with GST-TLS and the lncRNAs was performed followed with the Materials and Methods. The protein bands were detected with CBB staining.
(C) Mapping of the five lncRNAs to the GST-TLS fragment 1 (11,92 μg) with positive and negative controls. Binding assay with the GST-TLS fragment 1 and the lncRNAs was performed followed with the Materials and Methods. The protein bands were detected with CBB staining.
(D) Mapping of the five lncRNAs to the GST-TLS fragment 2 (13.08 μg) with positive and negative controls. Binding assay with the GST-TLS fragment 2 and the lncRNAs was performed followed with the Materials and Methods. The protein bands were detected with CBB staining.
(E) Mapping of the five lncRNAs to the GST-TLS fragment 3 (97.20 μg) with positive and negative controls. Binding assay with the GST-TLS fragment 3 and the lncRNAs was performed followed with the Materials and Methods. The protein bands were detected with CBB staining.
(F) Mapping of the five lncRNAs to the GST-TLS fragment 4 (6.21 μg) with positive and negative controls. Binding assay with the GST-TLS fragment 4 and the lncRNAs was performed followed with the Materials and Methods. The protein bands were detected with CBB staining.
(G) Mapping of the five lncRNAs to the putative inhibitory domain on the GST-TLS fragment 1 (11,92 μg) with competition of the GST-TLS fragment 4 (6.21 μg) with positive and negative controls. Binding assay with the GST-TLS fragments 1 and 4 was performed followed with Materials and Methods. The protein bands were detected with CBB staining.

4. Discussion

In this manuscript, we employed the affinity chromatography of GST-TLS to purify the RNAs binding to TLS. After t screening of t screening he lncRNA microarray, we have obtained sets of lncRNAs binding to TLS (Figure 6). The assay to assess the inhibitory activity in the TLS phase separation has been developed successfully. The lncRNAs 1 through 5 have been shown to exert inhibitory activity on the TLS phase separation and the resultant TLS precipitations. The first event of the precipitation inhibitions is interaction between lncRNAs and TLS. The binding affinity of TLS to the lncRNAs is determinant factor in the interaction. Our mapping experiments clearly indicate that the fragment 4 of TLS is interaction domain of TLS to the lncRNAs. The binding of the fragment 4 to the lncRNAs is stable and solid, while the fragment 1 binding to the same set of the lncRNAs seems fluctuated and fragile, because the fragment 1 contains wobbly intrinsically disordered region on it. Recently, it has been proposed that the IDR should function as a RNA-binding interface in biochemical systems and also in living cells [50, 51], although solid evidence remains to be refined.

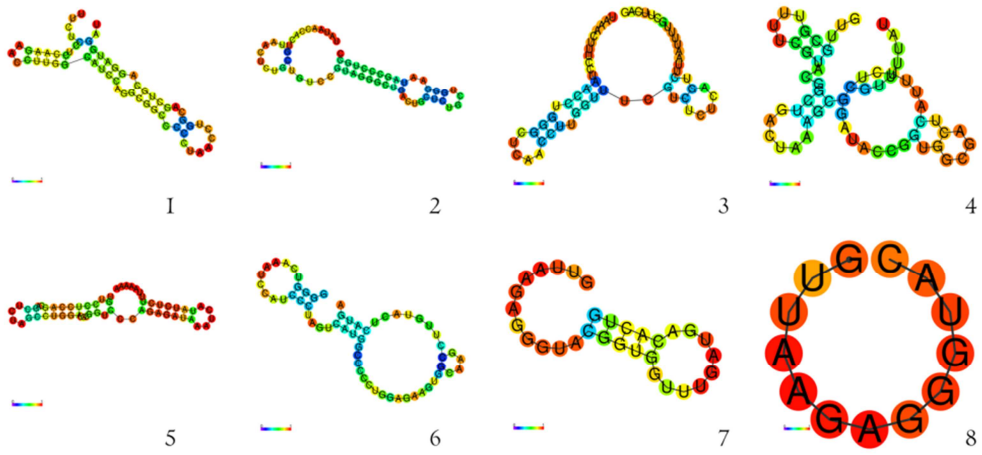

Figure 6. *Prediction of the three dimensional structures with analysis by the CentroidFold.*

The numbers 1 through 6 are lncRNA1, lncRNA2, lncRNA3, lncRNA4, lncRNA5, and lncRNA6, respectively. The numbers 7 and 8 are (1-1) that is pncRNA-D1 (32 to 62; 31mer), and the 5 (1-1) that is pncRNA-D1 (32 to 44, 13mer). The RNA secondary structures are predicted by the CentroidFold. The CentroidFold based on a centroid program is one of the most accurate tools for predicting RNA secondary structures. The predicted secondary structure is colored according to base pairing probabilities. http://rtools.cbrc.jp/centroidfold/

Binding of RNA is more divergent functions than that of DNA [34, 43, 64-68]. DNA-binding transcription factors recognize specific binding sites or responsive elements in a promoter or enhancer, like AGGTCA for nuclear receptor, usually bearing several base pairs in bases of DNA DNA with consensus motifs, and activate transcription of the target genes [69-73]. Transcription factors precisely recognize specific binding sites, and stringently regulate transcription upon binding to the sites [74-76]. Therefore, high affinity binding of transcription factors is essential for its appropriate missions for regulation of multiple gene networks.

On the other hand, RBPs have multiple functions including RNA metabolism, splicing, also construction of membrane less organelles. [28, 77-79]. Occasionally, RBPs form complexes with RNA to form cellular structures [52, 79, 80]. Broad ranges of affinities for RNA in RBPs are suitable for their multiple functions in living cells [81-83]. Promiscuous interactions of RBPs with RNA are partly forced with flexible conformations of RNA. Such kinds of protein-RNA interactions might be accommodated by IDR also because of its elasticity.

Recent our experiments have shown that RBPs are categorized into two groups regarding their affinities of RNA bindings [58-60]. Binding assays with completely random oligo nucleotides indicated that hnRNPU and hnRNPUL2 bind these random sequences of RNA. These two molecules are categorized as a low affinity RBPs. In the initiation complex of RNA polymerase II (pol II), hnRNPU functions as an inhibitor against pol II dependent RNA elongation step [84], and also works for DNA repair upon its phosphorylation with DNA-dependent protein kinase [85]. Failure of hnRNPUL2 functions causes cognitive impairment related to autism, and also stimulates accelerations of proliferation and migration in colorectal carcinoma and DNA repairs [86-88], with roles in generating cellular structures like chromosomal matrix [80]. Especially, hnRNPU forms with RNA and is incorporated into the chromatin matrix. In this series of functions, RBPs forms a complex with RNAs just as pieces of nuclear components and hold RNA as nucleic acid mass instead of recognition of specific RNA sequences.

Second, we have found that TLS binds to 5(1-1) and to a related sequence, 3(1-1), both sites located 5'end of pncRNA-D1 containing GGUG consensus of TLS [56, 60]. Dissociation constant (Kd) of TLS to 5(1-1) is 2 x 10^{-5}M, while Kd of 3(1-1) is 3.5 x 10^{-6} M [60]. Our experiments showed that hnRNPH1 binds 5(1-1) stronger than 3(1-1) [59]. Then, these

two RBPs are classified into a high affinity group. hnRNPH1 and TLS share the similar tendency toward RNA bindings. These two RBPs only bind poly G (100mers), but no binding to random RNA oligo nucleotides (from 5 to 12mer) and also to GUAC RNA oligomers (10mer). So far reported, poly G alone out of the rest of the three polymers is supposed to form a structure, G quadruplex which might generate specific binding surfaces to RBPs [89-91]. hnRNPH1 and TLS specifically recognize G quadruplex structures [92, 93]. Furthermore, TLS has been reported to bind more than 8000 species of RNA, most of them are mRNA, meaning that it could have also low affinity binding sites to wide ranges of affinity of RNA.

These findings and the present data indicate that binding profiles of RNAs are determined with four factors, their length, base composition, sequence, and structure. Affinity of RBPs to RNAs is another layer of generating specific profile of RNA bindings. The different profile of RBPs to RNAs might represent their physiological missions in living cells [94-96]. Analyzing biological consequence of functions of RBPs gives rise to more profound understanding of RNA biology.

Our mapping experiment indicated that the fragment 1 of TLS exhibited marginal bindings to RNAs with low affinity. This wobbly binding of the fragment 1 should be based upon its IDR. It has been reported that IDRs function as binding interfaces to RNAs with specific properties and exert biological activities through their RNA binding abilities [47, 48, 50, 51]. Actually, IDR-endowed TLS has been shown to bind to more than 8000 species of RNA molecules, suggesting that RBPs with IDRs could accommodate large numbers of RNAs [97]. These data imply that IDR could present flexible and fluctuated interfaces to divergent species of RNAs. For this recognition of divergent structures of RNAs, the IDR should suitable interfaces because of its flexibility. It has been reported that most of canonical RBPs contain IDR, although molecular mechanism of recognition by the IDRs remains to be cleared [49, 98-100].

Furthermore, extensive works have indicated a numbers of non-canonical RBP whose RNA-binding motif has not been identified. Some sorts of enzymes has RNA binding activity and also transcription factor estrogen receptor has been re-discovered as RBPs [43, 44]. For complete figure of RBP biology, more searches for unappreciated species of RBPs should be required.

5. Conclusion

Affinity chromatography of GST-TLS isolates the RNA fraction recognized by TLS from HeLa cell total RNA. The RNA has been processed for screening with the human long noncoding RNA microarray, providing the lncRNA sequences specific to TLS. Binding assays show that the lncRNAs tested specifically bind to TLS. Four lncRNAs has been found to exert inhibitory effect on the phase separation-induced precipitation. Mapping experiments using the four fragments across TLS indicate that the C-terminus of TLS is interaction domain for lncRNAs, while the N-terminus bearing IDR exhibits the marginal binding to lncRNAs and also the inhibitory activity to the TLS binding of lncRNAs. These data demonstrate that the affinity chromatography of GST-TLS successfully performs for identification of the functional lncRNAs specific to TLS. The establishment of GST-TLS affinity chromatography to isolate specific RNA has profound implication. As a GST-fusion construct, other RBPs, for instance, hnRNPA/B could be inserted into this framework to give GST-hnRNPA/B. This construct should be useful tool to isolate RNA specific to hnRNPA/B. Similar experiments are designed for divergent RNA-binding proteins. This application will open a door to next chapter of RNA biology.

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