

Affinity Profiles Categorize RNA-Binding Proteins into Distinctive Groups

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Abstract: Recently, we have established a novel assay to detect interaction of RNA-binding proteins (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 oligos). 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.

Keywords: TLS, FUS, Random RNA Oligos, hnRNPH1, hnRNPU, hnRNPUL2, DDX21, hnRNPA

1. Introduction

Physiology of RNA has been generating more impact on modern biomedical sciences extending the previous views for the RNA metabolism that has been just oriented to steps of the process of the Central Dogma [1-10]. The intensive investigations on transcriptomes of the human genome-derived RNAs have uncovered enormous numbers of RNAs transcribed from the noncoding regions [11-13]. Majority of the unidentified transcripts are found to be unannotated long noncoding RNAs (lncRNAs) of which biological activities remain largely unidentified [14], although their activities are supposed to be mediated by

RNA-binding proteins. RNA binding is much more intricate mechanism than DNA binding. RBPs are characterized by proficiency of RNA perception and possess divergent capability in living cells. Elucidation of mechanism of generating specificity of RBPs will give rise to developing more efficient technology to dissect them. Depending on technology in detection, experimental data indicate distinctive propensity.

RBPs form ribonucleoprotein (RNP) complexes primarily involving in regulation of gene expressions. RBPs exert their functions by interacting with RNAs via previously identified

RNA binding structures of RBPs [15], such as RNA recognition motif (RRM) [16], and DEAD box helicase domain [17]. However, prompt progress in exploring the structures of large RNP complexes like ribosome [18-20] and spliceosome [21, 22] indicate unprecedented interactions between proteins and RNAs without canonical RNA binding domains. These observations suggest that unconventional RNA bindings are more general events than previously perceived.

Most of RBPs possess intrinsically disordered regions (IDRs) or low complexity domains (LCDs) in their amino acid sequences [23]. These domains have been found to play critical roles in targeting RBPs into cellular bodies such as RNA granules and paraspeckles [24-26]. Their dynamic composition and amorphous structure still remain unsolved. Some RBPs have KH-rich peptide regions located in LCDs. These motifs possibly interact with RNA [27]. These observations indicate that LCD should be a target for RNA, suggesting that previously unidentified amino acid sequence functions as RNA binding motif.

There should be possible unexpected RBPs with novel RNA binding motif consisting of LCD in living cells. Most certain criterion for RBPs is just to detect binding ability to RNA. Recently, we have established a novel assay to detect binding of RNA-binding proteins, using biotinylated RNA oligos to capture RBPs with Western blot of specific antibodies against RBPs [28, 29]. The assay more confidently detects RNA binding than the traditional gel shift assay. For extensive analysis of the RNA bindings in RBPs, we start to categorize the RNA bindings according to their affinity to RNAs using our assay system. This sort of categorizing will give us an expedient technology to dissect RNA binding and be led to fruitful direction of the future investigation of RBPs.

2. Materials and Methods

2.1. Antibodies and Reagents

Mouse anti-TLS/FUS antibody (611385, Lot no.2209827) was purchased from BD Biosciences. Rabbit anti-hnRNPU antibody (ab20666, Lot no.825937) was purchased from Abcam. Rabbit anti-hnRNPUL2 antibody (A304-619A-T, A304-619A-T-1) was purchased from BETHYL. Rabbit anti-DDX21 antibody (ab126968, Lot no. GR166655-4) was purchased from Abcam. Rabbit anti-hnRNPAB antibody (AP5522a, Lot no. SA100907AO) was purchased from ABGENT. Rabbit anti-mouse HRP-conjugated IgG (P0161, 20017456) was purchased from Dako. Goat anti-rabbit HRP-conjugated IgG (7074S, 25) was purchased from Cell signaling. The RNase inhibitor (SIN-201) was purchased from Toyobo Life Science. HeLa cell NE was prepared as

previously described protocol [29-31].

2.2. RNA Binding Assay

The RNA binding assays were performed previously described [28]. Briefly, it has shown below. Dynabeads-M280 (Thermo Fisher) was washed with PBS containing 0.02% Tween 20. Ten pmol of biotinylated RNA oligos or RNAs was added to the beads and incubated for 15 min at room temperature with rotation. Subsequently, 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 applied to SDS-poly acrylamide gel following for stain with the SimplyBlue™ SafeStain (Thermo Fisher) or Western blotting analyses.

2.3. Protein Analysis

SDS-polyacrylamide gel electrophoresis was performed with 10% gels following coomassie brilliant blue staining [29]. Western blotting was done with anti-TLS monoclonal antibody from the BD bioscience, 611385 with the dilution ratio 1:2000 using standard protocol shown previously [29].

3. Results

Recently, we have established simple and solid technique of detection of the binding of RBPs, using biotinylated RNA oligos to precipitate RBPs by detection with Western blot of the antibodies against RBPs. This RBP assay more firmly detects RNA binding compared to gel shift assay with ³²P-labeled RNA oligos.

Using completely randomized RNA oligos from 5mer through 12mer, their binding was examined with proteins of HeLa cell NE (Figure 1A). These RNA oligos are usually employed to be negative control of typical RNA binding experiment. CBB-based protein staining did not detect any strong signal although some faint bands appeared (Figure 1A). Unexpectedly, the Western blot analysis of antibodies against typical six RBPs showed four RBPs bound with the random RNA oligos. All RNA oligos from 5mer to 12mer RNA were bound with hnRNPUL2, while hnRNPU bound RNA oligos from 6mer to 12mer (Figure 1B). These two RBPs exert minimal specificity so far tested. hnRNPAB bound to RNA oligos from 7mer to 11mer while DDX21 bound to RNA oligos from 9mer to 12mer (Figure 1C). hnRNPAB alone shows preferred binding to specific length of RNA oligos. Any TLS and hnRNPH1 signal was detected in these random RNA oligo samples (data not shown).

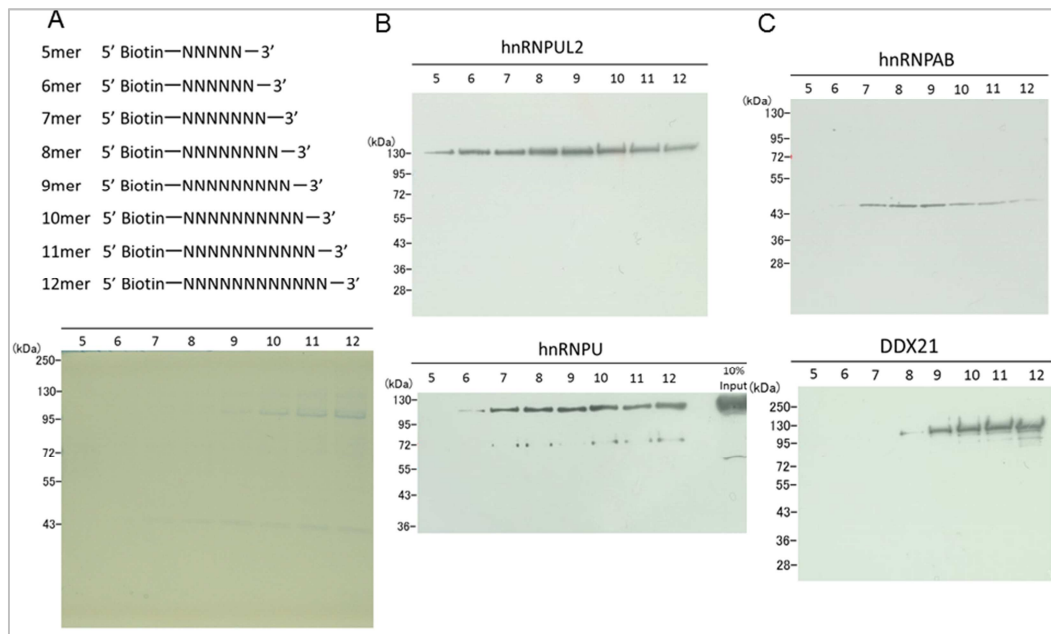


Figure 1. Low specificity binding of RNA binding proteins.

(A) Upper panel: Randomized RNA oligos are examined. Lower panel: Fifty µl of NE were tested for binding with the depicted randomized RNA oligos, and detected with CBB-staining. “5” represents “5mer”, and so on.

(B) Low specificity binding to random RNA oligos.

hnRNPUL2 binds random RNA oligos that are longer than 5mers. 10% input was at the similar intensity as hnRNPU.

hnRNPU binds random RNA oligos that are larger than 7mers.

(C) Moderate specificity binding to RNA oligos.

hnRNPAB and DDX21 bind RNA oligos with relatively narrow ranges of their length. 10% inputs were at the similar intensity as hnRNPU in Figure 1B.

Next, base specificity of RBPs was examined using sets of oligos of RNA fixed their base compositions at G (Guanine), A (Adenine), U (Uracil), and C (Cytosine) (GAUC RNA oligos). Oligomers, from 5mer, to 11mer of G RNA oligos were bound with the most prominent protein bands (Figure 2A). The same sets of A, U, and C of RNA oligos were bound to less numbers of proteins (Figure 2A). Based on scanning the oligos from 5mer to 11mer, we set the 10mer as a representative length of the RNA oligo sets. Western blot showed that hnRNPUL2 and hnRNPU bind all four oligos of GAUC at the 10mer length (Figure 2B). DDX21 bound G and U at 10mer while, hnRNPAB binds only a U at 10mer, indicating base preference on these two RBPs (Figure 2C). TLS and hnRNPH1 have no binding with these oligomers of GAUC (data not shown).

For further RNA binding assay, we employed poly G, poly A, poly U, and poly C which have approximately 100mer length of RNA to evaluate impact of the length of RNA. CBB staining of the fractions bound with these four polymers of RNA indicated that much more bands than oligomers in Figure 2 (Figure 3A). Especially, poly G and poly U were shown to have comparable numbers of bands, while poly A and poly C were found to have relatively smaller numbers of bands (Figure 3A). Western blot revealed that hnRNPU binds well to poly G, A, and, U, but less to poly C (Figure 3B). hnRNPUL2 and DDX21 indicated a similar binding profile to all four polymer RNAs with less intensity than hnRNPU (Figure 3B). hnRNPAB has preferred binding to poly G, A,

and U. but no binding to poly C (Figure 3C). Interestingly, hnRNPH1 and TLS were exclusively bound to poly G (Figure 3D).

Previously, we have identified specific RNA binding site for TLS in a long noncoding RNA, pncRNA-D, which is transcribed from the promoter for cyclin D1 gene [30]. The TLS-target RNA sequence at 32 to 44 of pncRNA-D is designated as 5'(1-1), and the adjacent sequence 3'(1-1) that has less binding to TLS (Figure 4A), were tested to see binding profile for RBPs detected in these experiments with negative controls of 6mer and 9mer of the random RNA oligos. Binding experiments using sets of RNA oligos indicated that hnRNPUL2 bind all RNA oligos, suggesting its low specificity (Figure 4B). hnRNPU binds all RNA oligos except 6mer of the randomized RNA oligos (Figure 4C). DDX21 has the similar profile to the hnRNPU, while hnRNPAB displays similar profile except less binding to 3'(1-1) (Figure 4C). hnRNPH1 and TLS has preferred bind to specific binding site, 5'(1-1) (Figure 4D).

These data demonstrate that RNA binding is regulated with three factors, length, base compositions, and sequence of RNA (Figure 5AB). Furthermore, hnRNPU and hnRNPUL2 have low specificity binding to RNA. DDX21 and hnRNPAB have mild specificity, while TLS and hnRNPH1 exert high specificity binding. These different propensities of each RBP are thought to represent each specific biological role in living cells.

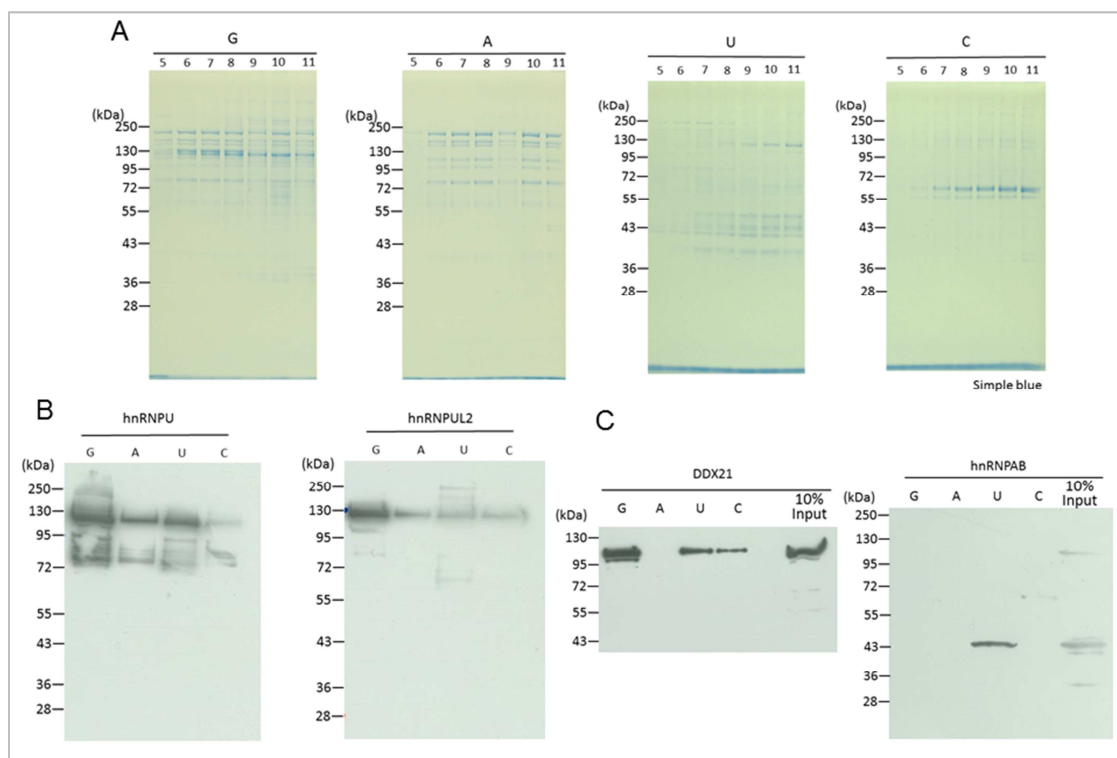


Figure 2. Binding profile of RNA oligos of GAUC from 5mers through 11mers.

(A) Distinct profiles are detected in each RNA oligo with each specific base composition (GAUC), and detected with CBB-staining.

Fifty μ l of NE were used in Figure 2. “5” represents “5mer”, and so on.

(B) hnRNPU and hnRNPU2 bind RNA oligos with divergent base compositions. Length of oligos is fixed at 10mer. 10% inputs were similar intensity as DDX21 at Figure 2C (data not shown).

(C) DDX21 and hnRNPA2 bind to RNA oligos with specific base compositions. Length of oligos is fixed at 10mer.

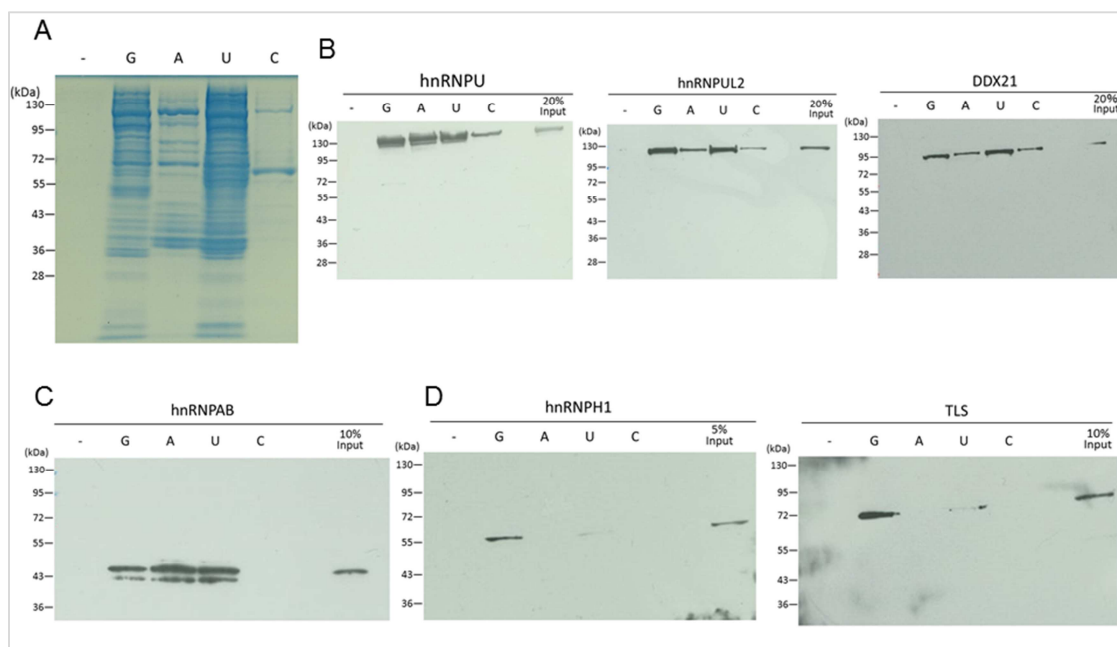


Figure 3. Bindings to Poly G, Poly A, Poly U, and Poly C RNA oligos (poly GAUC RNA oligos).

(A) Proteins are bound to poly GAUC RNA oligos, and detected with CBB-staining. Five μ l of NE were used for experiments in Figure 3.

(B) hnRNPU, hnRNPU2, and DDX21 bind to all four Poly GAUC RNA oligos.

(C) hnRNPA2 does not bind to poly C RNA oligos.

(D) hnRNPH1 and TLS have preferred binding to poly G RNA oligos.

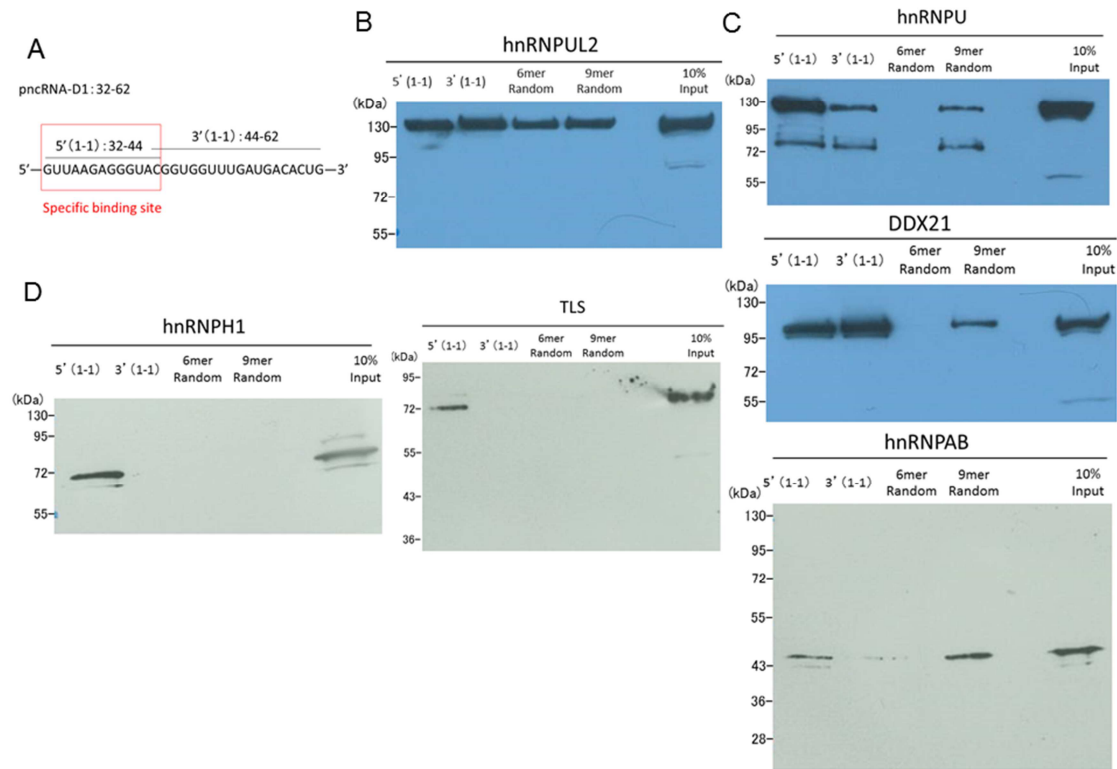


Figure 4. Specific and non-specific bindings of RBPs.

(A) pncRNA-D: 5'(1-1) and 3'(1-1) are depicted.
(B) hnRNPUL2 binds all four binding sites. Fifty µls of NE were used in Figure 4.
(C) hnRNP, DDX21, and hnRNPAB have similar binding profile.
(D) hnRNPH1 and TLS bind only high specific binding site, 5'(1-1).

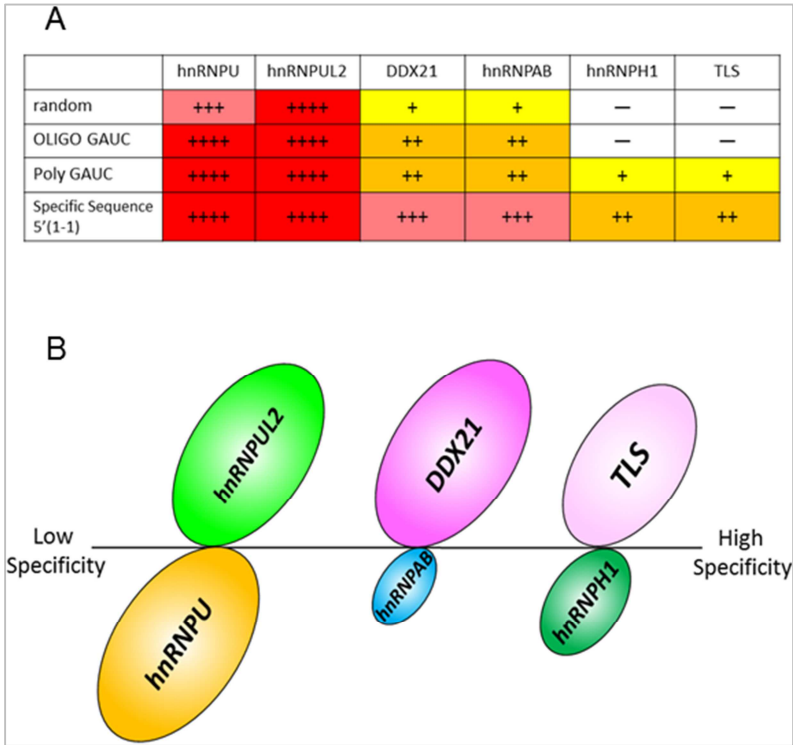


Figure 5. Length, base composition, and sequence of RNA determine affinity of RBPs.

(A) Summary of the binding assay.
(B) Visual summary of the propensity.

4. Discussion

RNA binding is more complex task than DNA binding. DNA-binding protein is mostly transcription factor that recognizes specific responsive elements typically composed of consensus motifs with several DNA oligomers and regulates (in many cases, activates) gene expression [32-35]. It is crucial for DNA-binding transcription factor to precisely recognize specific binding sites. These molecules execute stringent regulations on transcription upon specific binding to response elements on the promoter or the enhancer region [36-38]. Therefore, high affinity of the DNA-binding proteins requires for their proper function.

Contrarily, RBPs are a versatile molecule and have more divergent functions like RNA splicing, modulating RNA metabolism, builder for cellular structures [39-42]. In some cases, RBPs just connect RNAs to proteins to form cellular structures [24, 42, 43]. Versatility of RBP functions endows wide ranges of affinity of their binding capacity [44-46]. RBPs are characterized by proficiency of RNA perception and possess divergent capability in living cells.

Our binding assays have effectively demonstrated that RBPs have distinctive subgroups classified with criteria of their binding affinity [28, 29]. The experiments using random RNA oligos unpredictably indicated that hnRNPUL2 and hnRNP1 bind to these RNA oligos which are typically used for negative control of RNA binding assays [28, 29, 47]. It has been reported that hnRNPUL2 is a part of RNA polymerase II general transcription factor complex [48] and also works on DNA repair processes [49, 50], indicating roles in forming nuclear structures. These processes just require grasping RNA as a chemical compound, but do not need to exert specific perception of RNA sequences. hnRNP1 also works as backbone of various nuclear and cellular structures [43]. Secondly, hnRNPAB and DDX21 are found to have mild affinity to specific RNAs equipped with distinctive base compositions even they are relatively at short length. These two RBPs play each specific biological role in programs in live cells [51-53]. At the third category, we found hnRNPH1 and TLS which recognize a binding site, 5'(1-1), located in pncRNA-D transcribed from the cyclin D1 gene promoter. Intriguingly, 5'(1-1) has been identified as specific site for TLS [28-30]. Our experiments demonstrated that hnRNPH1 also binds the site specific to TLS. hnRNPH1 and TLS share the same tendency to binding RNA. These two RBPs only bind poly G, but no binding to random RNA oligos and to GAUC RNA oligomers. So far these RNAs tested, poly G alone is supposed to form a structure, G quadruplex which might generate specific bindings of RBPs [54-56]. hnRNPH1 and TLS specifically recognize G quadruplex structures [57, 58].

These data demonstrate that RNA binding is regulated with three factors, length, base compositions and sequence of RNAs. Furthermore, hnRNP1 and hnRNPUL2 have low specificity binding to RNAs. DDX21 and hnRNPAB have mild specificity to RNAs, while TLS and hnRNPH1 exert

high specific binding. These different tendencies of each RBP are supposed to represent each specific biological role in living cells [51, 59, 60].

Molecular mechanisms exerting these distinct affinities of RBPs are next issues to be investigated for RNA biology. One of clues for breakthrough is LCDs of these RBPs. Function of LCDs just has been recognized for their importance in cellular and molecular contexts. LCDs might possess their specific properties in their distinct molecular environments in cells. Solution of the structures of RBPs could provide more profound understanding of their functions in biological programs.

5. Conclusion

In this manuscript, we have demonstrated that RNA binding is regulated with three factors, length, base composition, and sequence. Furthermore, hnRNP1 and hnRNPUL2 have low specificity binding to RNAs. DDX21 and hnRNPAB has mild specificity to RNAs, while TLS and hnRNPH1 exert high specific binding. These different propensities in bindings of RBPs could represent specific biological roles in living cells.

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