

Chapter 17

Methods to Identify and Validate WT1–RNA Interaction

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Abstract

Tumor suppressor protein, Wt1 is a transcription factor that binds to DNA sequence similar to the Early Growth Response gene, EGR1 consensus binding sequence. Biophysical and biochemical validations have shown that the zinc fingers of Wt1 are capable of binding to both DNA and RNA albeit with different binding affinities which potentially is also isoform specific. SELEX based identification of the RNA binding motifs led to the identification of motifs which could not be translated into the in vivo context. With the advent of recent technologies that allow cross-linking of RNA and protein and high throughput sequencing techniques, it is now possible to analyze the in vivo RNA binding interactome of Wt1. This chapter outlines the initial studies that were aimed at addressing the Wt1 RNA interactome and also provides a detailed overview of some of the recent techniques used.

Key words RNA, NGS, IP, CLIP

1 Introduction

1.1 *Wt1 Binds to DNA and RNA*

Wilms' tumor suppressor protein, Wt1 is a transcription factor with four zinc fingers in the C-terminus. Several isoforms of this protein exist because of features like alternative start sites, RNA editing, and splicing. Of these different isoforms, the evolutionarily conserved isoforms are two, that differ from each other by the presence or absence of three amino acids KTS. An alternative splice site results in the insertion of these three amino acids between exon 9 and 10, referred to as the +KTS isoform (reviewed in ref. 1). The –KTS isoform binds to DNA sequences similar to the EGR1 consensus site. Due to the insertion of the amino acids, the +KTS isoform is structurally impeded in its DNA binding and hence has less affinity towards DNA. However, colocalization studies have shown the predominant +KTS isoform to be present in spliceosomes implicating an association with RNA. Biochemical validations have indeed shown that the +KTS isoform binds to RNA with greater affinity than the –KTS isoform which are summarized below.

1.2 *In Vitro and In Vivo RNA Interaction*

One of the compelling pieces of evidence of Wt1 and RNA interaction was the observation wherein the mouse +KTS of Wt1 in *Xenopus* oocytes was shown to be preferentially enriched on nascent transcripts [2]. It also led to the identification that zinc finger I is more important for the RNA binding ability because the absence led to the failure to sediment with the RNP components. Subsequent experiments also revealed that Wt1 associates with mRNP particles both in fetal as well as cancer cell lines [3]. There have also been data to show the localization of the Wt1 isoforms with snRNPs. It is also of interest to mention that it has been observed that the Wt1 consensus sequence is present in the 5' UTR of several candidate genes. A putative RNA recognition motif (RRM) was identified by structural modeling at the N-terminus but no functional study has shown its importance. There have been attempts to study this interaction using SELEX based approaches using the zinc fingers of Wt1. Biophysical binding studies have shown that the zinc fingers have RNA binding ability [4]. Some structural investigations have been done with the RNA aptamers, however their functional validation has not been done yet. Other experiments using IP followed by enrichment for RNAs has led to the identification of a structural protein associated network [5]. Based on all the above findings, it is clear that Wt1 and RNA interplay is involved in several steps of RNA metabolism with functional implications in different cellular events.

2 Materials

2.1 *Cell Lines*

Mesonephric cell line M15 and epicardial cell line, CoMEEC were used for the experiments.

2.2 *Reagents*

3' linker miRCat-33 linker (IDT) AppTGGAATTCTCGGG TGCCAAG/ddC/5' linkers (barcode marked red)L5Aa invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrArArGrC-OH L5Ab invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArUrUrArGrC-OHL5Ac invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrCrGrCrArGrC-OHL5Bb invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrUrGrArGrC-OHL5Bc invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrArCrUrArGrC-OHL5Bd invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrUrCrUrCrUrArGrC-OHL5Ca invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrCrUrArGrC-OHL5Cb invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrGrArGrC-OHL5Cc invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrArCrTrCrArGrC-OHL5Cd invddT-ACACrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrNrNrNrGrArCrTrArGrC-Barcode sequence together with short experiment title in each case is used in the sample name for the

case of data processing NNNTAAGC for L5AaNNNATTAGC for L5AbNNNGCGCAGC for L5AcNNNCGCTTAGC for L5AdNNNGTGAGC for L5BbNNNCACTAGC for L5BcNNNTCTCTAGC for L5BdNNNCTAGC for L5CaNNNTGGA GCforL5CbNNNACTCAGCforL5CcNNNGACTTAGCforL5Cd PCR primers miRCat-33 primer (IDT) CCTTGGCACCCGA GAATT primer for RLibrary Amplification Primers:PE_miRCat_PCR CAAGCAGAAGACGGCATAACGAGATCGGTCTCGGCAT TCCTGGCCTTGGCACCCGAGAATTCCP5 AATGATACGGC G A C C A C C G A G A T C T A C A C T C T T T C C C T A C ACGACGCTCTTCCGATCTOligos for EMSAs are ordered from SIGMA

2.3 Equipment

1. UV cross-linking is done in a Stratagene cross-linker.
2. Centrifugation is performed in an Eppendorf microfuge.

2.4 Buffer Composition

1. Binding buffer 10×: 100 mM Tris-HCl pH 7.9, 500 mM KCl, 10 mM DTT, 10 mM EDTA.
2. Lysis buffer: 50 mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate (before use add protease inhibitors and 40 U RNasin).
3. Wash buffer: 50 mM HEPES, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate (before use add 40 U RNasin per ml buffer).
4. CLASH buffers:
 - (a) Lysis buffer: (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.4% NP-40, 2 mM MgCl₂, 1 mM DTT, protease inhibitors (Roche, cOmplete, EDTA-free), RNase Inhibitor (Promega)).
 - (b) PBS-WB buffer: (PBS, +150 mM NaCl, 2 mM MgCl₂, 0.4% NP-40).
 - (c) EB (elution buffer: NuPAGE protein sample buffer plus 20 mM Tris-HCl, 1% SDS, 100 mM ME (β-mercaptoethanol)).
 - (d) HS-PBS-WB: (PBS, 0.3 M NaCl, 2 mM MgCl₂, 0.4% NP-40).
 - (e) UB: (20 mM Tris-HCl pH 7.4, 2 M UREA, 0.15 M NaCl, 0.4% NP-40).
 - (f) PNK buffer: (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.5% NP-40, 50 mM NaCl).
 - (g) Proteinase K buffer: (50 mM Tris-HCl pH 7.8, 50 mM NaCl, 0.4% NP-40, 0.5% SDS, 5 mM EDTA).
5. RNP Lysis buffer: (20 mM Tris-HCl, pH 8, 140 mM KCl, 1.5 mM MgCl₂, 0.5% NP-40, 0.5 mM DTT).
6. Interaction buffer: 20 mM Tris-HCl, pH 8.0, 140 mM KCl, 4 mM MgCl₂, 0.75 mM DTT, 0.1% NP-40, 0.1 u/μl *E. coli* tRNA.

3 Methods

3.1 RNA Immunoprecipitation (RIP)

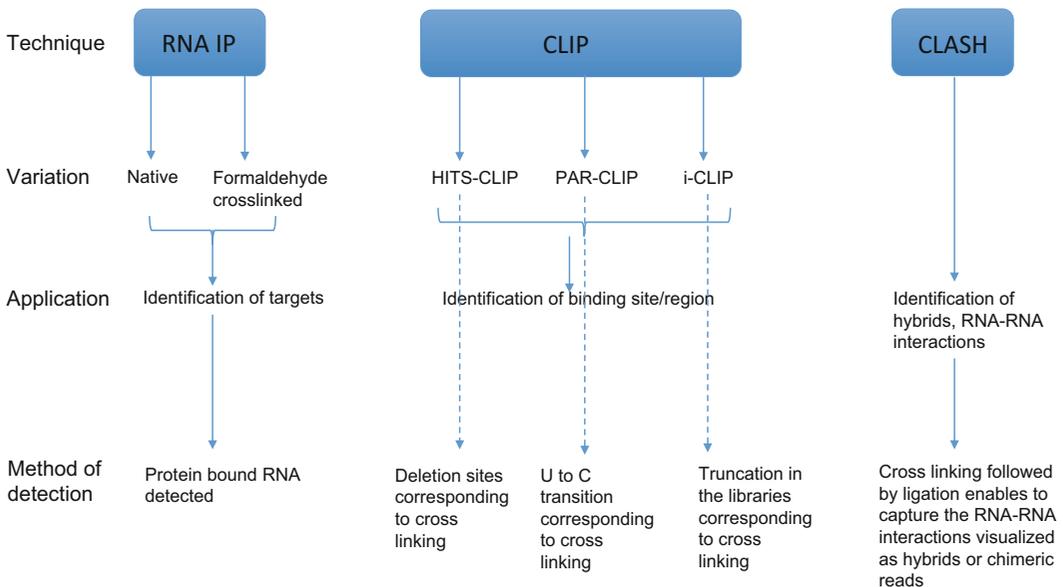
This protocol involves three distinct stages: (1) cross-linking, (2) immunoprecipitation, and (3) recovery of RNA.

3.1.1 Cross-Linking

This is variable based on the experimental strategy and can either be native when there is no cross-linking involved or formaldehyde cross-linking which leads to more general cross-linking or UV cross-linking which involves capturing interactions at distinct sites [6]. The UV cross-linking strategy can be further modified such that individual nucleotides can be cross-linked and identified. A representation of these different strategies is represented in Fig. 1.

(a) Formaldehyde Cross-Linking:

1. Confluent cells are washed with PBS (10 ml per 15 cm dish) after aspirating media.
2. The cells are cross-linked using 0.25 ml of 38% formaldehyde directly on the cells and incubated on shaker platform for 10 min at room temperature.
3. The cross-linking is neutralized by the addition of 1 ml of 2 M glycine and incubated on shaker platform for 10 min at room temperature.
4. The above solution is removed and the cells are scraped in 10 ml of ice-cold PBS, and centrifuged at $76 \times g$ for 10 min.



Different Approaches to Study RNA interactome

Fig. 1 Different approaches to study RNA interactome

5. The supernatant is discarded and the pellet is transferred to a 1.5 ml microfuge tube using 1 ml PBS.

(b) *UV Cross-Linking:*

1. Confluent cells are washed with PBS (10 ml per 15 cm dish) after aspirating media.
2. Cells are cross-linked using the cross-linker at (1200 μ joules/s) using the optimal cross-link function. (see Notes for further information)
3. The cells are scraped in minimal volume of PBS (1–2 ml) and centrifuged at $81.6 \times g$ for 10 min.
4. The pellet is washed with 5 ml PBS and processed as in (a), **step 5**.

(c) *Native Cross-Linking:*

1. Confluent cells are washed with PBS (10 ml per 15 cm dish) after aspirating media.
2. Cells are scraped in minimal volume of PBS (1–2 ml) and centrifuged at $81.6 \times g$ for 10 min.
3. The pellet is washed with 5 ml PBS and processed as in (a), **step 5**.

3.1.2 *RIP*

1. Cell pellet is resuspended in ice-cold PBS, and centrifuged at $117.5 \times g$ for 5 min at 4 °C.
2. Cell pellet is resuspended in lysis buffer (0.4 ml), and kept on ice for 5 min.
3. Sonicated at 5 sec on and 5 sec off pulse, six times.
4. To the sonicated extract, the following components are added, 25 mM $MgCl_2$, 5 mM $CaCl_2$, RNasin (3 μ l of 40u/ μ l stock), RNase free DNaseI (6 μ l of 1500 k Ustock), and incubated at 37 °C for 15 min.
5. The above reaction stopped by the addition of 20 mM EDTA and samples are centrifuged at $11752 \times g$ for 5 min.

Bead Preparation

1. Magnetic beads (Pierce A/G) resuspended by gentle shaking and pipetting.
2. Tubes labeled as per the IP conditions.
3. 25 μ l of the magnetic beads is added to each of the tubes, 0.25 ml of the RIP wash buffer is added to each tube and vortexed. Tubes placed on the magnetic rack. After solution has separated, the supernatant is removed.
4. The above step repeated once again to wash the beads.

IP

1. Tubes are removed from the magnetic stand; 0.1 ml of RIP lysis buffer added to the tube, 2 μ g of WT1 antibody added to the tube and incubated at RT for 30 min on end to end rotor.

2. After incubation with the antibody, tubes are centrifuged at $734 \times g$ for 2 min, placed on magnetic stand and supernatant discarded.
3. 0.5 ml of RIP lysis buffer is added to the beads, and vortexed. Placed on magnetic stand and supernatant discarded. The above step repeated twice.
4. IP done with RIP lysis buffer: 860 μ l + 0.5 M EDTA (35 μ l) and RNase inhibitor (5 μ l).
5. 0.6 ml of above RIP buffer added to each tube and 0.4 ml of lysate added to each tube (adjusted according to the lysate volume).
6. 10% of the above lysates is stored to be included as input sample in the Subheading 3.1.3.
7. IP was done overnight on end to end rotor at 4 °C.

Washes

1. Post IP the tubes are placed on the magnetic stand to facilitate separation.
2. 0.5 ml of RIP lysis buffer is used for wash, six times (each time, buffer added, vortexed and placed on magnetic stand for aiding in separation), followed by washes with wash buffer for three times.

3.1.3 RNA Precipitation

1. To the magnetic beads, the following is added, 150 μ l of proteinase K buffer, 117 μ l of RIP wash buffer +15 μ l of 10% SDS +18 μ l of 10 mg/ml proteinase K.
2. Input samples from **step 6**, Subheading 3.1.2.2 (10 μ l) + 107 μ l of RIP wash buffer +15 μ l of 10% SDS +18 μ l of proteinase K and incubated at 55 °C for 30 min, on thermomixer.
3. Reverse cross-linking is performed at 65 °C for 1 h, on a thermomixer.
4. Samples are centrifuged briefly to collect all the contents and placed on magnetic rack for separation. Supernatant transferred to fresh tube.
5. 0.25 ml of RIP wash buffer is added to each tube, and 0.4 ml of acidified phenol–chloroform is added, then vortexed for 15 s, centrifuged at $13792 \times g$ for 10 min.
6. Supernatant transferred to fresh tube, and to each of the tubes, the following is added, 40 μ l of sodium acetate +1 μ l of glycogen +850 μ l of absolute ethanol. Mixed well and left at –80 for minimum 1 h.
7. Samples centrifuged at $13792 \times g$ for 30 min at 4 °C. Pellet washed with 70% ethanol and air-dried.
8. Sample resuspended in 20 μ l of nuclease free water. Vortexed mildly and concentration estimated by bioanalyzer.

3.1.4 cDNA Library Preparation

Double stranded cDNA synthesis of RNA-IP samples.

First Strand cDNA Synthesis (Done in a Thermal Cycler)

1. IP RNA—9.5 μ l.
2. Random Primers—1 μ l (200 pmol in total).
3. Water—to a total volume of 10.5 μ l.

Incubate at 70 °C for 10 min, followed by incubation on ice. To the above reaction, the following components are added.

1. RT buffer 5X—4 μ l.
2. DTT—2 μ l.
3. AMV 25u/ μ l—1 μ l.
4. Protector RNase inhibitor—0.5 μ l.
5. dNTP mix—2 μ l.

Incubate at 42 °C for 60 min, followed by incubation on ice to terminate the reaction.

Second Strand Synthesis (Done in a Thermal Cycler)

1. cDNA from above step—20 μ l.
2. Second strand buffer—15 μ l.
3. dNTP mix—0.75 μ l.
4. Second strand enzyme mix—3.25 μ l.
5. Water—36 μ l.

(a) Total reaction volume of 75 μ l.

(b) Incubate at 16 °C for 2 h, followed by addition of 10 μ l of T4 DNA polymerase.

(c) Incubate at 16 °C for 5 min.

(d) Terminate reaction by addition of 8.5 μ l of 0.2 M EDTA, pH 8.0.

Digestion of RNA

1. 0.75 μ l of RNase I is added to the above reaction mix.
2. Incubate at 37 °C for 30 min.
3. 2.5 μ l of proteinase K is added to the reaction.
4. Incubate at 37 °C for 30 min.

Cleaning of dsDNA

1. Double stranded cDNA samples are subjected to phenol–chloroform extraction.
2. Supernatant is precipitated using 0.6 volume of 5 M ammonium acetate and 2.5 volumes of absolute ethanol.
3. Incubate at –80 for minimum 1 h
4. Centrifuge at $13792 \times g$ for 20 min in a mini centrifuge.
5. Pellet is washed with ethanol.
6. Pellet is air-dried and resuspended in 13 μ l of water; 2 μ l is used for bioanalyzer.

3.2 CLASH

CLASH refers to the Cross-linking and Association of Hybrids [7], a modified version of CLASH has been carried out to obtain endogenous interactions.

3.2.1 UV Cross-Linking

Mouse kidney mesonephric M15 cells are grown to 90% confluency and are UV cross-linked on ice with $\lambda = 254$ nm in Stratalinker 1800, at 400 mJ/cm².

3.2.2 Cell Lysis and Wt1 Bound Complexes Purification

1. M15 cells are lysed by addition of ice-cold CLASH lysis buffer. 10 μ l of RQ1DNase is added and the samples are mixed by pipetting and incubated for 10 min at room temperature.
2. Lysates are centrifuged in Eppendorf mini centrifuge at 14,000 rpm and 4 °C for 10 min and supernatant is collected.
3. Protein A beads conjugated with IgG and anti-WT1 C19 antibody are washed with PBS/0.4% NP40.
4. Cell lysates are incubated with IgG- or C19-beads for 60 min at 4 °C. Supernatant is discarded and the recovered beads are washed twice with PBS-WB buffer and once in 1x PBS with 2 mM MgCl₂.
5. RNase treatment: RNP complexes bound to the beads are treated with 0.5 unit RNaseA+T1 mix (RNase-IT, Stratagene) in 100 μ l PBS, 2 mM MgCl₂ buffer for 10 min at 20 °C. To remove indirect RNA and protein binding from WT1-RNA complexes the beads are washed again twice with PBS-WB buffer.
6. Beads were re-suspended in 100 μ l of PBS, followed by 100 μ l of 1% formalin in PBS and complexes cross linked for 1 min at room temperature. 50 μ l of 1M Tris-HCl pH 7.4 and 50 μ l of 2M Glycine (neutral pH) is added and incubated 5 min on ice, then washed twice with 4M UREA/PBS/0.4% NP40 buffer, twice with PBS-NP40 buffer, thrice with 1x PNK buffer.

3.2.3 Linkers' Ligation and RNA-Protein Complexes Recovery

1. To remove unwanted 3' phosphate groups from bound RNA fragments the complexes are treated with TSAP phosphatase in the phosphatase buffer for 40 min at room temperature.
2. To inactivate the enzyme, the beads are washed once with UB and 3 times with PNK buffer.
3. The complexes on the beads are incubated with 40 units T4 Polynucleotide kinase, first with P32 labeled ATP for 45 min, then for 20 min with 1 mM cold ATP, in PNK buffer with RNase inhibitors at room temperature. The beads are then washed as before once with UB and 4 times with PNK buffer.
4. WT1-bound RNA molecules are ligated together and with 3' linker (1 μ M miRCat-33), overnight using 40 units of T4 RNA ligase 1 in PNK buffer with RNase inhibitors at 16 °C. The next day, the beads are washed as before once with UB and four times with PNK buffer.

5. 40 units of RNA ligase 1, barcoded 5' linkers (final conc. 5 μ M; one for each sample) are ligated in RNA ligase 1 buffer with 1 mM ATP for 3–6 h at 20 °C. The beads are washed as before, once with UB and four times with PNK buffer.
6. The samples are eluted in NuPAGE protein sample buffer plus 20 mM Tris–HCl, 1 % SDS, 100 mM ME (β -mercaptoethanol). Boiled for 2 minutes at 98 °C. The samples are centrifuged for a minute to recover the supernatant with RNA–protein complexes.

3.2.4 SDS-PAGE and Transfer

1. Protein–RNA complexes in NuPAGE SB plus SDS, ME are resolved on a 4–12 % bis-tris NuPAGE gel in NuPAGE SDS MOPS running buffer followed by transfer to nitrocellulose membrane in NuPage transfer buffer with 10 % methanol for 1 h at 100 V.
2. Depending on the strength of the signal the membrane is exposed on film for 1 h or overnight at –70 °C.
3. The developed film is aligned with the membrane and the radioactive bands corresponding to the WT1–RNA complexes are excised.

3.2.5 Proteinase K Treatment and RNA Isolation

1. The excised bands are incubated with 150 μ g of Proteinase K (Roche) and proteinase K buffer for 2 h at 55 °C.
2. The RNA is extracted with phenol–chloroform–isoamyl alcohol (PCI) mixture and ethanol-precipitated overnight with 10 μ g of glycogen.

3.2.6 cDNA Library Preparation

1. The isolated RNA is dissolved in 12 μ l of distilled RNase-free water and reverse-transcribed using miRCat-33 primer with Superscript III Reverse Transcriptase in its buffer for 1 h at 50 °C.
2. RNA is then degraded by addition of RNase H for 30 min at 37 °C. cDNA is amplified using primers P5 and primer PE_miRCat_PCR and TaKaRa LA Taq polymerase.
3. PCR products are separated on a 2 % MetaPhor agarose gel with SYBRSafe in 1 \times TBE at 4 °C. The gel band corresponding to 150–200 bp is excised.
4. cDNA is purified with MinElute Gel Extraction Kit and the purified cDNA libraries are sent for high-throughput sequencing.

3.3 Electrophoretic Mobility Shift Assays (EMSA)

1. Nuclear extract aliquots are prepared using cytoplasmic nuclear fractionation kit as per manufacturer's protocols. The nuclear extracts are verified by western blotting to confirm Wt1 expression. The nuclear extracts (NE) are quantified for their protein content and stored as aliquots in –80 °C.
2. Competitor oligos and labeled oligos also referred to as the "hot" oligo is 3' boitin tagged are reconstituted to 100 μ M

concentration. Also needed are the complementary oligo and a non-tagged version.

3. 6 % acrylamide gel is prepared with 0.5 % TBE.
4. 100 μM oligos are diluted 1:1000 with water and the following annealing reaction is carried out.

F oligo	10 μl
R oligo*	10 μl
H restriction buffer	10 μl
Water	70 μl
The above components heated to 95 °C for 5 min, and allowed to cool to room temperature	

5. Competitor oligos are processed as follows:

F oligo	10 μl
R oligo	10 μl
H restriction buffer	10 μl
Water	70 μl
Heated to 95 °C for 5 min, and allowed to cool to room temperature	
The competition oligos are diluted 1:10 to give 50 \times excess	

6. Binding reaction is prepared as follows:

– NE	2 μl (4 μg of protein)
– dI/dC	1 μl (1 mg/ml solution)
– Binding buffer	1.5 μl
– Competitor if required	2 μl (50 \times excess, 1 pmol)
– Water	
– Oligo*	2 μl (20 fmols)
– Total	15 μl
All reagents except the hot* oligo are added together and incubated for 10 min on ice	

7. Hot oligo* is added and incubated at room temperature for 20 min.
8. The samples are mixed with 4 μl of 30 % sucrose/BPB loading dye and loaded on to the 6 % gel from **step 3**.
9. The samples are electrophoresed in 0.5 % TBE until dye front reaches the bottom of the gel and transferred in 0.5 % TBE on nitrocellulose (in the cold room).
10. Filters are subjected to UV cross-linking using the optimal cross-link option.

11. The filters are developed with Chemiluminescent Nucleic Acid Detection Kit as per the manufacturers' instructions.

3.4 RNA–Protein Cross-Linking Experiment

1. Cells (2×10^7) are pelleted and resuspended in 1 ml of RNP lysis buffer and centrifuged at $14,000 \times g$ for 10 min at 4 °C.
2. Glycerol is added to the supernatant at a final concentration of 5%.
3. Cytoplasmic extract is stored in –80 °C freezer, in aliquots at a concentration of 5–7 $\mu\text{g}/\mu\text{l}$.
4. Radiolabeled (RNA regions such as 5' UTR, fragments of coding regions, 3' UTR) fragments are synthesized at a 30 Ci uridine/mmol concentration.
5. Oligo template hybridized to T7 promoter primer.

T7 promoter primer	2 μl
DNA hyb buffer	6 μl
Oligo template (100 μM)	2 μl

6. Samples are heated to 70 °C for 5 min, left at room temperature for 5 min to hybridize.
7. Klenow filling

To the above reaction, the following components are added.	
10X Klenow reaction buffer	2 μl
10X dNTP mix	2 μl
Nuclease free water	4 μl
Exo-klenow	2 μl
Components are mixed well, centrifuged briefly, and incubated at 37 °C for 30 min.	

8. Transcription of probe:

10X transcription buffer	2 μl
dsDNA template	5 μl
A/C/G 10 mM	1 μl
Alpha-UTP*	5 μl
T7 RNA pol	2 μl
Water	5 μl
Incubated for 30 min at 37 °C.	
To the above, 1 μl of DNase I is added and further incubated for 10 min at 37 °C.	
Samples are heated for 1 min at 95 °C, placed on ice followed by addition of 10 ml of hybridization buffer added.	

9. Approximately, 15 μg of protein (lysate) is incubated with 100 nCi of labeled RNA fragments for 25 min at room temperature in 10 μl of the interaction buffer.
10. The above samples are irradiated with 254 nm wavelength UV for 30 min at 5.4 J/cm² on ice.
11. Excess probe is removed by digestion with 0.5 μg of RNase A at 37 °C for 25 min.
12. The samples are electrophoresed on 10% SDS-PAGE and subjected to autoradiography.

3.5 Discussion

Recent techniques coupled with next generation sequencing technologies have hugely impacted the field of RNA protein interactions. The earlier approaches such as SELEX identified a few potential RNA binding motifs; however, when these were used as baits, no physiological targets were identified except α -actinin. This was an indication towards the possibility that the endogenous interactions are likely to be tissue specific and also transient or dynamic thus necessitating approaches that can exploit endogenous interactions. The RNA IP approach and the CLASH approaches are precisely engineered towards these needs since RIP can provide information about Wt1 interacting RNA targets (an example depicted in Fig. 2), whereas the latter can provide information about binding sites as well as provide insights about the potential Wt1 RNA complex. Although CLASH relies on tagged proteins, it should be possible to adopt this to endogenous proteins using modifications to the cross-linking approaches.

3.6 Future Perspectives

The identification and elucidation of the Wt1 RNA interactome has only been possible by employing a variety of techniques including biochemical and sequencing approaches. This is very similar to the early advances in the field of protein–protein interactions which have now moved on to visualization of these interactions within the cell. There have been a few studies recently wherein the RNA–protein interaction have been attempted to be visualized in the

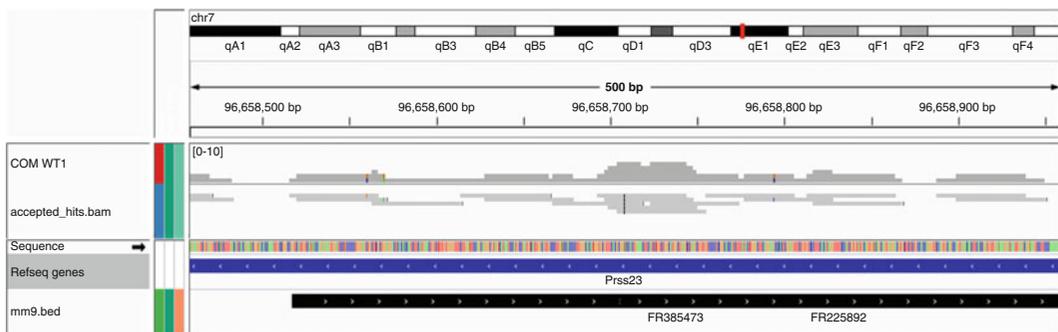


Fig. 2 CoMEEC cells that express endogenous Wt1 are subjected to RNA IP, followed by sequencing of the libraries. Represented above is an example of an alignment of the sequencing reads obtained to the noncoding regions within the Prss23 coding region

in vivo context. Although this involves fluorescent tagging of the molecules involved, thus not exactly a correlation of what is relevant in the physiological scenario, it is possible that with the CRISPR strategy, endogenous molecules can be visualized interacting in real time.

4 Notes

1. The cross-linking approaches can be varied depending on the need of the experiment. If the overall experimental strategy is towards identifying RNA targets of a particular protein, native or formaldehyde cross-linking suffice. However, UV cross-linking can provide more information on the binding sites.
2. Cells that are being processed for cross-linking should be always kept on ice so as to preserve any transient interactions. For cells that are to be subjected to UV cross-linking, it is advised to grow them on dishes rather than flasks, to maximize the cross-linking. The cells should be covered with minimal volume of PBS (about 1–2 ml) during the UV cross-linking procedure.
3. The samples during the processing for RNA interactions can be frozen at various time points such as after cross-linking in Subheading 3.1.1. The pellets can be subjected to flash freezing.
4. The fragmentation of RNA either before the IP or during the input sample preparation also influences the outcome of the experiment. Generally, if no fragmentation step has been included, the entire molecule is purified and thus impedes any binding motif identification. However, if sonicated fragments are used, or RNase digestion is performed, the overall enrichment of smaller fragments can lead to identification of potential binding motifs.

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