Chapter 16

WT1-Associated Protein–Protein Interaction Networks

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Abstract

Tumor-suppressor protein Wt1 has been shown to interact with specific proteins that influence its function. These protein interactions have been identified as direct individual interactions but with the potential to exist as a part of a multiprotein complex. In order to obtain the global proteome interaction map of Wt1, an unbiased label-free endogenous immunoprecipitation was performed followed by mass spectrometry to identify protein interactions that are Wt1 centric. This chapter details the different techniques that have been used to identify and characterize Wt1-interacting proteins.

Key words Immunoprecipitation, Proteome, Protein interaction, Mass spectrometry

1 WT1-Associated Proteome

Tumor-suppressor protein Wt1 is a transcription factor that has been shown to have both transcriptional activation and repression properties. Studies have shown that Wt1 interacts with other proteins with functional implications. Wt1-interacting proteins can be broadly classified into three functional categories, (a) transcription-related proteins, (b) cell cycle and apoptosis regulators, (c) splicing pathway components. These have been identified using several biochemical techniques such as yeast two-hybrid analysis, immuno pulldowns, and mass spectrometric analyses and validated by immunoprecipitation (IP) followed by immunoblots/western blotting approaches as well as by performing immunofluorescence to observe colocalization. A brief overview of these different functional categories of interacting proteins is followed by detailed protocols that have been used to identify and understand the significance of Wt1 interactome.

1.1 Functional Categories of Wt1-Interacting Proteins

(A) **Transcription-Related Proteins**: Tumor-suppressor protein p53 was one of the first Wt1-interacting proteins to be identified. This was later extended to include p63 and p73 as well. Several important transcription factors including STAT3, ERα, SRY, and

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Pax2 as well as general transcription factors such as TBP and TFIIB have been shown to physically interact with Wt1 (reviewed in ref. 1). Wt1 has also been shown to interact with transcription cofactors both coactivators and corepressors such as CBP, WTIP, and Basp1 to influence the transcriptional response.

(B) **Cell Cycle Regulators**: Wt1-interacting proteins with an implication towards cell cycle regulation such as p53 were identified initially. However, a few recent studies have identified Wt1 interaction with Htra2, a serine protease [2] with a role in apoptosis as well as interaction with a cell cycle checkpoint regulator MAD2 [3]. The heat-shock protein hsp70 which has several important regulatory roles including cell cycle is also a well-known Wt1-interacting protein [4].

(C) **Components of the Splicing Pathway**: Wt1 and its association with splicing factors and hnRNPs have been known for more than a decade. The study with U2AF65 was the outcome of a yeast two-hybrid assay, leading to the identification of a Wt1-interacting protein associated with the splicing machinery [5]. The +KTS isoform was seen to have better interaction with U2AF65. Incidentally, U2AF65 is essential for the recruitment of U2 snRNP. Yet another Wt1-interacting protein, WTAP, has also been implicated in alternative splicing events, especially the splice site selection. The Wt1 and RBM4 interaction is yet another study linking Wt1 and alternative splicing wherein minigenes were used to understand the contribution of Wt1 and RBM4 on their splicing (reviewed in ref. 6). The contribution of the isoforms as well as the actual mechanism in these processes needs further investigation.

(D) **Other Interacting Proteins**: Wt1 has also been shown to interact with structural proteins such as actin [7] and also with epigenetic factors such as DNMT1 [8] and the dioxygenase Tet2 [9] recruiting them to their target-binding sites to facilitate epigenetic modifications.

2 Materials

1. **Proteins:**

The protein–protein interaction studies have been mostly performed with overexpression constructs wherein tagged versions of the proteins were transfected and purified from cells with the help of a tag. Thus far, only few interaction and global proteomics experiments have been performed with endogenous Wt1-expressing cell lines. All the centrifugation steps have been performed in a microfuge.

2. **Reagents:**

   Agarose resins for tag purifications such as GST (Amersham), hexa-histidine (SIGMA), and anti-FLAG (SIGMA) have been used for enriching the tagged proteins as well as for interaction studies. Agarose-conjugated Wt1 antibody (sc-192) was used for the IP. Protein A/G agarose beads (sc-2003) used routinely for pre-clearing of lysates and control IPs.

2.1 Buffer Composition

1. **Gentle Lysis Buffer**: 10 mM Tris–HCl at pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1× protease inhibitor cocktail, 1 mM DTT, and 10 μg/ml of RNase A.
2. **RIPA Buffer**: 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, pH 8.0 with protease inhibitors (see Notes for further information)
3. **For RNA-binding proteins** (RBPs), the lysis buffer is modified to gentle lysis buffer, gentle lysis buffer: 10 mM Tris–HCl pH 7.5, 10 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 1 mM PMSF, 1× protease inhibitor cocktail, 1 mM DTT, and 10 μg/ml of RNase A. NaCl was added to the cleared lysate to a final concentration of 200 mM.
4. **Buffer 1**: 20 mM Tris–HCl (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM dithiothreitol (DTT),0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 M NaCl (DTT and PMSF added on the day of the experiment).
5. **Buffer 2**: 20 mM Tris–HCl (pH 7.9), 20% glycerol, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 1 M NaCl (add the DTT and PMSF on the day of the experiment).
6. **Buffer 3**: 20 mM Tris–HCl (pH 7.9), 20% glycerol, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 0.1 M NaCl (add the DTT and PMSF on the day of the experiment).

3 Methods

3.1 Yeast Two Hybrid

1. Plasmids were transformed into the respective strains using the lithium acetate method followed by analysis for interactions which was tested by growing leu–trp–his– media supplemented with 50 mM 3-amino triazole [5].
2. The β-galactosidase activity was tested by using filters from leu–trp– plates and liquid assays in leu–trp– media.
3. The activity of β-galactosidase was calculated using the following formula: $1000 \times A420 / (\text{volume} \times \text{time} \times \text{protein concentration})$.

3.2 Co-immunoprecipitation

1. To examine the interactions between Wt1 and interacting proteins, 8×10^6 cells that express Wt1 were collected by manual scraping using a scraper and pelleted by centrifugation.
2. Cell pellet was resuspended in 400 μl of gentle lysis buffer and incubated on ice for 15 min. Insoluble materials were removed by centrifugation at $13{,}400 \times g$ in a microcentrifuge at 4 °C for 15 min. NaCl was added to the cleared lysate to a final concentration of 200 mM.
3. Lysates are subjected to pre-clearing with 20 μl of protein A/G agarose beads at 4 °C for 1 h. Spin, at 735 × g for 5 min, supernatant transferred to fresh tube.
4. 350 μl of the lysate incubated with 2 μl of Wt1-agarose-conjugated antibody, pre-immune IgG at 4 °C overnight.
5. The next day, beads were washed and bound fractions eluted by 2× SDS-sample buffer by heating at 95 °C for 5 min.
6. Proteins were resolved by SDS-PAGE, followed by western blot analysis.

3.3 Silver Staining Protocol for Mass Spec-Compatible Gel Bands

1. Gel washed in water (dH_2O freshly autoclaved), 2×5 min.
2. Gel fixed in 30% ethanol: 10% acetic acid solution, 2×15 min.
3. Gel washed in 10% ethanol, 2×5 min, and in water, 2×5 min.
4. Gel sensitized in sensitizer working solution (50 μl sensitizer in 25 ml water), 1 min, washed in water, 2×1 min.
5. Gel stained in stain working solution (0.5 ml enhancer in 25 ml developer) for 5 min.
6. Gel developed in developer working solution (0.5 ml enhancer in 25 ml developer). Washed with water, 2×20 s. Developed for 1 min. Reaction stopped with the addition of 5% acetic acid for 10 min. Washed with water, 2×15 min (see Notes for further information).

3.4 Label-Free Proteomics

1. $20–30 \times 10^6$ cells at confluence, monolayer washed with PBS, cells scraped in 5 ml PBS, centrifuge, pellet washed with PBS.
2. Lysed in RIPA buffer (ten times pellet cell volume (PCV)).
3. Lysate pre-cleared for 1 h using agarose slurry/beads, 4 °C on end-to-end rotor, spin, $735 \times g$ for 5 min, supernatant transferred to fresh tube (see Notes for further information).
4. IP using 1–1.5 mg of whole-cell extract (WCE) with 2–4 μg of antibody, 4 °C on end-to-end rotor, overnight.
5. Samples centrifuged at 2000 rpm for 5 min. Supernatant transferred to fresh tube and discarded after confirmation of the IP.
6. Beads washed four times with RIPA buffer at 2000 rpm for 5 min, 4 °C.

7. Beads washed twice with PBS at 2000 rpm for 5 min, 4 °C.
8. The immunoprecipitated beads subjected to snap freezing using liquid nitrogen.

3.5 Mass Spec Acquisition

The samples were digested with trypsin and then analyzed on a Q-Exactive coupled to an LC with a homemade C18 ReproSil Aq 1.8 μm column as previously described in ref. 10. Peptides were eluted using a linear gradient from 2 to 32% acetonitrile over 40 min. Data analysis and label-free quantification were done by using MaxQuant searching against a mouse IPI database. The overall schematic of the label-free proteomic experiment is represented in Fig. 1a.

3.6 In Vitro Protein–Protein Interaction Assay

1. GST resin (10 μl packed volume so about 25 μl of resin) is taken per IP reaction.
2. Wash the resin three times with **buffer 1** (ten times volume of beads so 100 μl).
3. Protein GST and GST-Wt1 (500 ng each) mixed individually with the resin in buffer 1 and left on rotor in cold room for 1½ to 2 h.
4. Above samples centrifuged (735 × g, 4 °C, 5 min). Washed with **buffer 2**, three times, and **buffer 3**, three times (100 μl each).
5. Target protein diluted (His-tagged interacting protein X) in **buffer 3** such that there is 500 ng of protein in 5 μl volume. The washed beads from **step 4** mixed with the target protein and IP is performed in **buffer 3** in a total volume of 50 μl. In cold room, on rotor for 2 h. Samples centrifuged at 735 × g, 4 °C, 5 min. Supernatant to be used as unbound protein during western blotting.
6. Beads washed with **buffer 1,** four times (100 μl each). Sample eluted from the beads using 30 μl of SDS loading dye. Western to be probed with antibodies against the GST and His tag.

3.7 Colocalization by Immunofluorescence Analysis

1. Cells grown on cover slips, subjected to required treatment. Media aspirated. Washed with PBS (1×), three times for 2 min each.
2. Fixation with 4% PFA (use 0.2 ml if cells on cover slips in a 24-well plate/50 μl for cells in 16-well chamber slide), 10 min at RT on shaker. PFA removed. Washed with PBS (1×), three times for 5 min each.
3. Permeabilization with 0.25% Triton X 100 (use 0.2 ml if cells on cover slips in 24-well plate/50 μl for cells in 16-well chamber slide), 10 min RT on shaker (do not exceed the time). Triton removed, washed with PBS (1×), three times for 5 min each.
4. Blocking with 1% BSA in 1× PBST, 30 min at RT on shaker (0.2 ml for cover slips/50 μl for 16-well chamber slide). Blocking solution discarded.

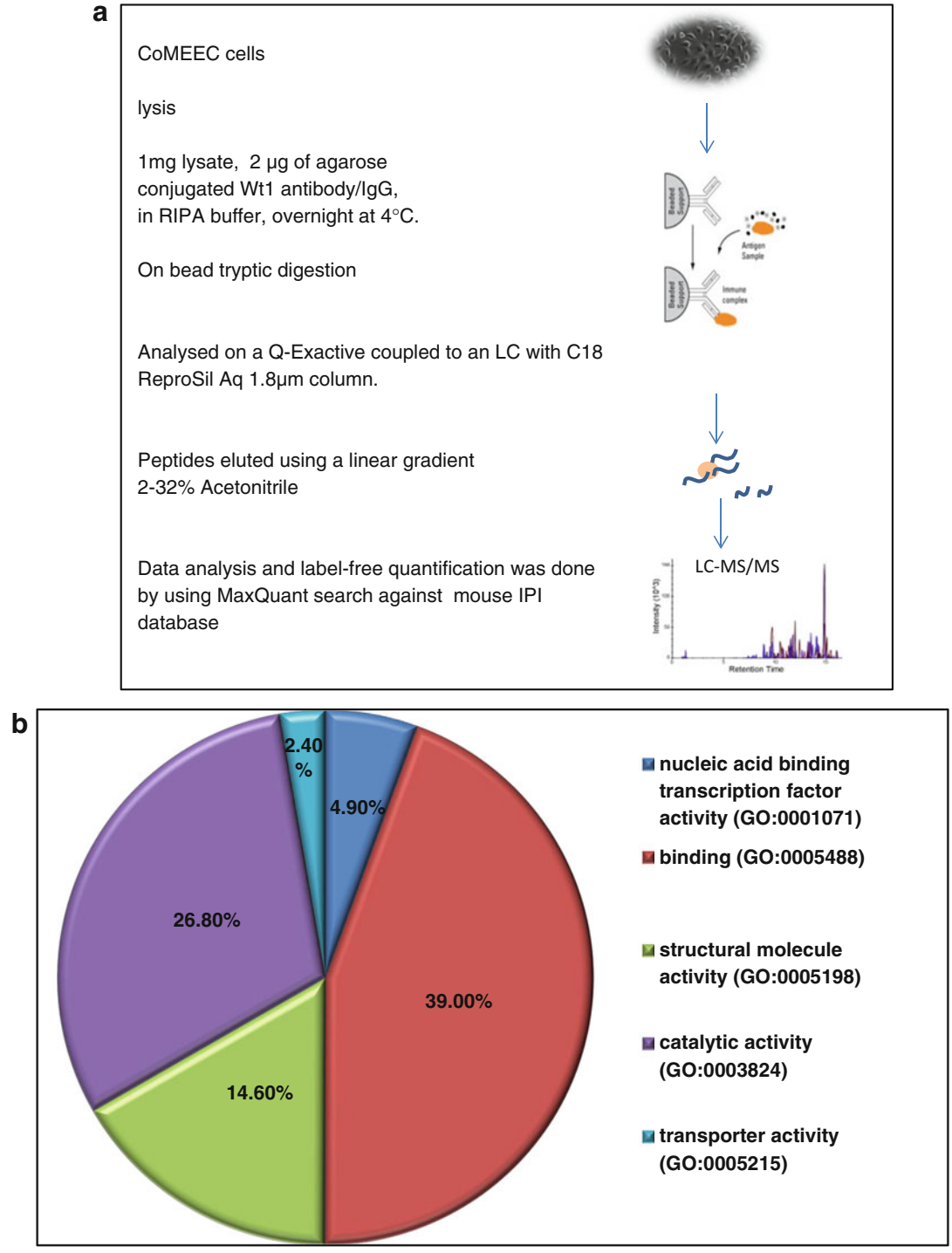

Fig. 1 An unbiased global WT1-interacting protein analysis by label-free mass spectrometry. (**a**) A brief schematic of the different steps involved in identifying Wt1-interacting proteins by label-free mass spectrometric approach. (**b**) A pie chart representation of the different gene ontology categories of Wt1-interacting proteome. The percentage in each category is also represented

5. Primary antibody to be added (if two different host antibodies are to be used, a mix of the two antibodies can also be used). 0.2 ml for cover slips/50 μl for 16-well chamber slide. Incubate at RT for 1 h. Aspirate antibody. Wash with 1× PBS (three times for 5 min each).
6. Secondary antibody or a mix added, 0.2 ml for cover slips/50 μl for 16-well chamber slide. Incubated at RT for 1 h wrapped in foil. Antibody aspirated. Washed with 1× PBS (three times for 5 min each), wrapped in foil.
7. If using chamber slide, wells and the protective seal removed. A drop of Vectashield H1200 added, cover slip placed on top. If using cover slips, on a glass slide, a drop of Vectashield H1200 added, the cover slip inverted on to the drop such that the cells are on the bottom side. Edges can be sealed with enamel for later visualization.

   Antibody solutions are made in 1% BSA in 1× PBST.

3.8 Discussion

The interactome analysis utilizes a series of different biochemical techniques to identify and characterize specific interactions. However, recent advances have allowed visualization of molecular interactions, thus providing spatial and temporal resolution to the protein interaction studies in the context of cell biology. Protein–protein interactions can now be visualized in living cells with the help of fluorescence-associated techniques such as FRET and FCS. FRET refers to the fluorescence/Förster resonance energy transfer which employs the differences in the excitation and emission energy of two fluorophores tagged to proteins as explained in ref. 11. FCS is fluorescence correlation spectroscopy which also relies on fluorophore-tagged proteins and their hydrodynamic diameter which represents changes based on interaction between the proteins [12]. These most often require additional cloning strategies to introduce the tagged version of the protein; however these techniques facilitate cellular biochemical interactions to be visualized in real time. Proximity ligation assays are yet another way of visualizing the dynamics of protein–protein interaction [13].

3.9 Protein Interaction Networks

Global protein–protein interactions can be depicted as interaction networks with the help of databases such as STRING. Alternatively, interaction data can also be analyzed for an over-representation of any particular category of proteins by using the gene ontology tools available online. An example of this is represented in Fig. 1b, where Wt1-interacting proteins identified in the epicardial cell line, enriched over a threshold of 2 in comparison to the control, were selected and submitted to the Panther database for analysis of distinct functional categories. This depicts an enrichment for Wt1-interacting proteins in the binding category, structural proteins, and catalytic activity.

4 Notes

1. Use agarose-conjugated antibodies wherever possible: If not antibodies need to be bound to the agarose slurry. (Beads equilibrated in IP buffer by washes at 326×g 3 min, twice, add antibody at required concentration in 1 ml of IP buffer, end-to-end rotor, 4 °C for 1 h, spin 326×g/3 min, remove supernatant, use these beads as the conjugated antibody for the IP.)
2. Overexpression strategies work well when matched with a good empty vector-transfected control.
3. Endogenous protein IPs are best since it does not perturb any interactions based on changes in transfection and/or expression efficiency. However, this approach requires additional controls so as to improve the efficiency of identifying true interactions.
4. Incorporation of DNase or RNase along with the lysis buffer and the IP buffer also facilitates the exclusion of nonspecific interactions.
5. PIERCE staining kit (24612) alternatively can also use the following kit (24600). Make all solutions fresh, use freshly autoclaved water (unopened before), wear gloves at all times, handle gel only with forceps if needed, and use new plates for the processing if possible.

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