

Original Article

Microscale thermophoresis analysis of the molecular interaction between small nuclear ribonucleoprotein polypeptide G and the RING finger domain of RBBP6 towards anti-cancer drug discovery

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Abstract: Regulatory core-splicing proteins are now becoming highly promising therapeutic targets for the development of anti-cancer drugs. SNRPG and RBBP6 are two good examples of regulatory core-splicing proteins involved in tumorigenesis and tumor development whose multi-functional role is primarily mediated by protein-protein interactions. Over the years, skepticism abutting from the two onco-proteins has been mounting. Suggestive evidence using yeast 2-hybrid technique observed possible involvement between SNRPG and the RING finger domain of RBBP6. However, the putative interaction remains elusive and yet to be characterized. In this study, we developed the first MST-based assay to confirm the interaction between SNRPG and the RING finger domain of RBBP6. The results demonstrated a strong binding affinity between SNRPG and the RING finger domain of RBBP6 with a K_D in the low nanomolar concentration range of 3.1596 nM. The results are congruent with previous findings suggesting possible involvement between the two proteins in cancer-cell networks, thereby providing a new mechanistic insight into the interaction between SNRPG and the RING finger domain of RBBP6. The interaction is therapeutically relevant and represents a great milestone in the anti-cancer drug discovery space. Identification of small molecule inhibitors to modulate the binding affinity between the two proteins would therefore be a major breakthrough in the development of new PPI-focused anti-cancer drugs.

Keywords: Anti-cancer drug discovery, cancer, microscale thermophoresis, RBBP6, RING finger, SNRPG

Introduction

The rational optimization of protein-protein interactions (PPIs) is becoming increasingly important in modern drug discovery processes [1-3]. This is currently driven by targeting the undruggable molecular space with the aim of designing new therapeutic agents that can selectively target intractable disease-specific molecular mechanisms or pathways. PPIs are an attractive class of molecular targets in the drug discovery parlance. Drugging the undruggable proteome space with the aim of designing new therapeutic agents is an indispensable arsenal in curbing pathophysiological cues and disease progression [4]. In this context, PPIs of cancer-implicated proteins are considered

high-value targets in drug development programmes [1]. Most cancer-implicated proteins possess structural domains that have a higher ratio of infidelity as compared to their non-cancer implicated counterparts, making them more prone to interaction with a wide diversity of proteins [4-6]. Cancer-implicated proteins have many interacting partners and occupy a central position in cancer-cell protein networks [6-8]. Thus, protein interactions between these macromolecules have a higher probability of being related to cancer processes than non-interacting proteins, making them therapeutically vulnerable for anti-cancer drug discovery [6-8].

Targeting cancer-implicated PPIs is a powerful arsenal to address mechanistic cues in tumori-

genesis and tumour development. Small Nuclear Ribonucleoprotein Polypeptide G (SNRPG) and Retinoblastoma Binding Protein 6 (RBBP6) are two good examples of cancer-implicated proteins whose functions are predominantly mediated by PPIs [9, 10]. The two proteins are connected by a wide array of biological processes and play critical roles in pathophysiological cues. SNRPG is a core-splicing protein that is essential in the biogenesis of small nuclear ribonucleoproteins (snRNPs), which are precursors of the spliceosome [11-14]. RBBP6 is a splicing-associated multi-domain and multi-functional nuclear protein known to play a role in mRNA splicing, cell cycle control and apoptosis. RBBP6 interacts with tumour suppressor proteins p53 and pRb in which the Really Interesting New Gene (RING) finger domain plays an essential role [15-17].

Varying expression levels of the two proteins have been reported in different types of cancer such as breast, lung, prostate and colon cancer, but very little is known about the putative interactions between RBBP6 and SNRPG in these different types of cancers [15, 18-20]. Chibi and co-workers [21] as well as Kappo and co-workers [16] predicted possible interactions between SNRPG and RBBP6 using the yeast 2-hybrid (Y2H) technique. The findings suggest possible involvement of SNRPG and RBBP6 through its RING finger domain in tumorigenesis and tumour development. However, the precise mechanisms involved remain elusive and yet to be characterised [22].

Robust and reliable determination of the binding affinity between SNRPG and its putative interactive partner, the RBBP6 RING finger domain, is a critical step in understanding the relationship between the splicing machinery, tumorigenesis and tumour development. More so, quantitative characterization of intermolecular interaction affinity between oncogenic core splicing proteins is highly necessary to develop novel and effective drugs for therapeutic interventions in cancer [23]. Most analytical techniques for PPIs are expensive (e.g., mass spectrometry), time-consuming [e.g., Surface Plasmon Resonance (SPR), Isothermal Titration Calorimetry (ITC)] and require high amounts of sample (e.g., Size-exclusion Chromatography, Isothermal Titration Calorimetry). However, MicroScale Thermophoresis (MST) is an attractive alternative technique with advantages of

speed, ultra-low sample consumption and high-throughput/cost-efficiency. Just like ITC and SPR, MST can be used to determine the equilibrium dissociation constant (K_d) and other thermodynamic parameters [2, 23-25].

MicroScale Thermophoresis (MST) is a versatile optical fluorescent technique used to quantify binding affinities in solution between a target molecule and its interactive partner [25]. This biophysical technique (**Figure 1**) detects variations in fluorescence signals resulting from infrared laser-induced temperature gradients. The variation in the fluorescence signal correlates with the binding of a ligand to the fluorescent target. This effect is known as TRIC (temperature-related intensity change) [2, 24]. The TRIC signals are additory and contribute to the high sensitivity and robustness of MST measurements in molecular binding events [2, 24].

However, the outstanding merit of utilising MST over other routinely used PPI methods is its ability to determine K_d values in complex sample matrices [2, 23, 24]. Although MST measurements is performed using intrinsic fluorescence of proteins, labelling of the target proteins with a suitable fluorophore is required [2, 24]. Different site-specific labelling strategies have been proposed and applied. The His-tag is the most popular and widely used affinity tag for purification, immobilization or detection of proteins. The application of tris-NTA-based labelling of His-tagged proteins is commonly used for MST measurements [24]. Thus, MST can be used to determine the binding affinity and binding strength between protein-protein biophysical interaction with very low sample consumption and high sensitivity. In this study, we used the innovative MST to establish an experimental assay for fast, precise, cost-efficient and quality-controlled characterization of the binding affinity, binding stoichiometry and interaction thermodynamics between SNRPG and the RING finger domain of RBBP6. The study provides novel insights into the molecular mechanisms between the two proteins towards PPI-focused anticancer drug discovery.

Experimental procedures

Bacterial expression and purification

Codon optimized DNA sequences incorporating *Bam*HI and *Xho*I restriction sites were amplified

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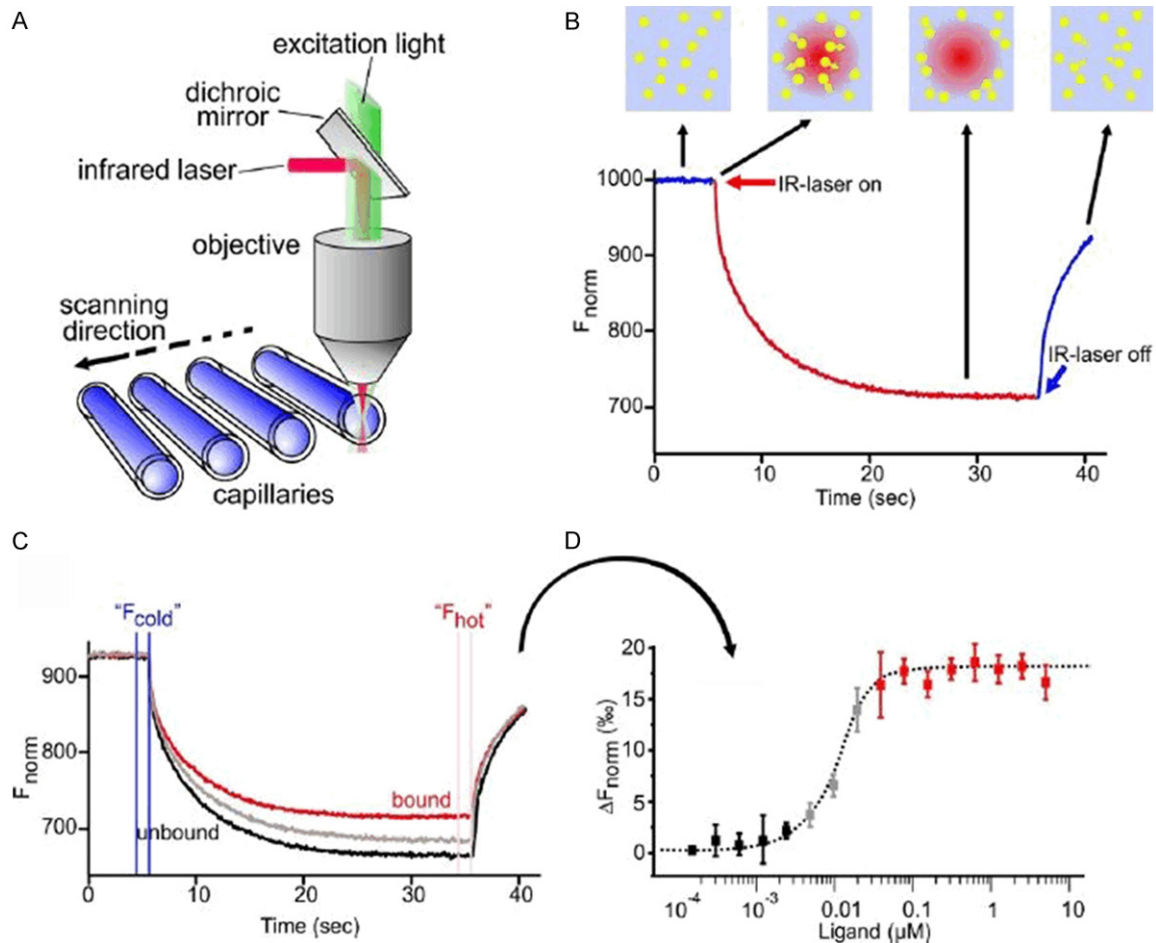


Figure 1. MicroScale Thermophoresis (MST) set-up. A. MST measurements are conducted in small glass capillaries. Infrared and fluorescence lasers trigger the MST effect and generate sample tracking. B. Temperature-related intensity change (TRIC) and thermophoresis account for the time-dependent change in fluorescence upon infrared-heating of the sample capillaries. C. Multiple MST traces are recorded for different mixture ratios of target and ligand molecules. D. Dose-response analysis of the MST traces allows for determination of the steady-state affinity of the target-ligand interaction (Figure extracted from Schubert and Langst [26]).

from the full-length cDNA sequences of SNRPG (UniProtKB-P62308 (RUXG_HUMAN)) and the RING finger domain (pdb: 3tzg) of RBBP6. The genes were cloned into the pQE30 and pGEX-6P-2 protein expression vectors and were purchased from GenScript (New Jersey, USA) to be used for protein expression. Expression of both proteins was induced with 0.5 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) concentration at 25°C. The expressed SNRPG protein was purified using a Nickel-NTA column recharged with cobalt, whereas the RING finger domain of RBBP6 was purified by the use of a glutathione-agarose (SIGMA® Aldrich) column and Econo® Chromatography Column (Amersham Pharmacia). The concentrations of the eluted proteins were determined using a

NanoDrop® ND2000 spectrophotometer (Thermo Fisher Scientific).

MicroScale thermophoresis measurement

A 100 μ l of 100 nM 6X His-tag-SNRPG protein labelled in 1X phosphate-buffered saline (pH 7.4) supplemented with 0.05% Tween-20 (PBS-T) was mixed with 100 μ l of 100 nM of Monolith His-Tag Labeling Kit RED-tris-NTA 2nd Generation (MO-L018) (NanoTemper Technologies, Munich, Germany) diluted in 1X PBS-T buffer to a final concentration where the fluorescent signals of the SNRPG proteins were similar and above the typical detection limit of the Monolith NT.115 instrument (NanoTemper Technologies, Munich, Germany). The mixture

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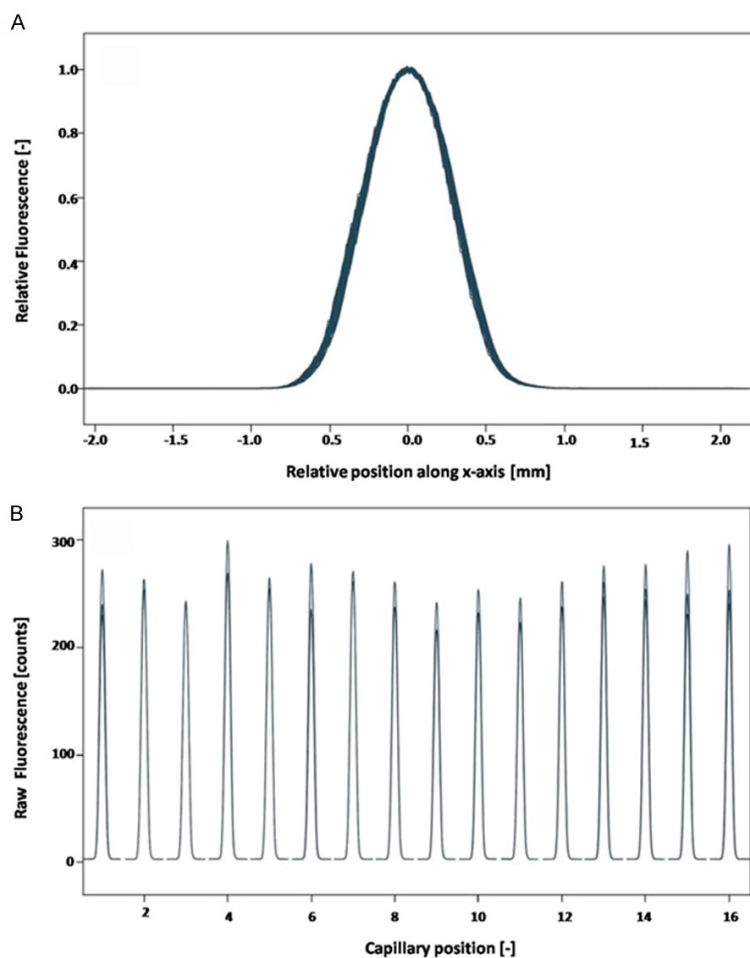


Figure 2. MST capillary scans for the 6X His-tag-SNRPG protein. A. The capillary scan graph overlaid perfectly suggesting no SNRPG protein adsorption onto the capillaries. B. The capillary scans output observed no fluctuations in protein fluorescence suggesting successful labelling of the SNRPG protein.

was incubated for 30 minutes at room temperature in the dark prior to the MST experiment. The final concentration of the fluorescently labelled 6X His-tag-SNRPG protein was 50 nM.

MicroScale thermophoresis experiments were performed on a NanoTemper® Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) as shown in **Figure 1**. Samples were prepared and loaded into premium treated capillaries. A 16-tube serial dilution of the non-fluorescent GST-RING finger domain of RBBP6 ranging from 1.11 μM to 3.39×10^{-5} μM was titrated against a fixed concentration (50 nM) of the fluorescent 6X His-tag-SNRPG. The sample was mixed and added to each tube to a final volume of 20 μl using low-bind pipette tips. Having prepared the serial dilutions, 4 μl

samples were filled into the capillaries through capillary action, resulting in low sample consumption. Triplicate MST measurements were performed on the NanoTemper® Monolith NT.115 (NanoTemper Technologies GmbH, Munich, Germany) in premium capillaries (MO-K025) at 25°C using 40% MST power and 40% light-emitting diode (LED) with laser off/on times of 5 seconds and 30 seconds, respectively. The system was operated with the latest version of the MO control software (v1.6) with data analysis performed using the NanoTemper® analysis software settings optimised for TRIC-sensitive dyes.

Results

The fluorescence labelling of the 6X His-tag SNRPG protein displayed a high affinity of 3.8 ± 0.5 nM using the RED-tris-NTA 2nd Generation dye. The novel Monolith His-Tag labelling Kit RED-tris-NTA 2nd Generation kit (MO-LO18) comparatively yields higher binding amplitudes and signal-to-noise ratios [2, 27]. As shown in **Figure 2A**, the MST

capillary scans for the 6X His-tag SNRPG protein depicted a perfectly overlaid graph, suggesting no protein adsorption of the fluorescently labelled His-tag SNRPG protein onto the capillaries. There were no fluctuations in protein fluorescence in the capillary scans output, suggesting successful labelling of the 6X His-tag SNRPG protein. It is always vital to check and avoid adsorption of proteins and reference ligands on the capillary walls as this apparently decreases or ablates ligand binding due to loss of material adversely, subsequently affecting the MST signal and results [2, 23, 27].

Following the successful fluorescence labelling of the 6X His-tag SNRPG, pre-test binding checks were conducted to confirm whether there was detectable binding between the 6X

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Table 1. Overview of the MST dataset between the SNRPG and RING finger domain of RBBP6

Description	Experiment
Target Name	SNRPG
Target Concentration	50 nM
Ligand Name	Ring finger domain of RBBP6
Ligand Concentration	1.11 μ M to 3.39×10^{-5} μ M
n	3
Excitation Power	40%
MST Power	40%
Temperature	25.0 °C
K_D	3.1596×10^{-9}
K_D Confidence	$\pm 7.627 \times 10^{-9}$
Response Amplitude	5.2101186
Target Concentration	5×10^{-8} [Fixed]
Unbound	861.21
Bound	866.42
Std. Error of Regression	1.0692345
Reduced χ^2	0.96629751
Signal to Noise	5.2341793

His-tag SNRPG and the GST-RING finger domain of RBBP6. The pre-test binding checks observed positive results suggesting detectable binding between the two proteins. Pre-tests are highly commended to facilitate adjustments to the labelling or concentrations, thus minimizing wastage of materials from failed or indeterminate binding affinity experiments due to insufficient fluorescence [2, 27, 28].

The subsequent characterization and binding affinity measurements of the binding event between the SNRPG and RING finger domain of RBBP6 were conducted and analysed by MO Affinity Analysis software v2.3. An MST on-time of 1.5 seconds was used for analysis and calculation of the K_D value (n=3 independent measurements, error bars represent the standard deviation). The MST raw dataset of the merged PPI dose-response between SNRPG and RBBP6 RING finger domain is summarised in **Table 1**. Additional MST raw data of merged dose-response of the PPIs are embedded as **Table S1**, while the resulting dose-response curves were fitted to a one-site binding model to extract K_D values from a K_D -binding model assuming a 1:1 binding stoichiometry.

As indicated on the raw MST trace plot, the relative change in fluorescence was observed as

the titration concentration of the RING finger domain of RBBP6 increased. The thermograph showed no signs of adhesion or aggregation (**Figure 3**). The result is a clear indication of the binding event between the two proteins, transiting from the unbound state to the bound state.

According to Mrozowich and co-workers [23], an accurate binding curve should observe three significant points denoting no binding, slope of the binding and complete binding. The titration curve as shown in **Figure 4** displays a typical sigmoidal shape with an atypical peak in the thermophoresis signal close to the apparent point of saturation. The observation suggests that the changes that affect fluorescence upon binding are identical and that different thermophoretic properties are formed during the binding event. The binding affinity measurement between SNRPG and the RING finger domain of RBBP6 observed a K_D of 3.1596 nM under aqueous buffer conditions.

Discussion

The identification of small molecule inhibitors that may be able to modulate the binding affinity between regulatory core-splicing proteins SNRPG and the RING finger domain of RBBP6 represents a major strategy in the development of new PPI-focused anti-cancer drugs. The two oncogenic and regulatory core-splicing proteins are variably expressed in different types of cancers and have been overlooked as potential therapeutic arsenals for many years. The molecular mechanisms by which SNRPG and the RING finger domain of RBBP6 mediate their oncogenic networks still remain unknown and uncharacterized [15, 18-20]. The understanding of PPIs of regulatory core-splicing proteins is biologically interesting and is essential for therapeutic optimization and modifications by small molecule inhibitors towards PPI-focused anticancer drug design [29, 30]. The inhibition of the oncogenic activity of the two splicing-associated proteins by developing PPI-focused anticancer modulators appears to be a promising therapeutic alternative in cancer.

The rational optimization of molecular interactions is becoming increasingly important in PPI-focused anti-cancer drug discovery processes. The putative interaction between SNRPG and the RING finger domain of RBBP6

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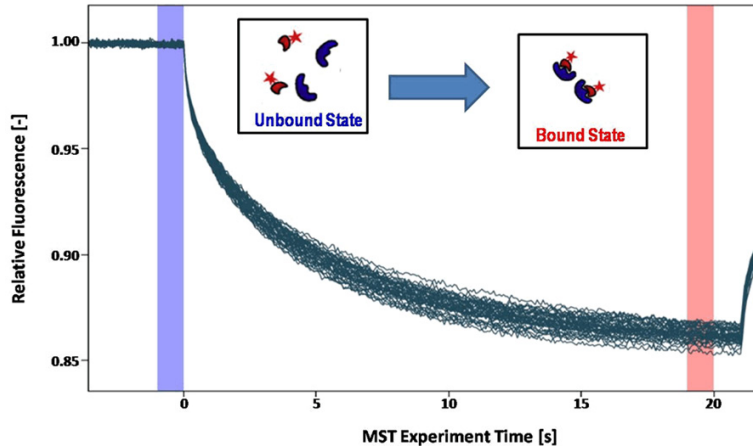


Figure 3. Thermograph of SNRPG binding to the RING finger domain of RBBP6 at 25 °C. Multiple MST traces were recorded for different mixture ratios of the SNRPG and RING finger domain of RBBP6. The cold region is set to 0 seconds (blue) and the hot region to 20 seconds (red) to determine the K_D of the interaction and to avoid potential convection phenomena.

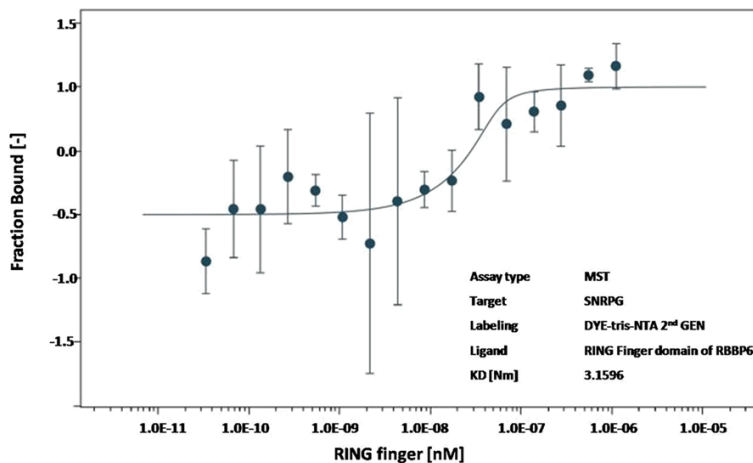


Figure 4. Dose-response curve for the binding interaction between SNRPG and the RING finger domain of RBBP6. The concentration of SNRPG protein was kept constant at 50 nM, while the ligand concentration varied from 1.11 μ M to 0.03 nM. The binding affinity measurement for the interaction observed a K_D of 3.1596 nM. The experiments were performed at 25 °C with a 30 min incubation at medium MST and 40% LED power.

has been suggested over the years using the yeast 2-hybrid (Y2H) technique. Two copies of SNRPG (conformational isomers of the same protein) were identified as part of the five substrates that bind to the RING finger domain of RBBP6 [16]. In another study, Chibi and co-workers [21] also suggested that SNRPG interacts with the N-terminal domain of RBBP6, which is a crucial component of the RNA processing machinery in the cell. The findings from the two studies are biologically interest-

ing considering that the two proteins are highly active in cancer-cell networks and vary in their expression profiles within different types of cancers. More so, the two proteins play active roles as core-splicing regulatory proteins in mRNA metabolism, which is a crucial process in tumor development and tumorigenesis. The findings suggest a high likelihood of the possible involvement between SNRPG and the RING finger domain of RBBP6 in tumorigenesis and tumor development.

Accordingly, PPIs identified using the Y2H assay are subject to verification by a series of other biochemical assays such as co-immunoprecipitation (co-IP), pull-down and colocalization experiments. This path works well only for strong PPIs. Weak binary PPIs might not be readily detectable by less-sensitive assays [31, 32]. Most analytical techniques for PPIs are expensive, time-consuming and require high amounts of sample. For this reason, a microscale thermophoresis analysis for the characterisation of the binding affinity between SNRPG and the RING finger domain of RBBP6 was developed.

This study provides the first evidence of quantitative interaction affinity measurement involving the two oncogenic proteins using the MST assay. It provides an optimized methodology suitable for studying other potential oncogenic regulatory core-splicing partners. The results from the MST experiment observed high binding affinity between SNRPG and the RING finger domain of RBBP6 with a K_D value in the low nanomolar concentration range of 3.1596 nM. The experiment was conducted at 25 °C with 30 minutes incubation time at medium MST and 40% LED power. The results confirm the findings by Kappo and co-

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workers [16] that suggested possible involvement between the SNRPG and the RING finger domain of RBBP6. Indeed, there is strong binding between the two proteins as depicted from the low nanomolar range K_D value.

Generally, weak PPIs in the low nanomolar range are poorly understood even though they are crucial in mediating therapeutically relevant biological processes in health and disease states. According to Salahudeen and Nishtala [33], the lower the K_D value in weak PPIs, the stronger the binding and the higher the binding affinity. Conversely, the higher the K_D value is, the weaker the binding and the lower the binding affinity. From a drug discovery point of view, the aim is to identify disease-relevant PPIs with lower K_D values (i.e., high binding affinity). Such PPIs have strong binding strength and can be used as targets for inhibition using small molecule inhibitors for PPI-focused anticancer drug discovery [33, 34]. Therefore, this study could be used as a starting point to perform high-throughput screening with SNRPG~RING finger domain of RBBP6 inhibitors to evaluate the ability of small molecules to modulate the affinity of the two proteins. The modulation of this interaction would represent a major breakthrough in the development of new strategies targeting immune escape in oncology.

The successful characterization of PPIs between oncogenic proteins and the determination of their binding affinity measurements using the MST technique has been reported in various cancer-related studies. Magnez and co-workers [35] have used the MST assay to determine the binding affinity between the transmembrane glycoprotein PD-1 and a type I transmembrane protein PDL1, which are involved in tumour escape processes towards designing small molecule inhibitors as anti-cancer drug agents. In another study, Liberelle and co-workers [36] provided the first MST interaction affinity measurement involving the oncogenic ErbB2 protein tyrosine kinase receptor and its membrane partner, the MUC4 mucin, to validate finding small molecule binding affinities for targeting the MUC4-ErbB2 protein complex for drug discovery in cancer. These studies are useful indices to justify the potential of using the results from this MST assay to further explore the interaction between SNRPG and the RING finger domain of RBBP6 as potential

anti-cancer drug targets in PPI-focused drug discovery.

The physical interaction between SNRPG and the RING finger domain of RBBP6 provides the first mechanistic insight of their structure-function relationship. The observation is of particular interest in pharmacological research since it provides a platform to study their possible involvement in cancer-cell networks towards anti-cancer drug discovery [37, 38]. An accurate deciphering of the binding affinity between the two proteins is essential for therapeutic optimization and modifications by small molecule inhibitors [29, 30]. Currently, there are no developed therapeutic approaches that target blockage of the SNRPG-RING finger domain of RBBP6 protein complex. Over the years, many similar studies in cancer reporting the successful modulation of therapeutically relevant onco-proteins have been conducted yielding promising results. Some drugs have already been approved, while others have entered clinical trials (summarised in **Table 2**).

PPI-focused anticancer drug strategies targeting interactions such as the MDM2/p53, Bcl-2/Bax, XIAP/caspase-9, Hsp90/Cdc37, c-Myc/Max, KRAS/PDE δ , CD40/CD40L, Skp2/Skp, Keap1/Nrf2 and PD-1/PD-L1 have shed light on the role of protein complexes in the quest to drug the once undruggable proteome space. The studies indicate that PPIs have great potential as intervention targets for novel treatments of refractory types of cancers and their regulation is an indispensably promising strategy in drug discovery. Blocking the two regulatory splicing proteins can help generate new anti-cancer 'lead' compounds and thus produce new treatment drugs [39, 40].

Targeting core-splicing regulatory proteins for anti-cancer drugs remains the epitome of future prospectives in PPI-focused drug discovery. Given the complexity of splicing regulation and its centrality in driving biological processes in pathological states, targeting the interaction between SNRPG and the RING finger domain of RBBP6 for drug discovery in cancer may provide a better understanding into the future of PPI-focused drug discovery from a different perspective. The biological connection between the splicing machinery and apoptosis, a phenomenon that allows the regulated

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Table 2. Examples of PPIs that have yielded modulators that are either approved or in clinical trials

PPI	Related disease	Drug	Status	References
PD-1/PD-L1	Non-small lung cancer	Keytruda	Approved-2014	[41]
PD-1/PD-L1	Non-small lung cancer	Opdivo	Approved-2014	[42]
PD-1/PD-L1	Non-small lung cancer	Tecentriq	Approved-2016	[43]
Bcl-2/Bax	Chronic lymphocytic leukemia	ABT-199	Approved-2016	[44]
PD-1/PD-L1	Merkel cell carcinoma	Bavencio	Approved-2017	[45]
PD-1/PD-L1	Non-small lung cancer	Imfinzi	Approved-2017	[46]
MDM2/p53	Acute myeloid leukemia	Idasanutlin	Phase III	[47]
MDM2/p53	Metastatic melanoma	AMG232	Phase I/II	[48]
MDM2/p53	Solid tumor with p53 wild type status	CGM097	Phase I	[49]
MDM2/p53	Advanced solid tumor, lymphoma	DS-3032b	Phase I	[50]
MDM2/p53	Neoplasm malignant	SAR405838	Phase I	[51]
MDM2/p53	Advanced solid tumors, lymphomas	ALRN-6924	Phase I/II	[52]
XIAP/caspase-9	Relapsed or refractory multiple myeloma	LCL-161	Phase II	[53]
XIAP/caspase-9	Recurrent head and neck squamous cell carcinoma	TL32711	Phase I	[54]
XIAP/caspase-9	Solid tumors, lymphoma	ASTX-660	Phase I/II	[55]
XIAP/caspase-9	Solid cancers	GDC-0917	Phase I	[56]
B-catenin/CBP	Liver cirrhosis	RPI-724	Phase I/II	[57]
PD-1/PD-L1	Prostatic neoplasms	CA-170	Phase II	[58]
CD40/CD40L	Advanced solid tumors	ABBV-428	Phase I	[59]

destruction and disposal of damaged or unwanted cells, remains an overlooked arsenal in designing anti-cancer therapies. Defects in the regulation of apoptosis have been associated with dysfunctional splicing patterns of a large number of apoptotic factors in tumorigenesis. Therefore, the modulation of anti-apoptotic and pro-apoptotic proteins via pharmaceutical manipulation of regulatory core-splicing proteins may open up new therapeutic avenues for the treatment of cancer. Thus, targeting the interaction between SNRPG and the RING finger domain of RBBP6 for drug discovery is a “bottom-up” approach in addressing the issues surrounding tumorigenesis and tumor development.

Conclusion

The panoply of using MST technology to detect and quantify high-affinity and therapeutically relevant PPIs towards PPI-focused drug discovery remains of particular interest in biomedical research. Deciphering the binding affinity of therapeutic proteins in cancer is essential for their rational optimization towards designing PPI-focused anti-cancer adjuvants. The MST analysis presented in this study provides the first mechanistic *in vitro* insight of the interaction between SNRPG and the RING finger

domain of RBBP6. The obtained results are coherent and in perfect agreement with previous suggestions implicating possible involvement between the two proteins. The study affirms and strongly establishes scientific pursuit insinuating the possible *in vivo* involvement between the two regulatory core-splicing proteins in cancer-cell networks. The study strongly disqualifies the oversight placed on the two onco-proteins in developing PPI-focused smart drugs, thus showing SNRPG and the RING finger domain of RBBP6 as potential anti-cancer drug candidates. Further exploration into their molecular and structural mechanism of action could significantly validate their efficacy as potential PPI-focused anti-cancer drug-gable targets. Identifying small molecule ‘lead’ compounds capable of modulating the interaction between SNRPG and the RING finger domain of RBBP6 could be the ‘missing link’ in the puzzle of the “quest for the cure”.

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Disclosure of conflict of interest

None.

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References

- [1] Plach MG, Grasser K and Schubert T. MicroScale Thermophoresis as a tool to study protein-peptide interactions in the context of large eukaryotic protein complexes. *Bio Protoc* 2017; 7: e2632.
- [2] Rainard JM, Pandarakalam GC and McElroy SP. Using microscale thermophoresis to characterize hits from high-throughput screening: a European lead factory perspective. *SLAS Discov* 2018; 23: 225-241.
- [3] Wienken CJ, Baaske P, Rothbauer U, Braun D and Duhr S. Protein-binding assays in biological liquids using microscale thermophoresis. *Nat Commun* 2010; 1: 100.
- [4] Nimmagadda A, Shi Y and Cai J. γ -AApeptides as a new strategy for therapeutic development. *Curr Med Chem* 2019; 26: 2313-2329.
- [5] Jonsson PF, Cavanna T, Zicha D and Bates PA. Cluster analysis of networks generated through homology: automatic identification of important protein communities involved in cancer metastasis. *BMC Bioinformatics* 2006; 7: 2.
- [6] Steinbrecher T and Labahn A. Towards accurate free energy calculations in ligand protein-binding studies. *Curr Med Chem* 2010; 17: 767-785.
- [7] Bhandari GP, Angdembe MR, Dhimal M, Neupane S and Bhusal C. State of non-communicable diseases in Nepal. *BMC Public Health* 2014; 14: 23.
- [8] Heneghan C, Blacklock C, Perera R, Davis R, Banerjee A, Gill P, Liew S, Chamas L, Hernandez J and Mahtani K. Evidence for non-communicable diseases: analysis of Cochrane reviews and randomised trials by world bank classification. *BMJ Open* 2013; 3: e003298.
- [9] Conte N, Charafe-Jauffret E, Delaval B, Adélaïde J, Ginestier C, Geneix J, Isnardon D, Jacquemier J and Birnbaum D. Carcinogenesis and translational controls: TACC1 is down-regulated in human cancers and associates with mRNA regulators. *Oncogene* 2002; 21: 5619-5630.
- [10] Shi Y, Di Giammartino DC, Taylor D, Sarkeshik A, Rice WJ, Yates JR 3rd, Frank J and Manley JL. Molecular architecture of the human pre-mRNA 3' processing complex. *Mol Cell* 2009; 33: 365-376.
- [11] Lührmann R, Kastner B and Bach M. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim Biophys Acta* 1990; 1087: 265-292.
- [12] Palfi Z, Lücke S, Lahm HW, Lane WS, Krufft V, Bragado-Nilsson E, Séraphin B and Bindereif A. The spliceosomal snRNP core complex of *Trypanosoma brucei*: cloning and functional analysis reveals seven Sm protein constituents. *Proc Natl Acad Sci U S A* 2000; 97: 8967-8972.
- [13] Stevens SW and Abelson J. Purification of the yeast U4/U6.U5 small nuclear ribonucleoprotein particle and identification of its proteins. *Proc Natl Acad Sci U S A* 1999; 96: 7226-7231.
- [14] Will CL and Lührmann R. Spliceosome structure and function. *Cold Spring Harb Perspect Biol* 2011; 3: a003707.
- [15] Hull R, Oosthuysen B, Cajee UF, Mokgohloa L, Nweke E, Antunes RJ, Coetzer TH and Ntwasa M. The drosophila retinoblastoma binding protein 6 family member has two isoforms and is potentially involved in embryonic patterning. *Int J Mol Sci* 2015; 16: 10242-10266.
- [16] Kappo MA, Eiso A, Hassem F, Atkinson RA, Faro A, Muleya V, Mulaudzi T, Poole JO, McKenzie JM, Chibi M, Moolman-Smook JC, Rees DJ and Pugh DJ. Solution structure of RING finger-like domain of retinoblastoma-binding protein-6 (RBBP6) suggests it functions as a U-box. *J Biol Chem* 2012; 287: 7146-7158.
- [17] Pugh DJ, Eiso A, Faro A, Luya PT, Hoffmann E and Rees DJ. DWNN, a novel ubiquitin-like domain, implicates RBBP6 in mRNA processing and ubiquitin-like pathways. *BMC Struct Biol* 2006; 6: 1.
- [18] Khan F, Allam M, Tincho MB and Pretorius A. Implications of RBBP6 in various types of cancer. *Proceedings IWBBIO* 2014.
- [19] Li L, Deng B, Xing G, Teng Y, Tian C, Cheng X, Yin X, Yang J, Gao X, Zhu Y, Sun Q, Zhang L, Yang X and He F. PACT is a negative regulator of p53 and essential for cell growth and embryonic development. *Proc Natl Acad Sci U S A* 2007; 104: 7951-7956.
- [20] Simons A, Melamed-Bessudo C, Wolkowicz R, Sperling J, Sperling R, Eisenbach L and Rotter V. PACT: cloning and characterization of a cellular p53 binding protein that interacts with Rb. *Oncogene* 1997; 14: 145-155.

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- [21] Chibi M, Meyer M, Skepu A, G Rees DJ, Moolman-Smook JC and Pugh DJ. RBBP6 interacts with multifunctional protein YB-1 through its RING finger domain, leading to ubiquitination and proteosomal degradation of YB-1. *J Mol Biol* 2008; 384: 908-916.
- [22] Mabonga L and Kappo AP. The oncogenic potential of small nuclear ribonucleoprotein polypeptide G: a comprehensive and perspective view. *Am J Transl Res* 2019; 11: 6702-6716.
- [23] Mrozowich T, Meier-Stephenson V and Patel TR. Microscale thermophoresis: warming up to a new biomolecular interaction technique. *Biochemist* 2019; 41: 8-12.
- [24] Bartoschik T, Galinec S, Kleusch C, Walkiewicz K, Breitsprecher D, Weigert S, Muller YA, You C, Piehler J, Vercruyse T, Daelemans D and Tschammer N. Near-native, site-specific and purification-free protein labeling for quantitative protein interaction analysis by MicroScale Thermophoresis. *Sci Rep* 2018; 8: 4977.
- [25] Berleth M, Berleth N, Minges A, Hänsch S, Burkart RC, Stork B, Stahl Y, Weidtkamp-Peters S, Simon R and Groth G. Molecular analysis of protein-protein interactions in the ethylene pathway in the different ethylene receptor sub-families. *Front Plant Sci* 2019; 10: 726.
- [26] Schubert T and Längst G. Studying epigenetic interactions using microscale thermophoresis (MST). *AIMS Biophys* 2015; 2: 370-380.
- [27] Bekic I, Molnar M and Tschammer N. Protein labeling-improved quantitation of biomolecular interactions by MST using the His-Tag labeling kit RED-tris-NTA 2nd generation. 2018.
- [28] Lata S, Reichel A, Brock R, Tampé R and Piehler J. High-affinity adaptors for switchable recognition of histidine-tagged proteins. *J Am Chem Soc* 2005; 127: 10205-10215.
- [29] Chen DS and Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* 2013; 39: 1-10.
- [30] Rueckert C and Guzmán CA. Vaccines: from empirical development to rational design. *PLoS Pathog* 2012; 8: e1003001.
- [31] Ito T, Ota K, Kubota H, Yamaguchi Y, Chiba T, Sakuraba K and Yoshida M. Roles for the two-hybrid system in exploration of the yeast protein interactome. *Mol Cell Proteomics* 2002; 1: 561-566.
- [32] Phizicky EM and Fields S. Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 1995; 59: 94-123.
- [33] Salahudeen MS and Nishtala PS. An overview of pharmacodynamic modelling, ligand-binding approach and its application in clinical practice. *Saudi Pharm J* 2017; 25: 165-175.
- [34] Kenakin T and Primer AP. Techniques for more effective and strategic drug discovery. Amsterdam, The Netherlands: Academic Press; 2014.
- [35] Magnez R, Thiroux B, Taront S, Segoula Z, Quesnel B and Thuru X. PD-1/PD-L1 binding studies using microscale thermophoresis. *Sci Rep* 2017; 7: 17623.
- [36] Liberelle M, Magnez R, Thuru X, Bencheikh Y, Ravez S, Quenon C, Drucbert AS, Foulon C, Melnyk P, Van Seuningen I and Lebègue N. MUC4-ErbB2 oncogenic complex: binding studies using microscale thermophoresis. *Sci Rep* 2019; 9: 16678.
- [37] Mellman I, Coukos G and Dranoff G. Cancer immunotherapy comes of age. *Nature* 2011; 480: 480-489.
- [38] O'Shea JJ, Kontzias A, Yamaoka K, Tanaka Y and Laurence A. Janus kinase inhibitors in autoimmune diseases. *Ann Rheum Dis* 2013; 72 Suppl 2: ii111-ii115.
- [39] Sharma P and Allison JP. The future of immune checkpoint therapy. *Science* 2015; 348: 56-61.
- [40] Sunshine J and Taube JM. Pd-1/pd-l1 inhibitors. *Curr Opin Pharmacol* 2015; 23: 32-38.
- [41] Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csósz T, Fülöp A, Gottfried M, Peled N, Tafreshi A and Cuffe S. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. *N Engl J Med* 2016; 375: 1823-1833.
- [42] Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhäufel M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crinò L, Blumenschein GR Jr, Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F and Brahmer JR. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med* 2015; 373: 1627-1639.
- [43] Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, Rodríguez-Abreu D, Moro-Sibilot D, Thomas CA and Barlesi F. Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. *N Engl J Med* 2018; 378: 2288-2301.
- [44] Korycka-Wolowicz A, Wolowicz D, Kubiak-Mlonka A and Robak T. Venetoclax in the treatment of chronic lymphocytic leukemia. *Expert Opin Drug Metab Toxicol* 2019; 15: 353-366.
- [45] Boyerinas B, Jochems C, Fantini M, Heery CR, Gulley JL, Tsang KY and Schlom J. Antibody-dependent cellular cytotoxicity activity of a novel anti-PD-L1 antibody avelumab (MSB-0010718C) on human tumor cells. *Cancer Immunol Res* 2015; 3: 1148-1157.
- [46] Antonia SJ, Villegas A, Daniel D, Vicente D, Murakami S, Hui R, Yokoi T, Chiappori A, Lee KH and de Wit M. Durvalumab after chemoradiotherapy in stage III non-small-cell lung cancer. *N Engl J Med* 2017; 377: 1919-1929.

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- [47] Lehmann C, Friess T, Birzele F, Kiialainen A and Dangl M. Superior anti-tumor activity of the MDM2 antagonist idasanutlin and the Bcl-2 inhibitor venetoclax in p53 wild-type acute myeloid leukemia models. *J Hematol Oncol* 2016; 9: 50.
- [48] Sun D, Li Z, Rew Y, Gribble M, Bartberger MD, Beck HP, Canon J, Chen A, Chen X, Chow D, Deignan J, Duquette J, Eksterowicz J, Fisher B, Fox BM, Fu J, Gonzalez AZ, Gonzalez-Lopez De Turiso F, Houze JB, Huang X, Jiang M, Jin L, Kayser F, Liu JJ, Lo MC, Long AM, Lucas B, McGee LR, McIntosh J, Mihalic J, Oliner JD, Osgood T, Peterson ML, Roveto P, Saiki AY, Shaffer P, Toteva M, Wang Y, Wang YC, Wortman S, Yakowec P, Yan X, Ye Q, Yu D, Yu M, Zhao X, Zhou J, Zhu J, Olson SH and Medina JC. Discovery of AMG 232, a potent, selective, and orally bioavailable MDM2-p53 inhibitor in clinical development. *J Med Chem* 2014; 57: 1454-1472.
- [49] Holzer P, Masuya K, Furet P, Kallen J, Valat-Stachyra T, Ferretti S, Berghausen J, Bouisset-Leonard M, Buschmann N, Pissot-Soldermann C, Rynn C, Ruetz S, Stutz S, Chène P, Jeay S and Gessier F. Discovery of a dihydroisoquinolinone derivative (NVP-CGM097): a highly potent and selective MDM2 inhibitor undergoing phase 1 clinical trials in p53wt tumors. *J Med Chem* 2015; 58: 6348-6358.
- [50] Arnhold V, Schmelz K, Proba J, Winkler A, Wünschel J, Toedling J, Deubzer HE, Künkele A, Eggert A and Schulte JH. Reactivating TP53 signaling by the novel MDM2 inhibitor DS-3032b as a therapeutic option for high-risk neuroblastoma. *Oncotarget* 2018; 9: 2304-2319.
- [51] De Weger V, Lolkema M, Dickson M, Le Cesne A, Wagner A, Merqui-Roelvink M, Varga A, Tap W, Schwartz G and Demetri G. 378 A first-in-human (FIH) safety and pharmacological study of SAR405838, a novel HDM2 antagonist, in patients with solid malignancies. *Eur J Cancer* 2014; 50: 121-122.
- [52] Carvajal LA, Neria DB, Senecal A, Benard L, Thiruthuvanathan V, Yatsenko T, Narayanagari SR, Wheat JC, Todorova TI, Mitchell K, Kenworthy C, Guerlavais V, Annis DA, Bartholdy B, Will B, Anampa JD, Mantzaris I, Aivado M, Singer RH, Coleman RA, Verma A and Steidl U. Dual inhibition of MDMX and MDM2 as a therapeutic strategy in leukemia. *Sci Transl Med* 2018; 10: eaao3003.
- [53] West A, Martin BP, Andrews D, Hogg S, Banerjee A, Grigoriadis G, Johnstone R and Shortt J. The SMAC mimetic, LCL-161, reduces survival in aggressive MYC-driven lymphoma while promoting susceptibility to endotoxin shock. *Oncogenesis* 2016; 5: e216.
- [54] Benetatos CA, Mitsuuchi Y, Burns JM, Neiman EM, Condon SM, Yu G, Seipel ME, Kapoor GS, LaPorte MG, Rippin SR, Deng Y, Hendi MS, Tirunahari PK, Lee YH, Haimowitz T, Alexander MD, Graham MA, Weng D, Shi Y, McKinlay MA and Chunduru SK. Birinapant (TL32711), a bivalent SMAC mimetic, targets TRAF2-associated cIAPs, abrogates TNF-induced NF- κ B activation, and is active in patient-derived xenograft models. *Mol Cancer Ther* 2014; 13: 867-879.
- [55] Ward GA, Lewis EJ, Ahn JS, Johnson CN, Lyons JF, Martins V, Munck JM, Rich SJ, Smyth T, Thompson NT, Williams PA, Wilsher NE, Wallis NG and Chessari G. ASTX660, a novel non-peptidomimetic antagonist of cIAP1/2 and XIAP, potently induces TNF α -dependent apoptosis in cancer cell lines and inhibits tumor growth. *Mol Cancer Ther* 2018; 17: 1381-1391.
- [56] Wong H, Gould SE, Budha N, Darbonne WC, Kadel EE, La H, Alicke B, Halladay JS, Erickson R and Portera C. Learning and confirming with preclinical studies: modeling and simulation in the discovery of GDC-0917, an inhibitor of apoptosis proteins antagonist. *Drug Metab Dispos* 2013; 41: 2104-2113.
- [57] Kimura K, Ikoma A, Shibakawa M, Shimoda S, Harada K, Saio M, Imamura J, Osawa Y, Kimura M, Nishikawa K, Okusaka T, Morita S, Inoue K, Kanto T, Todaka K, Nakanishi Y, Kohara M and Mizokami M. Safety, tolerability, and preliminary efficacy of the anti-fibrotic small molecule PRI-724, a CBP/ β -catenin inhibitor, in patients with hepatitis C virus-related cirrhosis: a single-center, open-label, dose escalation phase 1 trial. *EBioMedicine* 2017; 23: 79-87.
- [58] Musielak B, Kocik J, Skalniak L, Magiera-Mularz K, Sala D, Czub M, Stec M, Siedlar M, Holak TA and Plewka J. CA-170-a potent small-molecule PD-L1 inhibitor or not? *Molecules* 2019; 24: 2804.
- [59] Ye S, Cohen D, Belmar NA, Choi D, Tan SS, Sho M, Akamatsu Y, Kim H, Iyer R and Cabel J. A bispecific molecule targeting CD40 and tumor antigen mesothelin enhances tumor-specific immunity. *Cancer Immunol Res* 2019; 7: 1864-1875.

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Table S1. MST raw data of merged dose-response of the PPIs between SNRPG and RING finger domain of RBBP6

Dose	Response (Average)	Std. Dev.	N
1.11E-06	867.28155	0.9235	3
5.55E-07	866.9055	0.26835	2
2.775E-07	865.66652	1.66455	2
1.3875E-07	865.42005	0.81742	2
6.9375E-08	864.91562	2.33324	2
3.46875E-08	866.01389	1.33826	3
1.734375E-08	862.59946	1.25891	2
8.671875E-09	862.22974	0.74229	2
4.335938E-09	861.75527	4.2119	2
2.167969E-09	860.03	5.31867	2
1.083984E-09	861.10854	0.89908	2
5.41992E-10	862.19	0.63159	2
2.70996E-10	862.75342	1.93067	3
1.35498E-10	861.43567	2.59197	3
6.7749E-11	861.44066	1.97434	3
3.3875E-11	859.30191	1.3296	3