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Biochemical characterization of TRIM72 E3 ligase and its interaction with the insulin receptor substrate 1

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ABSTRACT

TRIM family of E3 ubiquitin ligases have an amino-terminal conserved tripartite motif consisting of RING, B-Box, coiled-coil domain and different C-terminal domain leading it to classification into 11 subclasses. TRIM72 is an E3 ligase of class IV and subclass 1 with its role in a multitude of cellular processes. Despite being crucial in multiple cellular processes, TRIM72 still hasn't been biochemically characterized. In the present study, we have characterized the oligomeric status of TRIM72 and found that it forms both monomers, dimers, and tetramers. We have screened a set of 12 E2s and identified two novel E2 enzymes (Ubch5c and Ubch10) that work in cooperation with TRIM72. Nevertheless, E3 ligase activity is minimal and we propose that additional regulation is required to enhance its E3 ligase activity. We have also used surface plasmon resonance to study interaction with one of its substrate proteins, IRS1, and identified the PH domain of IRS1 is mediating interaction with the TRIM72 E3 ligase while the PTB domain of IRS1, does not show any interaction.

1. Introduction

Protein ubiquitination is a posttranslational modification playing a crucial role in maintaining homeostasis of the cell [1]. It regulates a multitude of cellular processes like apoptosis, DNA replication, and repair, cell cycle progression, transcription and autophagy [2–4]. Ubiquitination process involves the covalent attachment of a ubiquitin (~76 amino acid) tag to a target protein through an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue of a target protein [5]. The ubiquitin conjugation process involves the three important enzymes: ubiquitin activating enzyme (E1) that forms an ATP-dependent labile thioester linkage with the carboxyl-terminal group of ubiquitin through its cysteine thiol group, thereby activating the C-terminus of ubiquitin for nucleophilic attack; a conjugating enzyme (E2) that transiently carries the activated ubiquitin molecule as a thiol ester; and an ubiquitin ligase (E3) that finally transfers the activated ubiquitin from the E2 to the ε-amino group of an acceptor lysine residue of the substrate [6]. E3 ligases play a pivotal role in recognizing the substrate and in determining the target of the substrate depending on the distinct ubiquitin chains attach to lysine residue. They have been classified on the basis of their catalytic domain as HECT, RING, U Box and RBR E3 ligases [7–9].

The TRIpartite Motif containing family of proteins or TRIMs, constitute a subset of RING E3 ligases that possess an amino-terminal conserved tripartite motif consisting of RING, B-Box, and coiled-coil domains [10]. They also have a C-terminal domain, which is majorly a PRY-SPRY, NHL or Bromo domain, leading to a classification of these into 11 sub-classes [11]. The RBCC motif is well conserved in TRIM class family, with ~70 TRIMs identified in humans [12]. However, very few of them have been fully characterized. The E3 ligase activity is within the RING domain which is coordinated to two zinc ions. Of the B-box domains; B1 and B2 may be present together or B2 is found alone. The B-boxes have zinc finger motifs with conserved cysteine and histidine residues, but their function is still not discovered. The next domain is the coiled coil domain, formed by multiple intertwining alpha helices, with a foremost role in homo and hetero oligomerization [13]. TRIM family of proteins are known to be involved in many biological processes and are associated with developmental disorders, neurodegenerative diseases, viral infections, cardiovascular diseases and cancer [41,42].[43][44]

Abbreviations: E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligases; IRS1, Insulin receptor substrate 1; MALLS, Multi-angle Laser Light scattering; SPR, Surface Plasmon Resonance

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TRIM72 is an E3 ligase of class IV subclass 1, with its role defined in many different cellular processes [14–16]. Membrane repair is one such process wherein it is recruited to an oxidative environment, and complex formation occurs with dysferlin and Caveolin-3 [17,18]. It is also shown to help in the wound healing process by regulating the TGF-beta signaling. The overexpression of TRIM72 is known to induce diabetic cardiomyopathy in mice, accompanied by compromised glucose uptake, increased lipid accumulation, myocardial hypertrophy, fibrosis, and cardiac dysfunction [19,20]. Functioning as an E3 ligase, it degrades the insulin receptor and insulin receptor substrate 1 (IRS1) resulting in insulin resistance, obesity, hypertension, and dyslipidemia, which constitute metabolic syndrome in mice models [19]. Thus, the E3 ligase activity of TRIM72 plays a crucial role in insulin resistance, but in vitro, this activity has not yet been characterized.

Here, we have performed autoubiquitination assays in vitro, to gain insight into TRIM72 E3 ligase activity and identifed novel sets of E2 enzymes working in cooperation with TRIM72. We have used a surface plasmon resonance approach to identify the interaction of TRIM72 with one of its substrate proteins IRS1.

2. Material and methods

2.1. Cloning, expression, and purification of proteins

Provided in supplementary material and methods

2.1.1. Molecular weight determination using MALLS

Purified catalytic RING domain ($TRIM72^{RING}$) and full length protein ($TRIM72^{FL}$) were run on gel filtration column coupled to Multi-angle Laser Light scattering (MALS) using the miniDawn Tristar light scattering detector (Wyatt technologies, USA). After equilibration of the Superdex 75 10/300 column (GE Healthcare) and Superdex 200 10/300 GL column with buffer (10mM Hepes pH7.5, 150mM NaCl and 1mM DTT) respectively, proteins sample were loaded. The molecular weight was determined by using the refractive index signal with the manufacture's software (Astra software).

2.3. Thermal unfolding of proteins

Tryptophan fluorescence based thermal unfolding experiment was performed using the Prometheus NT.48. $TRIM72^{FL}$ protein concentration of 10μM was used to screen 24 different buffer conditions with variation in pH 7.5, 8.0 and 8.5, salt concentration 50mM, 100mM, 150mM and 200mM NaCl, reducing agent 1mM DTT and additives 10% glycerol. The capillaries containing 10μl of the $TRIM72^{FL}$ protein in respective buffers were inserted into the machine, the temperature was increased at a rate of 1 °C/min from 20 °C to 90 °C and the fluorescence at emission wavelengths of 330nm and 350nm was measured. The ratio of fluorescence intensities at 350nm and 330nm as a function of temperature was used to determine the transition temperature, which is interpreted as the melting temperature. Simultaneously, the aggregation onset temperature (T_{agg}) of the protein samples was detected by measuring back reflection intensity of a light beam that passes through the capillary twice [21].

2.4. Fluorescent labelling of ubiquitin

Ubiquitin with cysteine residue introduced at the N-terminus after the methionine position 1 was prepared as described before [22] and labelled using maleimide-linked TAMRA dye (Thermo Scientific, T6027). 20μM of protein was diluted in buffer 25mM Tris pH8.0, 150mM NaCl and 50mM DTT and incubated at room temperature for 30 min. Buffer exchange was carried out using a desalting column to remove the DTT. Protein was then incubated with TAMRA dye (50μM) and incubated at 4 °C for 2 h. The excess dye was removed by passing through a desalting column (PD-10 Columns, GE Healthcare) and buffer exchange via Centricon (3MWCO, Millipore).

2.5. Auto-ubiquitination assay

$TRIM72^{FL}$ and different domains were ubiquitinated using 500nM hUBA1, 5μM E2, 10μM TAMRA labelled ubiquitin, 10μM E3 ligase and 10mM ATP in 20mM Tris pH8.0, 150mM NaCl, 1mM TCEP, 10mM $MgCl_2$ for different time incubation at 37 °C. Samples were collected by stopping the reaction at given times by adding non-reducing SDS-PAGE dye. Samples were run on 4–12% NuPAGE gels (Invitrogen) in MOPS buffer (Life Technologies) and the fluorescent TAMRA signal was read out using the ChemiDoc XRS system (Biorad).

2.6. Biotinylation of $IRS1^{PH}$, $IRS1^{PTB}$ for SPR measurements

PH and PTB domain of IRS (hereafter $IRS1^{PH}$ and $IRS1^{PTB}$) at 25μM were biotinylated overnight at 4 °C by mixing with 25μM EZ-link-Sulfo–NHS–LC-Biotin (Thermo Fischer, 21338) in a reaction volume of 500μl. Excess of biotin was removed by passing through the Zeba Spin Desalting column (Thermo Scientific, 89890) according to the manufacturer's protocol.

2.7. Surface plasmon resonance (SPR) binding

Surface Plasmon Resonance experiments were carried in the Biacore T200 instrument (GE Healthcare) at 25 °C. The binding experiments were performed in buffer HBS-P+ buffer (10mM HEPES pH 7.4, 150mM NaCl, and 0.005% Surfactant P20). $IRS1^{PH}$ and $IRS1^{PTB}$ labelled by NHS biotin were captured on the SA sensor chip (GE Healthcare) to an immobilization level of approximately 1500 RU on flow cells 2 (Fc2) and (Fc4) respectively. The binding experiments were carried out in a single cycle kinetics mode. $TRIM72^{FL}$ and TRIM72 PRY SPRY domain ($TRIM72^{PRYSPRY}$) were serially diluted in running buffer, and injected at a flow rate of 30 μl/min across both surface for 60 s and dissociation was set up for 60 s. Data from the reference flow cell were subtracted for all runs. The analysis was done using GraphPad Prism 7 software (GraphPad Software Inc, USA).

3. Results and discussion

3.1. Molecular cloning and expression of proteins

Ubiquitin (cysteine introduced at N-terminal) from (pETNKI-hisSUMO3-LIC) [21] vector and Ubc5Hc from (pGEX6p) vector were subcloned to pETM11 vector using Nco1 and Kpn1 restriction sites. $TRIM72^{FL}$ and $TRIM72^{PRYSPRY}$ were amplified by the polymerase chain reaction and cloned with Nco1 and Kpn1 restriction sites in pETM41 and pETM30 vectors. $TRIM72^{RING}$ and $TRIM72^{RLBCC}$ were cloned with N-terminal His tag in pETM11 using respective primers (Supplementary Table S1). All the clones were screened with restriction digestion and they produced the band of the respective sizes. The expression of the proteins was done in different expression strains at 18 °C for overnight. All the proteins were purified to homogeneity using affinity purification and ion exchange columns. The purified protein was analyzed by size exclusion chromatography on Superdex 75 10/300 or Superdex 200 10/300 depending upon the molecular weight.

3.2. Biophysical characterization of proteins using MALLS

For the in vitro analysis of TRIM72, we assess the oligomeric state of the $TRIM72^{RING}$, and $TRIM72^{FL}$ by analytical size-exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS). Samples were run at concentrations as indicated to define the oligomeric status of the proteins. $TRIM72^{RING}$ was run at two different concentrations (500μM and 1mM) and found to elute as a monomer,

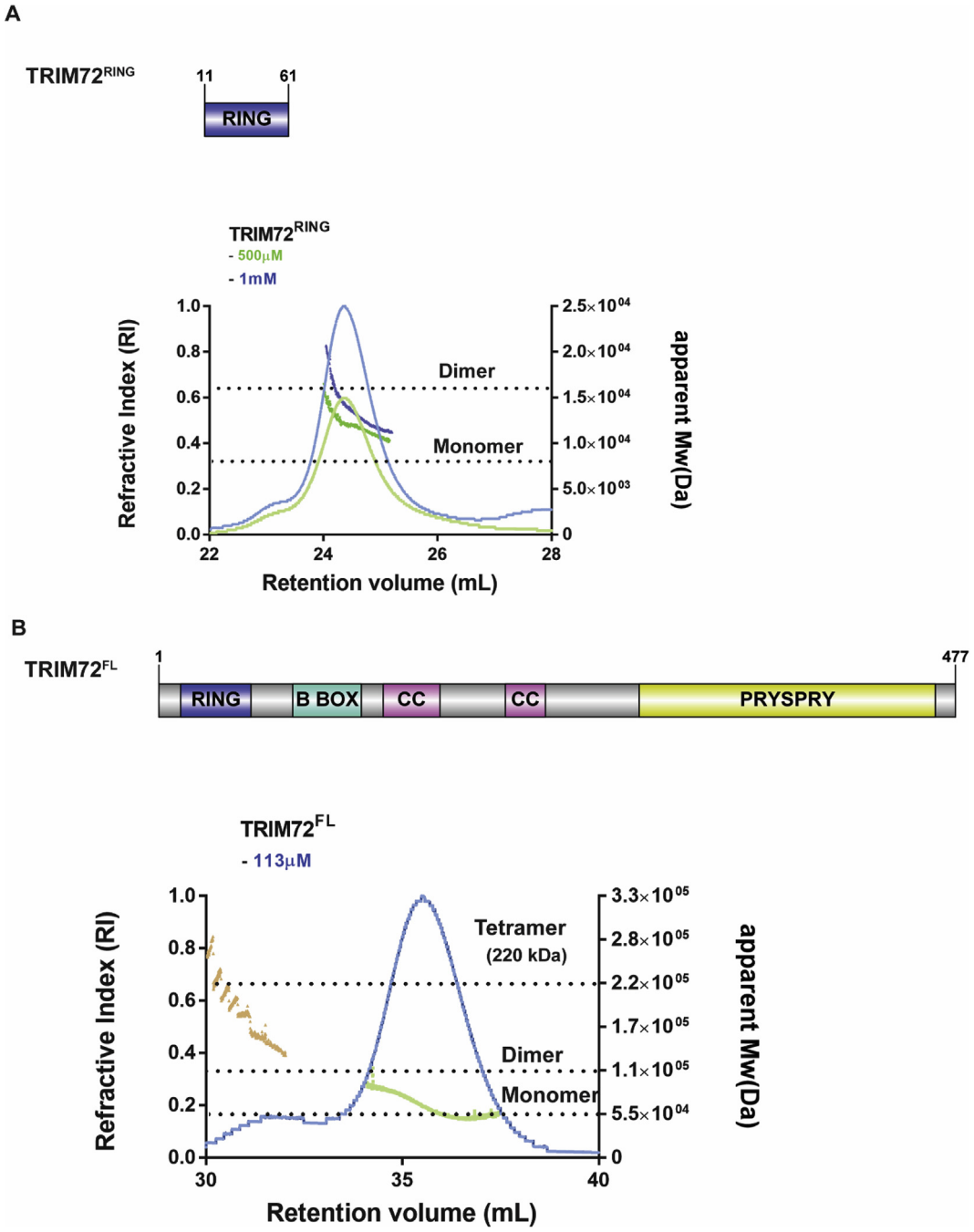

Fig. 1. (A) TRIM72^{RING}, MALLS signal as the refractive Index (RI) is plotted against the retention volume. (B) TRIM72^{FL} domain representation. RING domain, BBox domain, CC- Coiled Coil and PRYSPRY domain. MALLS signal as the refractive Index (RI) is plotted against the retention volume.

but with an increase in concentration, it shifts towards the dimeric form. This suggests TRIM72^{RING} exists in dynamic monomer-dimer equilibrium (Fig. 1A). TRIM72^{FL} (113μM) eluted in two peaks, one small peak with a mass that corresponds to a tetramer form (220 kDa) and the major peak whose mass conforms to a dimer-monomer equilibrium (Fig. 1B). The Ring domain in the E3 ligases are known to homodimerize while the coiled coil domain in the TRIM is known to form an anti-parallel dimer [23–26]. This suggests that the tetramer observed here could be due to the combined effect of the coiled coil induced dimerization and the RING mediated dimerization.

3.3. Thermal unfolding of TRIM72

Buffer screening was performed at different pH, salt concentration and with the addition of glycerol to determine a buffer that would enhance the stability of TRIM72. The ratio of 330/350nm and its first derivative was used to determine the onset temperature of unfolding (T_{on}) and melting temperature (T_m) of the protein. The addition of glycerol increased the T_{on} from 42.5 °C to 48.3 °C and T_m significantly from 50.7 °C to 54.7 °C (Fig. 2A and B). At pH 8.0 and 8.5, salt concentration 150mM, 200mM and addition of glycerol, no aggregation during heating was detected, indicating high colloidal stability of protein in the above condition (Fig. 2C). At pH 7.5 with and without glycerol, the samples form aggregates and is detected by increase in signal of aggregation detector, indicating low protein stability (Supplementary Fig. S1A). The best buffer tested was Tris pH 8.0, 150mM NaCl, 1mM DTT and 10% glycerol with maximum stability and no aggregation (Supplementary Table 2).

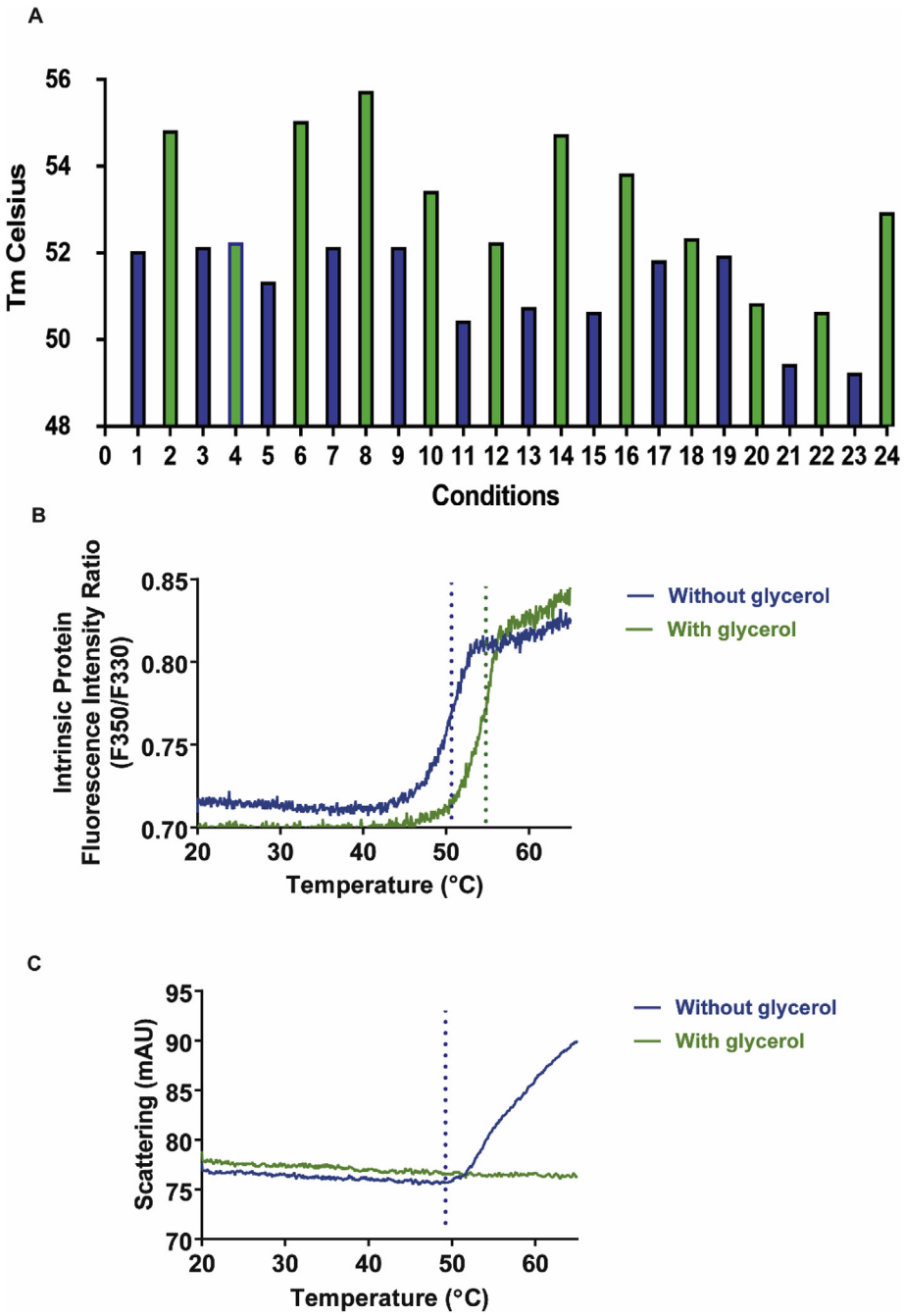

Fig. 2. (A) Bar Graph showing different buffers condition and the melting temperature of TRIM72^{FL}. Blue color bar represent buffer condition without glycerol and green color bar with the addition of glycerol. The buffer conditions are listed in Supplementary Table 2 (B) Thermal stability of two buffer conditions with and without glycerol. Two wavelengths, 330 and 350nm are recorded and ratio of the wavelength is plotted against temperature. The dotted line represents the melting temperature (T_m) of protein. (C) Analysis of protein stability with back reflection aggregation detection. The back reflection scattering intensity (mAU) is plotted against the temperature and onset temperature of aggregation (T_{agg}) is represented by dotted line. The represented buffer condition is Tris pH 8.0, 150mM NaCl, 1mM DTT without glycerol and with 10% glycerol.

3.4. Analysis of TRIM72 E3 ligase activity

To assess the catalytic activity of TRIM72, we carried out auto-ubiquitination assays with UbE2H, an E2 that was identified in previous studies [5]. The assay was carried out at 37 °C for different time points (Supplementary Fig. S2 A). We noticed very minimal catalytic activity compared to other E3 ligases as reported in the literature with their subsequent E2 enzymes (Supplementary Fig.S2 B).

We hypothesized that UbE2H may not be the best E2 for this TRIM72 activity and other E2s might enhance its activity. To address this, we screened ~12 E2 enzymes including UbE2H at 37 °C for 60 min (Supplementary Fig. S3 B and D). Control experiments were carried out to confirm that ubiquitin chains are dependent on the presence of the E3 and not by the E2 alone in an E3 ligase-independent activity (Supplementary Fig. S3 A and C). In this assay, we found that TRIM72 preferred UbcH5c and Ubch10 as the ubiquitin conjugating enzyme for in vitro autoubiquitination.

Further in order to compare the catalytic activity of TRIM72 with the previously established E2, UbE2H relative to the new E2s (UbcH5c and Ubch10), ubiquitination assays were set up at timepoint 15, 30, 60 and 90 min. We observed the activity of the TRIM72 was enhanced in the presence of the E2s; Ubch5c and Ubch10 (Fig. 3A, B, and C). But it was considerably less compared to other TRIM E3 ligases activity reported in the literature. Control experiments were set where only E2s dependent ubiquitin chains were observed as E2-Ub. The E2-Ub chains were quite prominent with UbcH10 and were shown as E2-Ub_2 and E2-Ub_3. These experiments helped to differentiate the E3-Ub chains with E2-Ub chains and are critical to determine E2-E3 specificity. The E3-Ub chains were detected at 15 min with all the three E2 enzymes (UbE2H, Ubch5c and Ubch10) and was observed to increase with time.

We have predicted autoinhibition by the C-terminal TRIM72^{PRYSPRY} may be the cause of the low enzymatic activity of TRIM72^{FL}. It has been

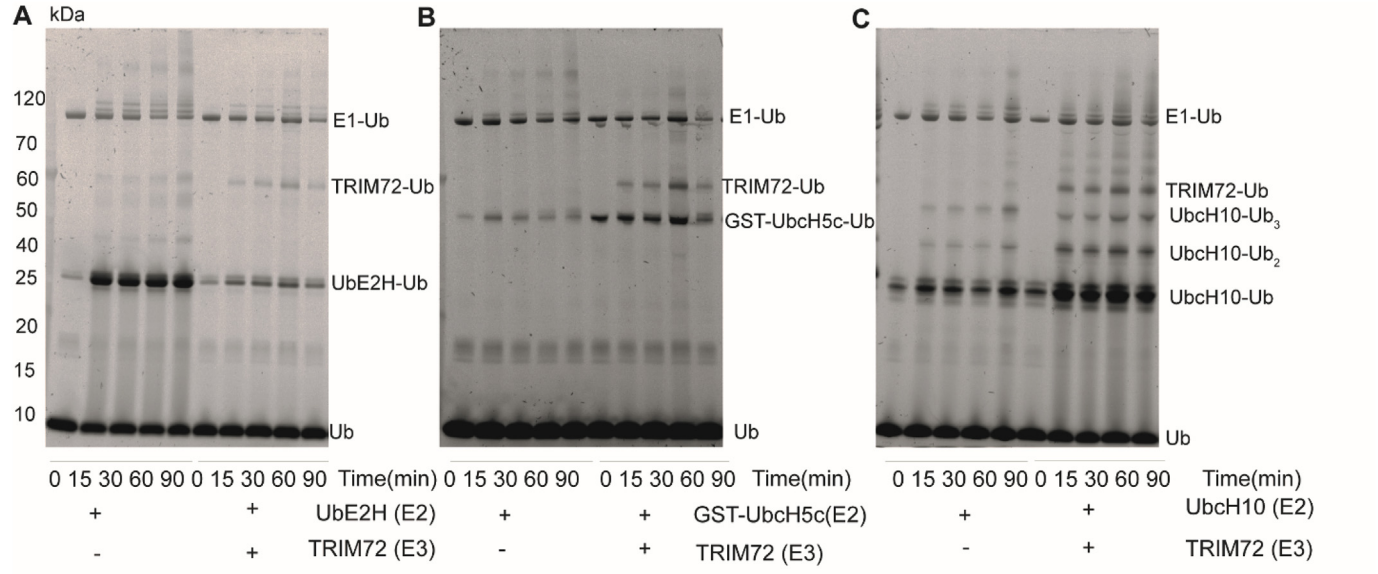

Fig. 3. (A) Comparison of TRIM72 invitro ubiquitination activity with UbE2H (B) UbcH5c (C) UbcH10. The TAMRA signal is visualized on the gel by using TAMRA labelled ubiquitin in assay. The reversible covalent bonds are indicated as E1-Ub, E2-Ub and E3-Ub (TRIM72-Ub). Time course of ubiquitination reaction is conducted at different time points in presence (+) or absence (-) of E2 enzymes. The number on the left represents the molecular marker in kDa.

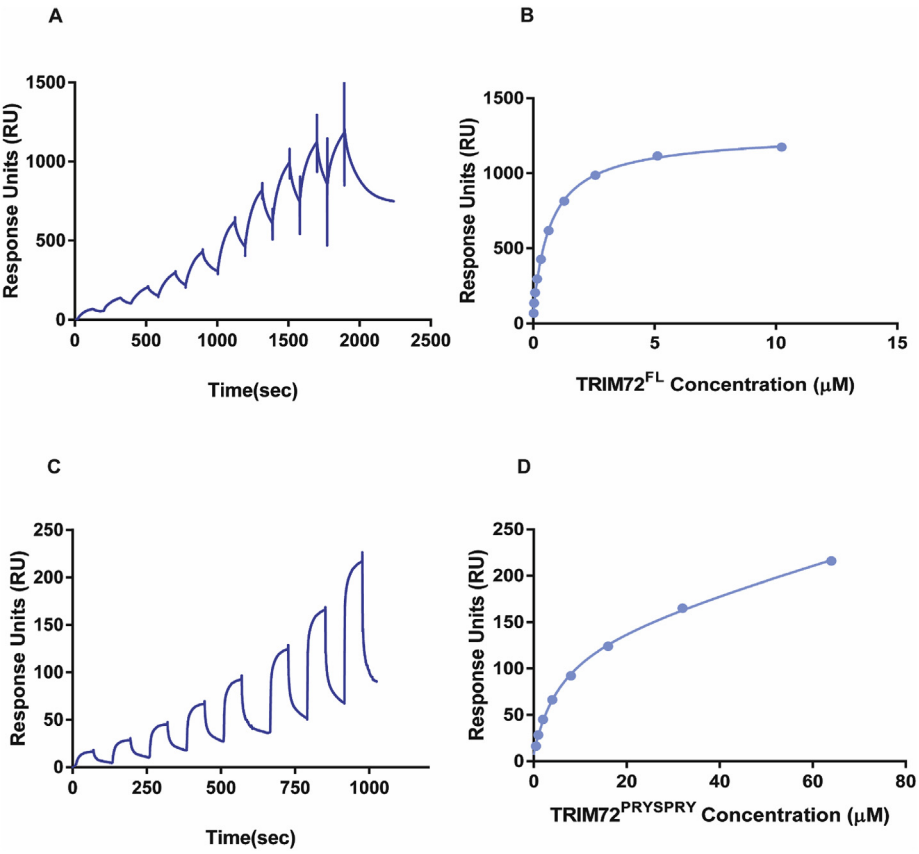

Fig. 4. (A) Single-cycle Kinetics profile of $TRIM72^{FL}$ binding with the $IRS1^{PH}$. (B) Affinity profile plotted with concentration and Response Units (RU) of $TRIM72^{FL}$ binding with the $IRS1^{PH}$. (C) Single-cycle Kinetics profile of $TRIM72^{PRYSPRY}$binding with the $IRS1^{PH}$. (D) Affinity profile plotted with concentration and Response Units (RU) of $TRIM72^{PRYSPRY}$ binding with the $IRS1^{PH}$.

shown by recent studies that E3 ligase HECT WWP2, activity is auto-inhibited by the linker region present between WW2 and WW3 domains [27]. To gain better insight into the molecular basis we generated a construct of $TRIM72^{RLBCC}$ and carried out a ubiquitination assay at 37 °C (Supplementary Fig.S4 A). Again, our result suggested, no change in the activity and therefore we ruled out an autoinhibition mechanism for low enzymatic activity for TRIM72.

Another possible mechanism we postulated is the dimerization of the E3 ligase for its activity. Dimerization could be mediated by either formation of homodimers or heterodimers. We used a GST-fused $TRIM72^{RLBCC}$ construct for setting up a ubiquitination assay at two different concentration 20 and 40μM. As GST is an obligate dimer, this may help to oligomerize $TRIM72^{RLBCC}$ and potentially increase its activity if homodimerization is required (Supplementary Fig.S4 B). In

practice, however, this construct does not change TRIM72 activity.

In all these studies the TRIM72 E3 ligase activity was still very weak, relative to other TRIMs [28,29]. Our results ruled out the possibility of auto inhibition and homodimerization as one of the mechanisms adapted by TRIM72 to influence its E3 ligase activity. However, we assumed the minimal E3 ligase activity might be dependent on the other sets of E3 ligases where it forms a heterodimer and become active otherwise exist in an inactive form. In skeletal muscle TRIM72 is highly expressed and plays crucial role in membrane repair, there are other set of E3 ligases; TRIM32, TRIM62 and TRIM63 that are highly expressed in skeletal muscle and they might play a major role in enhancing the E3 ligase activity of TRIM72 [30–33]. Recently TRIM32 is identified to invitro ubiquitinate TRIM72, suggesting to influence the E3 ligase activity of TRIM72 [34]. A recent study has also shown a particular class of TRIM E3 ligase (TRIM28, TRIM33, and TRIM24) functioning as chromatin regulators to be working as an inactive E3 ligase that may require additional E3s to stimulate its E3 ligase activity [35]. We speculate that TRIM72 E3 ligase is also one of the inactive E3 ligases.

3.5. $TRIM72^{FL}$ and $TRIM72^{PRYSPRY}$ show preferred binding to $IRS1^{PH}$

To study the interactions of the $TRIM72^{FL}$ or its protein-protein interacting domain $TRIM72^{PRYSPRY}$ with IRS1 domains PH and PTB (IRS^{PH} and IRS^{PTB}), surface plasmon resonance (SPR) was performed. PH and PTB domain were biotinylated and captured on the SA sensor chip, $TRIM72^{FL}$ and $TRIM72^{PRYSPRY}$ was flowed over the chip at different concentrations. We observed that $IRS1^{PH}$ shows relatively high binding affinity (700nM) to $TRIM72^{FL}$ (Fig. 4A) while more moderate affinity (5.8μM) for $TRIM72^{PRYSPRY}$ (Fig. 4B) suggesting that other domains of TRIM72 (RING and B-Box coiled coil) also contribute towards binding of $IRS1^{PH}$. To further study the interaction of individual domains (RING and B-Box coiled coil) within TRIM72 with $IRS1^{PH}$, experiments were set up, but due to non-specific binding of these proteins on reference SA sensor surface, no binding curves could be obtained. Repeated attempts with the addition of 5mM $MgCl_2$, 0.05mM EDTA, 0.05% Tween 20, 1 mg/ml BSA and 1 mg/ml Dextran in buffer was done to reduce non-specific binding but no success was obtained. Different available sensor surface such as CM5, C1, NTA, and SA were also used by using different binding chemistry to capture the protein, but non-specificity still could not be reduced. We also attempted to immobilize TRIM72 domains and flow the IRS^{PH} but it also showed non-specific binding to the reference surface. Therefore, we were not able to conclude the role of the other domains of TRIM72 towards $IRS1^{PH}$ binding. Interestingly, no binding of $IRS1^{PTB}$ was observed with either $TRIM72^{FL}$ or $TRIM72^{PRYSPRY,}$ mapping the binding of TRIM72 primarily to the $IRS1^{PH}$ domain.

4. Conclusion

TRIM72 is the E3 ligase of class IV subclass, has multiple domains such as Ring, B-box, coiled coil and PRY SPRY domain. We have identified oligomerization behavior of TRIM72; due to the presence of coiled coil domain. Interestingly, TRIM72 exists as tetramer and dimer-monomer equilibrium suggests its dynamic nature of interaction with other proteins and diverse role in the cell [26,36]. TRIM E3 ligase has a RING catalytic domain whose activity depends on the cooperating with the E2 enzymes; therefore, we have performed a screening of different sets of E2 enzymes along with the known E2 (UbE2H), in order to know the potential E2 for TRIM72 activity. Our results also ruled out the possibility of auto inhibition and homodimerization as one of the mechanisms adapted by TRIM72 to influence its E3 ligase activity. However, we assumed the minimal E3 ligase activity might be dependent on the other sets of E3 ligases where it forms a heterodimer and become active otherwise exist in an inactive form. Heterodimeric E3 ligases are also quite common as BRCA1-BARD1, Mdm2-MdmX, and RING1B-Bmi1 [26,36]. In these complexes, the E3 ligase activity for BARD1, MdmX, and Bmi1 is absent and they become active only after the heterodimerization with their partners. TRIM E3 ligase also have the ability to self-associate due to the presence of coiled coil domain and able to form and functionally active as a heterodimer [37]. Another reason influencing the catalytic activity of TRIM72 E3 ligase could be due to the post-translational modifications it requires prior to ubiquitination such as phosphorylation [38]. An additional possibility to influence E3 ligase activity of TRIM72 is role of the deubiquitinating enzymes which are known to stabilize TRIM72 and further enhance its activity. Few deubiquitinating enzymes are identified to influence the E3 ligase activity such as USP8 is known to interact and stabilize Nrdp1 Ring E3 ligase, and USP19 stabilizes HRD1 E3 ligase [39,40]. These assumptions, however, require further clarification and exploration. TRIM72 is known to mediate the ubiquitin mediated destruction of IRS1. This protein has two main domains characterized by PH and PTB. Through our study, we have been able to identify that the IRS1 PH domain can interact with the $TRIM72^{FL}$ and its C-terminal PRY SPRY domain.

Declaration of competing interest

The authors declare they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100729.

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