

Structural and functional characterization of Class IV TRIM E3 ligases

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MPhil Thesis

Statement of Originality

I declare that all the work presented in this thesis is my own work, performed in the Rittinger lab while at the Francis Crick Institute, London, UK. I also certify that where necessary information that was sourced outside of this thesis is correctly referenced.

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Abstract

Ubiquitination is a highly abundant post-translation modification that is involved in the control of a large number of cellular processes. Target ubiquitination is achieved through the action of three separate enzymes; the E1, ubiquitin-activating enzyme, the E2, ubiquitin-conjugating enzyme and the E3, ubiquitin ligase. TRIM E3 ligases are the largest family of RING-type E3 ligases and are classified by a N-terminal tripartite motif consisting of the catalytic RING domain, one or two B-box domains, B1 and B2, and a coiled-coil domain. In addition, most TRIMs possess a C-terminal substrate-binding domain, which classifies them into one of eleven TRIM classes. The PRYSPRY domain is the most common substrate-binding domain in humans and links class IV TRIMs to roles in cellular innate immunity. TRIM22 and TRIM6, are Class IV TRIMs that share high sequence identity with the well-studied HIV restriction factor, TRIM5 α , and have also been implicated in the anti-viral response. TRIM22 is reported to function directly as a viral restriction factor against RNA viruses such as, IAV, HCV and EMCV. While TRIM6 functions to activate the innate immune signalling pathway through activation of the immune signalling factor, IKK ϵ . Aspects of TRIM22 and TRIM6 function remain understudied, including their biochemical and biophysical properties and this is the focus of this study. The results described herein outline key differences in the self-association properties of these proteins in comparison to TRIM5 α . Furthermore, they highlight discrepancies between the ubiquitination profiles of TRIM22 and TRIM6 presented in the literature and the activity observed in this study. Overall, this emphasizes the need for further study of the roles of TRIM22 and TRIM6, to verify current proposed functions, as well as identify potential additional functions within the cell.

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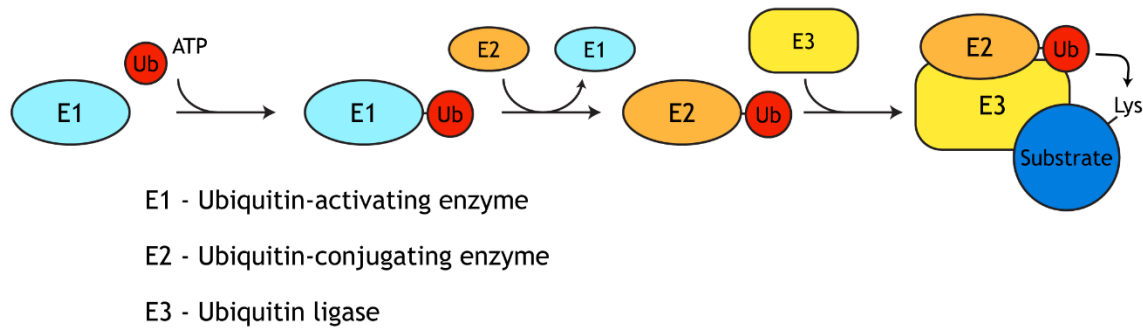
1. Introduction

1.1 Ubiquitination

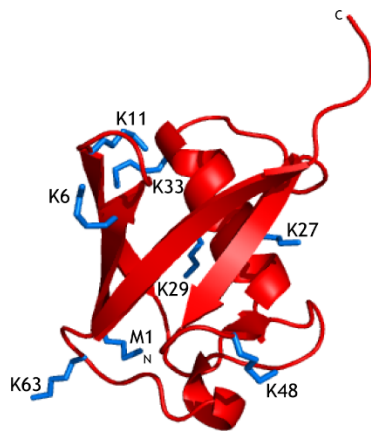
Ubiquitination is a fundamental and highly conserved post-translational modification, which is present in all eukaryotes, with analogous systems also existing in bacteria and archaea [1]. Ubiquitination describes the covalent modification of proteins with a 76 amino acid protein, ubiquitin (Ub), which shows remarkable sequence similarity across eukaryotes [2]. Ubiquitination is involved in the control of most cellular processes including cell cycle progression, immune signaling and proteasomal degradation, through which it was originally discovered [3].

Ubiquitination occurs via a three-enzyme cascade involving ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes (Fig. 1A). Initially, the ubiquitin molecule is activated by the E1 enzyme in an ATP-dependent manner resulting in the conjugation of the ubiquitin C-terminal carboxyl group to the E1 active site cysteine via a thioester bond (represented by ~) [4]. From the E1~Ub conjugate, the ubiquitin is transferred through a transthioesterification reaction to the active site cysteine of the E2 enzyme. Finally, the E3 enzyme interacts with both the E2~Ub conjugate (E2~Ub) and the substrate, facilitating the transfer of ubiquitin onto the target amino group [5]. The modified target can be any exposed lysine residue on the surface of the substrate, one of the seven lysine residues that exist within the ubiquitin molecule (K6, K9, K11, K27, K33, K48, K63) or alternatively the N-terminal amino group of the target protein (Fig. 1B) [3][6]. Modification of one or multiple lysine residues within a target protein is referred to as mono or multi-monoubiquitination and is often responsible for altering the cellular localisation of substrates (Fig. 1C) [3]. In contrast, polyubiquitination describes the formation of chains of ubiquitin through ubiquitination of amino groups within ubiquitin itself. Eight types of homotypic ubiquitin chains exist, in addition to more recent discoveries of heterotypic and branched chains containing multiple lysine linkages (Fig. 1C) [3]. This diversity allows translation of a wide variety of distinct messages to downstream partners, most of which are not entirely understood. However, certain ubiquitin chains have been well-studied including K48-linked chains, which produce the well-defined proteasomal degradation signal, as well as K63-linked chains which trigger protein signaling cascades [7].

A.



B.



C.

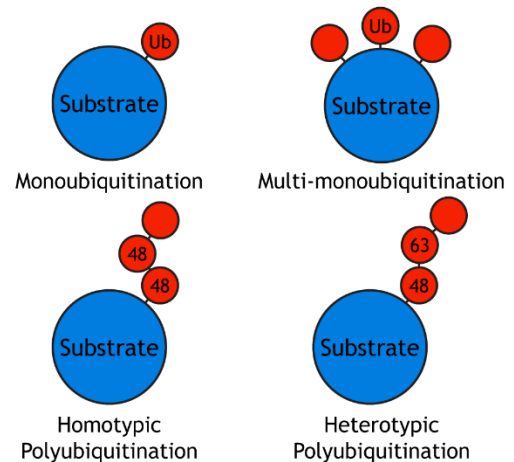


Figure 1 - Ubiquitination and the ubiquitin cascade. A. Ubiquitination occurs in a three-step pathway; initially the ubiquitin molecule is activated in an ATP-dependent manner by the ubiquitin-activating enzyme (E1) leading to conjugation to the E1 active-site cysteine. The ubiquitin is then shuttled from the E1 active site cysteine to that of the ubiquitin-conjugating enzyme (E2) forming an E2-ubiquitin conjugate (E2~Ub), before the ubiquitin ligase (E3) acts as an adaptor bringing both the E2~Ub and the substrate together to enabling ubiquitin transfer to the substrate. B. Ubiquitin is an 8.6 kDa protein that forms a unique β -grasp fold, it possesses seven internal lysines residues and an N-terminal amino group of the starting methionine (side chains shown in blue and labelled with residue number), all of which can be modified by ubiquitin itself. PDB: 1UBQ C. Ubiquitin modifications come in three different forms, mono-ubiquitination, multi-monoubiquitination, and polyubiquitination. Polyubiquitination itself also comes in variety of forms including homotypic, heterotypic and branched chains.

Within the human genome, the E1, E2 and E3 genes are organised in a pyramidal manner, with two E1, around 40 E2, and over 300 E3 ligase genes [8]. In the cell, the two E1s, UBA1 and UBA6, play a major and minor role respectively [8]. UBA6 is responsible for ubiquitin charging of UBE2Z, whereas all other E2s are charged by UBA1 [9]. At cellular steady state, most E2s exist in their charged form ready for activation by cognate E3s. The choice of E2 by a given E3 can determine the biological consequence of ubiquitination. Different E2s have different reactivity profiles and in some cases control aspects of ubiquitination such as chain linkage, chain length and

chain processivity. The E2, UBE2K, for example, can only form ubiquitin chains of K48 linkage, while the E2, UBE2W, is only capable of chain initiation via monoubiquitination of the N-terminal α -amino group of substrates [9][10]. In contrast, the heterodimeric E2, UBE2N/UEV1 is incapable of protein target lysine modification and instead conjugates ubiquitin to another ubiquitin molecule and hence either forms unanchored chains or extends pre-existing ubiquitin modifications, via K63 linkages [12]. *In vitro*, individual E2s can interact with a variety of E3s and vice versa, however this biochemically observed reactivity isn't necessarily biologically relevant due to additional factors such as protein expression levels and spatiotemporal representation. This area of research is still largely unexplored and despite some well characterized E2-E3 partners, *in vivo* E2-E3 coupling is still not well understood.

1.2 RING E3 ligases

It is the E3 enzymes in most part, that achieve substrate specificity of the ubiquitination pathway and they are divided into three different families: RING, HECT and RBR ligases [13]. Of these three families, the RING E3 ligases represent the largest one and they achieve target ubiquitination through direct transfer of the ubiquitin from the E2~Ub onto the substrate [14]. RING E3 ligases consist minimally of a catalytic RING domain and a substrate-binding domain. The RING domain, which shares a conserved pattern of zinc-coordinating cysteine and histidine residues, is responsible for interaction with both components of the E2~Ub intermediate. Interactions of the RING domain with the E2~Ub conjugate bias the otherwise flexible ensemble towards an active ("closed") conformation in which the C-terminal tail of Ub is extended along the surface of the E2 exposing the E2~Ub thioester for nucleophilic attack by the target lysine residue (Fig. 2A) [5].

RING domains have multiple mechanisms of promoting the closed state of the E2~Ub. Some RING domains are active in their monomeric form, in which case, additional elements outside of the RING core, such as a phosphorylated tyrosine residue (Y363) in the case of Cbl-b, are utilized to stabilise the E2~Ub closed conformation (Fig 2B)[15]. Additionally, a large number of RING domains utilise either homo or hetero-dimerisation to achieve catalytic activity. In homodimeric RINGs, such as BIRC7 (Fig. 2C), both RING monomers are active and each interact with an E2 molecule, while the conjugated ubiquitins contact both protomers within the dimer [16]. Heterodimeric RINGs on the other hand, often consist of one active and one inactive RING domain, that come together to form a functional dimer, as is the case for the BRCA1-BARD1 heterodimer (Fig. 2D)[17], [18]. The coupling of the inactive RING to the active RING, increases ubiquitination activity, while in certain cases providing additional functions such as protein localization and increased protein stability [5]. Finally, RING domain-containing subunits also form part of large multi-domain E3 ligase complexes such as cullin-RING ligases (CRLs) (Fig. 2E) or the anaphase-promoting complex (APC) which are flexible in their subunit composition and recruit different substrates to be ubiquitinated by the resident RING E3 [19]-[21].

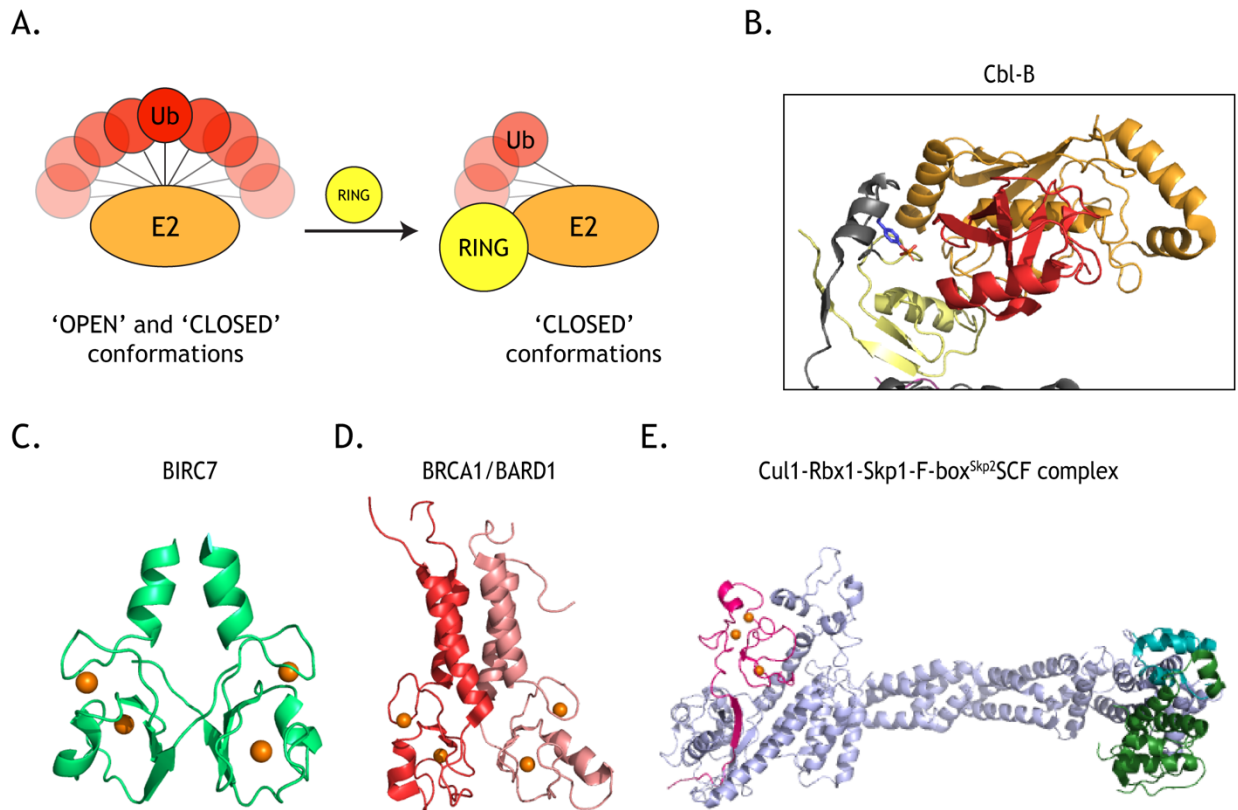


Figure 2 - RING E3 ligases. **A.** The mechanism of action of RING E3 ligases is achieved through interaction with both the E2 and ubiquitin molecules, biasing the E2~Ub conformational ensemble towards the closed conformation exposing the E2~Ub thioester bond for nucleophilic attack. **B.** Close-up view of the crystal structure of the monomeric RING pCbl-b in complex with UbCH5B-Ub and a ZAP70 peptide substrate. The RING domain of Cbl-B is shown in pale yellow while the substrate-binding domain is shown in dark grey. The side chain of the phosphorylated Y363 is highlighted in blue. The E2, UbCH5B, and ubiquitin are highlighted in orange and red, respectively. PDB: 3ZNI. **C.** Structure of the homodimeric RING domains of the E3 ligase, BIRC7, PDB: 4AUQ. **D.** Structure of the RING domains of the heterodimeric E3 enzyme, BRCA1/BARD4 (BRCA1 RING shown in salmon, BARD1 RING shown in tv red) PDB: 1JM7. **E.** Structure of the multi-subunit cullin-RING ligase (CRL), comprising the RING domain subunit Rbx1 (shown in hot pink), Cul1 (shown in light blue), Skp1 (shown in forest) and the F box of Skp2 (shown in teal) PDB: 1LDK.

1.3 TRIM E3 ligases

The largest sub-family of RING E3 ligases is the TRIM E3 ligase family with over 70 TRIM genes present in the human genome [22]. TRIM E3 ligases share a conserved domain architecture located at the N-terminus of the protein referred to as the tripartite motif, consisting of the catalytic RING domain, one or two B-box domains (B-box 1 (B1) and B-box 2 (B2)) and a coiled-coil region (CC) [23]. In addition to the tripartite motif most TRIMs possess one or more C-terminal domains that dictate substrate specificity and allow classification of TRIM E3 ligases into eleven classes in humans (Fig. 3) [24][25].

Although TRIM E3 ligases are present in all metazoans, their number are greatly increased in vertebrates, coinciding with the expansion of the adaptive immune system [22][26]. TRIM proteins have been implicated in a wide range of cellular processes, however their link to adaptive immunity is illustrated by their predominant role in cellular immune responses [24][25]. Disease-related mutations identified in TRIM proteins and the existence of viral antagonists to combat TRIM protein function, further outlines the fundamental role of this sub-family of E3 ligases [29]-[32].

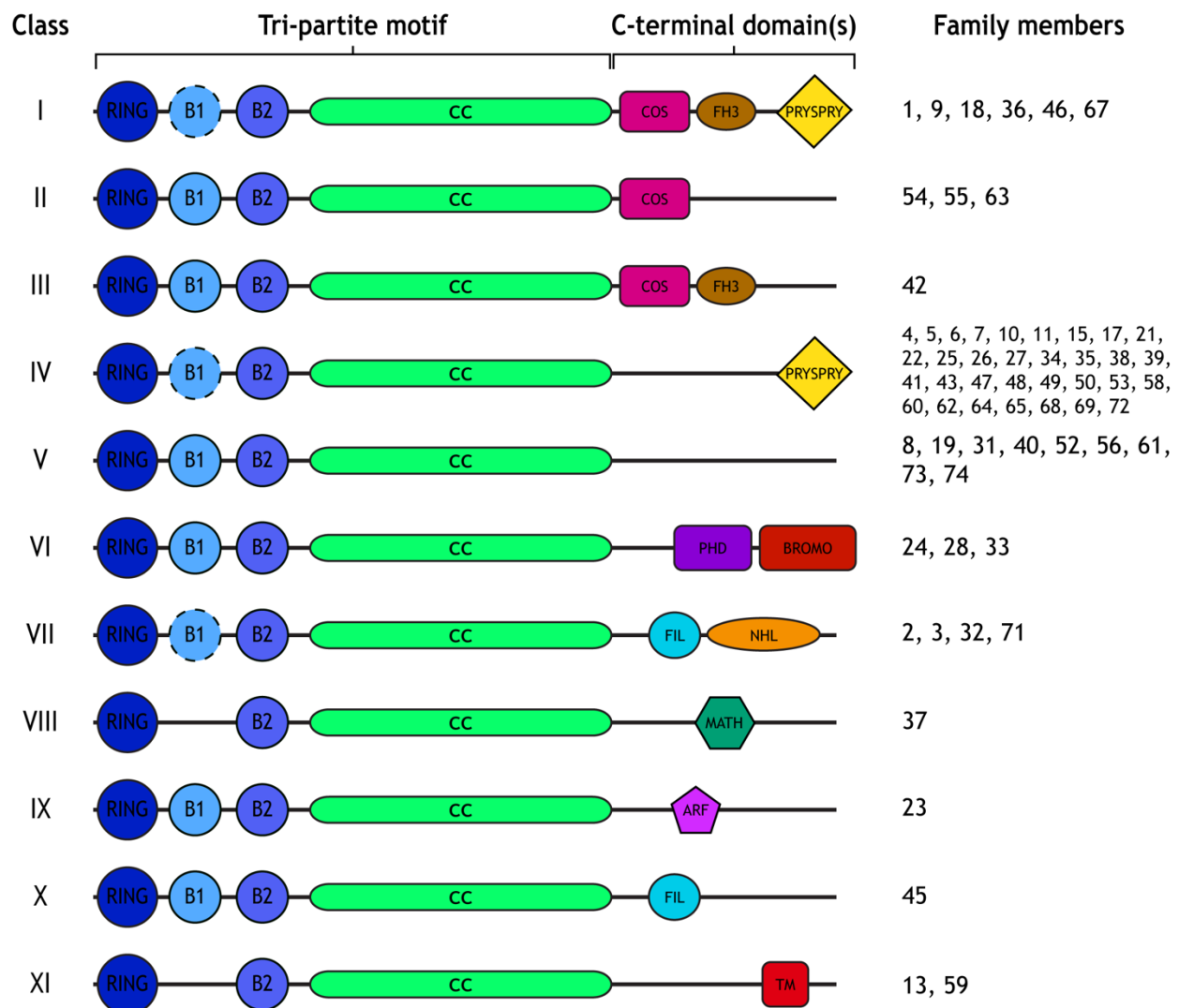


Figure 3 - TRIM E3 ligases. TRIM E3 ligases (TRIMs) are separated into 11 separate classes (I - XI) based on the identity of their C-terminal domains. TRIMs share a common tripartite motif consisting of a RING domain, followed by one or two B-boxes (labelled B1 or B2) and a coiled-coil domain (CC). Downstream of this tripartite motif, TRIMs possess one of the eleven existing C-terminal domains (PRYSPRY, COS, FN3, FIL, NHL, PHD, BROMO, ARF, MATH, ACID, TM). Dotted outlines indicate domains that are not present in all members of an individual class. The numbers of the individual family members are shown on the right.

One of the first and most extensively-studied TRIM E3 ligases is TRIM25. TRIM25 was initially identified as an essential regulator of innate immune signaling pathways through ubiquitination of the pattern recognition receptor, RIG-I [33]. Formation of K63-linked ubiquitin chains on the CARD domain of RIG-I were suggested to trigger downstream signaling cascades leading to IFN induction and promotion of a cellular anti-viral state [27], [34], [35]. The essential role of TRIM25 has been a matter of dispute over the past few years, with an increasing number of studies suggesting that another E3 ligase, Riplet, which also ubiquitinates the RIG-I receptor, is equally important if not more dominant in immune signaling pathway activation [36]-[39]. The precise division of labour between the two enzymes is still disputed, however it is clear from extensive studies that TRIM25 plays an important role in RIG-I mediated IFN induction.

Another well studied TRIM E3 ligase that functions in innate immunity is the splice variant alpha of TRIM5 (TRIM5 α), which functions as a species-specific retroviral restriction factor that in rhesus macaques (rhTRIM5 α) is a potent inhibitor of HIV infection [40]. rhTRIM5 α recognizes the HIV viral capsid through its C-terminal PRYSPRY domain triggering oligomerization and formation of a hexameric assembly that coats the viral capsid [41]. Restriction is then achieved in a dual manner, through inhibition of reverse transcription and induction of premature viral capsid disassembly, as well as through activation of immune signaling cascades. Both processes have been shown to require the ubiquitination activity of rhTRIM5 α [40], [42], [43]. Additionally, TRIM21 also has a well-characterised role in innate immunity, with studies outlining its function as a cytosolic antigen receptor that recognizes endocytosed, antibody-bound pathogens [44]. TRIM21 coating of these opsonised pathogen particles, triggers both pathogen degradation through the proteasome and anti-viral signaling pathways via the release of unanchored K63-linked ubiquitin chains upon pathogen recruitment to the proteasome [44], [45]. As previously mentioned, TRIMs also play key roles in other cellular processes and an example of this is TRIM28 which functions as a transcriptional co-regulator. TRIM28 interacts via its CC domain with the KRAB-domain containing zinc-finger proteins (KRAB-ZFPs) and is subsequently recruited to promoter regions of DNA where it is responsible for repressing the transcription of transposable elements [46], [47]. Intriguingly, this activity doesn't require ubiquitination activity and instead uses the unique SUMOylation capabilities of TRIM28 [48].

1.4 TRIM domain function

The RING domain is responsible for the E3 ligase activity of TRIMs and interacts with both constituents of the E2~Ub conjugate. Catalytic activity of the RING domain requires dimerization in those TRIMs studied in detail so far and this is achieved through formation of a four-helical bundle formed by additional N and C-terminal helices outside of the core RING fold (Fig. 4A) [49], [50]. Deletion or mutation within these regions abrogates activity, while TRIM E3 ligases that lack these secondary structure elements are intrinsically inactive [51]-[53]. Structures exist of multiple different TRIM E3 ligase RING domains, including in complex with the charged E2~Ub conjugate and

have illustrated that dimerisation provides additional contacts from the distal RING to the E2~Ub that promote the required closed E2~Ub conformation (Fig. 2A) [28][47][49]-[52]. Intriguingly, some studies of TRIM21 suggest that the monomeric RING is able to activate the E2~Ub, despite this, the RING domain still shows a tendency to dimerise in solution and crystallises in its dimeric form [52], [58]. Biophysical studies of a number of isolated TRIM E3 ligase RING domains have been performed and illustrate a range of dimerisation affinities across the family. RING dimerisation is also influenced by interactions with the E2~Ub, and the overall activity profile of each TRIM E3 ligase is contributed to by this dimerization affinity but also the degree to which the RING promotes the closed E2~Ub conformation.

Two different B-box domains exist in TRIM E3 ligases, B1 and B2, both of which are zinc-coordinating domains (Fig. 4B) [59]. The B-box domain is one of the defining features of this family and all TRIMs possess at least a B2 domain. In addition, a quarter of TRIMs contain tandem B-box domains in a conserved B1-B2 order. Studies of B1 and B2 domains have so far outlined roles in higher-order oligomerisation, substrate recruitment and auto-inhibition through protein-protein interactions, which might constitute a general function of these domains. However, more studies are needed before this can be assumed, likewise the reason for the presence of either single or tandem B-box domains is also still unclear [52], [60]-[62]. The best understood example of B-box domain function is that of rhTRIM5 α , for which the B2 domain provides the interaction interface for homotypic trimerisation, allowing it to form hexagonal arrays which interact with higher avidity with the HIV capsid restricting viral replication [60], [63], [64].

The CC domain is located in the central portion of TRIM E3 ligases and confers the ability to form dimers or higher order oligomers on these proteins. Several structures have reported an anti-parallel arrangement through a long central α -helix of approximately 170Å in length (Fig. 4C) [47], [64]-[67]. In these structures, additional smaller helices fold back onto the central helix and form a four-helical bundle (4HB). Secondary structure predictions and conserved heptad and hendecad sequence repeats within this region suggest that the central antiparallel dimer and the 4HB are common features across the TRIM family [67]. Importantly, this antiparallel arrangement places the two RING domains within the homodimer at opposite ends and therefore potentially presents a barrier to dimerization and subsequent ubiquitination activity. An increasing number of recent studies have illustrated a potential role for the CC as a protein-protein interaction motif, suggesting additional roles for this domain above and beyond its role as an antiparallel dimerization motif [31], [68]-[70].

The C-terminal domain(s) is the main determinant of substrate recognition and specificity for TRIMs and diverges greatly across the family (Fig. 3). The most common C-terminal domain is the PRYSPRY domain which is present in 60% of human TRIMs and is responsible for the family's expanded role in innate immunity (Fig. 4D) [71]. The PRYSPRY domains of certain TRIMs interact directly with viral proteins allowing targeted degradation while others interact with pattern recognition receptors and stimulate innate immune signaling pathways [28], [72], [73]. Other C-terminal domains, including

the COS-box, are responsible for targeting TRIMs to microtubules, while the PHD and BROMO domains contribute to transcriptional regulation in class VI TRIMs [48][74].

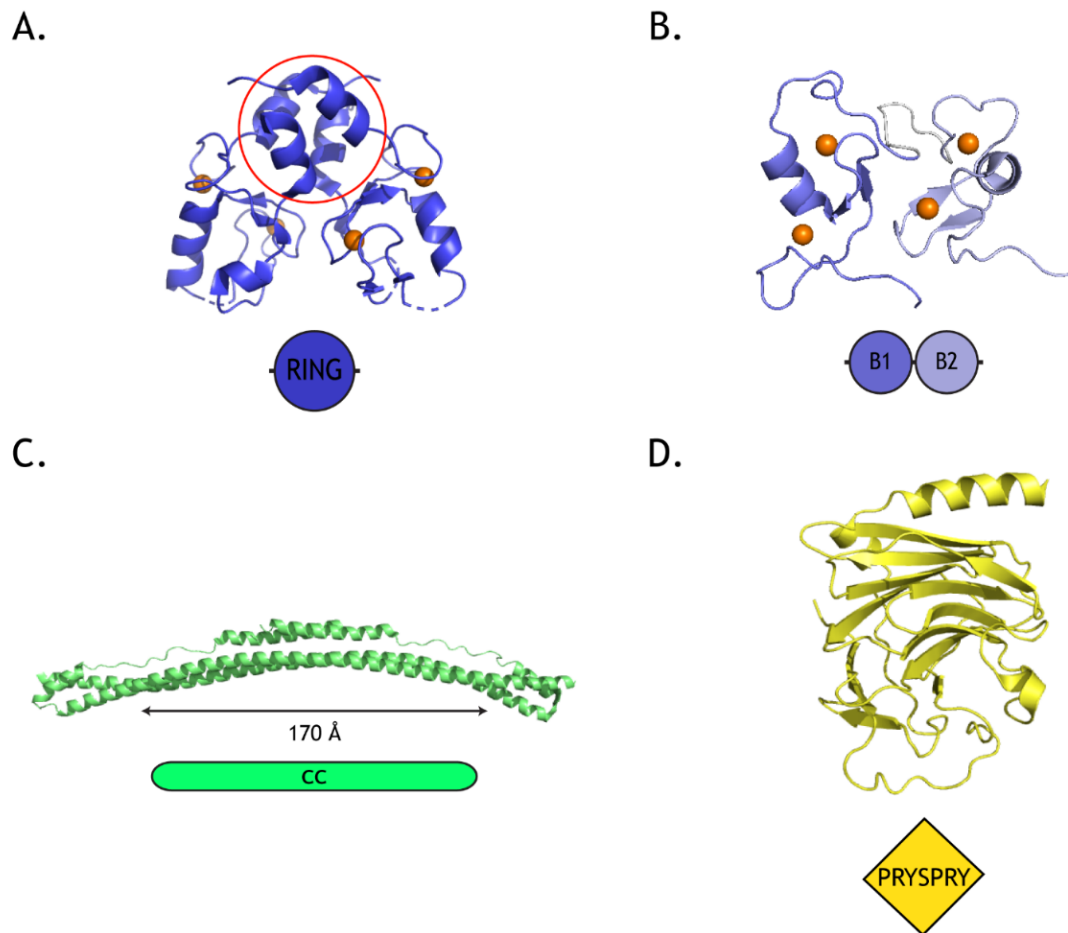


Figure 4 - Structures of individual domains of TRIM E3 ligases. **A.** Structure of the dimeric RING domain of TRIM32, highlighting the dimerisation interfaces formed by the N and C-terminal helices with the zinc molecules shown as orange spheres. PDB: 5FEY. **B.** Structure of the tandem B-box domains of MID-1 (TRIM18), B1 (shown in slate) and B2 (shown in light blue) the structure shows packing of the two domains against each other in a similar fashion to heterodimeric RING E3 ligases. Zinc molecules are labelled as in A. PDB: 2JUN **C.** Structure of the anti-parallel coiled-coil dimer of TRIM25 showing a long central alpha helix of approximately 170 Å, in addition to two smaller helices that packing against the longer helix forming three-helical and four-helical bundles respectively. PDB: 4CFG. **D.** Structure of the PRYSPRY C-terminal domain of TRIM25 illustrating a β -strand fold comprising of thirteen β -sheets and a series of variable loops connecting each β -sheet. PDB: 6FLM.

Structural information of the isolated domains of TRIM E3 ligases has steadily increased over the years and has been important in understanding and comparing the roles of each subdomain in individual TRIM E3 ligases. It is, however, the concerted role of the multiple subdomains that controls activity and function and ultimately differentiates the family members. In this regard, the limited availability of cryo-EM and X-ray crystal structures of composite TRIM E3 ligase domains, are a barrier to our understanding.

A conundrum for TRIM E3 ligase activity is the anti-parallel arrangement of the tripartite motif coupled with the dimerization requirements of RING domain activity. The use of existing structural information and biophysical and biochemical tools has outlined the solution for specific TRIM E3 ligases and illustrated a remarkable diversity in mechanism. For TRIM25, the X-ray crystal structure and SAXS analysis of the CC-PRYSPRY and RBCC constructs respectively, propose an arrangement in which the domains both N and C-terminal of the coiled-coil fold-back along the alpha helix. The tandem B-boxes and long linkers between domains, provide extra length and allow RING and PRYSPRY domains to sit at the centre of the CC; such a scenario would facilitate RING dimerization in an intra-molecular or alternatively, inter-molecular (through higher-order oligomerisation) fashion, in close proximity to the substrate interaction surface on the PRYSPRY domain (Fig. 5A) [31], [51]. Further diversity has been provided by TRIM32, for which the RBCC region has been shown to form tetramers, opening up the possibility of RING dimerization at both ends of the tetrameric assembly (Fig. 5B) [51].

In the case of TRIM5 α , formation of hexagonal arrays through B-box mediated self-association brings three RING domains into close proximity at the trimeric interface; in this arrangement it has been suggested that dimerization occurs between two of these domains while the third is used as the substrate for ubiquitination (Fig. 5C) [75]. A similar mechanism of RING clustering is used by TRIM21 and has been proposed for TRIM65 and RIPLET (a TRIM-like protein). In these scenarios multiple binding epitopes are present within the same substrate complex either as a result of substrate oligomerisation, in the case of TRIM65 and RIPLET, or through multiple antibodies binding to the surface of a single virion, in the case of TRIM21 (Fig. 5D) [76], [77]. This anchoring of numerous TRIM molecules to the same substrate complex increases the local concentration of RING domains and allows dimerization across neighbouring molecules in an intermolecular fashion. RING clustering also presents a scenario in which RING dimerization could be controlled by interaction with the substrate, ensuring tight control of ubiquitination activity. In this regard, other TRIMs may exhibit similar mechanism, although similar characteristics may not exist for all TRIM substrates. Likewise, only a quarter of TRIM E3 ligases possess tandem B-boxes, as present in TRIM25. Hence, additional mechanisms of RING dimerization are likely to exist and emphasizes the clear need for further studies into this family of RING E3 ligases.

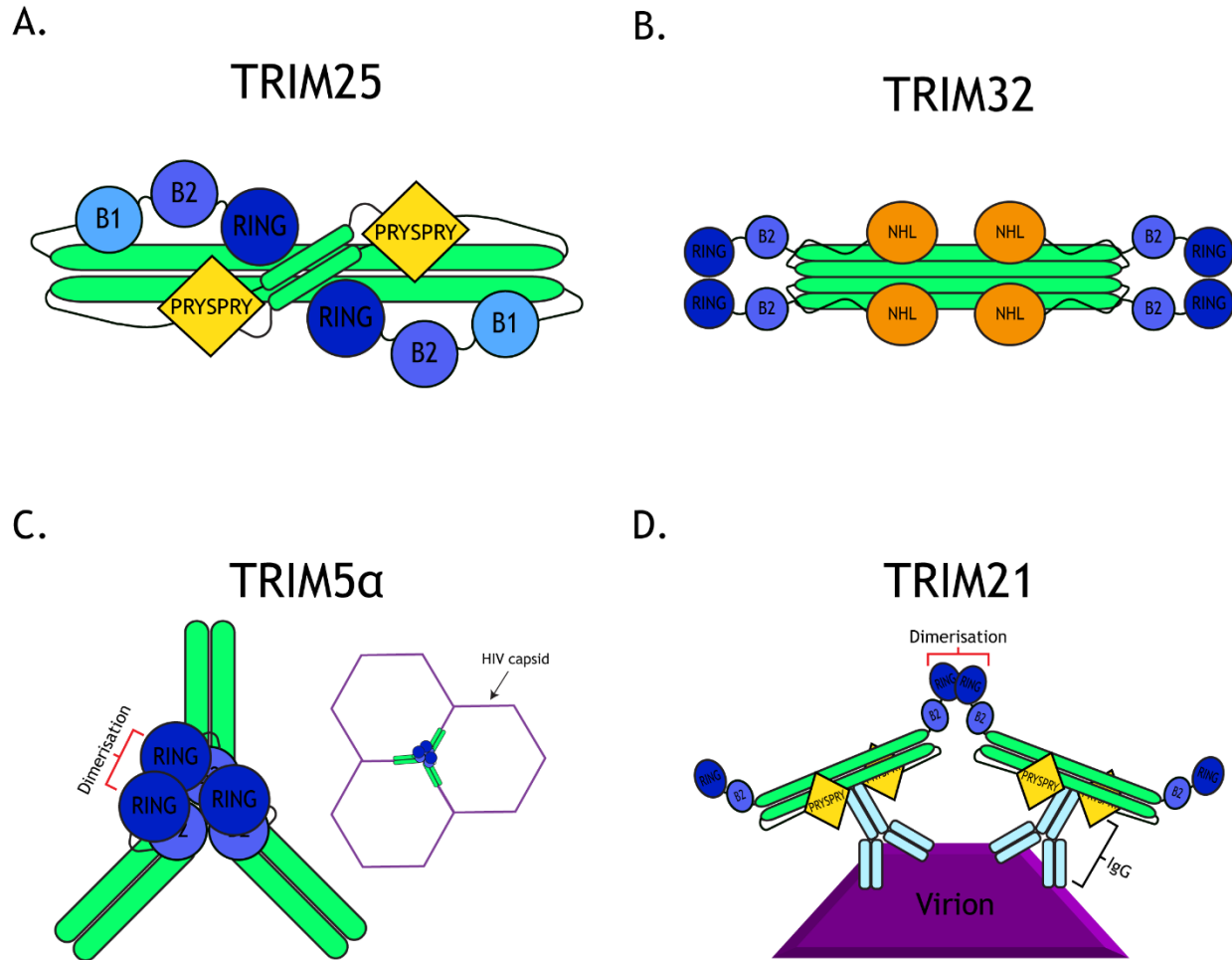


Figure 5 - Mechanisms of RING dimerization in TRIM E3 ligases. **A. TRIM25.** Crystal structures and SAXS data of TRIM25 suggest a compact dimeric structure in which the RING, B-box and PRYSPRY domains fold back along the CC and enable intramolecular or intermolecular RING dimerization in close vicinity to the substrate-binding PRYSPRY domain. **B. TRIM32.** SAXS and SEC-MALLS data of the TRIM32 RBCC construct have illustrated that it forms elongated tetrameric complexes, with the RING and B2 domains extended like beads on a string, placing two RING domains at each end of the CC allowing for RING dimerization. **C. TRIM5 α .** Electron micrographs and crystal structures of different TRIM5 α constructs have illustrated the formation of higher-order oligomers through the B-box domain that situates three RING domains in close proximity allowing dimerization of two of these (Left). This trimerization coupled with CC dimerization allows formation of hexameric assemblies that match the assembly of the HIV capsid (Right). **D. TRIM21.** The RING domains of TRIM21 remain inactive within the dimeric molecule due to separation by the CC. Binding of TRIM21 dimers to multiple copies of antibodies present on the surface of endocytosed virions, clusters the RING domains of neighbouring molecules allowing for intermolecular dimerization.

1.5 Class IV TRIMs

Class IV TRIM E3 ligases, the largest class of TRIMs, are classified by the presence of the C-terminal PRYSPRY domain and contain the highest number of human-specific TRIM genes [78]. The PRYSPRY domain is common to proteins that function in innate immune responses and consistently, a predominant role for this class of TRIM E3 ligases is in such processes [79]. In addition, the expression of a large percentage of class IV TRIM E3 ligases is sensitive to type I or type II IFN secretion, further illustrating their involvement in cellular innate immunity [80], [81]. The most well-studied TRIMs in this class are TRIM25, TRIM5 α and TRIM21, the roles for which have been explained above. However, additional roles as immune signaling and viral restriction factors have been proposed for an increasing number of Class IV members [28], [72].

The structure of the PRYSPRY domain has been solved for multiple TRIM E3 ligases (rhTRIM5 α , TRIM14, TRIM20, TRIM21, TRIM25, TRIM65, TRIM72) [77], [82]-[87]. Despite low sequence conservation, PRYSPRY domains share a common distorted β -sandwich fold consisting of thirteen β -strands, connected by a series of hypervariable loop regions responsible for substrate interactions (Fig. 4D) [88]. The combination of the conserved structural core with variable ligand-binding surfaces is similar to that of the variable domains of IgM molecules, and provides PRYSPRY domains with a flexible platform for substrate interactions [83], [89]. The small number of PRYSPRY-substrate complex structures available illustrate the use of different combinations of variable loops to recognise structurally diverse targets and outline the generally weak affinity of these substrate interactions [77], [84], [88], [90], [91]. The exception to this is the PRYSPRY domain of TRIM21 which binds with extremely high affinity to the Fc region of IgG [84]. However, these low affinity interactions can be overcome by use of binding avidity, which is provided minimally by the dimeric TRIM arrangement and further built upon, in certain cases, by higher order oligomerization, for example in TRIM5 α [92].

The focus of this study are the Class IV TRIM E3 ligases, TRIM22 and TRIM6 which exist within a cluster of TRIM genes on chromosome 11 alongside TRIM5 with which they share high sequence similarity; 50.2% (TRIM22) and 53.8% (TRIM6), respectively [93]. Expression of both, TRIM22 and TRIM6, is upregulated in response to IFN expression, and both have been implicated in the cellular anti-viral response [81]. TRIM22 and TRIM6 were selected for this study due to their sequence similarity to TRIM5 α and their simpler domain composition in comparison to other TRIM family members as well as their intriguing reported cellular functions (Fig. 6A).

Studies on TRIM22 have reported a direct function as a multi-valent viral restriction factor for RNA viruses, Influenza (IAV), Hepatitis C (HCV) and Encephalomyocarditis Virus (EMCV) [94]-[96]. This function relies on the ability of TRIM22 to interact with individual proteins from each of the aforementioned viruses, NP (IAV), NS5A (HCV) and 3C protease (EMCV) and induce ubiquitination and in most cases degradation of the specific proteins. In contrast, a more indirect role as an innate immune signaling factor has been reported for TRIM6. TRIM6 has been reported to function in conjunction with the E2, UBE2K, to produce unanchored K48-linked ubiquitin chains which activate IKK ϵ

for target phosphorylation leading to downstream interferon-stimulated gene (ISG) induction [97]. Importantly, certain viruses have evolved mechanisms to counter this activity such as the Nipah virus (NiV) which encodes the antagonist, NiV-M, that promotes degradation of TRIM6 and hence inhibits host IFN responses [98]. While, the Ebola virus encodes a protein, VP35, that is reported to hijack TRIM6 ubiquitination activity to promote its own replication [99].

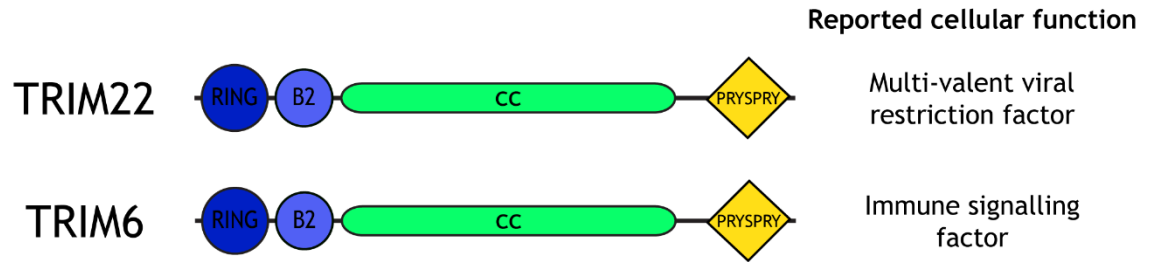
Alternative functions in other cellular processes have also been proposed for these TRIM E3 ligases, most notably in cancer. TRIM22 expression is controlled by the tumour suppressive transcription factor, p53, and related reports have suggested its expression is linked to reduced clonogenic growth in leukemic cells [100], [101]. Conversely, a more recent study has shown TRIM22 to be significantly upregulated in non-small cell lung cancer specimens correlating with poor patient prognosis [102]. TRIM6 has also been implicated in multiple cancer scenarios, outlining oncogenic properties through ubiquitination and degradation of key cellular proteins involved in preventing cancer progression in both colorectal and breast cancer cells [103], [104].

Despite these reported functions, structural information on TRIM22 and TRIM6 full-length or sub-domain constructs is not available, and only limited biochemical and biophysical analysis has been performed. On top of this, little information is known about the nature of substrate interactions. On a sequence level, the B-box domains of TRIM22 and TRIM6 share 63% and 87% sequence identity respectively, with that of rhTRIM5 α , including certain residues important in forming the homotrimeric interface (Fig. 6C) [63]. Therefore, it is possible that TRIM22 and TRIM6 could utilize a B-box domain mediated higher-order oligomerization to facilitate RING activity.

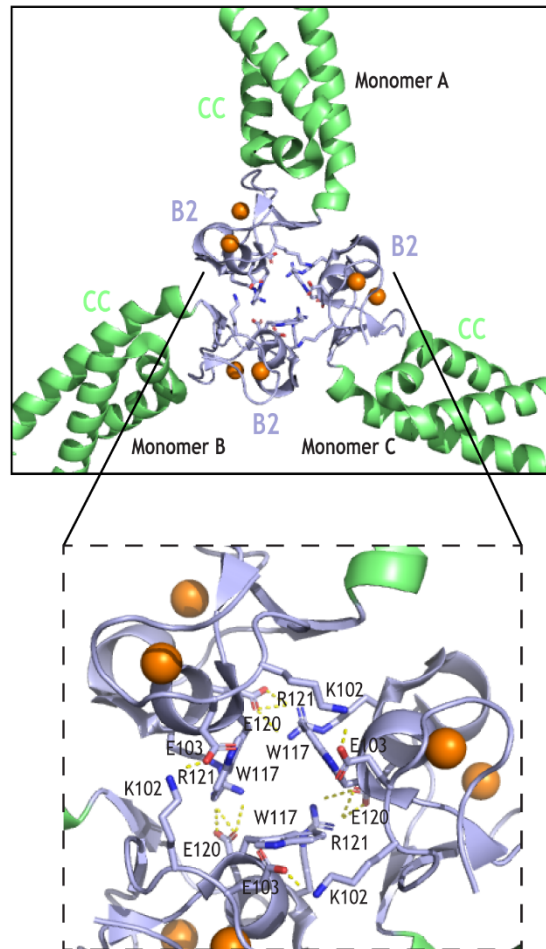
Ubiquitination activity has also been proposed for full-length TRIM22 in *in vitro* assays with the E2 UDE2D2 (Ubch5B), while TRIM6, as previously mentioned, is reported to use UBE2K, as its cognate E2 to achieve immune signaling pathway activation [97], [105].

The target of this study is to build upon the limited data available on TRIM22 and TRIM6 using a combination of biophysical and biochemical tools, to provide information on the ubiquitination activities, self-association profiles and substrate interactions of these proteins. The work on TRIM22 project was started *ab initio* as part of this study, whereas the work on TRIM6 was built upon preliminary work performed by a former PhD student in the lab, Rebecca Stevens.

A.



B.



C.

		Seq. ID
rhTRIM5α	KVDHCA RHG E K L L L F C Q E D S K V I C W L C E R S Q E H R G H H T F L	100.0%
TRIM22	K R D V C E H H G K K L Q I F C K E D G K V I C W V C E L S Q E H Q G H Q T F R	63.4%
TRIM6	K A V L C A D H G E K L Q L F C Q E D G K V I C W L C E R S Q E H R G H H T F L	86.5%
	93 132	

Figure 6 - Class IV TRIM E3 Ligases: TRIM22 and TRIM6. **A.** Domain composition of Class IV TRIM E3 ligases, TRIM22 and TRIM6 alongside their reported cellular roles. **B.** Subsection of the crystal structure of the trimeric rhTRIM5 α BCC MT construct showing the homotrimeric interactions mediated by the B-box 2 domains. Individual monomers and specific domains are labelled. The boxed panel shows an expanded view of the B-box 2 interactions formed, most importantly salt bridges between K102 & E103 and E120 & R121 of neighbouring molecules, as well as a hydrophobic core formed by the three W117 residues. **C.** Sequence alignment of the B-box domains of rhTRIM5 α , TRIM22 and TRIM6, illustrating the percentage sequence identity across the domain. The key residues responsible for trimerization in TRIM5 α , outlined in **B.**, and their equivalents in TRIM22 and TRIM6 are highlighted in red.

2. Materials & Methods

2.1 Plasmids & Cloning

All the plasmids used for expression and purification, or transfection, in *E. coli* and insect cells or in mammalian cells respectively, are listed in Table 1. Constructs that were cloned for this study were either bought as synthetic genes from IDT or amplified by PCR from plasmids or cDNA samples present in the lab. DNA inserts were then cloned via ligation independent cloning using the Gibson Assembly method (NEB). To enable this, homologous overhang regions of 15-20 base pairs, optimized for Gibson Assembly with the relevant linearized vectors, were either added at each end of the synthesised gene or, at the 5' end of the forward and reverse primers. Once amplified, 0.5 µl of the DNA insert and 0.5 µl of the linearized vector were combined with 1 µl of 2x Gibson Assembly Master Mix and incubated at 50°C for 30-60 mins. For mutagenesis, overlapping primers were designed containing the relevant nucleotide substitutions along with 15 base pairs on either side of the mutated codon, and were used to amplify a mutated version of the template plasmid by PCR. PCR reactions were then treated with *Dpn1* endonuclease FastDigest (NEB) for 15 mins at 37°C to remove the unmutated template plasmid, before transformation. 100 ng of the mutated plasmid or the whole Gibson Assembly reaction was then used to transform 25 µl of DH5α *E. coli* competent cells (NEB) and plated on agar plates supplemented with the relevant antibiotic for the specific plasmid vector (Ampicillin, Kanamycin or Gentamycin). Single colonies were picked, and the plasmid DNA purified using the Qiaprep Spin Miniprep kit (Qiagen). DNA samples were then sent for sequencing by Genewiz/GATC to validate successful clones.

Protein	Boundaries	Source	Vector	Tag(s)
TRIM22 RING WT	2-87	Provided by Diego Esposito	pET49	His ₆ -SUMO
TRIM22 RING M1Y	1-87	Mutated by mutagenesis from pET49-T22 RING WT plasmid	pET49	His ₆ -SUMO
TRIM22 RB2	2-133	Provided by Diego Esposito	pET49	His ₆ -SUMO
TRIM22 RBCC MT W	1-265 Δ CC	Ordered as synthetic gene	pET49	His ₆ -SUMO
TRIM22 RBCC MT G	1-261 Δ CC	Ordered as synthetic gene	pET49	His ₆ -SUMO
TRIM22 BCC MT G	88-261 Δ CC	Cloned from pET49-T22 RBCC MT G plasmid	pET49	His ₆ -SUMO
TRIM22 RBCC	1-295	Provided by Diego Esposito	pET49, pACEBac1	His ₆ -SUMO (pET49), 3xFLAG (pACEBac1)
TRIM22 CC	132-295	Cloned from pET49-T22 FL vector	pET49	His ₆ -SUMO
TRIM22 CC-PRYSPRY	135-498	Cloned from pET49-T22 FL vector	pET49	His ₆ -SUMO
TRIM22 PRYSPRY	283-498	Cloned from pET49-T22 FL vector	pET49	GST, MBP (pET49)
TRIM22 FL	1-498	pET49-T22-FL vector provided by Diego Esposito, other vectors cloned from this	pET49, pACEBac1, pcDNA3.1	His ₆ -SUMO (pET49), His ₆ , His ₆ -SUMO, 3xFLAG (pACEBac1), 3xFLAG (pcDNA3.1)
TRIM6 RING	1-87	Cloned & used by Rebecca Stevens	pET49	His ₆ -TRX
TRIM6 RB2	1-133	Cloned & used by Rebecca Stevens	pET49	His ₆ -TRX
TRIM6 RBCC MT G	1-261 Δ CC	Ordered as synthetic gene	pET49	His ₆ -SUMO
TRIM6 BCC MT G	88-261 Δ CC	Cloned from pET49-T6 RBCC MT G plasmid	pET49	His ₆ -SUMO
TRIM6 RBCC	1-293	Cloned & used by Rebecca Stevens	pET49	His ₆ -TRX
TRIM6 PRYSPRY	283-488	Cloned from pET49-T6 FL	pET49	His ₆ -SUMO
TRIM6 FL	1-488	Provided by Rebecca Stevens as pET49-HT-T6 FL and used as a template for cloning into pACEBac1 & pcDNA3.1	pET49, pACEBac1, pcDNA3.1	His ₆ -TRX (pET49), His-SUMO (pACEBac1), 3xFLAG (pcDNA3.1)
TRIM21 RING	1-85	Cloned from pCMV-GFP-TRIM21	pET49	His ₆ -SUMO
TRIM23 FL	1-574	Provided by Filippo Fiorentini	pcDNA3.1	3xFLAG
TRIM2 FL	1-744	Cloned from pET49-T2 FL provided by Diego Esposito	pcDNA3.1	3xFLAG

Table 1. Details of all TRIM protein constructs, and their respective vector backbones, used in this study.

2.2 Protein expression & purification

Construct	Residues	MW (g.mol ⁻¹)	E (M ⁻¹ cm ⁻¹)	Source (where not produced in this study)
TRIM22				
TRIM22 RING WT	2-87	9801.4	No aromatic residues	-
TRIM22 RING M1Y	1-87	9964.6	1490	-
TRIM22 RB2	2-133	15460.8	5500	-
TRIM22 RBCC MiniTRIM	1-261ΔCC	21873.2	5500	-
TRIM22 BCC MiniTRIM	88-261ΔCC	13128.0	5500	-
TRIM22 CC	132-295	19416.2	12490	-
TRIM6				
TRIM6 RING	1-87	9831.3	1490	R.Stevens
TRIM6 RB2	1-133	15102.4	6990	R.Stevens
TRIM6 RBCC MiniTRIM	1-261ΔCC	21833.8	8480	-
TRIM6 BCC MiniTRIM	88-261ΔCC	12231.7	6990	-
Other TRIMs				
TRIM21 RING	1-85	9711.3	5500	-
TRIM23 RBB	2-213	23225.7	9970	F.Fiorentini
TRIM2 RBCC	2-288	32198.8	5960	D.Esposito

Table 2. Details of purified TRIM E3 ligase constructs used in this study.

Protein	MW (kDa)	Source
Ubiquitin (Ub)	8.6	Sigma
Ub ^{atto}	8.6	I.Tsai
UBA1	117.8	C.Wong
UBE2D1 (UbcH5A)	16.6	C.Wong
UbcH5A~Ub ^{Cy3}	25.2	C.Kennedy
UBE2K	22.4	R.Stevens
UBE2N	17.1	C.Wong
UBE2V2	16.4	C.Wong

Table 3. Details of additional purified proteins used in this study.

2.2.1 Bacterial protein expression

For all bacterial protein purifications, plasmids containing the relevant protein construct were transformed into BL21-GOLD (DE3) (Agilent), BL21-CodonPlus (DE3) (Agilent), C41 (DE3) (Sigma), T7 Express *lysY* (NEB) or ArticExpress (DE3) (Agilent) cells and plated on agar plates supplemented with the appropriate antibiotic to select for positive colonies. Positive colonies were then used to inoculate a starter culture of LB media also supplemented with antibiotic (and 200 μ M ZnCl₂ for expression of constructs containing Zn-coordinating domains) and the cells were grown overnight at 37°C and 220 rpm.

Small-Scale purification

For small-scale purifications, a 5 ml starter culture was prepared of which 2.5 ml was used to inoculate a larger 50 ml culture (supplemented with antibiotic and 200 μ M ZnCl₂ where necessary) and grown at 37°C and 250 rpm until an OD₆₀₀ of $\approx$ 0.6-0.8 was reached. Protein expression was then induced by addition of 0.25 mM IPTG and cultures were incubated for a further 4 hrs at 25°C and 250 rpm. Cells were then harvested by centrifugation at 4500 rpm for 25 mins at 4°C in a Heraeus Multifuge 3 and stored at -20°C. Cell pellets were resuspended in 5 ml lysis buffer most commonly containing: 50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP, 0.2 mg/ml lysozyme, 2 μ g/ml DNase I, 2.5 mM MgCl₂ & 10x protease inhibitor stock solution, in addition to 20 mM Imidazole (IZ) for Ni-NTA affinity purifications, and lysed by sonication using a program of 4 seconds on, 6 seconds off, for 1 minute in total. Clarification of the lysate was performed at 5000 rpm for 30 mins at 4°C and the supernatant collected. 500 μ l of Ni-NTA agarose (ThermoFisher) or 500 μ l Glutathione Sepharose 4 Fast-Flow (Cytiva) resin was then loaded onto a small gravity column, washed with MilliQ water and equilibrated with lysis buffer, to which the supernatant was then added. The flowthrough was collected, and the resin washed with 40 column volumes (CV) standard wash buffer: 50 mM HEPES pH 7.5, 300 mM NaCl & 0.5 mM TCEP, with the addition of for 20 mM Imidazole for His-affinity purifications. The protein was then eluted with 1 ml of wash buffer, supplemented with 500 mM IZ or, 25 mM glutathione (GSH), for His and GST affinity purifications respectively, reloaded twice. Samples from throughout the purification were analysed by SDS-PAGE to assess expression and purification, including the insoluble fraction (dissolved in buffer containing: 8M Urea, 100 mM K₂PO₄ and 10 mM TRIS pH 7.5 at 95°C for 1 h) and the resin (resuspended in 1 ml wash buffer).

Large-Scale purification

For large-scale purifications, a starter culture of 20 ml was grown which was then used to inoculate larger cultures of 1L LB media in 2L flasks, at a ratio of 1/1000 (again supplemented with antibiotic and 200 μ M ZnCl₂ where necessary). Cultures were then grown at 37°C and 220 rpm as for the small-scale cultures. For most large-scale protein purifications, 2-6L of LB media was used. Protein expression was then induced with 0.25 mM IPTG at 18°C and 220 rpm overnight. The cells were harvested the following day by centrifugation at 4500 rpm, 4°C for 25 mins in a Beckman J6-M1 rotor. Cell pellets were then either used directly for protein purification or washed with PBS, flash frozen and stored at -80°C.

For the cell lysis, cell pellets were resuspended in the following lysis buffer: 50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP, 0.2 mg/ml lysozyme, 2 µg/ml DNase I, 2.5 mM MgCl₂ & 1 protease inhibitor tablet per 50 ml, in addition to 20 mM IZ for Ni-NTA affinity purifications, at a ratio of 7 ml/g of cell pellet. Lysis was performed on ice by sonication using the same protocol as for smaller cultures, with total lengths of 5-8 mins, depending on the volume of lysate. Clarification was achieved by centrifugation at 21,000 rpm, for 1h at 4°C in a Beckman JA 25.50 rotor and the supernatant was collected for downstream purification. The first step for all purifications was affinity purification using either Ni-NTA agarose (ThermoFisher) or Glutathione Sepharose 4 Fast-Flow (Cytiva) resin. 3-5 ml resin slurry was placed in a gravity column, washed with MilliQ water, and equilibrated with lysis buffer. The supernatant was loaded onto the column and the resin was then washed with 10 CV standard wash buffer: 50 mM HEPES pH 7.5, 300 mM NaCl & 0.5 mM TCEP, including 20 mM IZ for His-affinity purifications, followed by 10 CV high salt wash buffer (wash buffer + 1M NaCl) before 20 CV standard wash buffer again. For Ni-NTA affinity purifications, protein samples were then cleaved, either in-solution or on-column. For in-solution cleavage, the protein was eluted with elution buffer (wash buffer + 500 mM IZ), using Quick Start Bradford reagent (Bio-Rad) to determine when all the sample had been eluted and cleaved using 3C protease (Human Rhinovirus) at 4°C overnight, along with dialysis in a large volume of wash buffer (without IZ) to remove the high concentration of imidazole. For on-column cleavage, the Ni-NTA resin was resuspended in 1 CV wash buffer and 3C protease added directly to the column, which was then sealed and incubated at 4°C on the rocker, overnight. Reverse affinity of cleaved samples was then performed by either reloading the dialysed sample onto fresh Ni-NTA resin or collection of the flowthrough from the on-column cleavage sample. For GST purification, the sample was left uncleaved and eluted with 25 mM Glutathione which was then removed by downstream gel filtration. Samples of cleaved and/or eluted proteins were analysed by SDS-PAGE, pooled and concentrated in 5-30 kDa molecular weight cut-off concentrators (Amicon) for further purification steps.

For some constructs an ion-exchange step was required following the affinity purification to clean up the sample before gel filtration. The predicted pI of the protein (calculated by ExPASy ProtParam) was used to determine whether anion or cation exchange was necessary, and these were performed using the HiTrap Q HP and HiTrap SP FF 1 ml cation and anion exchange columns (Cytiva). The sample was first diluted into a low salt ion-exchange buffer: 50 mM NaCl, 50 mM HEPES pH 7.5 and 0.5 mM TCEP, to allow binding to the resin and loaded onto pre-equilibrated columns using a peristaltic pump. Once the FT had been collected, the columns were attached to an ÄKTA PRIME system and proteins were eluted using a 700 mM NaCl gradient over 20 CVs. Fraction samples were analysed by SDS-PAGE, pooled, and concentrated for gel filtration.

Size-exclusion chromatography (SEC) was used as a final purification step for all purifications, for buffer exchange, quality control and additional removal of contaminants. Depending on protein size and predicted oligomeric state, either the HiLoad Superdex 75 or HiLoad Superdex 200 16/600 prep grade columns (Cytiva) were

used. The columns were attached to an ÄKTA PRIME or Purifier system and pre-equilibrated in SEC buffer: 50 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM TCEP. Samples were concentrated to <5 ml volume and injected onto the columns using a 5 ml sample loop. Fraction samples were finally analysed by SDS-PAGE, pooled, and concentrated before being flash frozen and stored at -80°C.

2.2.2 Insect cell protein expression

Viral production and amplification

Upon cloning of the protein of interest into the pACEBac1 vector (Geneva BIOTECH), transposition to produce a recombinant bacmid was achieved using EmBacY cells, which possess the bacmid, containing a GFP gene, used to visualize transfection, and a *lacZ* gene, encoding the β -galactosidase protein, for blue-white screening. The EmBacY cells also possess a helper plasmid, which expresses the site-specific recombinase required for recombination. EmBacY cells were transformed and plated onto selection plates from which positive white colonies were picked and amplified. The bacmid DNA was purified using a modified Miniprep protocol and used to transfect *Sf9* insect cells. 2 ml of *Sf9* cells at a density of 0.4 m/ml were plated in 6-well plates and transfected with a transfection mixture containing 3 μ g bacmid DNA and 8 μ l Cellfectin II transfection reagent, diluted in Sf900 III media. The cells were subsequently incubated for 72 hrs at 28°C, after which, successful transfection was assessed via observation of GFP expression. 0.5 ml of the supernatant (P0 viral stock) from the successful transfections was used to infect a larger 25 ml culture of *Sf9* cells at 2 m/ml cell density and incubated for a further 72 hrs at 28°C. This cell culture was centrifuged at 2500 rpm for 10 mins to remove cell debris. 50 μ l of the supernatant (P1 viral stock) was used to infect a fresh 25 ml culture of *Sf9* cells at 2 m/ml and incubated again for 72 hrs at 28°C. These cells were again centrifuged at 2500 rpm for 10 mins, the supernatant (P2 viral stock) was stored in black 50 ml falcon tubes and the cell pellet was flash frozen to be used for an initial expression trial.

Small-Scale purification

For expression trials, the P2 cell pellet was resuspended in 5 ml of insect cell lysis buffer: 50 mM HEPES pH 7.5, 300 mM NaCl, 10% Glycerol, 0.5 mM TCEP, 1 mM AEBSF, 2 μ g/ml DNase I, 10 mM MgCl₂ and protease inhibitor tablets, in addition to 20 mM IZ for His-tagged protein purifications. TRITON detergent, added at a final concentration of 0.5%, was then used to lyse the cells at 4°C for 30 mins on a rotator. Cell debris was then removed by centrifugation at 13000 rpm, for 10 mins at 4°C and the lysate was loaded onto 500 μ l of pre-equilibrated Ni-NTA agarose resin or anti-FLAG M2 affinity gel (SIGMA). The resin was then washed with 100 ml of wash buffer: 50 mM HEPES pH 7.5, 300 mM NaCl and 0.5 mM TCEP and the protein was eluted with 500 μ l of wash buffer supplemented with either 500 mM IZ or 250 μ g/ml FLAG peptide for His-tagged and FLAG-tagged proteins respectively, reloaded onto the resin twice to ensure all protein was eluted. Samples from the expression trial were then analysed by SDS-PAGE, including the insoluble fraction.

Large-Scale purification

For large-scale purifications, larger cultures of 1 - 3 litres of *Sf9* cells were used. For this, stocks of *Sf9* cells were split to 1.1 million cells/ml (m/ml) in 500 ml cultures and incubated at 28°C for 24 hrs, to obtain a cell culture of approximately 2 m/ml at the time of infection. 24 hrs after splitting, each 500 ml culture was infected with 1 ml of P2 viral stock and left to incubate for between 72 - 96 hrs at 28°C, until the cell viability was <85%. Cells were then harvested by centrifugation at 2500 rpm for 25 mins at 4°C, resuspended in 100 ml insect cell lysis buffer and flash frozen for storage at -80°C. For purification, cells were then thawed and lysis was performed by addition of 0.5% TRITON, followed by incubation on a rotating shaker at 4°C for 1 h. Clarification was then achieved by centrifugation at 21000 rpm, for 1 h at 4°C. The supernatant was collected and diluted in lysis buffer to a final volume of 150 ml/L of cells, before being loaded onto a gravity column containing 1.5 ml or 5 ml of pre-equilibrated Ni-NTA agarose or anti-FLAG M2 affinity resin, respectively. The resin was subsequently washed with 10 CV wash buffer: 50 mM HEPES pH 7.5, 300 mM NaCl & 0.5 mM TCEP, including 20 mM IZ for His-affinity purifications, followed by 10 CV high salt wash buffer (wash buffer + 1M NaCl) before an additional 30 CV of wash buffer. The samples were then eluted via addition of between 10-40 ml of elution buffer, supplemented with 500 mM IZ for His-tagged protein and 250 µg/ml FLAG peptide for FLAG-tagged constructs. Samples were analysed by SDS-PAGE, however purifications were not continued to due to insufficient protein expression yield.

2.3 Antibodies

All the antibodies used in this study are listed in Table 4 along with their working dilutions. All primary antibodies were diluted in PBS supplemented with 0.1% TWEEN (PBS-T) and 5% w/v skimmed milk powder and used at 4°C before being frozen and stored at -20°C. Primary antibodies were re-used a maximum of three times. All secondary antibodies were also diluted in PBS-T + 5% (w/v) milk and used at room temperature. All secondary antibodies were used only once.

Antibodies	Ratio used	Source	Supplier
Anti-FLAG M2	1:2000	Mouse	Sigma
Anti-Ubiquitin (Ubi-1)	1:1000	Mouse	ThermoFisher
Anti-GAPDH	1:10000	Mouse	Sigma
Anti-TRIM22	1:1000	Rabbit	Sigma
Anti-TRIM6	1:1000	Rabbit	Proteintech
Anti-STAT1	1:1000	Rabbit	Cell Signalling (Bishop Lab)
Anti-mouse HRP	1:2000	Goat	Agilent Technologies
Anti-rabbit HRP	1:3000	Swine	Agilent Technologies

Clean-Blot IP Detection Reagent HRP	1:200	N/A	ThermoFisher
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Table 4. Antibodies and their relevant dilutions used in this study

2.4 SDS-PAGE electrophoresis & Western Blotting

SDS-PAGE gel electrophoresis was used to separate protein samples for analysis with either Coomassie staining, fluorescence or western blot detection. Samples were run on NuPAGE 4-12% Bis-TRIS gradient gels (Invitrogen) for 35 minutes at 200 volts in 1 X NuPAGE MES buffer (Invitrogen). Gels were either stained directly using Quick Coomassie stain (NeoBiotech) or transferred to nitrocellulous membrane for western blot analysis. For Coomassie-stained gels, the Mark12 unstained standard (ThermoFisher) protein ladder was used, while for western blot analysis, the SeeBlue Plus2 Pre-stained (ThermoFisher) protein ladder was used. For western blotting, the protein samples were transferred to nitrocellulous membranes using either the iBlot (ThermoFisher) or Trans-blot (BioRAD) transfer systems. Transfer efficiency was assayed using the reversible Ponceau S stain (Merck) which was removed by washing with PBS-T. The membranes were subsequently blocked using the blocking solution (PBS-T + 5% milk (w/v)) for up to 30 minutes at room temperature (RT), before primary antibody staining overnight at 4°C. Following primary antibody staining, the membranes were washed three times, 5 mins each, in PBS-T, before staining with HRP-conjugated secondary antibody for 1 h at RT. Membranes were then washed a further three times in PBS-T, dried briefly, and detected via chemiluminescence. The enhanced chemiluminescence western blotting detection reagent (Amersham) was used for detection at a ratio of 1.5 ml detection reagent per mini SDS-PAGE gel. The membrane was incubated for 2 min with the solution before being imaged using the ChemiDoc MP Imaging system (BioRad) at exposure times of 10 - 300 seconds.

2.5 Cell culture

For mammalian cell expression, pcDNA3.1 mammalian expression vectors containing an N-terminal 3 x FLAG tag and cloned with the relevant protein constructs, were transformed into DH5α *E. coli* competent cells and extracted using the Qiaprep Spin Miniprep kit for transfection into the relevant mammalian cell line. HEK293T, U2OS and A549 cell lines were supplied by the Cell Services Scientific Technology Platform at The Francis Crick Institute and maintained in Dulbecco's Modified Eagle Medium (DMEM - ThermoFisher) supplemented with 10% fetal bovine serum (FBS - Gibco), 500 U penicillin and 0.5 mg streptomycin.

For activity assays, 2 x 10⁶ HEK293T cells were plated on a 10 cm² plate and incubated at 37°C for 24 hrs. After 24 hrs the media was replaced, and each plate was transfected with the relevant plasmid DNA. The transfection mixture consisted of 5 µg of plasmid DNA mixed with FuGENE transfection reagent (Promega) at a ratio of 3:1, µl of FuGENE: µg of DNA and diluted up to 200 µl in Opti-MEM media (Gibco). The transfection mixture was incubated for 20 minutes at RT before addition to the cells in a drop-wise manner. The cells were then incubated for a further 24 hrs at 37°C before harvesting. To harvest

the cells, media was first removed from the plates and the cells were washed with 3 ml PBS to remove excess media, before 2 ml trypsin was added and incubated at 37°C for 2-3 mins. Cells were then resuspended and transferred to 15 ml falcon tubes and spun down at 700 g, for 5 minutes at 4°C. The supernatant was then removed, and the cells were resuspended in 1 ml PBS and transferred to 2 ml Eppendorf tubes before a further round of centrifugation at 700 g and 4°C, for 5 minutes. The PBS was then removed, and the cell pellets were either lysed directly or flash frozen and stored at -80°C. The lysis buffer used consisted of: 50 mM HEPES pH 7.5, 150 mM NaCl, 1% IGEPAL, 0.1% SDS, 0.5 mM TCEP + cOmplete EDTA-free protease inhibitor tablets (Roche). Cell pellets from 3 x 10cm² plates were resuspended in 1 ml lysis buffer and lysed on ice for 30-60 minutes before centrifugation at 13000 rpm, for 10 minutes at 4°C, to remove cell debris. The lysates were then transferred to fresh 2 ml Eppendorf tubes and a 40 µl input sample was taken and combined with 20 µl 4x SDS sample buffer, of which 15 µl (1%) was loaded onto an SDS-PAGE gel for western blot analysis, before being boiled for 3 minutes at 95°C and flash frozen. 10 µl of anti-FLAG M2 magnetic bead slur (SIGMA), washed with deionized water and equilibrated with lysis buffer, using the MagRack 6 (GE Healthcare), was then added to each lysate sample and incubated overnight on a rotator at 4°C. The beads containing the immobilized protein were subsequently washed three times with lysis buffer and three times with reaction buffer using the MagRack 6, before being used in *in vitro* ubiquitination assays (see Section 2.11).

For the IFN-stimulation assays, 3.5 x 10⁵ HEK293T and U2OS cells or, 4.5 x 10⁵ A549 cells were seeded into each well of a 6-well plate and left to incubate for 24 hrs at 37°C. After 24 hrs the media was exchanged in each well and, for each cell line, cells were either left unstimulated, stimulated with 1000 U/ml IFN-β (PBL assay science), added directly to the media, or, stimulated with 4.5 µg poly(I:C) low MW (Invivogen) transfected as part of a transfection mixture with FuGENE at the same 3:1 ratio mentioned previously, supplemented with 50 µl of Opti-MEM media. Cells were incubated for a further 16 h at 37°C post-stimulation before harvesting. The media was then removed from each well and the cells were washed with 1 ml PBS, before addition of 1 ml trypsin and incubation for 5 mins at 37°C. Cells were then resuspended and transferred to 1.5 ml Eppendorf tubes before being spun down and washed as mentioned previously. The cell pellet from each well was resuspended in 100 µl lysis buffer and lysed as explained above. The lysates were transferred to fresh 2 ml Eppendorfs and 75 µl was combined with 25 µl SDS sample buffer for further analysis. Each condition was repeated in triplicate and 12.5 µl (10%) of each sample was run on three separate SDS-PAGE gels for analysis by western blotting using anti-GAPDH, anti-TRIM22, anti-TRIM6 and anti-STAT1 antibodies.

2.6 Circular Dichroism

Circular dichroism (CD) is a technique that measures the differential absorbance of left and right-handed circularly polarized light by optically active molecules and can be used to assess the secondary structure content of proteins in solution. In this study, CD was used to compare the secondary structure of different length constructs of TRIM6 and TRIM22 expressed and purified in *E. coli*. Samples were prepared at a concentration

of 0.15 mg/ml in buffer containing: 20 mM HEPES pH 7.5, 150 mM NaCl and 0.5 mM TCEP. Samples were measured at a range of wavelengths from 260 nm to 200 nm, alongside a buffer control, using the Jasco spectrophotometer. CD units in millidegrees (S) were then converted to either molar CD extinction coefficient ($\Delta\epsilon_M$) or mean residue CD extinction coefficient ($\Delta\epsilon_{MRW}$) using the equations below. Experimental set-up, data processing and data analysis were carried out with the help of Dr. Laura Masino (The Francis Crick Institute).

$$\Delta\epsilon_M = \frac{S}{32,980 \times C_M \times L} = M^{-1}cm^{-1}$$

$$\Delta\epsilon_{MRW} = \frac{S \times MRW}{32,980 \times C_{mg/ml} \times L} = deg.M^{-1}cm^{-1}$$

L = pathlength (cm)

C_M = molar concentration

$C_{mg/ml}$ = concentration in mg/ml

MRW = mean residue weight (Molecular weight/No of residues)

2.7 Protein crystallisation

Crystallisation trials were set-up for purified protein constructs at a range of concentrations from 3 - 20 mg/ml depending on the construct. Crystallisation conditions were screened using either, 96-well commercial screens or manual screens set-up using the Formulatrix (Formulatrix). The sitting-drop vapour diffusion method was used for crystallization and drops containing 100 nl of protein sample, and 100 nl of precipitant solution, were set-up using the Mosquito LCP crystallization robot (TP Labtech). 96-well plates were stored at either 20°C or 4°C and imaged at regular intervals using the Rock Imager (Formulatrix). For those plates where seeding stocks were used to aid crystallization, crystal seeds were prepared from an initial hit using the Hampton Research protocol. Crystals were viewed using the Leica MZ16 microscope, crushed using a crystal crusher tool and resuspended in a final volume of 50 µl of reservoir solution. The seed stock was vortexed and either used directly for crystallization set-up or flash-frozen and thawed for further attempts at ratios of 1:3-10, volume of protein sample: volume of seed stock solution.

2.8 SEC-MALLS

Size exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was used to determine the absolute molar mass of protein samples separated by analytical gel filtration. For SEC-MALLS, samples are loaded onto a gel filtration column and the eluted fractions flow through light scattering and refractive index detectors, which measure molar mass and protein concentration respectively. Light scattering measurements are recorded at 16 different angles and produce an accurate calculation of average molar mass that is independent of the shape of the protein. Meanwhile, the refractive index measurement gives a protein concentration that is independent of UV absorbance and extinction coefficient.

For these experiments, 120 µl of filtered protein sample was used, at a range of concentrations (1 - 6 mg/ml to assess concentration dependent self-association) and

applied to either a Superdex 75 Increase or Superdex 200 Increase 10/300 column attached to a Jasco HPLC system. The gel filtration columns were pre-equilibrated in the buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP and 3 mM NaN₃, and experimental runs were performed at a flow rate of 0.5 ml/min. Scattering intensity was measured by a DAWN-HELIOS II laser photometer (Wyatt Technology), while the refractive index was measured by an OPTILAB T-rEX differential refractometer (Wyatt Technology). The ASTRA software package (WYATT technology) was used to determine the average molecular mass and polydispersity across the individual elution peaks. Data collection and analysis were performed by Dr. Ian Taylor (The Francis Crick Institute).

2.9 *In vitro* ubiquitination assays

In vitro ubiquitination assays were performed using either full-length FLAG-tagged TRIM E3 ligases purified from HEK293T cells or recombinant N-terminal TRIM E3 ligase constructs purified from *E. coli*. All reactions were performed with the required components of the ubiquitination cascade: E1, E2, ATP & Ubiquitin, in addition to Ub^{atto} for the full-length TRIM E3 ligase reactions. The following reaction buffer was used: 50 mM HEPES pH 7.5, 150 mM NaCl and 20 mM MgCl₂. For the reactions with the recombinant E3 protein purified from *E. coli*, E3 concentrations of 1 µM, 4 µM & 10 µM were used, along with 1 µM E1, 2.5 µM of each E2, 50 or 100 µM Ubiquitin and 3 mM ATP. Reactions were incubated in the thermomixer at a range of temperatures: 25°C, 30°C & 37°C, at agitation speeds of either 600 or 1000 rpm. For the reactions with full-length FLAG-tagged TRIM proteins, the protein was expressed in HEK293T cells before being immunoprecipitated from the whole cell extract using anti-FLAG beads. The immobilised protein on the magnetic beads was then washed and resuspended in reaction buffer containing the relevant reaction components at the following concentrations of 1 µM E1, 0.5 µM of each E2, 50 µM Ubiquitin, 1 µM Ub^{atto} and 3 mM ATP. For all reactions a timepoint 0 sample was taken before addition of ATP and quenched by addition of 2 x SDS sample buffer supplemented with 500 mM DTT, before being boiled at 95°C for 3 min. Thereafter, samples were taken at stated timepoints and treated as the timepoint 0 sample and resolved using SDS-PAGE. For assays with purified recombinant protein, samples were analysed by either Coomassie staining or western blot detection. For assays with full-length TRIM E3 ligases, ubiquitination activity was analysed via western blot or fluorescence detection, while input samples were analysed by western blot detection.

2.10 Lysine discharge assays

Lysine-discharge assays were carried out with the E2, UbCH5A, using the precharged UbCH5A-Ub^{Cy3} adduct. Reactions were performed with E3s, TRIM22 RING and TRIM2 RBCC (positive control) at a concentration of 4 µM, along with a no E3 control, in addition to 1 µM UbCH5A-Ub^{Cy3} and 20 mM L-Lysine in the following reaction buffer: 50 mM HEPES pH 7.5 and 150 mM NaCl. A sample was taken before initiation of discharge with E3 and L-Lysine and subsequently at stated timepoints. The samples were mixed with 2 x SDS sample buffer before being resolved with SDS-PAGE and analysed by fluorescent detection using the LI-COR Odyssey imaging machine. For quantification, the Image Studio Lite Version 5.2 software was used to integrate the intensity of the UbCH5A-Ub^{Cy3} adduct band over the course of the reaction, using the no E3 control

reaction as a standard. The reactions were performed in triplicate and plotted as an average of relative amount of Ubch5A~Ub^{Cy3} against time, using the Graphpad Prism software.

2.11 E2 scan analysis

E2 Scans were performed with purified recombinant protein constructs of TRIM22 and TRIM6 E3 ligases. The E2 Scan Kit version 2 (UBIQUIGENT) contains purified samples of 34 different E2 conjugating enzymes provided in duplicate in a 96-well plate format. To these samples of E2, a master mix of E1 and Ub, with and without E3, to provide a 'no E3 ligase' control, was added in a reaction buffer of 25 mM HEPES pH 7.5, 150 mM NaCl and 20 mM MgCl₂. The following final concentrations of the reaction components were used: 0.3 µM E1, 2.5 µM E2, 10 µM E3 and 100 µM Ub. The reaction was initiated via addition of ATP, at a concentration of 2 mM, to each reaction well on the plate before incubation at 30 °C and 600 rpm for 1 h. The reaction was quenched by adding 10 µl of sample to 4x SDS sample buffer supplemented with 100 mM DTT and boiled for 10 minutes at 95 °C. Samples were run on large 25-well 4-12% Bis-TRIS gradient gels and analysed by western blot using the anti-ubiquitin (Ubi-1) antibody.

3. Results

3.1 Protein purification and biophysical characterization

3.1.1 Construct design and protein purification

Different lengths TRIM22 constructs, containing either single or multiple consecutive domains within the protein, were cloned and expression trials carried out in either small or large-scale format. These constructs were designed to assess the contribution of the individual domains to ubiquitination activity and/or substrate binding, as well as to identify those well-expressed and soluble, amenable for structural and biophysical characterization. In the case of TRIM6, the design and purification of different constructs was previously carried out by Rebecca Stevens, although additional attempts to purify the full-length protein and the substrate-binding domains of the protein were performed in this study.

Initially, bacterial expression systems were used for recombinant protein production as these are simpler and more efficient than eukaryotic expression systems. However, for constructs that were insoluble upon bacterial expression, in particular the longer constructs, insect cell-based expression systems, that have a more complex protein folding machinery, were also tested. A summary of the outcome of these expression trials is shown in Table 5.

For both TRIM22 and TRIM6, only shorter constructs containing the RING and the B-box domains, along with the CC domain for TRIM22, were soluble and could be expressed to high levels in *E. coli* (Fig. 7D). Unfortunately, the complete tripartite motif and constructs containing the substrate binding domain were insoluble in *E. coli* and use of insect cell expression systems did not improve protein solubility. As a result, alternative strategies were applied including the use of artificial constructs (“Mini TRIMs”) that have been used in previous studies of other TRIM E3 ligases (described in detail below).

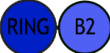
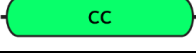
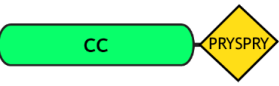
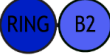
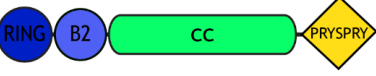
Construct		Affinity tag	Expression system	Results
TRIM22				
Schematic	Name			
	RING	His ₆ -SUMO	<i>E. coli</i>	Well expressed and soluble
	RB2	His ₆ -SUMO	<i>E. coli</i>	Well expressed and soluble
	RBCC	His ₆ -SUMO, 3xFLAG	<i>E. coli</i> , Sf9	Insoluble in small scale expression test
	CC	His ₆ -SUMO	<i>E. coli</i>	Well expressed and soluble
	CC-PRYSPRY	His ₆ -SUMO	<i>E. coli</i> , Sf9	Insoluble in large scale expression test (<i>E. coli</i>), Small band seen from small scale Sf9 expression test
	PRYSPRY	His ₆ -GST, His ₆ -MBP	<i>E. coli</i>	Insoluble upon removal of solubility tag
	Full-length	His ₆ -SUMO, His ₆ , 3xFLAG	<i>E. coli</i> , Sf9	Insoluble in small-scale expression test (<i>E. coli</i>), insoluble as His ₆ -SUMO fusion protein (Sf9), small band for His ₆ & 3xFLAG-tagged protein however scale-up showed no increase in soluble protein.
TRIM6				
Schematic	Name			
	RING	His ₆ -TRX	<i>E. coli</i>	Well expressed and soluble
	RB2	His ₆ -TRX	<i>E. coli</i>	Partially degraded but can be purified
	RBCC	His ₆ -TRX	<i>E. coli</i>	Insoluble in small scale expression test
	PRYSPRY	His ₆ -SUMO	<i>E. coli</i>	Insoluble in small scale expression test
	Full-length	His ₆ -TRX, His ₆ -SUMO	<i>E. coli</i> , Sf9	Insoluble in small-scale expression test (<i>E. coli</i>), insoluble as His ₆ -SUMO fusion protein (Sf9), small band for His ₆ & 3xFLAG-tagged protein however scale-up showed no increase in soluble protein.

Table 5. Summary of TRIM22 and TRIM6 purification trials attempted in this study. Constructs for which purifications were attempted are shown for both TRIM22 and TRIM6. The affinity and solubility tag(s) used for each construct are also shown; all protein constructs were cloned with the tags present N-terminal of the construct along with a 3C protease cleavage site present in between. The outcome of the purification is summarized in the final column, with the successful purifications labelled in **bold**.

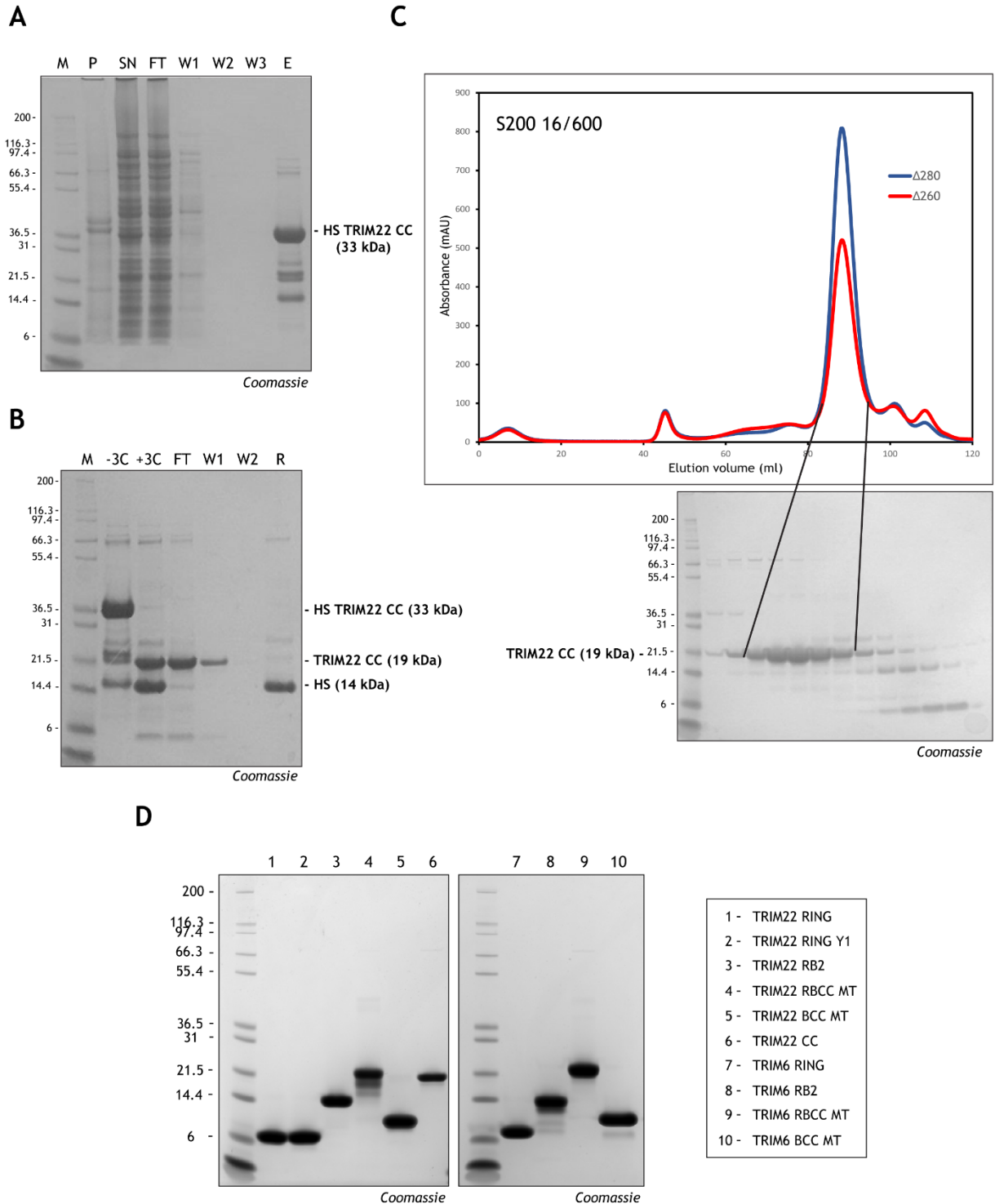


Figure 7. TRIM22 and TRIM6 protein construct purifications

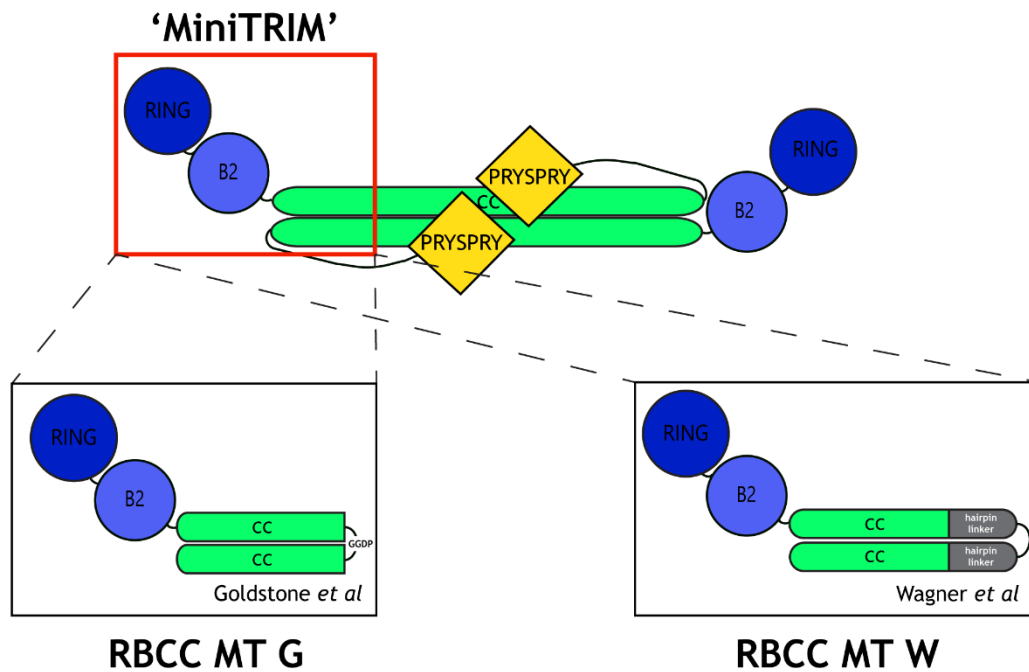
A - C Example purification of TRIM22 CC construct. **A.** SDS-PAGE gel of the fractions from the initial Ni-NTA affinity chromatography step with each sample labelled above the respective gel lane. P = cell pellet, SN = supernatant, FT = flowthrough, W = wash, E = elution. Relevant protein bands are annotated; HS = His₆-SUMO. **B.** SDS-PAGE gel of the samples from the reverse affinity Ni-NTA purification step, following cleavage by 3C protease. The sample in each well is labelled above and protein bands annotated also. -3C = before 3C cleavage, +3C = after 3C cleavage, R = Ni-NTA resin. **C.** Elution profile of TRIM22 CC from the Superdex 200 16/600 column, alongside SDS-PAGE gel of relevant fractions. Absorbance at both 280 and 260 nm is shown in blue and red respectively. **D.** SDS-PAGE gel of all purified protein samples for both TRIM22 and TRIM6 that were used for downstream experiments. Individual lanes are numbered with the specific sample corresponding to each number annotated on the right.

3.1.2 MiniTRIM constructs

MiniTRIM constructs, schematics shown in Figure 8A, are designed to represent a monomeric tripartite motif dimer and have previously been used in the study of rhTRIM5 α [63], [106]. MiniTRIM constructs consist of the RING and B-box domains, in addition to N and C-terminal regions of the coiled-coil, connected by an anti-parallel hairpin linker. This design uncouples interactions mediated by the RING and B-box domains from coiled-coil dimerization, while maintaining the B-box domain in a more native environment, with its hydrophobic region packed against the N-terminal end of the coiled-coil. The smaller size of these constructs compared to the full-length tripartite motif also makes them more amenable to high levels of expression in *E. coli*. In studies of rhTRIM5 α , miniTRIM constructs were used to probe the role of the B-box domain in higher order self-association and identified the key residues involved in self-association, some of which are also present in TRIM22 and TRIM6.

Two different miniTRIM designs have been used in the literature for rhTRIM5 α , differing in linker sequences and the regions of the coiled-coil included. Wagner *et al*, used longer coiled-coil sequences and an anti-parallel coiled-coil linker derived from a bacterial seryl-tRNA synthetase. This design was successfully used to crystallise the BCC miniTRIM of rhTRIM5 α [63]. In contrast, Goldstone *et al*, used shorter coiled-coil sequences and a simpler four residue GGDP linker in their rhTRIM5 α MiniTRIM design [106]. Both design concepts were applied to TRIM22 to produce two MiniTRIM constructs termed RBCC MT G and RBCC MT W, for the Goldstone *et al* and Wagner *et al* designs, respectively. Expression and purification trials were performed with TRIM22 using both designs for which the gel filtration profiles of both purifications, alongside the SDS-PAGE gels of the respective fractions, are shown in Fig. 8B. These results illustrate that the solubility, expression levels, and purity of the RBCC MT G construct were much higher than that of the RBCC MT W. As a result, the BCC MT G construct for TRIM22 was cloned, lacking the RING domain, and both RBCC MT G and BCC MT G constructs (hereafter referred to as RBCC MT and BCC MT respectively) were also cloned for TRIM6. These constructs showed similar levels of solubility and expression levels as the equivalent TRIM22 constructs (Final samples shown in Fig. 7D).

A.



B.

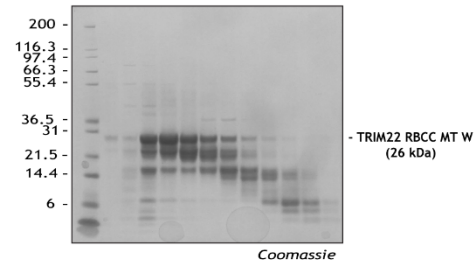
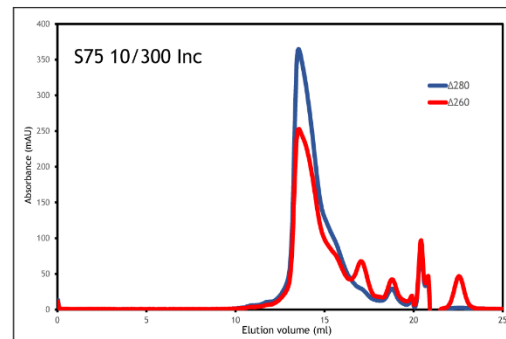
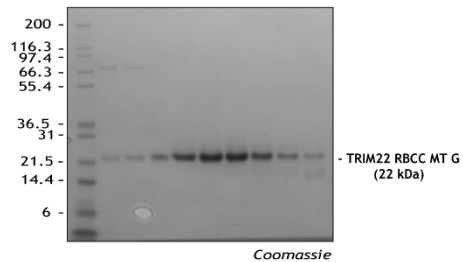
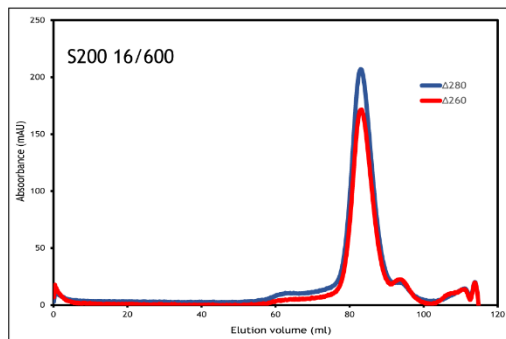


Figure 8. MiniTRIM construct designs and purification trials. A. Schematic of a dimeric TRIM molecule outlining the regions represented by the MiniTRIM constructs (Top). Diagrams of the two different MiniTRIMs, RBCC MT G (Bottom left) and RBCC MT W (Bottom right), illustrating the presence of the RING and B-box 2 domains in addition to sections of the CC separated by two different linker sequences. B. Elution profiles for TRIM22 RBCC MT G (Left) and RBCC MT W (Right), from the size-exclusion chromatography steps of the purification trials, along with the corresponding SDS-PAGE gel of the fractions.

Since there is no structural information for either TRIM22 or TRIM6, construct boundaries of the miniTRIM constructs were designed based on sequence alignment with rhTRIM5 α . To investigate if the regions of the coiled-coil selected in construct design, in particular the C-terminal sequences following the linker region, are correctly folded, scanning CD spectra of the RING, RB2 and RBCC MT constructs of both TRIM22 and TRIM6 were performed and compared using both molar ($\Delta\epsilon_M$) and mean residue ($\Delta\epsilon_{MRW}$) CD extinction coefficients (Fig. 9). The miniTRIM constructs of TRIM22 and TRIM6 should theoretically contain additional secondary structure compared to the RB2 constructs that would translate to stronger signal when probed by CD. In this regard, a clear increase in secondary structure, specifically alpha helical content, can be seen for TRIM6 RBCC MT using both metrics inferring correct folding of the miniTRIM construct. TRIM22 RBCC MT, in contrast, appears to lack any additional secondary structure beyond that of the RB2 construct, despite retaining the spectrum of a folded protein.

Since these experiments were performed, the AlphaFold 2 software has been released and protein homology models have been made available through the AlphaFold protein structure database. Structural predictions for TRIM22 show that construct boundaries chosen for the C-terminal region of the coiled-coil domain, have the same secondary structure as those used in rhTRIM5 α , providing confidence that this region is folded and correctly assembled in the TRIM22 MiniTRIM construct, much like rhTRIM5 α . Considering this, I decided to continue with downstream characterization of both of these constructs.

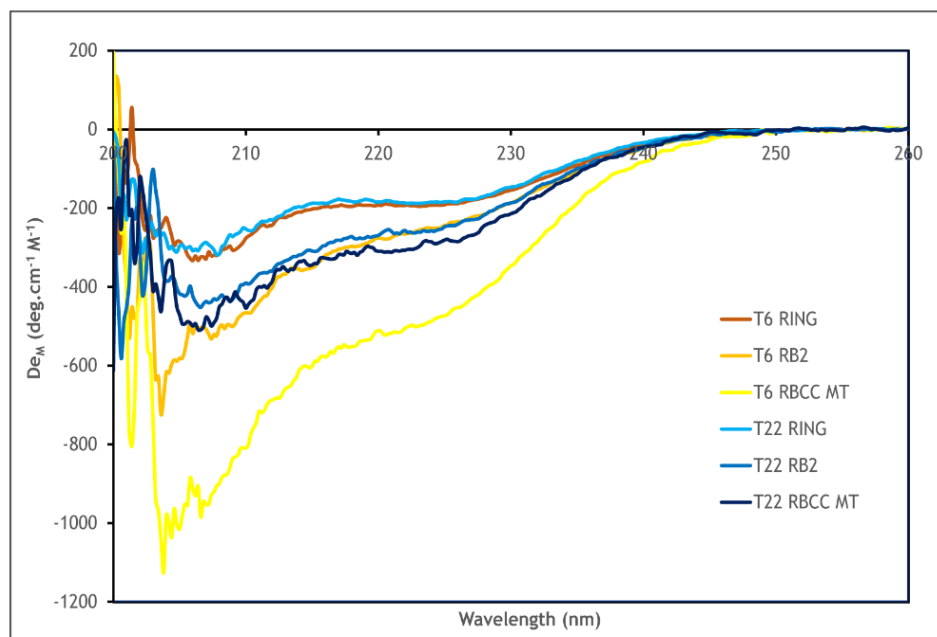
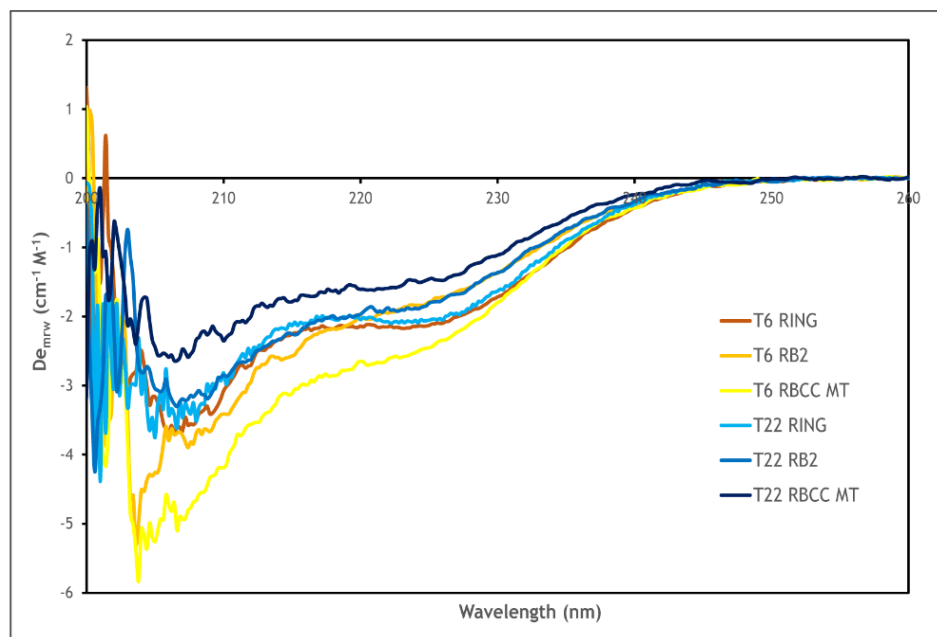
A**B**

Figure 9. Far-UV CD spectra of TRIM22 and TRIM6 RING, RB2 and RBCC MT construct. The far-UV CD spectra of all TRIM22 and TRIM6 constructs is shown, plotted as both molar and mean residue CD extinction coefficients in A. and B. respectively. The molar CD extinction coefficient illustrates the overall CD signal of each individual protein construct and indicates the contribution of each domain to the total CD signal. The mean residue extinction coefficient, on the other hand, illustrates the CD signal per residue and is comparable between constructs of different molecular weights.

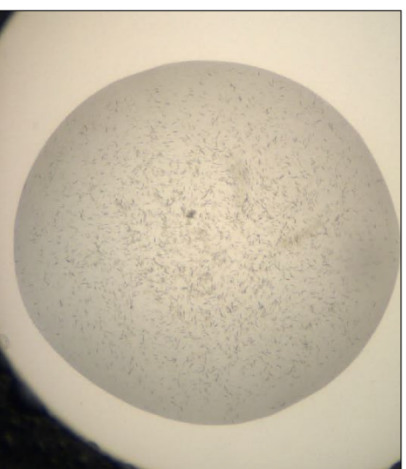
3.1.3 TRIM22 and TRIM6 crystallisation trials

Crystallisation trials with all purified TRIM22 and TRIM6 constructs were set-up at multiple concentrations with a range of at least six available 96-well commercial crystallization screens (all conditions tested are listed in Fig. 10A). However, only the TRIM22 coiled-coil domain produced tractable crystals. Initial needle-like crystals were produced in the Wizard 1 & 2 sparse matrix (Jena Bioscience) in the following reservoir condition: 0.2 M NaCl, 35% w/v MPD and 0.1 M TRIS pH 7 (Fig. 10B). Secondary commercial screens and manual screens were subsequently set-up to optimize these crystals, screening for buffer pH and MPD concentration. This led to more conditions containing mostly microcrystals and needles, in addition to one drop with larger individual crystals (Fig. 10C). The largest of these crystals was fished, frozen and shot using our in-house X-ray beam, however, the crystal didn't diffract. The remaining crystals were crushed to make seeds for future crystallization attempts. Further optimization was performed without success, in part due to the low surface tension of the MPD precipitant which affected consistency of drop formation.

A

Construct	Crystallisation Screens	Concentration (mg/ml)
TRIM22		
RING (1-87)	Batch 1: AmSO4, Index, JSCG+, PEG RX, W 1&2, W 3&4	Batch 1: 10 & 12 mg/ml
	Batch 2: Index HT, JSCG Core I, JSCG+, PACT, PEG Suite I, W 3&4 Index HT, JSCG Core I-IV, JSCG+, Wizard 1&2, Wizard 3&4	Batch 2: 4.9 & 7.2 mg/ml
RING M1Y (1-87)	AmSO4, Index HT, JSCG+, Morpheus, PEG Suite I, Wizard 1&2	All screens: 15 & 20 mg/ml
RB2 (1-133)	AmSO4, Index HT, JSCG+, Morpheus, PEG Suite I, Wizard 1&2	All screens: 9.8 mg/ml AmSO4 & PEG Suite I screens: 6.2 & 9.3 mg/ml
RBCC MT (1-261ΔCC)	AmSO4, Index HT, JSCG+, Morpheus, PEG Suite I, Wizard 1&2	AmSO4 & PEG Suite I screens: 3.9, 5.2 & 9.7 mg/ml Other screens: 8.0 mg/ml
BCC MT (88-261ΔCC)	AmSO4, PEG Suite I, Index HT, JSCG+, W 1&2, W 3&4	AmSO4 & PEG Suite I: 5.5 & 7.8 mg/ml Other screens: 4.4 & 7.9 mg/ml
CC (132-295)	AmSO4, Index HT, JSCG+, PACT, PEG RX, W 1&2, MPD Suite (RT & 4 °C), Morpheus I & II Manual Screens: 10-65% MPD, 0.1 M MES/TRIS/HEPES pH 6 - 8.2	AmSO4 & PACT screens: 8.5 mg/ml Index HT, JSCG+, PEG RX & Wizard 1&2 screens: 5.4, 6.6 & 8.5 mg/ml Morpheus I & II screens: 5.8 mg/ml +/- seed stock (1:20) MPD Suite screen: 4.7 & 7.0 mg/ml Manual Screens: 4.7 or 5.8 mg/ml +/- seed stock (1:3-10)
TRIM6		
RBCC MT (1-261ΔCC)	AmSO4, Index HT, JSCG+, JSCG Core I, JSCG Core II, PEG Suite I, pH clear II, W 1&2, W 3&4	All screens: 8.2 mg/ml AmSO4 & PEG Suite I also at: 12.1 mg/ml Other Screens also at: 12.5 mg/ml
BCC MT (88-261ΔCC)	Index HT, JSCG Core I, JSCG+, PACT, PEG Suite I, W 3&4	All screens: 3.2 & 4.8 mg/ml

B



C



Figure 10. List of crystallization screening trials.

A. A table listing each of the protein constructs for which crystallization trials were made alongside the crystallization screens tested and concentrations tested. **B.** Initial TRIM22 CC crystal hit from the commercial sparse matrix Wizard 1 & 2 crystallisation screen. **C.** Follow-up TRIM22 crystal hit from the secondary commercial crystallization screen, MPD suite.

3.1.4 Characterisation of self-association properties of TRIM22 & TRIM6

To investigate the self-association properties of the individual domains of TRIM22 and TRIM6, solution-based experiments were performed. As outlined in the introduction, self-association, specifically dimerization of the RING domain, is a pre-requisite for TRIM E3 ligase activity. Additionally, interactions between other domains in the protein can also contribute to the function of TRIM E3 ligases, as reported for TRIM5 α [60]. To assess the self-association properties, I used size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) which provides accurate molar masses of the different species within a protein sample separated by size-exclusion chromatography. All available purified protein samples of TRIM22 and TRIM6 were tested, using two different columns, the S75 or S200 10/300 Increase columns, both with different resolution ranges. The choice of column was based on the molecular weight and the predicted oligomeric state of each sample and multiple concentrations were tested to assess the concentration effect on self-association. The SEC-MALLS profiles of TRIM22 and TRIM6 constructs are shown in Fig. 11 and Fig. 12, respectively. TRIM22 and TRIM6 share high sequence similarity with rhTRIM5 α within the B-box trimerization interface, presenting the possibility of higher order oligomerization for TRIM22 B-box containing samples. With this in mind, all TRIM22 samples, including the TRIM22 RING domain for consistency, were run on the S200 10/300 Increase column which has a better resolution for higher molecular weight samples. This initial analysis suggested that the RING domain of TRIM22 existed as a single species at all concentrations tested, while the RB2 and RBCC MT constructs exhibited concentration dependent monomer-dimer equilibria (Fig. 11A, C-D). Removal of the RING domain in the BCC MT construct disrupted this equilibrium, showing a largely single, monomeric species, indicating that the RING domain of TRIM22 was responsible for self-association (Fig. 11E).

This discrepancy between the RING and the RB2 and RBCC MT profiles was unexpected and inferred disruption of RING domain dimerization by the B-box domain. Another puzzling observation was that the single peak from the RING domain profile eluted at a molecular weight between that of the monomer and the dimer (Fig. 11A). One possibility was that the resolution of the S200 10/300 increase column, which is relatively weak at this molecular weight (10-20 kDa), was unable to separate the monomeric and dimeric species. As a result, I decided to run the RING domain sample again on the S75 10/300 Increase column, with better resolution at smaller molecular weights. In agreement with this hypothesis, the second run of the TRIM22 RING domain produced two separate peaks for the monomeric and the dimeric RING species,

indicating that the RING domain of TRIM22 exists in a monomer-dimer equilibrium (Fig. 11B).

Despite separation of the monomeric and dimeric species, the calculated molar mass of the dimeric RING sample was still below the expected molecular weight, and this was also the case for the RB2 and RBCC MT constructs (Fig. 11B-D). In contrast, the CC domain, which was dimeric at all concentrations tested, appeared at a molecular weight much closer to the expected dimeric molecular weight (Fig. 11E). One possible explanation could be that the reduced molecular weight was the result of degradation during purification. To investigate this, the purified RING, RB2 and RBCC MT constructs were analysed by mass spectrometry. The resulting calculated molecular weights of the TRIM22 constructs were within 0.3 daltons of the expected molecular weight and confirmed that degradation is not the reason for this unusual behaviour in SEC-MALLS (Supplementary Fig. 1).

As a result of the improved resolution of the S75 10/300 Increase column, the RING and BCC MT constructs of TRIM6 were also analysed using this column. Figure 12A-B shows that in comparison to TRIM22, the RING and RBCC MT constructs of TRIM6 show much stronger dimeric tendencies with no significant shift in molecular weight with changes in concentration. Analysis of the TRIM6 BCC MT construct shows disruption of this strict dimerization, however a clear titration effect could still be seen (Fig. 12C). This suggests that the B-box domain may still contribute to TRIM6 oligomerization, despite possessing weaker self-association properties than the RING domain (Fig. 12A-B). The improved resolution of the S75 10/300 column at lower molecular weights makes this observation clearer and puts into question the conclusions for the TRIM22 BCC MT construct which, when analysed with the S200 10/300 column, appears to show only marginal titration, however this may be the result of the reduced resolution (Fig. 11E).

Overall, the self-association properties of TRIM22 and TRIM6 align with other TRIM E3 ligases that show a range of dimerization affinities of the RING domain. Interestingly however, TRIM22 and TRIM6 do not show B-box trimerization as observed in rhTRIM5 α despite possessing some of the key residues responsible for this interaction. In contrast, both show stronger self-association within the RING domain [106]. This could relate to their physiological function, since neither TRIM22 or TRIM6 are reported to bind to oligomeric structures such as viral capsids, which triggers oligomerisation of rhTRIM5 α and subsequent RING dimerization. Instead, TRIM22 and TRIM6 likely require different control mechanisms for E3 ligase function. The tendency to dimerise within the RING domains of both TRIM22 and TRIM6 suggests that they have E3 ligase activity, and the characterization of this activity is the focus of the subsequent experiments of this study.

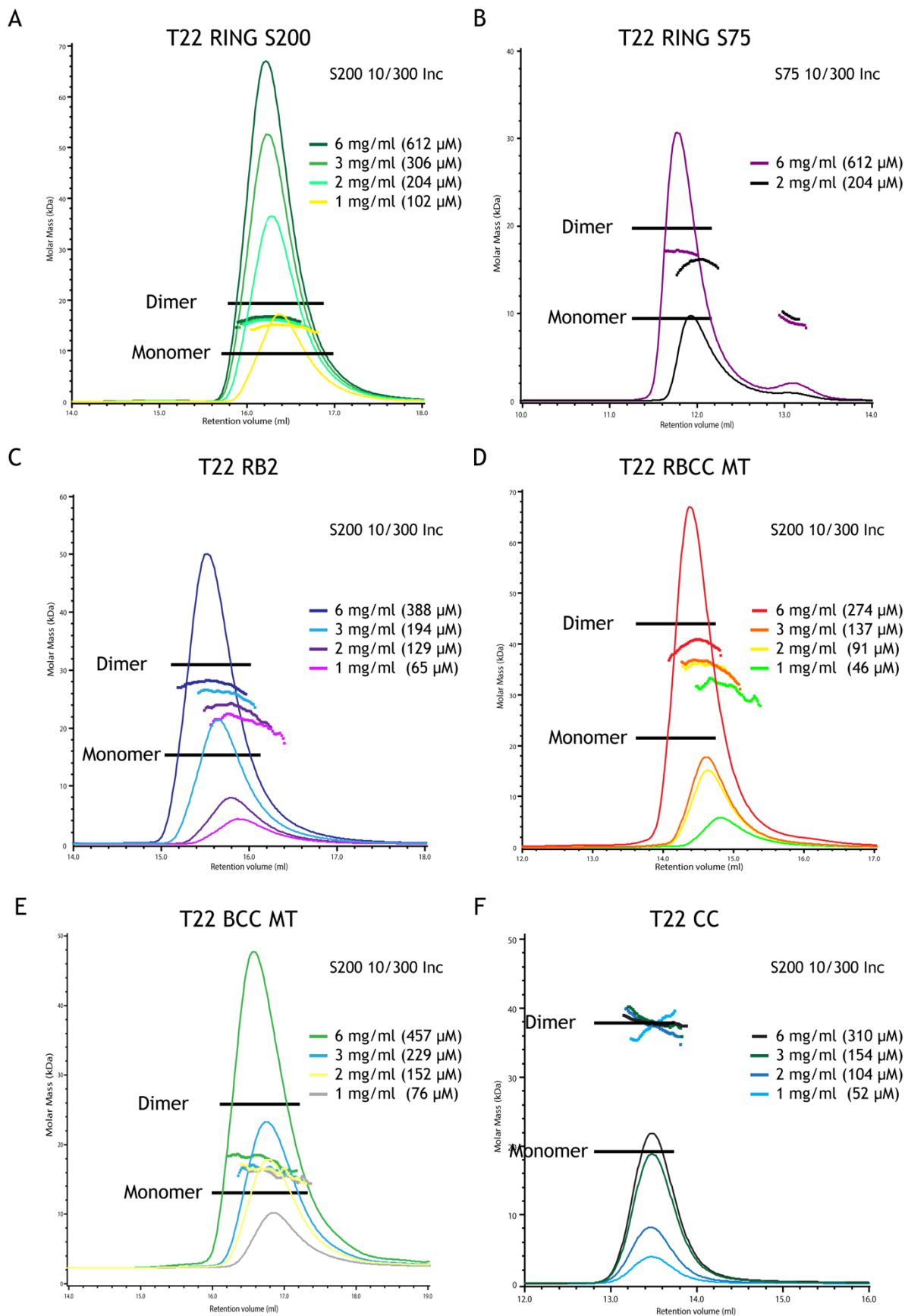


Figure 11. The SEC-MALLS profiles of purified TRIM22 constructs.

The SEC-MALLS profiles, tested at different concentrations, for the TRIM22 constructs, **A.** TRIM22 RING S200, **B.** TRIM22 RING S75, **C.** TRIM22 RB2, **D.** TRIM22 RBCC MT, **E.** TRIM22 BCC MT, and **F.** TRIM22 CC. The specific concentration, size-exclusion column used, and the expected molecular weights of the monomeric and dimeric species are annotated on each profile.

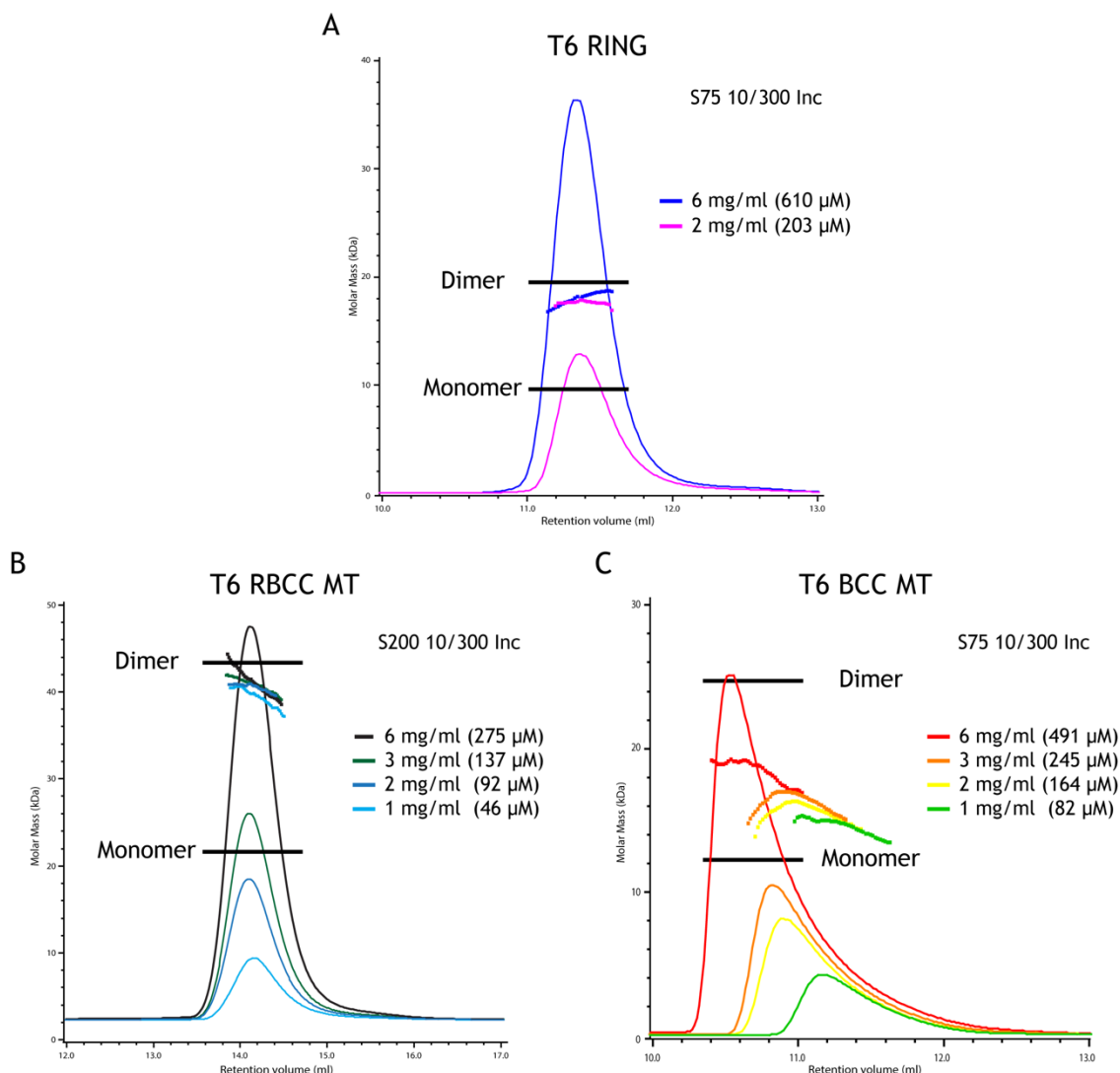


Figure 12. The SEC-MALLS profiles of purified TRIM6 constructs.

The SEC-MALLS profiles of **A.** TRIM6 RING, **B.** TRIM6 RBCC MT and **C.** TRIM6 BCC MT annotated with the specific concentrations tested, along with the size-exclusion column used as well as the expected molecular weights of the monomeric and dimeric species.

3.2 Characterisation of the ubiquitination activities of TRIM22 and TRIM6 E3 ligases

3.2.1 Assessment of previously reported TRIM22 and TRIM6 ubiquitination activity

Reports in the literature suggest that TRIM22 and TRIM6 possess ubiquitination activity in conjunction with the E2s, UBE2D2 (Ubch5B) and UBE2K respectively [97], [105]. In both cases, activity assays were performed with the full-length proteins expressed in mammalian cells and not with purified protein expressed in *E. coli*. Therefore, I decided to test activity of the purified RING domain containing constructs of TRIM22 and TRIM6, from *E. coli*, in *in vitro* ubiquitination assays with the relevant E2s. For TRIM22 constructs, activity assays were performed with UBE2D1 (Ubch5A) as opposed to Ubch5B since they share 89% sequence identity, and the former is more generally used in ubiquitination assays, in both the literature and in our lab.

Ubiquitination assays with Ubch5A and UBE2K have different activity readouts due to their different enzymatic properties. Ubch5A is capable of conjugating ubiquitin onto lysine residues within a substrate protein including the E3 itself and therefore autoubiquitination of the E3 construct can be used as a readout. UBE2K in contrast, produces unanchored polyubiquitin chains making the readout, the sequential elongation of these polyubiquitin chains. These distinct readouts also allow different conclusions; whereas unanchored polyubiquitin chain formation can be used efficiently to compare constructs of different sizes, autoubiquitination is affected by the number of available lysine residues within the E3 construct and therefore is biased towards larger constructs. Furthermore, all *in vitro* assays were performed alongside a negative control as E2s possess different levels of background E3-independent ubiquitination activity.

Initial experiments were performed under the standard assay conditions used previously in the lab, with E3 concentrations of 4 μ M and at a temperature of 25 °C (further details are outlined in the methods section). Analysis was performed by Coomassie blue staining of SDS-PAGE gels as is protocol in our lab since it enables robust comparison of different reactions. For autoubiquitination assays with TRIM22 RING, RB2 and RBCC MT constructs, a TRIM23 RING-B-box1-B-box-2 (RBB) construct, that was available in the lab, was used as a positive control. TRIM23 RBB is a similar size to the largest TRIM22 construct and has a similar number of lysine residues. Furthermore, previous studies have shown it to exhibit a similar monomer-dimer equilibrium within the RING domain as seen for TRIM22 [55].

TRIM22

Surprisingly, Figure 13 shows that under these experimental conditions no E3 ligase activity could be detected for any of the TRIM22 constructs, in comparison to robust autoubiquitination after 30 mins for the positive control, suggesting that TRIM22 RING,

RB2 and RBCC MT constructs are incapable of autoubiquitination under these assay conditions.

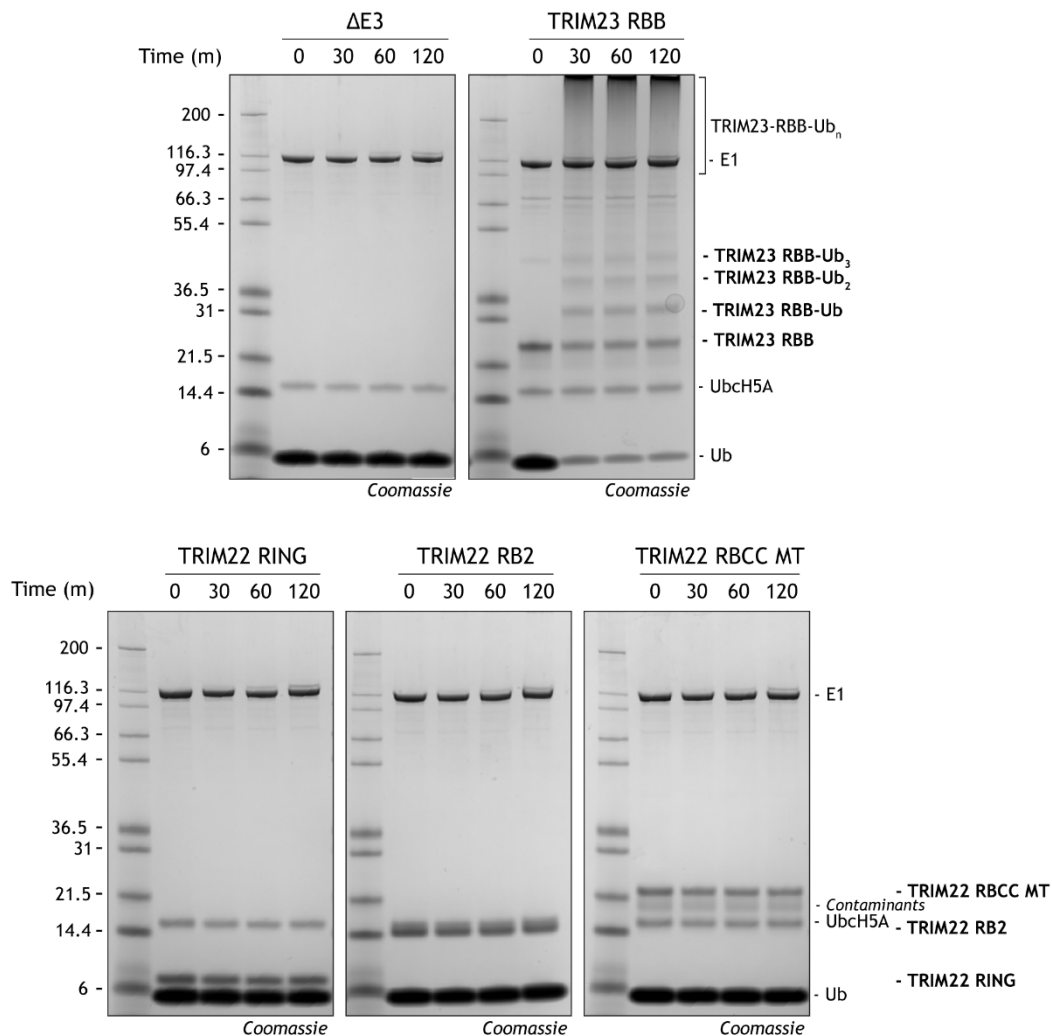


Figure 13. *In vitro* autoubiquitination assays with TRIM22 RING domain containing constructs in conjunction with UbCH5A.

SDS-PAGE gels of the samples from autoubiquitination assays with different purified TRIM22 constructs, including positive (TRIM23 RBB) and negative ($\Delta E3$) reaction controls. The respective construct tested is labelled above the gel. Reactions were performed at 25°C, with E3 concentrations of 4 μ M. Samples were taken at timepoints: 0, 30, 60 and 120 mins. The protein bands for the components of the reaction, in addition to ubiquitinated products, are annotated. The results in this figure are representative of n = 3 experiments.

Following this outcome, I decided to test different conditions including lower E3 concentrations of 1 μ M at physiological temperature, 37°C, alongside a much higher E3 concentration of 10 μ M, to push the equilibrium towards the dimeric species. In addition, I tested the more sensitive western blot detection method for these assays using the anti-ubiquitin antibody (Ubi-1) commonly used in the ubiquitination field. For

these assays only the longest construct of TRIM22 was used (TRIM22 RBCC MT) since it exhibits the same self-association properties as the smaller constructs; TRIM23 RBB was used again as a positive control at a concentration of 1 μ M. Figure 14 shows that despite increased temperature and E3 concentration, no detectable ubiquitination activity could be seen for TRIM22 RBCC MT at either concentration, in comparison to high levels of TRIM23 RBB autoubiquitination.

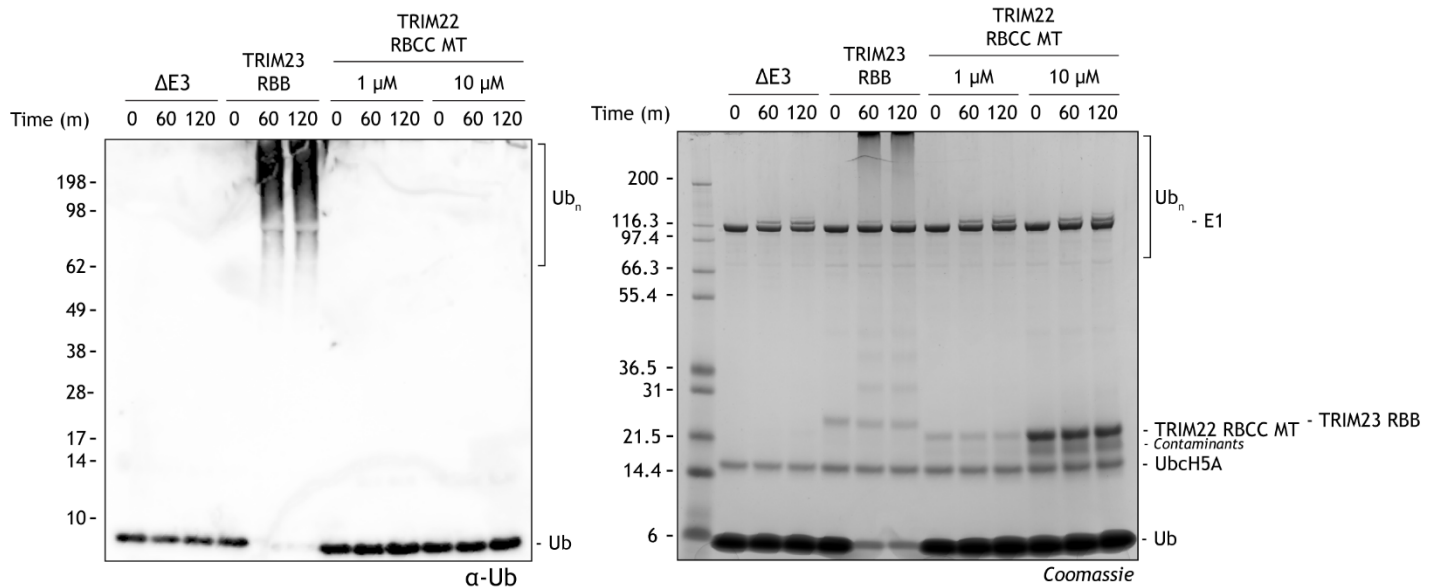


Figure 14. *In vitro* TRIM22 autoubiquitination assays using Ubch5A, at physiological temperature.

In vitro autoubiquitination assays for TRIM22 RBCC MT alongside Ubch5A performed at both 1 μ M and 10 μ M E3 concentrations, at 37°C, alongside positive (TRIM23 RBB) and negative (Δ E3) controls. Reactions were analysed by both western blot (left) and Coomassie blue (right) detection and protein bands for reaction components or ubiquitinated species were annotated. The data presented is representative of $n = 2$ experiments.

As mentioned previously, the drawback of these assays is their dependence on available lysine residues within the E3 construct. An alternative method of quantifying activity is the use of lysine-discharge assays; in these assays the E2 enzyme of choice is pre-charged with a ubiquitin molecule and subsequently incubated with the E3 enzyme, along with an excess of free lysine. A readout of ubiquitination activity is then achieved by monitoring the rate of discharge of the ubiquitin from the E2-ubiquitin conjugate onto the free lysine, in the presence and absence of the E3. This setup overcomes the bias due to the available number of lysine residues in autoubiquitination assays and therefore can be used to assay any construct regardless of lysine availability. The use of fluorescent dyes also enables quantification of the discharge activity. In this instance, Cy3-labelled ubiquitin (Ub^{Cy3}) was loaded onto Ubch5A and the purified Ubch5A- Ub^{Cy3} adduct was used in the discharge assays. The assays were performed with TRIM22 RING domain, using the TRIM2 RBCC construct this time as a positive control, along with a negative ‘no E3’ control. Quantification was performed using the Image

Studio Lite Version 5.2 software, by integration of the E2-ubiquitin conjugate at different timepoints, normalized against the intensity of the UbCH5A-Ub^{Cy3} band at timepoint 0. Despite this unbiased approach, Figure 15B, shows that T22 RING domain has no significant discharge activity above that of the negative control in comparison to the positive control for which approximately all ubiquitin was transferred onto free lysine after 2 mins. These results suggest that the T22 RING domain alone is inactive in conjunction with UbCH5A in *in vitro* ubiquitination assays.

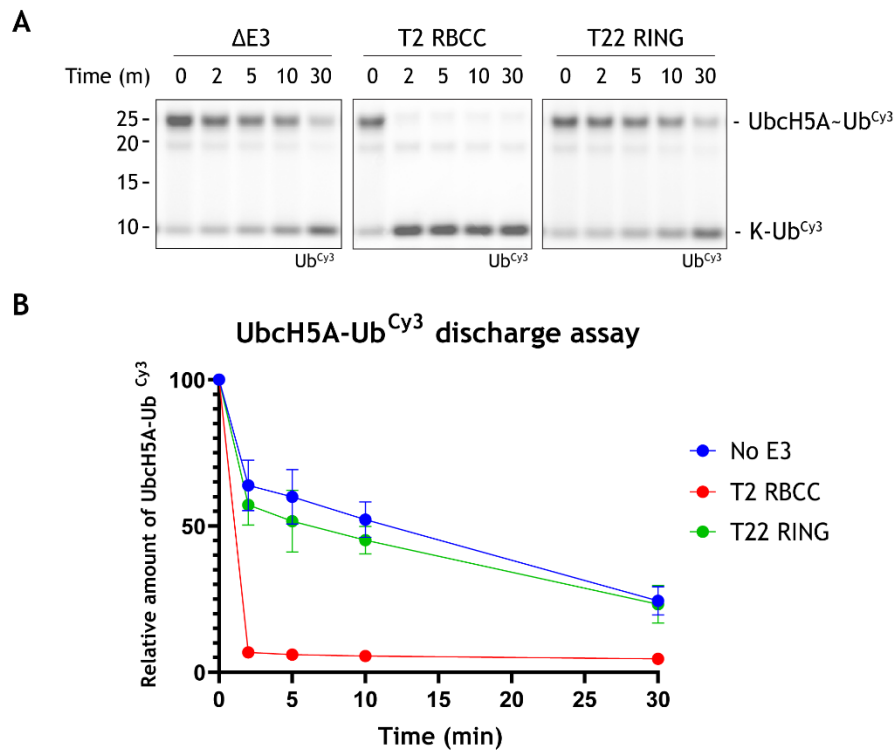


Figure 15. UbCH5A-Ub^{Cy3} lysine discharge assays with TRIM22 RING domain.

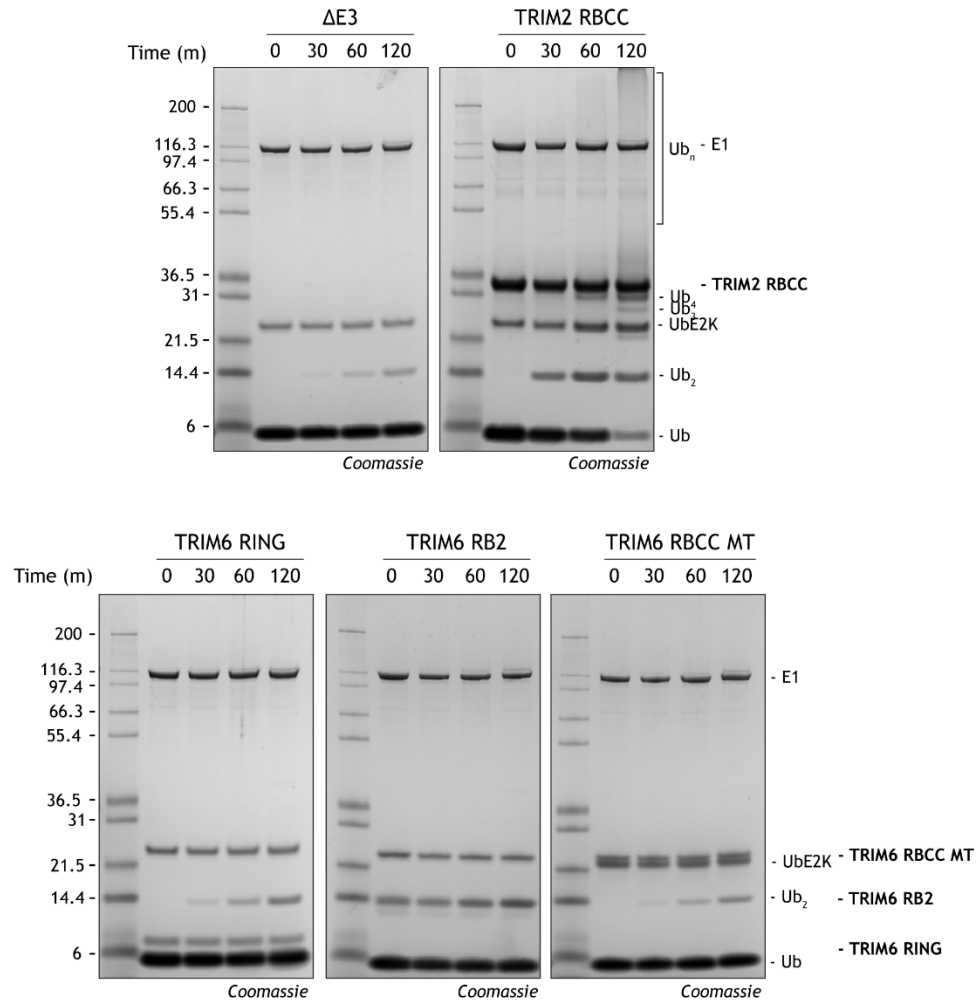
A. SDS-PAGE gels of samples from the lysine-discharge reactions with TRIM22 RING domain, along with both positive (TRIM2 RBCC) and negative controls (ΔE3), analysed by fluorescent detection at 569 nm. Protein bands representing the UbCH5A-Ub^{Cy3} conjugate and K-Ub^{Cy3} are annotated. **B.** Graphical representation of the results from the lysine-discharge assays with TRIM22 RING and controls using the intensity of the UbCH5A-Ub^{Cy3} band over time as a readout.

TRIM6

Similar activity assays were performed with the RING domain containing constructs of TRIM6 in conjunction with UbE2K. As previously mentioned, polyubiquitin chain formation assays can be performed with UbE2K and therefore different length constructs can be directly compared without the need for lysine-discharge assays. Initial assays were carried out under standard conditions ([E3] = 4 μM at 25 °C) using TRIM2 RBCC as a positive control. Figure 16A, shows significant ubiquitination activity after 120 mins, for the positive control, however no activity can be seen for any of the TRIM6 constructs under these conditions. The RB2 construct of TRIM6 co-migrates with the di-ubiquitin species making interpretation by Coomassie blue staining impossible.

Subsequent attempts were made to visualize the ubiquitination activity using increased temperatures (37°C) and E3 concentration (10 µM), in addition to western blot detection. Despite these attempts, it can clearly be observed from Figure 16B, that TRIM6 shows no ubiquitination activity in comparison to strong smears of polyubiquitin chains present in the positive control reaction after 60 minutes incubation. These results suggest that, like for TRIM22, activity seen in the literature with the E2 enzyme, UbE2K, cannot be reproduced in our experimental setting.

A



B

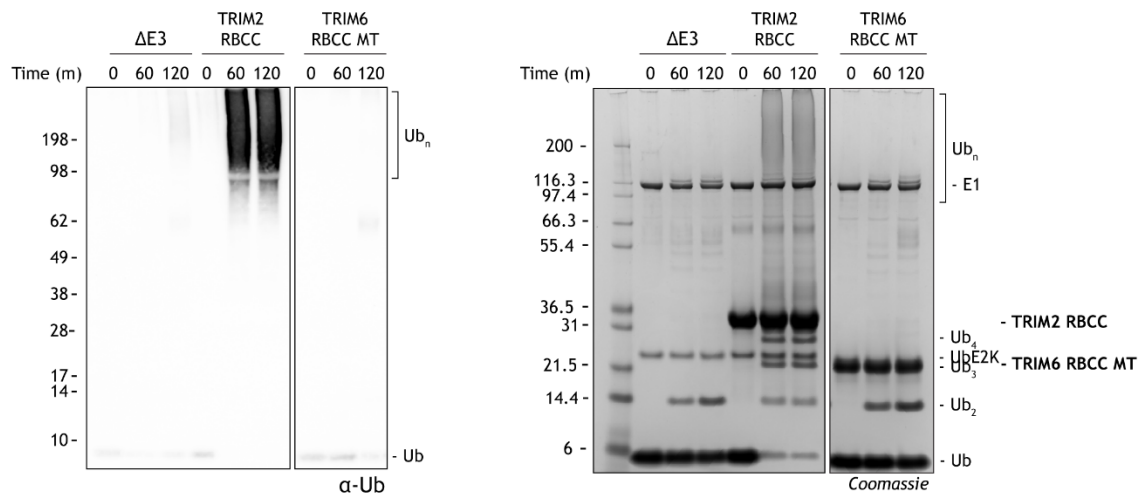


Figure 16. *In vitro* TRIM6 polyubiquitination assays alongside the E2, UBE2K.

A. SDS-PAGE gels of *in vitro* polyubiquitination assays for TRIM6 RING, RB2 and RBCC MT constructs in conjunction with UBE2K, alongside positive (TRIM2 RBCC) and negative (Δ E3) controls, performed under standard assay conditions of 4 μ M E3 concentration, and at 25°C. Relevant protein bands are annotated. **B.** *In vitro* polyubiquitination assays with TRIM6 RBCC MT in conjunction with UBE2K, using conditions of 10 μ M E3 concentration and at 37 °C, analysed by both western blot (left) and Coomassie blue (right) detection. The results in this figure are representative of n = 2 experiments.

3.2.2 E2 screening experiments

The experiments described in the previous section did not detect any ubiquitination activity for either TRIM22 or TRIM6 RBCC MT constructs with the two E2 enzymes referenced in the literature, neither through Coomassie blue or western blot detection methods. Nonetheless, it is possible that TRIM22 and TRIM6 possess *in vitro* E3 ligase activity in conjunction with other E2 enzymes. To explore this possibility, TRIM22 and TRIM6 RBCC MT constructs were used in commercial E2 screens which contain purified samples of all 35 human E2 enzymes in a 96-well format, allowing comprehensive analysis of potential E2-E3 partners. The conditions for this screen are incubation at 30°C for 1 hour and detection using the anti-ubiquitin antibody. All reactions were performed in duplicate, with and without E3, to distinguish between E3-dependent and E3-independent ubiquitination activity. In contrast to the recommended E3 concentrations of 1 μ M, both TRIM22 and TRIM6 RBCC MT were used at 10 μ M, to ensure low levels of activity were highlighted.

TRIM22

Figure 17 shows the results of the TRIM22 RBCC MT E2 screen. Low levels of activity can be seen in conjunction with the E2s, UBE2S and UBE2Q, that are slightly higher than that of the 'no E3' control. In addition, faint high molecular weight smears could be detected in the presence of the E3, for all members of the UBE2D family of E2s; not present in TRIM6 (see below). This implies TRIM22 may have very low levels of ubiquitination activity with the UBE2D family along with UBE2S and UBE2Q, although considering the reaction conditions, how relevant this is in a physiological setting is unclear.

In contrast, the ubiquitin smears seen in the reactions with the heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2, are clear signs of activity. The heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2 share similar *in vitro* activity profiles despite distinct cellular roles [8]. They are present in high concentrations within the cell in one form or the other and produce K63-linked polyubiquitin chains which are linked to a variety of non-proteolytic functions, including activation of cell signaling cascades.

TRIM6

Similar results to those with TRIM22, were seen for TRIM6 RBCC MT (Figure 18), with minimal levels of activity in conjunction with the E2s, UBE2S, UBE2R1, UBE2Q and

UBE2Q2. However, again, clear activity could be detected in conjunction with heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2. For TRIM6 RBCC MT the smears of ubiquitination activity extended to higher molecular weights than for TRIM22 RBCC MT, suggesting TRIM6 RBCC MT is potentially more active in K63-linked polyubiquitin chain formation.

Since the E3 concentration used in these assays was much higher than in previous studies of other TRIM E3 ligases in our lab, I decided to follow-up these results with additional assays at lower E3 concentrations.

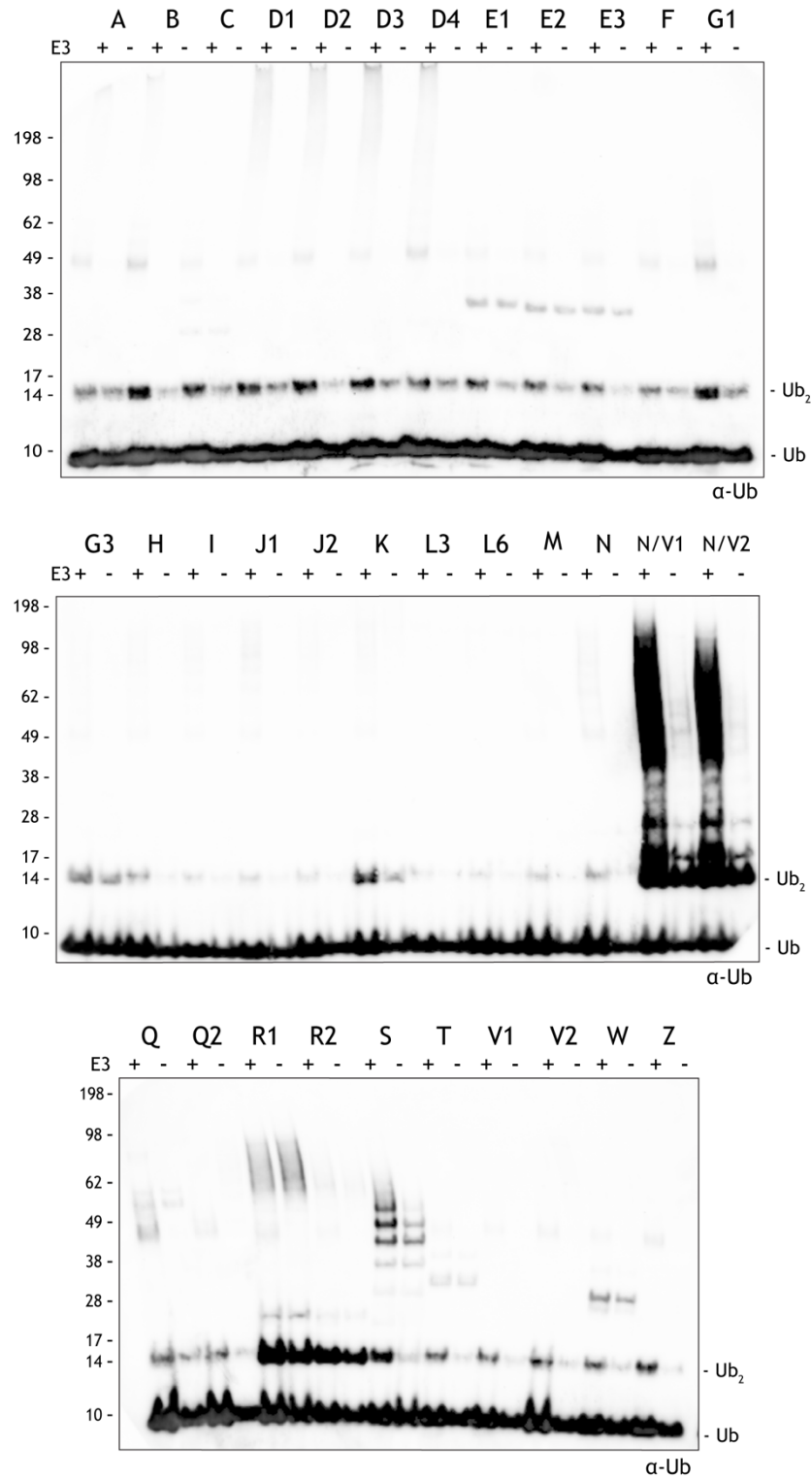


Figure 17. TRIM22 RBCC MT E2 Scan

Results from the E2 scan experiments with TRIM22 RBCC MT analysed by western blot detection using the Ubi-1 antibody. The blots are labelled with the individual E2 suffixes minus the 'UBE2' prefix, for example, UBE2D1 is labelled simply as D1. Two samples are present for each E2, with and without E3. All reactions were performed with E3 concentrations of 10 μ M at 30°C.

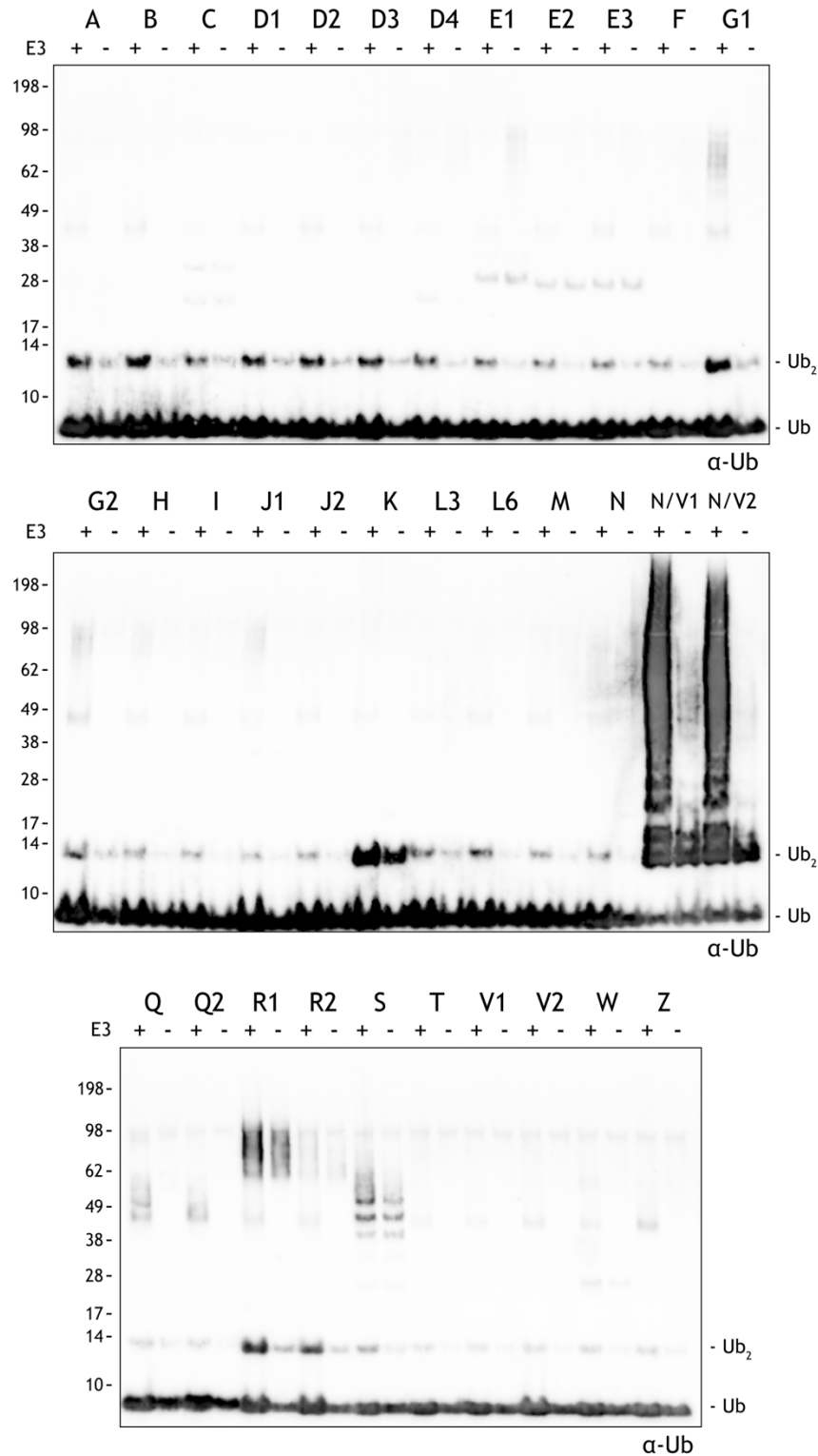


Figure 18. TRIM6 RBCC MT E2 Scan

TRIM6 RBCC MT E2 scan results analysed by western blot detection with the Ubi-1 antibody. Reactions for each E2 were performed in duplicate with and without the E3 and samples for each individual E2 are labelled with the suffixes minus the 'UBE2' prefix, for example, UBE2G2 is labelled simply as G2. Reactions were performed at 30°C and with an E3 concentration of 10µM.

To follow up on the results from the E2 scans, activity of the different constructs of TRIM22 and TRIM6 in conjunction with UBE2N/UEV2 was tested under our standard assay conditions of 4 μ M E3 at 25°C. For these experiments, a TRIM21 RING construct was used as a positive control, due to the availability of experimental data on this E3 in combination with UBE2N/UEV2, in the literature [58]. Figure 19 shows that no activity could be seen at this concentration for TRIM22 constructs, while only low levels of activity were visible for TRIM6 constructs with subtle decreases in activity as the length of the construct increased.

Subsequently, ubiquitination assays at E3 concentrations of 1 μ M and at physiological temperature, 37°C, were performed using Coomassie blue and western blot staining methods. For this experiment, only the RBCC MT constructs of TRIM22 and TRIM6 were used, as activity levels in the experiment shown in Figure 19 were either the same or very similar for all constructs tested. Figure 20 shows that under these conditions TRIM22 and TRIM6 RBCC MT constructs show clear activity, visible by both detection methods suggesting K63 polyubiquitin chain formation activity for these two TRIM E3 ligases. Nonetheless, the activity for both constructs was lower than when tested at 10 μ M, this may well be the result of changes in the monomer-dimer ratios of these proteins.

In summary, the results from the *in vitro* ubiquitination assays using RING domain-containing constructs of TRIM22 and TRIM6 expressed in *E. coli*, clearly differ from those that have been reported in the literature. While both TRIM E3 ligases were inactive at a range of conditions with their suggested E2 partners, the UbcH5 family (TRIM22) and UBE2K (TRIM6); both showed reproducible activity with the heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2. However, the observed activity of both TRIM22 and TRIM6 was much weaker than the positive control, TRIM21. Despite these results, additional factors could influence the activity profile such as, the arrangement of the full-length protein, post-translational modifications and/or potential binding partners. To address these possibilities, I decided to test the ubiquitination activity of the full-length TRIM22 and TRIM6 proteins. Since full-length protein expression had not been successful in *E. coli* or insect cell expression systems, mammalian expression systems were used for these experiments. The advantage of this expression system is also the presence of native post-translation modification systems and potential interaction partners.

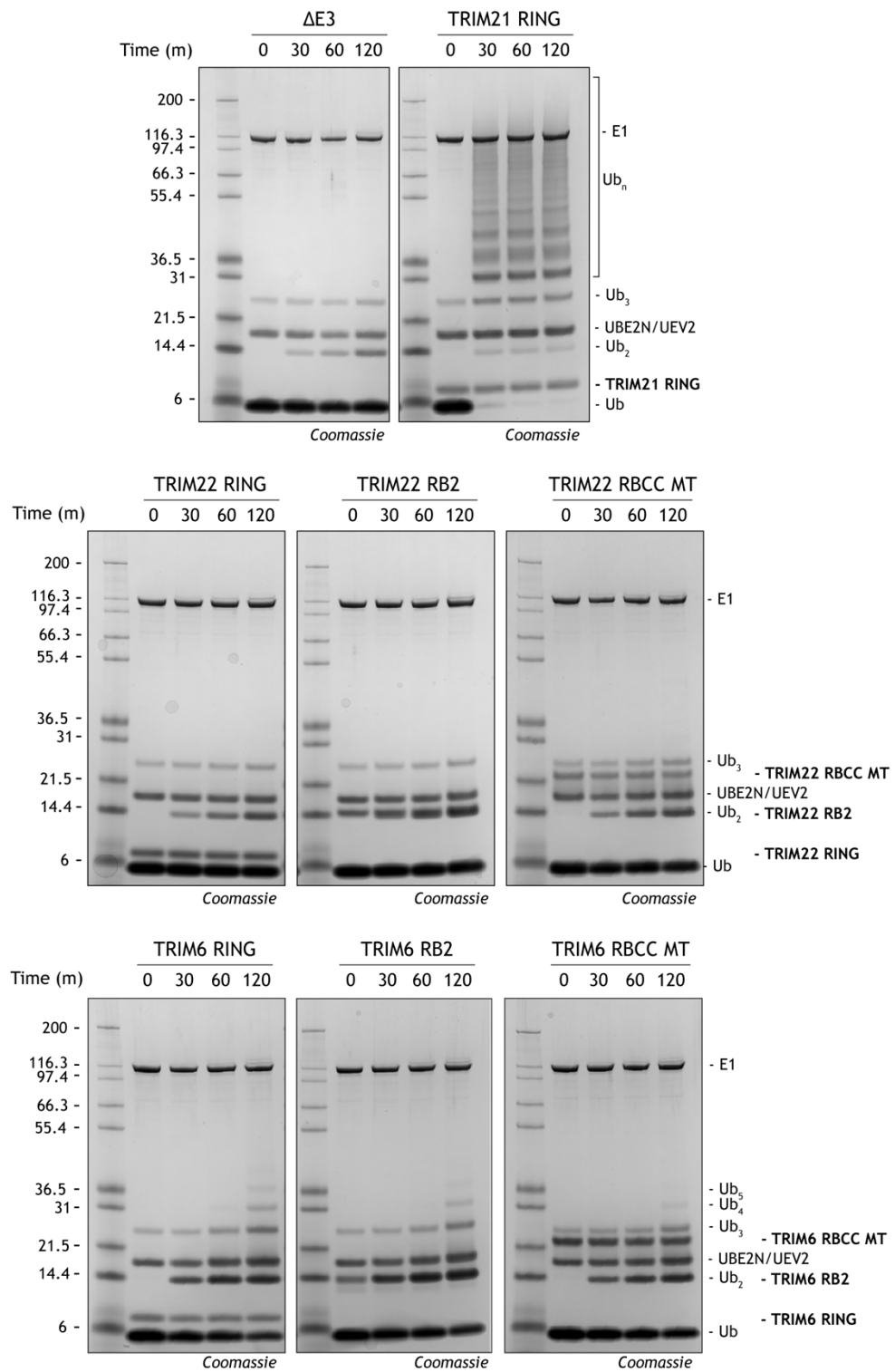


Figure 19. *In vitro* polyubiquitination assays using the heterodimeric E2, UBE2N/UEV2 with TRIM22 and TRIM6 constructs.

SDS-PAGE gels for *in vitro* polyubiquitination reactions with TRIM22 and TRIM6 RING, RB2 and RBCC MT constructs in conjunction with the E2, UBE2N/UEV2. Reactions were performed under standard conditions ($[E3] = 4\mu\text{M}$ and 25°C) alongside positive (TRIM21 RING) and negative ($\Delta E3$) controls. Protein bands representing reaction components, E3 constructs and ubiquitinated species are annotated. The data presented within this figure are representative of $n = 3$ and $n = 2$ experiments for TRIM22 and TRIM6 constructs, respectively.

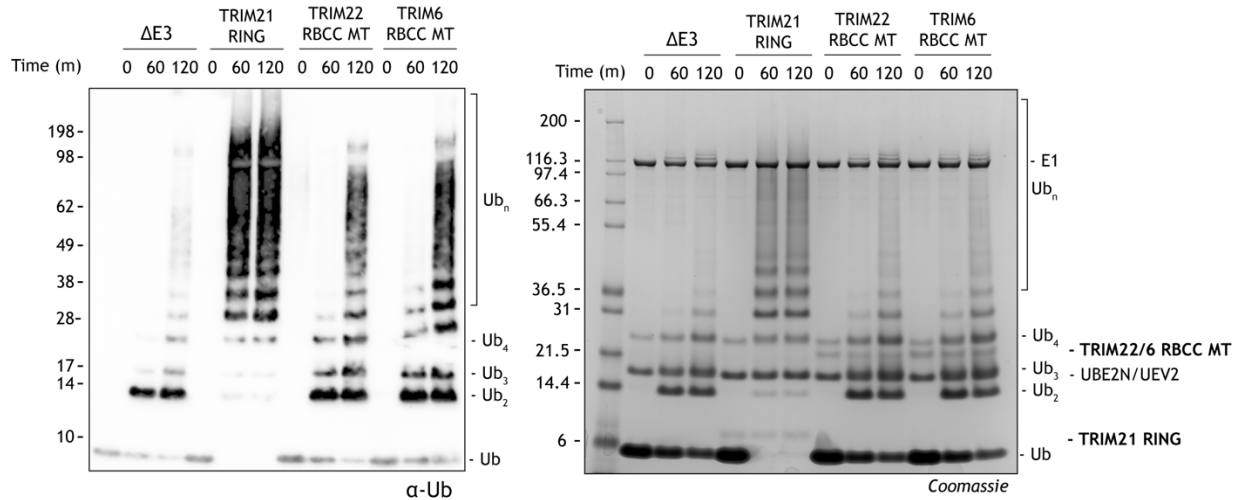


Figure 20. *In vitro* TRIM22 and TRIM6 RBCC MT polyubiquitination assays in conjunction with UBE2N/UEV2, at lower E3 concentrations.

Results from *in vitro* TRIM22 and TRIM6 RBCC MT polyubiquitination assays with UBE2N/UEV2 performed at lower E3 concentrations of $1\mu\text{M}$, at 37°C . Samples were analysed by both western blot (left) and Coomassie blue (right) staining, alongside the positive control, TRIM21 RING, and the negative control, $\Delta E3$. The results in this figure are representative of $n = 2$ and $n = 1$ experiments for TRIM22 and TRIM6 RBCC MT constructs, respectively.

3.2.3 TRIM22 and TRIM6 full-length activity in HEK293T cells

Both studies that reported E3 ligase activity for TRIM22 and TRIM6 used full-length protein expressed and isolated from HEK293T cells via pull-down or immunoprecipitation [97], [105]. This set-up has also been used previously in our lab to assay the activity of other TRIM E3 ligases. Therefore, I decided to set-up a similar assay using FLAG-tagged full-length constructs of TRIM22 and TRIM6. Mammalian expression vectors were used to transiently transfect HEK293T cells, and the proteins were immunoprecipitated from the lysate using anti-FLAG M2 magnetic beads. The immobilized E3 samples were then used for *in vitro* ubiquitination assays via addition of the relevant components of the ubiquitination cascade: E1, E2, ubiquitin and ATP, directly to the magnetic beads. Initial reactions conditions were taken from previous

protocols from the lab using other TRIM E3 ligases and positive controls were used to compare the assay readout and validate the reaction efficiency.

Initial experimental conditions used a relatively low concentration of ubiquitin (10 μM) spiked with 0.5 μM of fluorescently labelled Ub^{atto} and transfected a single 10 cm² plate of HEK293T cells per reaction. The presence of fluorescently labelled ubiquitin allowed detection of ubiquitination that specifically occurred during the *in vitro* reaction and not background signal coming from ubiquitinated species potentially bound non-specifically to the anti-FLAG M2 beads. In addition, the fluorescent signal was compared with western blot detection using the anti-ubiquitin antibody. Over-expression levels and immunoprecipitation efficiency for each construct was also assessed by western blot staining of the whole-cell extract (WCE) as well as the reaction sample timepoint 0, using the anti-FLAG M2 antibody.

These conditions were first tested with FLAG-tagged TRIM22 in an *in vitro* Ubch5A autoubiquitination assay using FLAG-tagged TRIM23 full-length as a positive control (Figure 21). The results from these assays showed autoubiquitination for the positive control while no autoubiquitination could be seen for TRIM22. The signal produced from this assay was not very strong for the positive control in comparison to similar ubiquitination assays from the literature, as well as the previous assays I performed with a TRIM23 RBB construct (Figure 14) [105]. In addition, the wild-type ubiquitin concentration appears to be a limiting factor in ubiquitination activity, based on the result from the anti-ubiquitin staining (Figure 21B). Finally, potential degradation of FLAG-tagged constructs is also visible, more so for TRIM22 than for TRIM23, and could also be affecting the final output (Figure 21C). As a result, changes to the reaction conditions were made to improve the assay. These included changes to the transfection protocol and concentrations of wild-type ubiquitin, Ub^{atto} and E3 ligase. Moreover, assays were also performed with the FLAG-tag re-cloned at the C-terminus of the construct to assess whether restriction of the RING domain via N-terminal immobilization was affecting activity. The assay with this latter construct showed little improvement compared to the N-terminal FLAG-tagged TRIM22 and therefore was not continued. The final ubiquitination assays were performed with N-terminal FLAG-tagged TRIM constructs transfected into three 10 cm² plates of HEK293T cells per reaction (to increase the E3 concentration) alongside concentrations of 50 μM and 1 μM , for wild-type ubiquitin and Ub^{atto} respectively.

The improved reaction conditions were tested again in a Ubch5A autoubiquitination assay with FLAG-tagged TRIM22 using TRIM23 full-length as a positive control. The results are shown in Figure 22, illustrating robust autoubiquitination of TRIM23 full-length seen by both fluorescence and western blot detection similar to previous assays (Figure 14). In Figure 22C, the concentration of TRIM23 appears to be lower than in Figure 21C, however the increased concentrations of wild-type ubiquitin and Ub^{atto} allow greater levels of ubiquitination. Unfortunately, no improvement could be achieved with regards to construct stability. Figure 22C, shows higher concentration of FLAG-tagged TRIM22 input, however this is coupled to stronger and more numerous degradation products than seen in Figure 21C. Overall, from these results it can be

concluded that, in addition to purified N-terminal TRIM22 constructs, full-length TRIM22 expressed in eukaryotic expression systems is inactive with Ubch5A, contrary to previous reports.

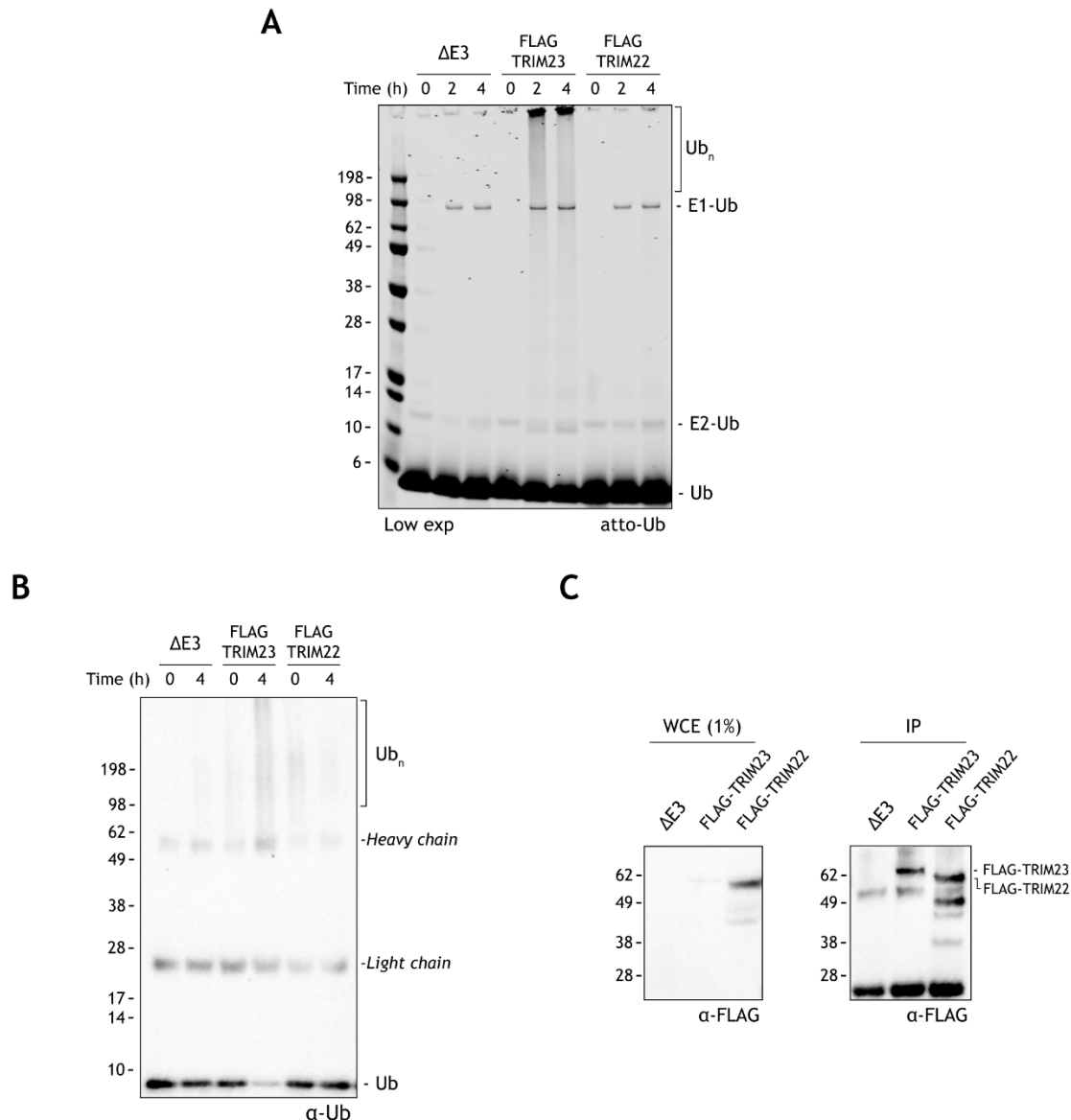
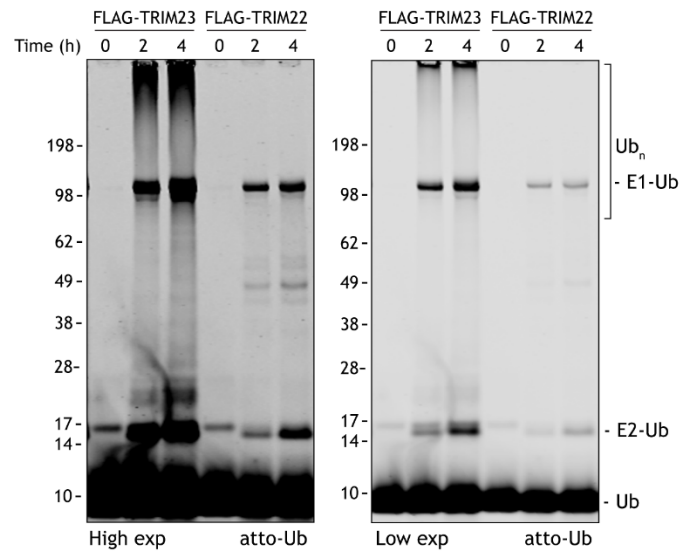


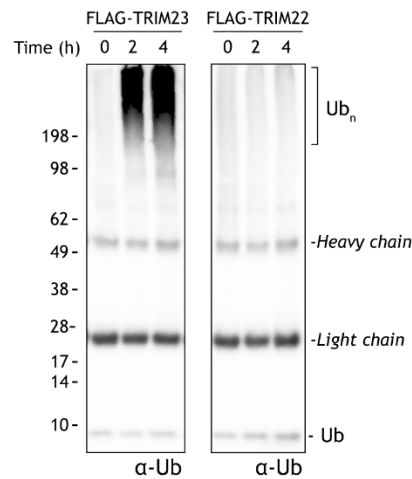
Figure 21. *In vitro* autoubiquitination assays with FLAG-tagged TRIM22 full-length from HEK293T cells

A. *In vitro* FLAG-tagged TRIM22 autoubiquitination assay with Ubch5A over a time course of 4 hours, using FLAG-tagged TRIM23 and no E3 as controls. Ubiquitination was analysed by fluorescence detection of the atto moiety. **B.** Reaction samples from **A.** detected via western blot using the anti-Ub antibody. **C.** WCE and immunoprecipitated (IP) samples were analysed by western blot using the anti-FLAG antibody to confirm expression and bead immobilization for each reaction. Annotations are positioned at the expected molecular weight of the full-length FLAG-tagged species. The data in this figure is representative of n = 2 experiments.

A



B



C

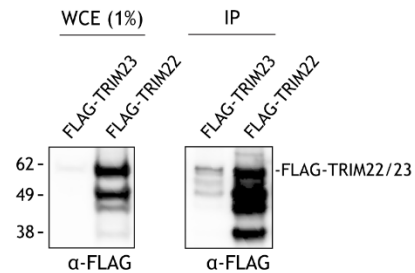


Figure 22. Optimised *in vitro* Ubch5A autoubiquitination assays with TRIM22 full-length from HEK293T cells

A. *In vitro* FLAG-tagged TRIM22 autoubiquitination assay in conjunction with Ubch5A, alongside FLAG-tagged TRIM23 as a positive control. Reaction samples were analysed by fluorescence detection of the atto moiety as both high exposure (left) and low exposure (right), to allow visualisation of high molecular weight smears and better resolution of the di-ubiquitin bands, respectively. **B.** Reaction samples from **A.** were also blotted for using the Ubi-1 antibody. **C.** WCE and IP samples from each reaction were blotted for using the anti-FLAG antibody to analyse expression levels. The results in this figure are representative of $n = 2$ experiments.

The optimised conditions were subsequently used to test the activity of FLAG-tagged TRIM6 full-length in conjunction with UBE2K. For this assay, FLAG-tagged TRIM2 full-length was used as a positive control, as in the previous assays with UBE2K using purified constructs from *E. coli*. The optimized conditions in these experiments, however, did not produce robust ubiquitination activity for the positive control, unlike previous assays with TRIM2 constructs in conjunction with UBE2K (Fig. 16B). In addition, the lack of a negative control means the level of E3-independent ubiquitination is unknown, although previous *in vitro* assays with UBE2K have detected small amounts of E3-independent activity (Fig. 16). In Figure 23A, the fluorescent gel at both high and low exposures, suggests slightly higher levels of ubiquitination activity for TRIM6 than TRIM2, evidenced by the stronger band for di and tri-ubiquitin species, as well as stronger smears at higher molecular weights. In contrast, anti-ubiquitin staining, shows stronger signal for TRIM2, with a faint high molecular weight smear present in the 2-hour sample that is absent for TRIM6 (Fig. 23B). The difference in activity between FLAG-tagged TRIM2 full-length and previous ubiquitination assays performed with the purified TRIM2 RBCC construct suggests the potential contribution of post-translation modifications and/or binding partners to activity. Nonetheless, *in vitro* ubiquitination assays with FLAG-tagged TRIM6 full-length in conjunction with UBE2K, do not show strong ubiquitination activity.

Following *in vitro* ubiquitination assays using the reported E2 partners from the literature, I decided to also test the ubiquitination activity of TRIM22 and TRIM6 identified in the E2 scan. FLAG-tagged TRIM22 and TRIM6 full-length proteins were assayed in conjunction with UBE2N/UEV2, alongside FLAG-tagged TRIM2 as a positive control. The same reaction conditions were used as for the UbcH5A and UBE2K assays with HEK293T samples. The results of this assay are shown in Figure 24. Similar to Figure 23, the signal from the positive control is weaker than expected. In this case, some TRIM2 ubiquitination activity can be seen above the 'no E3' control, notably in the 2-hour sample of the anti-Ub blot (however this signal is lacking in the 4 hour sample, possibly due to higher MW species being unable to enter the gel), although minimal activity is observed in the fluorescent gel. As with the UBE2K assays, post-translational modifications and/or cellular binding partners resulting from mammalian cell expression could modulate TRIM2 full-length activity, however, it is also possible that the set-up of the assay is not optimal and needs to be further improved. In conclusion, these preliminary results suggest that the *in vitro* E3 ligase activity in conjunction with UBE2N/UEV2, observed for the purified TRIM22 and TRIM6 RBCC MT constructs cannot be reproduced with full-length protein produced in HEK293T cells.

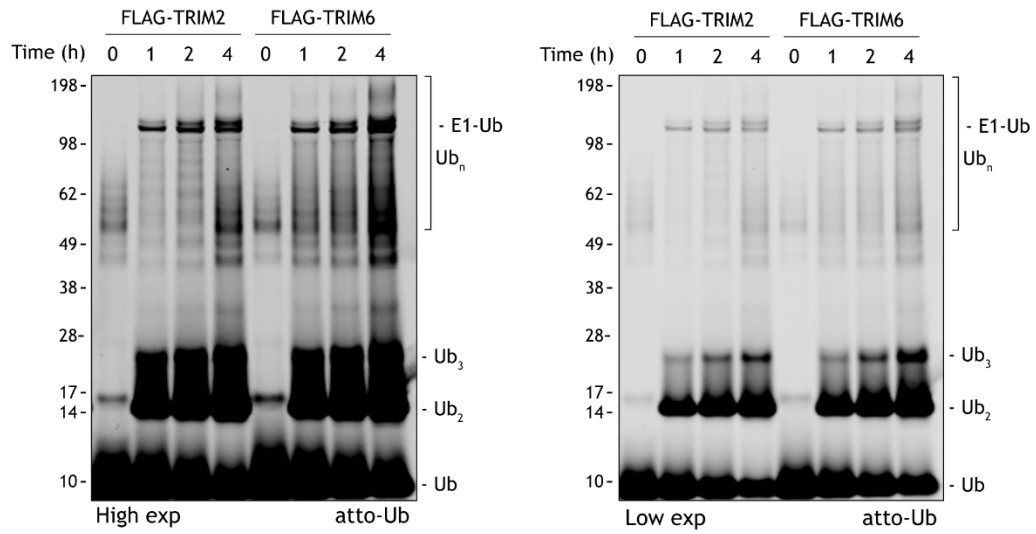
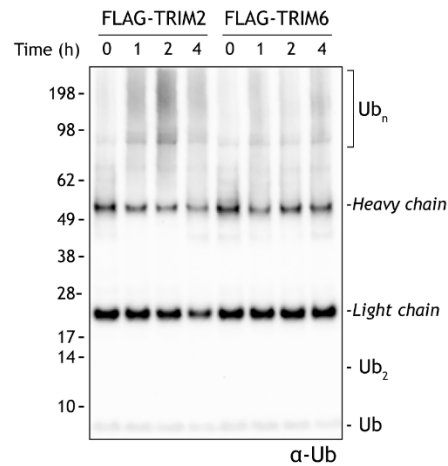
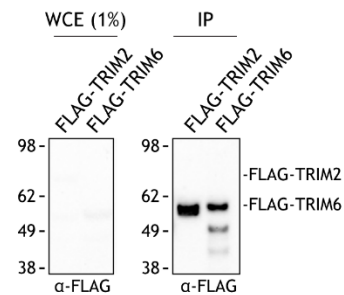
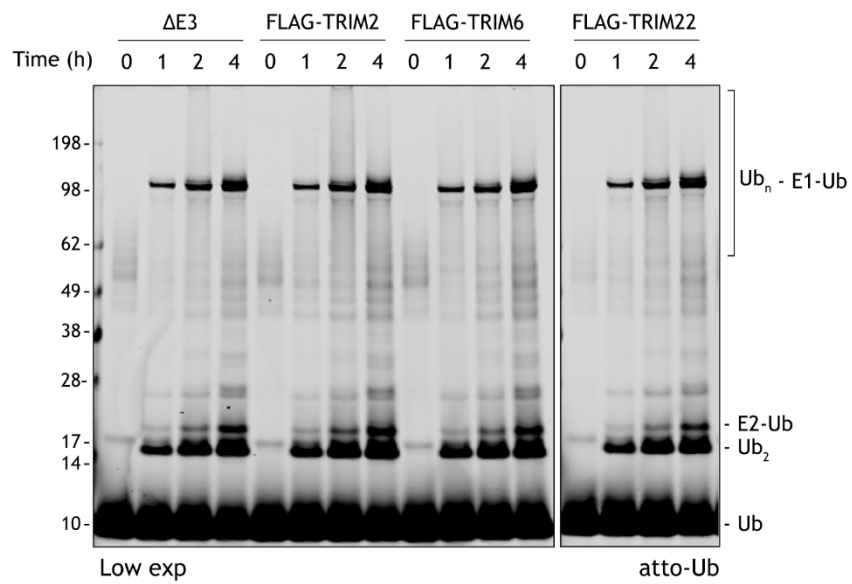
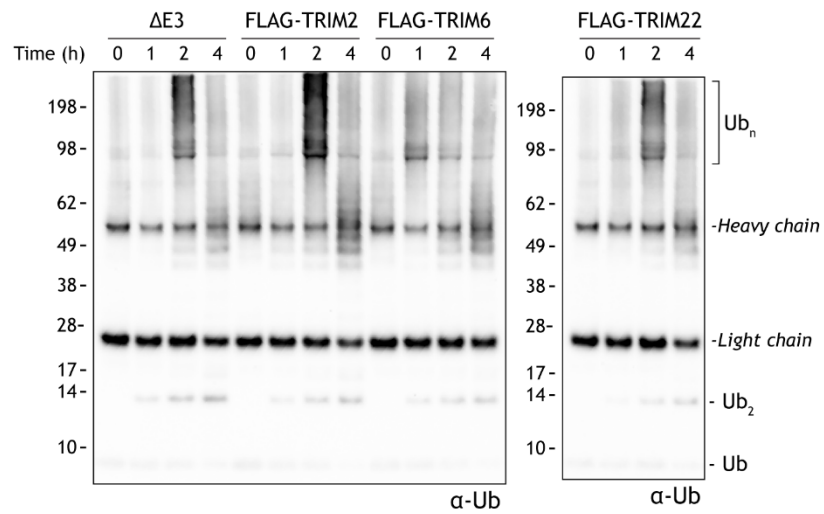
A**B****C**

Figure 23. *In vitro* UbE2K ubiquitination assays with TRIM6 full-length from HEK293T cells
A. *In vitro* FLAG-tagged TRIM6 polyubiquitination assay with the E2, UbE2K, alongside FLAG-tagged TRIM2 as a control over a time course of 4 hours. Ubiquitination was visualized using fluorescence detection via the atto moiety at both high exposure (left) and low exposure (right). **B.** *In vitro* FLAG-tagged TRIM6 and TRIM2 polyubiquitination levels were also analysed by western blotting using the Ubi-1 antibody. **C.** Expression levels and immobilization efficiency of the WCE and IP samples was further analysed by western blotting using the anti-FLAG antibody. The data presented in this figure is representative of $n = 3$ experiments.

A



B



C

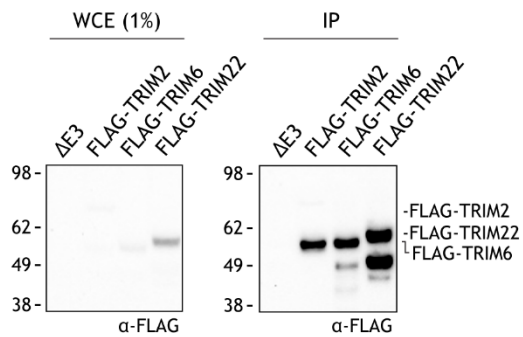


Figure 24. *In vitro* UBE2N/UEV2 polyubiquitination assays with TRIM22 and TRIM6 full-length from HEK293T cells

In vitro polyubiquitination assays with FLAG-tagged TRIM22 and TRIM6 in conjunction with the heterodimeric E2, UBE2N/UEV2, using FLAG-tagged TRIM2 and no E3 as positive and negative controls, respectively. Ubiquitination levels were analysed by **A.** fluorescence detection using the atto moiety and **B.** western blotting using the Ubi-1 antibody. **C.** WCE and IP samples were stained for expression levels and immobilisation efficiency, using the anti-FLAG antibody, with the expected molecular weight of the full-length FLAG-tagged constructs annotated. The data in this figure is the result of a single experiment.

Overall, *in vitro* ubiquitination activity assays with FLAG-tagged TRIM22 and TRIM6 full-length proteins, in the presence of the reported E2 partners as well as putative cognate E2s identified in the E2 scan, showed little or no activity. It remains possible that binding partners that are washed off during these assays or not present under these expression conditions or specific cell line, could play a role in ubiquitination activity. In this respect, it is important to note that expression of both, TRIM22 and TRIM6, is stimulated by interferons, along with many other interferon-stimulated genes and this cellular scenario could provide the conditions required for functional ubiquitination activity. Development of an experimental set-up to test this hypothesis is the focus of the final section of this study.

3.3 IFN stimulation studies

IFN-stimulation occurs when interferons, produced by cells in response to infection with foreign bodies, bind to extracellular receptors and trigger signaling cascades resulting in changes in transcription of a large number of interferon-stimulated genes. As mentioned previously, the close association of TRIMs with cellular innate immune responses, has resulted in ~25% of TRIM genes being sensitive to IFN expression [80], [81]. This includes TRIM22 and TRIM6, whose gene transcription is up-regulated approximately 2-5 fold and >10 fold respectively, in response to type-I interferons. IFN-stimulated up or down-regulation of protein expression may be a way of controlling TRIM ubiquitination activity. However, it is also feasible that IFN-stimulation leads to increased expression of an essential binding partner or specific post-translational modifications that may modulate activity. Before testing this hypothesis for TRIM22 and TRIM6, I planned first to identify a relevant cellular model in which efficient IFN-induced up regulation could be seen for both proteins. The readout for these experiments was protein expression levels of TRIM22 and TRIM6, analysed by western blot. Additionally, protein levels of STAT1, an IKK ϵ -independent ISG, were also blotted for as a control for IFN-stimulation.

Different stimulants exist to trigger a cellular IFN response and act at different points along the IFN signaling pathway. For these experiments I decided to test two different stimulants that have been previously used in TRIM-related IFN studies, polyI:C and IFN- β [77], [97]. polyI:C is a synthetic dsDNA mimic Toll-like receptor 3 (TLR3) agonist that acts at the beginning of the IFN signaling pathway to activate both an IFN response and

a pro-inflammatory response within cells. IFN- β , meanwhile, is a type-I IFN that functions downstream of polyI:C, binding to the extracellular interferon alpha and beta receptor (IFNAR) and triggering IFN-stimulated gene expression. Concentrations of stimulant and duration of stimulation were chosen based on previous reports in the literature and are outlined in the Materials and Methods section. Importantly, since polyI:C is an intracellular agonist, it had to be delivered via transfection as opposed to directly added to the media, as was the case for IFN- β .

A range of cell lines were tested as differential stimulation phenotypes are often observed. In this instance, HEK293T, A549 and U2OS cell lines were chosen for analysis. The HEK293T cell line is an extremely common cancer cell line and has previously been used in TRIM cell stimulation experiments [77], while A549 and U2OS cell lines are lung epithelial and mesenchymal cancer cell lines respectively, that are often used in IFN-stimulation and viral challenge studies in the literature.

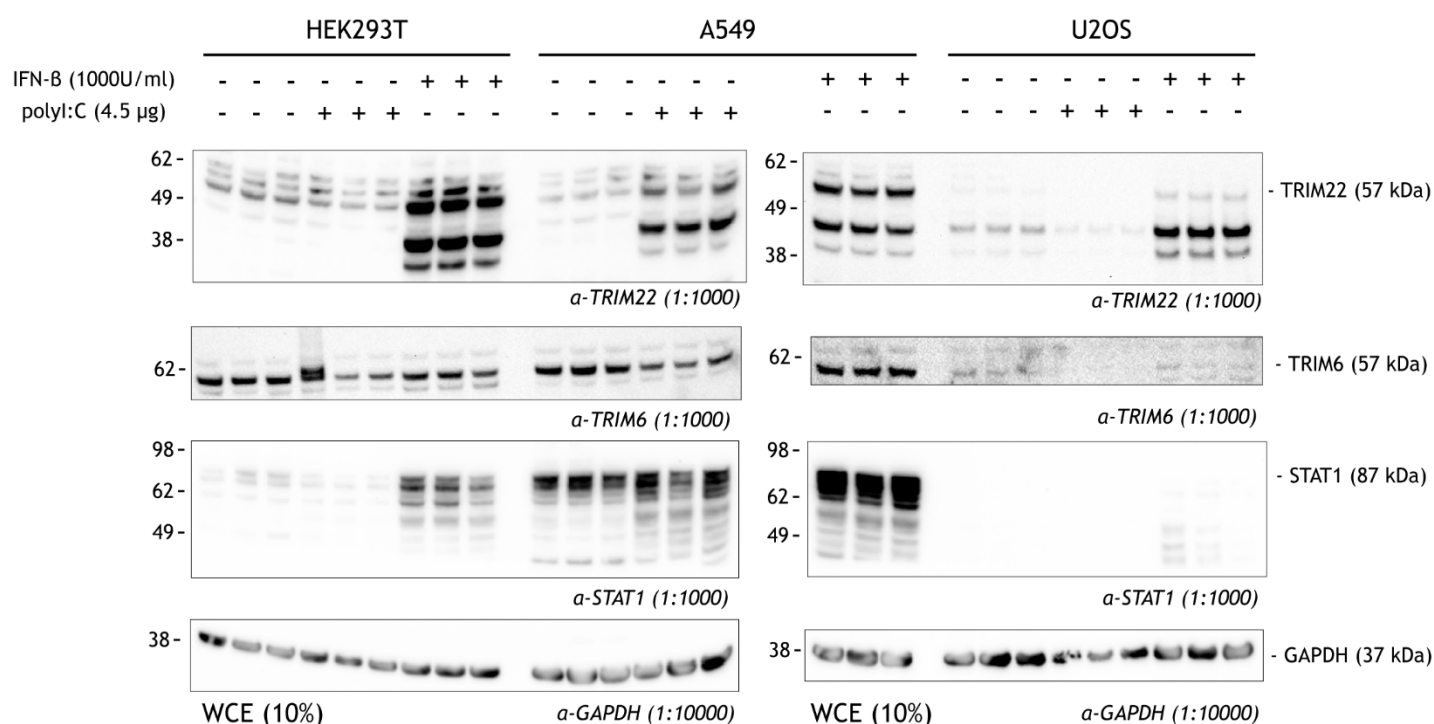


Figure 25. IFN stimulation trials in HEK293T, A549 and U2OS cells, for TRIM22 and TRIM6. HEK293T, A549 and U2OS cell lines were stimulated with either polyI:C (4.5 μ g) or IFN- β (1000 U/ml) for 24h and protein levels of TRIM22, TRIM6 and STAT1 assessed by western blot detection using the relevant antibodies. Each condition was performed in triplicate and α -GAPDH was used as a loading control. The expected molecular weights of the individual proteins are annotated beside each blot.

The results of the IFN stimulation experiments are shown in Figure 25. Each condition for each cell line was tested in triplicate and western blot detection of GAPDH was used as input control between the samples. The results suggest that IFN- β is a more effective

stimulant in protein up-regulation than polyI:C, illustrated by the levels of TRIM22, TRIM6 and STAT1 expression with and without stimulation in the different cell lines. This is most apparent in the A549 cells where strong increases in expression of both TRIM22 and the control gene STAT1 can be seen in response to IFN- β , as well as small increases in TRIM6 expression. HEK293T cells also showed increases in TRIM22 expression, however up-regulation of TRIM6 and STAT1 was less dramatic compared to the A549 cells. Unexpectedly, levels of TRIM6 expression in A549 and U2OS cells and TRIM22 in U2OS cells appear to decrease in response to polyI:C stimulation. PolyI:C has previously been reported to reduce the expression of specific proteins following stimulation, however its more commonly known to up-regulate ISG expression through the interferon response, as is predicted for TRIM22 and TRIM6 [107]. Alternative reasons for this observation could relate to reduced concentrations of polyI:C due to transfection inefficiency or toxicity of the transfection reagent in these particular cell lines. The overall health of the cells is also a potential reason for the lower levels of protein expression in U2OS cells compared to the other two cell lines, where STAT1 expression in particular, is extremely low in all conditions. Finally, the anti-TRIM22 blot shows multiple bands either corresponding to unspecific binding of the antibody or different length TRIM22 constructs, which may be the result of protein degradation or different protein isoforms. To verify the specificity of these bands, future experiments could utilise of siRNAs against TRIM22 that would remove all specific bands. Degradation or differential isoform expression could contribute to the activity of TRIM22 and therefore is important to address. Overall, the results from these preliminary experiments suggest that IFN- β stimulation in A549 cells could be a suitable experimental set-up for future IFN-stimulation experiments to be performed with TRIM22 and TRIM6.

4. Discussion

The focus of this study was the biochemical and biophysical characterization of the class IV TRIM E3 ligases, TRIM22 and TRIM6. These proteins share high sequence identity with the well-studied retroviral restriction factor, TRIM5 α , and have proposed roles in viral restriction and immune signaling, respectively. Studies of TRIM22 have suggested that it restricts the replication of RNA viruses, IAV, HCV and EMCV, through direct interaction with the viral proteins NS5A (HCV), NP (IAV) and 3C protease (EMCV) [94]-[96]. Interaction with TRIM22 was suggested to induce mono and polyubiquitination of substrates leading predominantly to proteasomal degradation. In literature reports, assays with full-length TRIM22 purified from COS7 cells have shown ubiquitination activity in conjunction with the promiscuous E2, UbCH5B, known to be indiscriminate in its ubiquitination specificity [105]. On the other hand, TRIM6 isolated from HEK293T cells was shown to cooperate with the UBE2K E2 enzyme to produce unanchored K48-linked ubiquitin chains [97]. These free chains were proposed to activate the immune signaling factor, IKK ϵ , leading to downstream stimulation of ISGs. Despite these reported biological roles, structural insights and characterization of the mechanisms of action of TRIM22 and TRIM6, is either limited or unavailable.

Generally, information on PRYSPRY-mediated substrate interactions amongst TRIM E3 ligases is still lacking and this is also the case for TRIM22 and TRIM6. Specific questions such as how interactions dictate the precise ubiquitination of the substrate, as well as how certain PRYSPRY domains interact with multiple reported interaction partners remain unanswered. Unfortunately, attempts to express the substrate-binding PRYSPRY domain and PRYSPRY domain-containing constructs of both proteins in this study were unsuccessful and therefore substrate-binding studies of TRIM22 and TRIM6 could not be continued. As a result, a more concentrated focus was placed on deciphering the mechanism of action of TRIM22 and TRIM6, notably their ubiquitination activity profiles and self-association properties, known to contribute to TRIM E3 ligase activity.

First, to investigate the properties of the individual and composite domains of these proteins, different length constructs were cloned, and purification trials performed. Unfortunately, only the RING, RB2, RBCC MT and BCC MT constructs for both proteins, in addition to the CC domain of TRIM22, could be purified with sufficient yields for biophysical and biochemical experiments. In contrast, the full-length proteins, PRYSPRY domains, PRYSPRY domain-containing constructs, as well as the entire tripartite motifs, for TRIM22 and TRIM6 were either insoluble or not well-expressed using both *E. coli* and insect-cell based expression systems. Overall, full-length and tripartite motifs of TRIM proteins can be hard to express recombinantly due to their complex domain architecture. Furthermore, the variable loop regions present within the PRYSPRY domain can also inhibit solubility, as seen for TRIM5 α [108]. To obtain structural information on these TRIMs, crystallization trials were set-up for all protein constructs that were purified in this study. However, these experiments proved unsuccessful and only initial crystal hits could be produced for the TRIM22 CC domain, which did not diffract and could not be further optimized.

Analysis of the self-association properties of the purified protein constructs of TRIM22 and TRIM6, was carried out using the solution-based SEC-MALLS technique. These experiments illustrated that the RING domains of both TRIM22 and TRIM6 are capable of dimerization with varying affinities. TRIM6 RING-domain containing constructs were strictly dimeric at all concentrations tested, while equivalent constructs of TRIM22 were present in monomer-dimer equilibrium. The RING domain of the closely-related TRIM5 α has been shown to be monomeric even at high protein concentrations and in this regard both TRIM22 and TRIM6 show stronger RING domain dimerization [106]. In contrast, B-box2-mediated trimerization, which has been identified for TRIM5 α , was not observed in TRIM22 and TRIM6, despite high conservation of key residues within the TRIM5 α trimeric B-box2 interface (Fig. 6C). In the case of TRIM22, this can be explained by the loss of R121 (L121 in TRIM22), which in TRIM5 α , forms a key salt bridge with E120 of the neighbouring B-box2 domain. Furthermore, overlap of the AlphaFold prediction of the TRIM22 B-box2 domain with the trimeric BCC MiniTRIM structure of rhTRIM5 α , identifies R132 (L132 in TRIM5 α) as a potential steric hindrance to recruitment of the third B-box2 domain. For TRIM6, the reasons behind the lack of B-box2 trimerization are less obvious, however it is possible that the tighter dimerization of the RING domain may restrict the flexibility of the B-box domain and prevent such interactions. Trimerization for TRIM22 and TRIM6 has been previously reported in the literature, however, these were qualitative studies in which mammalian cell lysates over-expressing either TRIM protein were incubated with cross-linking reagents and shown to migrate at higher molecular weights on SDS-PAGE gels, approximately the molecular weight of the trimeric species [93]. Given the propensity for self-association and the fact that both TRIM22 and TRIM6 have been shown to form cytoplasmic punctates when over-expressed in mammalian cells, it is unsurprising that incubation with cross-linking reagents produces higher molecular weight species [109]. Finally, the CC domain of TRIM22 was also shown to be dimeric at all concentrations tested and had no tendency to form higher-order oligomers as seen in other TRIM E3 ligases (unpublished data, Rittinger lab).

Ubiquitination activity of TRIM22 and TRIM6 was initially assessed through *in vitro* assays with the purified RING, RB2 and RBCC MT constructs from *E. coli*, and focused on reproducing data from the literature. In the case of TRIM22, a range of autoubiquitination assays were performed with Ubch5A, a homologue of the Ubch5B E2 enzyme, used in the assays reported in the literature. Surprisingly, no autoubiquitination activity could be seen for TRIM22 constructs at all concentrations and temperatures tested, using both Coomassie Blue and western blot detection methods. Likewise, *in vitro* polyubiquitination assays were performed with TRIM6 RING, RB2 and RBCC MT constructs in conjunction with the E2, Ube2K. Similarly, no polyubiquitin chain formation could be detected at multiple concentrations and temperatures, again using both Coomassie blue and western blot detection methods.

The E3 ligase activity of TRIM22 and TRIM6 was hence tested more generally against all 35 human E2 enzymes using commercially available E2 screens. Surprisingly, even at elevated E3 concentrations (10-fold higher than standard conditions) these experiments showed that only faint ubiquitination activity could be detected for both TRIM22 RBCC

MT and TRIM6 RBCC MT, with several E2s, including the Ubch5 family for TRIM22. However, these experiments did identify significant polyubiquitin chain formation for both TRIMs in conjunction with the heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2. These two heterodimeric E2s share similar *in vitro* activity profiles despite distinct cellular functions and are responsible for the formation of specifically K63-linked chains [8]. Follow-up assays were performed at conditions closer to those within the cell and confirmed that, even at 1 μ M E3 concentration and at 37°C, ubiquitination activity in conjunction with UBE2N/UEV2 could be clearly observed. This ubiquitination activity was slightly stronger for TRIM6 than TRIM22, however notably weaker than TRIM21, used as a positive control. The biological meaning of this observation is discussed in more detail below.

Subsequent attempts were made to assess the activity of the full-length proteins expressed and isolated from mammalian cells, much like previous activity assays in the literature. These experiments showed little or no detectable activity for TRIM22 and TRIM6 in conjunction with both reported E2 partners, Ubch5A and Ube2K, respectively, as well as UBE2N/UEV2. The results with Ubch5A and Ube2K further contradict reports in the literature of TRIM22 and TRIM6 activity and suggest that the full-length proteins and any basal post-translational modifications are not sufficient to rescue activity of, in combination with Ubch5A and Ube2K, respectively. Surprisingly, the results from these assays also contradicted the activity seen previously in the E2 screens with TRIM22 and TRIM6. In this case, the context of the full-length protein and any potential post-translational modifications greatly reduced activity. Similarly, TRIM2 used as a control in the assays with both Ube2K and UBE2N/UEV2, showed much weaker activity when compared to previous assays with the purified RBCC construct.

Taken together, the findings from these experiments illustrate that TRIM22 and TRIM6 have different self-association properties to their closely related homologue, TRIM5 α . Despite conservation of a large number of residues that form the TRIM5 α B-box2 trimeric interface, neither protein shows efficient B-box2-mediated trimerisation. Trimerisation through the B-box2 domain allows TRIM5 α to form hexameric assemblies which mirror the symmetry of target viral capsids and enables substrate interaction and RING domain activity. Since the substrates of TRIM22 and TRIM6 are not viral capsid structures, hexameric assembly may not be required. Instead, loss of the trimeric B-box2 interface, which promotes RING dimerization, is replaced by stronger RING dimerization propensities for both TRIM22 and TRIM6 and opens up the possibility of alternative RING dimerization mechanisms. The substrate context could still influence such mechanisms, for example via target-induced RING domain clustering, as evidenced for TRIM21. At present, the stoichiometry of TRIM22 and TRIM6 substrate interactions is unknown, however self-association, which could aid RING clustering, has been reported for some of the putative TRIM22 and TRIM6 targets, such as NP (TRIM22), NS5A (TRIM22) and IKK ϵ (TRIM6) [97], [110], [111].

Unexpectedly, despite RING dimerization for both TRIM22 and TRIM6, purified RING-domain containing constructs were inactive with previously reported E2 enzymes, including the Ubch5 family. The ability to catalyse *in vitro* ubiquitination in conjunction

with the promiscuous UbcH5 family has been illustrated for all TRIM E3 ligases studied thus far, in which the RING domain is capable of dimerization, and therefore this inactivity for TRIM22 and TRIM6 is unique [51], [54], [58], [112]. Instead, the purified RING-domain containing constructs of TRIM22 and TRIM6 showed modest K63-linked polyubiquitin chain formation in conjunction with the heterodimeric E2s, UBE2N/UEV1 and UBE2N/UEV2. UBE2N-coupled activity for TRIM22 and TRIM6 was previously predicted by Kiss *et al* [58]. This study described a common tri-ionic motif within certain RING domains, including TRIM22 and TRIM6, responsible for specific catalytic activation of the UBE2N-Ub conjugate. Importantly, mutation of this tri-ionic motif had no effect on the activity of TRIM21 and TRIM5 α in conjunction with UbcH5A. Cellular concentrations of UBE2N and its constituent E2 partners UEV1 and UEV2, have been shown to be high in multiple studies and therefore it is possible that this *in vitro* observed activity could occur in a cellular context [8], [113]. However, reports of TRIM22 and TRIM6 cellular activity do not immediately align with K63-linked polyubiquitin chain formation, which is predominantly linked to activation of cell signaling pathways. Instead, TRIM22, has been reported to induce degradation of substrate targets, for which K48-linked ubiquitin chains are the predominant chain linkage [97], while TRIM6 has been reported to form unanchored K48-linked ubiquitin chains.

Intriguingly, results from the assays with full-length TRIM22 and TRIM6 proteins isolated from cells illustrated little or no E3 ligase activity. A continued lack of activity was observed alongside the reported E2 partners, UbcH5A and UBE2K, with TRIM22 and TRIM6 respectively. Meanwhile, a reduction in activity was observed for both full-length proteins with the heterodimeric UBE2N/UEV2 enzyme. This suggests that the full-length context and any potential post-translational modifications are either incapable of recapitulating reported activity or reduce the activity of TRIM22 and TRIM6 compared with purified MiniTRIM constructs from *E. coli*. Possible reasons for this include lack of relevant PTMs as well as absence of required interaction partners. This could relate to incorrect protein subcellular localization or absence of specific cellular stimuli, for example IFN-stimulation. In this regard, the subcellular localization of TRIMs, which was not assessed during these experiments, has been shown to be dynamic and sensitive to factors including, viral infection, cell cycle phase and presence of ubiquitin chains, as well as specific affinity tag [23], [97], [114], [115]. Furthermore, approximately 25% of TRIM E3 ligase genes have been reported to be sensitive to IFN-stimulation, including TRIM22 and TRIM6, while ~ half of all TRIMs have been shown to enhance the innate immune response [81], [115]. Heterodimerisation of TRIMs has also been suggested for certain TRIMs which could modulate *in vivo* activity, however clear evidence for this is still lacking and the contribution to catalytic activity remains to be seen [93], [116]. Overall, it seems possible that these factors could alter the processing, interactions, and activity profiles of TRIM22 and TRIM6. Alternatively, the lack of activity could be related to the self-association properties of the full-length proteins. The oligomerization states of full-length TRIM22 and TRIM6 are unknown since they could not be purified and analysed by SEC-MALLS. As a result, it is possible that the full-length context disrupts the RING dimerization observed in the RING-domain containing constructs purified from *E. coli*. Furthermore, the concentrations of E3 used in these

assays are low and may shift the equilibrium towards the monomeric form of the protein, more so for TRIM22 than TRIM6. Finally, multiple TRIMs have been reported to form cytoplasmic punctates when overexpressed in mammalian cells, which may facilitate activity *in vivo* by increasing the local concentration of TRIM E3 ligases; conditions that are not replicated in these assays. Altogether, these results along with the results and findings within the literature, outline the complex activity profiles of TRIM E3 ligases and the need for further experiments.

To assess the effect of IFN-stimulation on the activity profile of these proteins, preliminary experiments were first performed to validate reports of TRIM22 and TRIM6 up-regulation in the literature. These experiments showed that protein expression of both TRIM22 and TRIM6 is increased upon IFN-stimulation in A549 cells, a lung epithelial cancer cell line. Stimulation with IFN- β , a type-I IFN, lead to robust up-regulation of TRIM22 protein levels, as well as marginal increases in TRIM6 protein expression, in line with previous studies that suggested increases in mRNA levels of between 2-5 fold and >10 fold for TRIM6 and TRIM22, respectively, in response to IFN stimulation [81]. These results outline an appropriate cellular model for future studies.

In summary, this study has addressed the biochemical and biophysical properties of the class IV TRIM E3 ligases, TRIM22 and TRIM6. This analysis identified certain biophysical characteristics that differ from closely related TRIMs, notably the lack of B-box2-mediated trimerization, as well as stronger RING dimerization affinities. Biochemical activity assays also identified a novel activity for TRIM22 and TRIM6 in K63-linked polyubiquitin chain formation, while attempts to reproduce previous activity profiles in the literature were unsuccessful. The importance of this newly discovered K63 chain forming activity on the cellular function of these proteins is unclear and should be the subject of future studies. In addition, the effect of IFN-stimulation on TRIM22 and TRIM6 activities should also be further analysed.

References

- [1] A. Zuin, M. Isasa, and B. Crosas, "Ubiquitin Signaling: Extreme Conservation as a Source of Diversity," *Cells*, vol. 3, no. 3, pp. 690-701, 2014.
- [2] A. Ciechanover, Y. Hod, and A. Hershko, "A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes.," *Biochem. Biophys. Res. Commun.*, vol. 425, no. 3, pp. 565-570, 1978.
- [3] D. Komander and M. Rape, "The Ubiquitin Code," *Annu. Rev. Biochem.*, vol. 81, no. 1, pp. 203-229, 2012.
- [4] B. A. Schulman and J. Wade Harper, "Ubiquitin-like protein activation by E1 enzymes: The apex for downstream signalling pathways," *Nat. Rev. Mol. Cell Biol.*, vol. 10, no. 5, pp. 319-331, 2009.
- [5] Y. Pylayeva-Gupta, "RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination," *Bone*, vol. 23, no. 1, pp. 1-7, 2011.
- [6] K. M. Scaglione *et al.*, "The ubiquitin-conjugating enzyme (E2) ube2w ubiquitinates the N terminus of substrates," *J. Biol. Chem.*, vol. 288, no. 26, pp. 18784-18788, 2013.
- [7] M. Akutsu, I. Dikic, and A. Bremm, "Ubiquitin chain diversity at a glance," *J. Cell Sci.*, vol. 129, no. 5, pp. 875-880, 2016.
- [8] M. J. Clague, C. Heride, and S. Urbé, "The demographics of the ubiquitin system," *Trends Cell Biol.*, vol. 25, no. 7, pp. 417-426, 2015.
- [9] J. Jin, X. Li, S. P. Gygi, and J. W. Harper, "Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging," *Nature*, vol. 447, no. 7148, pp. 1135-1138, 2007.
- [10] A. J. Middleton and C. L. Day, "The molecular basis of lysine 48 ubiquitin chain synthesis by Ube2K," *Sci. Rep.*, vol. 5, pp. 1-14, 2015.
- [11] E. G. Jaffray, H. Salmen, R. T. Hay, M. H. Tatham, and A. P. A., "Ube2W conjugates ubiquitin to α -amino groups of protein N-termini," vol. 145, pp. 137-145, 2013.
- [12] M. J. Eddins, C. M. Carlile, K. M. Gomez, C. M. Pickart, and C. Wolberger, "Mms2-Ubc13 covalently bound to ubiquitin reveals the structural basis of linkage-specific polyubiquitin chain formation," *Nat. Struct. Mol. Biol.*, vol. 13, no. 10, pp. 915-920, 2006.
- [13] L. Buetow and D. T. Huang, "Structural insights into the catalysis and regulation of E3 ubiquitin ligases," *Nat. Rev. Mol. Cell Biol.*, vol. 17, no. 10, pp. 626-642, 2016.
- [14] N. Zheng, P. Wang, P. D. Jeffrey, and N. P. Pavletich, "Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases," *Cell*, vol. 102, no. 4, pp. 533-539, 2000.
- [15] H. Dou, L. Buetow, G. J. Sibbet, K. Cameron, and D. T. Huang, "Essentiality of a non-RING element in priming donor ubiquitin for catalysis by a monomeric E3," *Nat. Struct. Mol. Biol.*, vol. 20, no. 8, pp. 982-986, 2013.
- [16] H. Dou, L. Buetow, G. J. Sibbet, K. Cameron, and D. T. Huang, "BIRC7-E2 ubiquitin conjugate structure reveals the mechanism of ubiquitin transfer by a RING dimer," *Nat. Struct. Mol. Biol.*, vol. 19, no. 9, pp. 876-883, 2012.
- [17] P. S. Brzovic, P. Rajagopal, D. W. Hoyt, M. King, and R. E. Klevit, "Structure of

- a BRCA1 - BARD1 heterodimeric RING - RING complex," *Nat. Publ. Gr.*, pp. 833-837, 2001.
- [18] D. E. Christensen, P. S. Brzovic, and R. E. Klevit, "E2-BRCA1 RING interactions dictate synthesis of mono- or specific polyubiquitin chain linkages," *Nat. Struct. Mol. Biol.*, vol. 14, no. 10, pp. 941-948, 2007.
 - [19] A. Schreiber *et al.*, "Structural basis for the subunit assembly of the anaphase-promoting complex," *Nature*, vol. 470, no. 7333, pp. 227-235, 2011.
 - [20] D. M. Duda, D. C. Scott, M. F. Calabrese, E. S. Zimmerman, N. Zheng, and B. A. Schulman, "Structural regulation of cullin-RING ubiquitin ligase complexes," *Curr. Opin. Struct. Biol.*, vol. 21, no. 2, pp. 257-264, 2011.
 - [21] N. Zheng *et al.*, "Structure of the Cul1-Rbx1-Skp1-F box Skp2 SCF ubiquitin ligase complex," *Nature*, vol. 416, no. April, pp. 703-709, 2002.
 - [22] G. Meroni, *TRIM/RBCC Proteins*. 2012.
 - [23] A. Reymond *et al.*, "The tripartite motif family identifies cell compartments," *EMBO J.*, vol. 20, no. 9, 2001.
 - [24] S. Hatakeyama, "TRIM Family Proteins: Roles in Autophagy, Immunity, and Carcinogenesis," *Trends Biochem. Sci.*, vol. 42, no. 4, pp. 297-311, 2017.
 - [25] I. Marín, "Origin and Diversification of TRIM Ubiquitin Ligases," *PLoS One*, vol. 7, no. 11, 2012.
 - [26] Z. Pancer and M. D. Cooper, "the Evolution of Adaptive Immunity," *Annu. Rev. Immunol.*, vol. 24, no. 1, pp. 497-518, 2006.
 - [27] M. Van Gent, K. M. J. Sparrer, and M. U. Gack, "TRIM proteins and their roles in antiviral host defenses," *Annu. Rev. Virol.*, vol. 5, pp. 385-405, Sep. 2018.
 - [28] S. van Tol, A. Hage, M. I. Giraldo, P. Bharaj, and R. Rajsbaum, "The TRIMendous role of TRIMs in virus-host interactions," *Vaccines*, vol. 5, no. 3, Sep. 2017.
 - [29] A. Trockenbacher *et al.*, "MID1, mutated in Opitz syndrome, encodes an ubiquitin ligase that targets phosphatase 2A for degradation," *Nat. Genet.*, vol. 29, no. 3, pp. 287-294, 2001.
 - [30] J. Muller *et al.*, "Identification of 28 novel mutations in the Bardet-Biedl syndrome genes: The burden of private mutations in an extensively heterogeneous disease," *Hum. Genet.*, vol. 127, no. 5, pp. 583-593, 2010.
 - [31] M. G. Koliopoulos *et al.*, "Molecular mechanism of influenza A NS1-mediated TRIM25 recognition and inhibition," *Nat. Commun.*, vol. 9, no. 1, 2018.
 - [32] M. Scherer *et al.*, "Crystal Structure of Cytomegalovirus IE1 Protein Reveals Targeting of TRIM Family Member PML via Coiled-Coil Interactions," *PLoS Pathog.*, vol. 10, no. 11, 2014.
 - [33] M. U. Gack *et al.*, "TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity," *Nature*, vol. 446, no. 7138, pp. 916-920, Apr. 2007.
 - [34] M. U. Gack *et al.*, "TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity," *Nature*, vol. 446, no. 7138, pp. 916-920, 2007.
 - [35] J. G. Sanchez *et al.*, "Mechanism of TRIM25 Catalytic Activation in the Antiviral RIG-I Pathway," *Cell Rep.*, vol. 16, no. 5, pp. 1315-1325, 2016.
 - [36] T. J. Hayman *et al.*, "RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses," *Immunol. Cell Biol.*, vol. 97, no. 9, pp. 840-852, Oct. 2019.
 - [37] H. Oshiumi, M. Matsumoto, S. Hatakeyama, and T. Seya, "Riplet/RNF135, a

- RING finger protein, ubiquitinates RIG-I to promote interferon- β induction during the early phase of viral infection,” *J. Biol. Chem.*, vol. 284, no. 2, pp. 807-817, Jan. 2009.
- [38] H. Oshiumi, M. Miyashita, N. Inoue, M. Okabe, M. Matsumoto, and T. Seya, “The ubiquitin ligase riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection,” *Cell Host Microbe*, vol. 8, no. 6, pp. 496-509, Dec. 2010.
 - [39] H. Oshiumi, M. Miyashita, M. Matsumoto, and T. Seya, “A Distinct Role of Riplet-Mediated K63-Linked Polyubiquitination of the RIG-I Repressor Domain in Human Antiviral Innate Immune Responses,” *PLoS Pathog.*, vol. 9, no. 8, Aug. 2013.
 - [40] M. Stremlau, C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski, “The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys,” *Nature*, vol. 427, no. 6977, pp. 848-853, 2004.
 - [41] B. K. Ganer-Pornillos, V. Chandrasekaran, O. Pornillos, J. G. Sodroski, W. I. Sundquist, and M. Yeager, “Hexagonal assembly of a restricting TRIM5 α protein,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 108, no. 2, pp. 546-550, 2011.
 - [42] M. Stremlau *et al.*, “Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5 α restriction factor,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 103, no. 14, pp. 5514-5519, 2006.
 - [43] T. Pertel *et al.*, “TRIM5 is an innate immune sensor for the retrovirus capsid lattice,” *Nature*, vol. 472, no. 7343, pp. 361-365, Apr. 2011.
 - [44] A. J. Fletcher and L. C. James, “Coordinated Neutralization and Immune Activation by the Cytosolic Antibody Receptor TRIM21,” *J. Virol.*, vol. 90, no. 10, pp. 4856-4859, May 2016.
 - [45] D. L. Mallery, W. A. McEwan, S. R. Bidgood, G. J. Towers, C. M. Johnson, and L. C. James, “Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21),” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 46, pp. 19985-19990, Nov. 2010.
 - [46] S. Iyengar and P. J. Farnham, “KAP1 protein: An enigmatic master regulator of the genome,” *Journal of Biological Chemistry*, vol. 286, no. 30, pp. 26267-26276, 29-Jul-2011.
 - [47] G. A. Stoll, S. ichiro Oda, Z. S. Chong, M. Yu, S. H. McLaughlin, and Y. Modis, “Structure of KAP1 tripartite motif identifies molecular interfaces required for retroelement silencing,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 116, no. 30, pp. 15042-15051, 2019.
 - [48] A. V. Ivanov *et al.*, “PHD Domain-Mediated E3 Ligase Activity Directs Intramolecular Sumoylation of an Adjacent Bromodomain Required for Gene Silencing,” *Mol. Cell*, vol. 28, no. 5, pp. 823-837, Dec. 2007.
 - [49] D. Esposito, M. G. Koliopoulos, and K. Rittinger, “Structural determinants of TRIM protein function,” *Biochem. Soc. Trans.*, vol. 45, no. 1, pp. 183-191, 2017.
 - [50] F. Fiorentini, D. Esposito, and K. Rittinger, “Does it take two to tango? RING domain self-association and activity in TRIM E3 ubiquitin ligases,” *Biochem. Soc. Trans.*, vol. 0, pp. 1-10, 2020.
 - [51] M. G. Koliopoulos, D. Esposito, E. Christodoulou, I. A. Taylor, and K. Rittinger, “Functional role of TRIM E3 ligase oligomerization and regulation of catalytic activity,” *EMBO J.*, vol. 35, no. 11, pp. 1204-1218, 2016.

- [52] C. Dickson *et al.*, “Intracellular antibody signalling is regulated by phosphorylation of the Fc receptor TRIM21,” *Elife*, vol. 7, pp. 1-22, 2018.
- [53] R. V. Stevens, D. Esposito, and K. Rittinger, “Characterisation of class VI TRIM RING domains: Linking RING activity to C-terminal domain identity,” *Life Sci. Alliance*, vol. 2, no. 3, pp. 1-14, 2019.
- [54] Z. Yudina *et al.*, “RING Dimerization Links Higher-Order Assembly of TRIM5 α to Synthesis of K63-Linked Polyubiquitin,” *Cell Rep.*, vol. 12, no. 5, pp. 788-797, 2015.
- [55] D. M. Dawidziak, J. G. Sanchez, J. M. Wagner, B. K. Ganser-Pornillos, and O. Pornillos, “Structure and catalytic activation of the TRIM23 RING E3 ubiquitin ligase,” *Proteins Struct. Funct. Bioinforma.*, vol. 85, no. 10, pp. 1957-1961, 2017.
- [56] E. Fiskin, S. Bhogaraju, L. Herhaus, S. Kalayil, M. Hahn, and I. Dikic, “Structural basis for the recognition and degradation of host TRIM proteins by Salmonella effector SopA,” *Nat. Commun.*, vol. 8, 2017.
- [57] J. R. Keown, J. Yang, M. M. Black, and D. C. Goldstone, “The RING domain of TRIM69 promotes higher-order assembly,” *Acta Crystallogr. Sect. D Struct. Biol.*, vol. 76, pp. 954-961, 2020.
- [58] L. Kiss *et al.*, “A tri-ionic anchor mechanism drives Ube2N-specific recruitment and K63-chain ubiquitination in TRIM ligases,” *Nat. Commun.*, vol. 10, no. 1, 2019.
- [59] L. Micale, “The Tripartite Motif - Structure and Function,” *TRIM/RBCC Proteins*, pp. 38-79, 2012.
- [60] X. Li and J. Sodroski, “The TRIM5 α B-Box 2 Domain Promotes Cooperative Binding to the Retroviral Capsid by Mediating Higher-Order Self-Association,” *J. Virol.*, vol. 82, no. 23, pp. 11495-11502, 2008.
- [61] Y. Li *et al.*, “B1 oligomerization regulates PML nuclear body biogenesis and leukemogenesis,” *Nat. Commun.*, vol. 10, no. 1, 2019.
- [62] H. Du *et al.*, “MID1 catalyzes the ubiquitination of protein phosphatase 2A and mutations within its Bbox1 domain disrupt polyubiquitination of Alpha4 but not of PP2Ac,” *PLoS One*, vol. 9, no. 9, 2014.
- [63] J. M. Wagner *et al.*, “Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5 α ,” *Elife*, vol. 5, no. JUNE2016, pp. 1-26, 2016.
- [64] D. C. Goldstone *et al.*, “Structural studies of postentry restriction factors reveal antiparallel dimers that enable avid binding to the HIV-1 capsid lattice,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 26, pp. 9609-9614, 2014.
- [65] C. Weinert, D. Morger, A. Djekic, M. G. Grütter, and P. R. E. Mittl, “Crystal structure of TRIM20 C-terminal coiled-coil/B30.2 fragment: Implications for the recognition of higher order oligomers,” *Sci. Rep.*, vol. 5, no. April, pp. 1-10, 2015.
- [66] Y. Li *et al.*, “Structural insights into the TRIM family of ubiquitin E3 ligases,” *Cell Res.*, vol. 24, no. 6, pp. 762-765, 2014.
- [67] J. G. Sanchez, K. Okreglicka, V. Chandrasekaran, J. M. Welker, W. I. Sundquist, and O. Pornillos, “The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 111, no. 7, pp. 2494-2499,

- 2014.
- [68] J. R. Keown *et al.*, "A helical LC3-interacting region mediates the interaction between the retroviral restriction factor Trim5 and mammalian autophagy-related ATG8 proteins," *J. Biol. Chem.*, vol. 293, no. 47, pp. 18378-18386, 2018.
 - [69] X. Wu *et al.*, "Inhibition of influenza A virus replication by TRIM14 via its multifaceted protein-protein interaction with NP," *Front. Microbiol.*, vol. 10, no. FEB, pp. 1-14, 2019.
 - [70] J. G. Sanchez *et al.*, "TRIM25 binds RNA to modulate cellular anti-viral defense," *J. Mol. Biol.*, no. xxxx, pp. 1-14, 2018.
 - [71] S. Nisole, J. P. Stoye, and A. Saïb, "TRIM family proteins: Retroviral restriction and antiviral defence," *Nat. Rev. Microbiol.*, vol. 3, no. 10, pp. 799-808, 2005.
 - [72] M. I. Giraldo, A. Hage, S. van Tol, and R. Rajsbaum, "TRIM Proteins in Host Defense and Viral Pathogenesis," *Curr. Clin. Microbiol. Reports*, 2020.
 - [73] R. Rajsbaum, A. García-Sastre, and G. A. Versteeg, "TRIMmunity: The roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity," *J. Mol. Biol.*, vol. 426, no. 6, pp. 1265-1284, 2014.
 - [74] K. M. Short and T. C. Cox, "Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding," *J. Biol. Chem.*, vol. 281, no. 13, pp. 8970-8980, 2006.
 - [75] A. J. Fletcher *et al.*, "Trivalent RING Assembly on Retroviral Capsids Activates TRIM5 Ubiquitination and Innate Immune Signaling," *Cell Host Microbe*, vol. 24, no. 6, pp. 761-775.e6, Dec. 2018.
 - [76] J. Zeng *et al.*, "Target-induced clustering activates Trim-Away of pathogens and proteins," *Nat. Struct. Mol. Biol.*, vol. 28, no. 3, pp. 278-289, Mar. 2021.
 - [77] K. Kato *et al.*, "Structural analysis of RIG-I-like receptors reveals ancient rules of engagement between diverse RNA helicases and TRIM ubiquitin ligases," *Mol. Cell*, pp. 1-55, 2020.
 - [78] M. Sardiello, S. Cairo, B. Fontanella, A. Ballabio, and G. Meroni, "Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties," *BMC Evol. Biol.*, vol. 8, no. 1, pp. 1-22, 2008.
 - [79] D. A. Rhodes, B. De Bono, and J. Trowsdale, "Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence," *Immunology*, vol. 116, no. 4, pp. 411-417, 2005.
 - [80] R. Rajsbaum, J. P. Stoye, and A. O'Garra, "Type I interferon-dependent and -independent expression of tripartite motif proteins in immune cells," *Eur. J. Immunol.*, vol. 38, no. 3, pp. 619-630, 2008.
 - [81] L. Carthagen *et al.*, "Human TRIM gene expression in response to interferons," *PLoS One*, vol. 4, no. 3, 2009.
 - [82] M. G. Koliopoulos *et al.*, "Molecular mechanism of influenza A NS1-mediated TRIM25 recognition and inhibition," *Nat. Commun.*, vol. 9, no. 1, p. 1820, Dec. 2018.
 - [83] N. Biris *et al.*, "Structure of the rhesus monkey TRIM5α PRYSPRY domain, the HIV capsid recognition module," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 33, pp. 13278-13283, 2012.
 - [84] A. H. Keeble, Z. Khan, A. Forster, and L. C. James, "TRIM21 is an IgG receptor

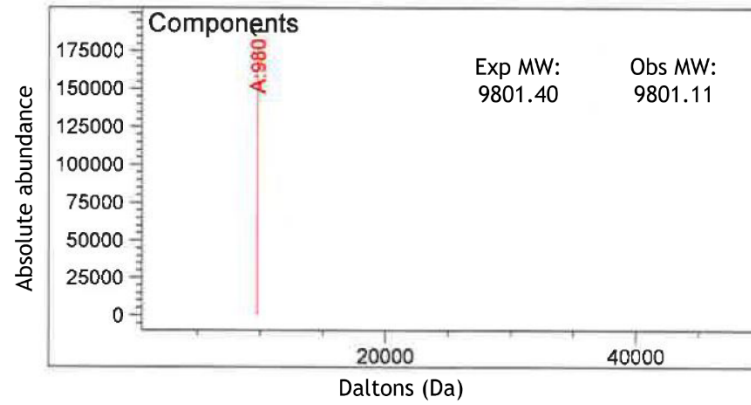
- that is structurally, thermodynamically, and kinetically conserved,” vol. 105, no. 16, 2008.
- [85] C. Weinert, D. Morger, A. Djekic, M. G. Grütter, and P. R. E. Mittl, “Crystal structure of TRIM20 C-terminal coiled-coil/B30.2 fragment: Implications for the recognition of higher order oligomers,” *Sci. Rep.*, vol. 5, no. February, pp. 1-10, 2015.
 - [86] Y. Yu, L. Liang, Y. Jin, and Y. Yin, “The TRIM14 PRYSPRY domain mediates protein interaction via its basic interface,” *FEBS Lett.*, vol. 593, no. 10, pp. 1122-1129, 2019.
 - [87] E. Y. Park *et al.*, “Crystal structure of PRY-SPRY domain of human TRIM72,” *Proteins Struct. Funct. Bioinforma.*, vol. 78, no. 3, pp. 790-795, 2010.
 - [88] J. S. Woo, J. H. Imm, C. K. Min, K. J. Kim, S. S. Cha, and B. H. Oh, “Structural and functional insights into the B30.2/SPRY domain,” *EMBO J.*, vol. 25, no. 6, pp. 1353-1363, 2006.
 - [89] L. C. James, A. H. Keeble, Z. Khan, D. A. Rhodes, and J. Trowsdale, “Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 104, no. 15, pp. 6200-6205, 2007.
 - [90] C. Cadena *et al.*, “Ubiquitin-Dependent and -Independent Roles of E3 Ligase RIPLET in Innate Immunity,” *Cell*, vol. 177, no. 5, pp. 1187-1200.e16, 2019.
 - [91] C. J. Muñoz Sosa, F. M. Issoglio, and M. E. Carrizo, “Crystal structure and mutational analysis of the human TRIM7 B30.2 domain provide insights into the molecular basis of its binding to glycogenin-1,” *J. Biol. Chem.*, p. 100772, 2021.
 - [92] A. K. Kar, F. Diaz-Griffero, Y. Li, X. Li, and J. Sodroski, “Biochemical and Biophysical Characterization of a Chimeric TRIM21-TRIM5 α Protein,” *J. Virol.*, vol. 82, no. 23, pp. 11669-11681, Dec. 2008.
 - [93] X. Li *et al.*, “Unique features of TRIM5 α among closely related human TRIM family members,” *Virology*, vol. 360, no. 2, pp. 419-433, 2007.
 - [94] A. Di Pietro *et al.*, “TRIM22 Inhibits Influenza A Virus Infection by Targeting the Viral Nucleoprotein for Degradation,” *J. Virol.*, vol. 87, no. 8, pp. 4523-4533, 2013.
 - [95] C. Yang *et al.*, “Interferon alpha (IFN α)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A,” *Cell. Mol. Immunol.*, vol. 13, no. 1, pp. 94-102, 2016.
 - [96] P. Eldin, L. Papon, A. Oteiza, E. Brocchi, T. G. Lawson, and N. Mechti, “TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus,” *J. Gen. Virol.*, vol. 90, no. 3, pp. 536-545, 2009.
 - [97] R. Rajsbaum *et al.*, “Unanchored K48-Linked Polyubiquitin Synthesized by the E3-Ubiquitin Ligase TRIM6 Stimulates the Interferon-IKK ϵ Kinase-Mediated Antiviral Response,” *Immunity*, vol. 40, no. 6, pp. 880-895, 2014.
 - [98] P. Bharaj *et al.*, “The Matrix Protein of Nipah Virus Targets the E3-Ubiquitin Ligase TRIM6 to Inhibit the IKK ϵ Kinase-Mediated Type-I IFN Antiviral Response,” *PLoS Pathog.*, vol. 12, no. 9, pp. 1-27, 2016.
 - [99] K. C. Prins, W. B. Cárdenas, and C. F. Basler, “Ebola Virus Protein VP35 Impairs the Function of Interferon Regulatory Factor-Activating Kinases IKK ϵ and TBK-1,” *J. Virol.*, vol. 83, no. 7, pp. 3069-3077, 2009.
 - [100] S. Obad, H. Brunnström, J. Vallon-Christersson, Å. Borg, K. Drott, and U.

- Gullberg, "Staf50 is a novel p53 target gene conferring reduced clonogenic growth of leukemic U-937 cells," *Oncogene*, vol. 23, no. 23, pp. 4050-4059, 2004.
- [101] Y. Sun *et al.*, "Down-regulation of Tripartite-motif containing 22 expression in breast cancer is associated with a lack of p53-mediated induction," *Biochem. Biophys. Res. Commun.*, vol. 441, no. 3, pp. 600-606, 2013.
 - [102] L. Liu *et al.*, "TRIM22 confers poor prognosis and promotes epithelial-mesenchymal transition through regulation of AKT/GSK3B/ β -catenin signaling in non-small cell lung cancer," *Oncotarget*, vol. 8, no. 37, pp. 62069-62080, 2017.
 - [103] S. Zheng, C. Zhou, Y. Wang, H. Li, Y. Sun, and Z. Shen, "TRIM6 promotes colorectal cancer cells proliferation and response to thiostrepton by TIS21/FoxM1," *J. Exp. Clin. Cancer Res.*, vol. 39, no. 1, pp. 1-17, 2020.
 - [104] C. Wei, J. Wu, W. Liu, J. Lu, H. Li, and B. Hai, "Tripartite motif-containing protein 6 facilitates growth and migration of breast cancer through degradation of STUB1," *Eur. J. Histochem.*, vol. 65, no. 1, pp. 1-2, 2021.
 - [105] Z. Duan, B. Gao, W. Xu, and S. Xiong, "Identification of TRIM22 as a RING finger E3 ubiquitin ligase," *Biochem. Biophys. Res. Commun.*, vol. 374, no. 3, pp. 502-506, 2008.
 - [106] J. R. Keown, J. X. Yang, J. Douglas, and D. C. Goldstone, "Characterisation of assembly and ubiquitylation by the RBCC motif of Trim5 α ," *Sci. Rep.*, vol. 6, no. May, pp. 1-11, 2016.
 - [107] T. Ohkuni *et al.*, "Poly(I:C) reduces expression of JAM-A and induces secretion of IL-8 and TNF- α via distinct NF- κ B pathways in human nasal epithelial cells," *Toxicol. Appl. Pharmacol.*, vol. 250, no. 1, pp. 29-38, 2011.
 - [108] H. Yang *et al.*, "Structural insight into HIV-1 capsid recognition by rhesus TRIM5 α ," *Proc. Natl. Acad. Sci. U. S. A.*, vol. 109, no. 45, pp. 18372-18377, 2012.
 - [109] A. Reymond *et al.*, "The tripartite motif family identifies cell compartments," *EMBO J.*, vol. 20, no. 9, pp. 2140-2151, 2001.
 - [110] Q. Ye, R. M. Krug, and Y. J. Tao, "The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA," *Nature*, vol. 444, no. 7122, pp. 1078-1082, 2006.
 - [111] S. Shanmugam, A. K. Nichols, D. Saravanabalaji, C. Welsch, and M. K. Yi, *HCV NS5A dimer interface residues regulate HCV replication by controlling its self-interaction, hyperphosphorylation, subcellular localization and interaction with cyclophilin A*, vol. 14, no. 7. 2018.
 - [112] I. Gupta, S. Dharadhar, T. Sixma, and S. Khan, "Biochemical characterization of TRIM72 E3 ligase and its interaction with the insulin receptor substrate 1," *Biochem. Biophys. Reports*, vol. 21, no. January, p. 100729, 2020.
 - [113] M. Beck *et al.*, "The quantitative proteome of a human cell line," *Mol. Syst. Biol.*, vol. 7, no. 549, pp. 1-8, 2011.
 - [114] G. Sivaramakrishnan, Y. Sun, S. K. Tan, and V. C. L. Lin, "Dynamic localization of tripartite motif-containing 22 in nuclear and nucleolar bodies," *Exp. Cell Res.*, vol. 315, no. 8, pp. 1521-1532, 2009.
 - [115] G. A. Versteeg *et al.*, "The E3-Ligase TRIM Family of Proteins Regulates

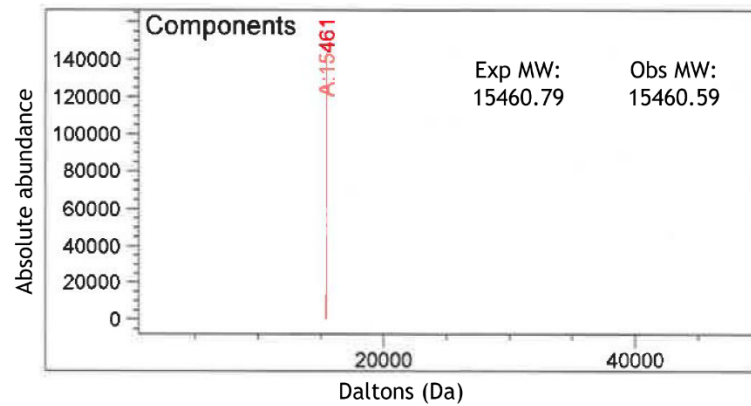
- Signaling Pathways Triggered by Innate Immune Pattern-Recognition Receptors,” *Immunity*, vol. 38, no. 2, pp. 384-398, 2013.
- [116] L. M. Napolitano and G. Meroni, “TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation,” *IUBMB Life*, vol. 64, no. 1, pp. 64-71, 2012.

Supplementary Figures

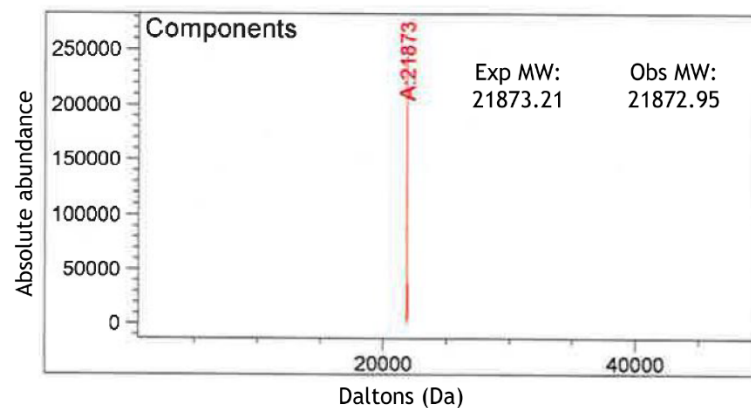
A.



B.



C.



Supplementary Figure 1. Intact mass spectra of TRIM22 RING, RB2 and RBCC MT constructs
Intact mass spectra for **A.** TRIM22 RING 1-87 **B.** TRIM22 RB2 2-133 **C.** TRIM22 RBCC MT 1-261ΔCC. Expected (Exp) and observed (Obs) molecular weights are annotated on each figure.