Chapter

Zinc-Binding B-Box Domains with RING Folds Serve Critical Roles in the Protein Ubiquitination Pathways in Plants and Animals

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Abstract

Protein ubiquitination is an essential cellular process that maintains protein homeostasis, regulates protein, and cell functions, and removes aggregated and misfolded protein. Disruption in function of any of the protein components of the ubiquitination pathway is associated with human diseases including cancers. An important member in the ubiquitination cascade is the very large E3 ligase family that directs substrate modification. The RING-type E3 ligases possess a cysteine/histidine-rich zinc-binding RING domain that confers ligase functionality. RING domains adopt a canonical ββα-fold. TRIM proteins represent a novel class of RING-type E3 ligase. TRIM proteins consist of an N-terminal RING domain followed by one or two B-box domains. The two types of B-box domains play essential roles in protein ubiquitination by contributing to substrate targeting, ligase activity enhancement, and redundancy of ligase activity. This review presents a general background of the B-box domains, a structural and functional comparison with RING domains, and a summary of recent work demonstrating their role in proteolysis. We discuss new findings that reveal B-box domains which are ubiquitous and are found in non-TRIM plant proteins without the adjacent RING domain, indicating that B-boxes are members of RING-class E3 ligases.

Keywords: zinc-finger, ubiquitination, E4 ligases, MID1, TRIM, BBX

1. Introduction

Protein ubiquitination is an essential cellular process that maintains protein homeostasis (proteostasis) and removes aggregated and misfolded protein that could recruit other proteins away from their normal cellular functions. It serves to regulate protein and cellular functions. Dysregulation of any of the protein component usually result in human diseases including cancers and birth defects. As such, the focus on protein ubiquitination has grown significantly in the past 2 decades, leading to extensive knowledge and new insights. A member of the ubiquitination cascade that has received considerable attention involves the large and growing family of E3 ligases. This family directs the last step in the reaction cascade by facilitating the ubiquitination of protein substrates. It is generally accepted that each E3 ligase has a specific or group of substrates (**Figure 1**).

There are several subgroups of E3 ligases, with the largest consisting of proteins with a RING domain that confer ligase functionality. The RING E3 ligase domains are cysteine- and histidine-rich sequences that bind two zinc ions in a unique cross-brace manner and adopt a canonical ββα-fold (**Figure 1**). *Tri*partite *m*otif (TRIM) proteins represent a new class of RING E3 ligases. TRIM proteins are characterized by their N-terminal RING domain followed by one or two cysteine/histidine-rich

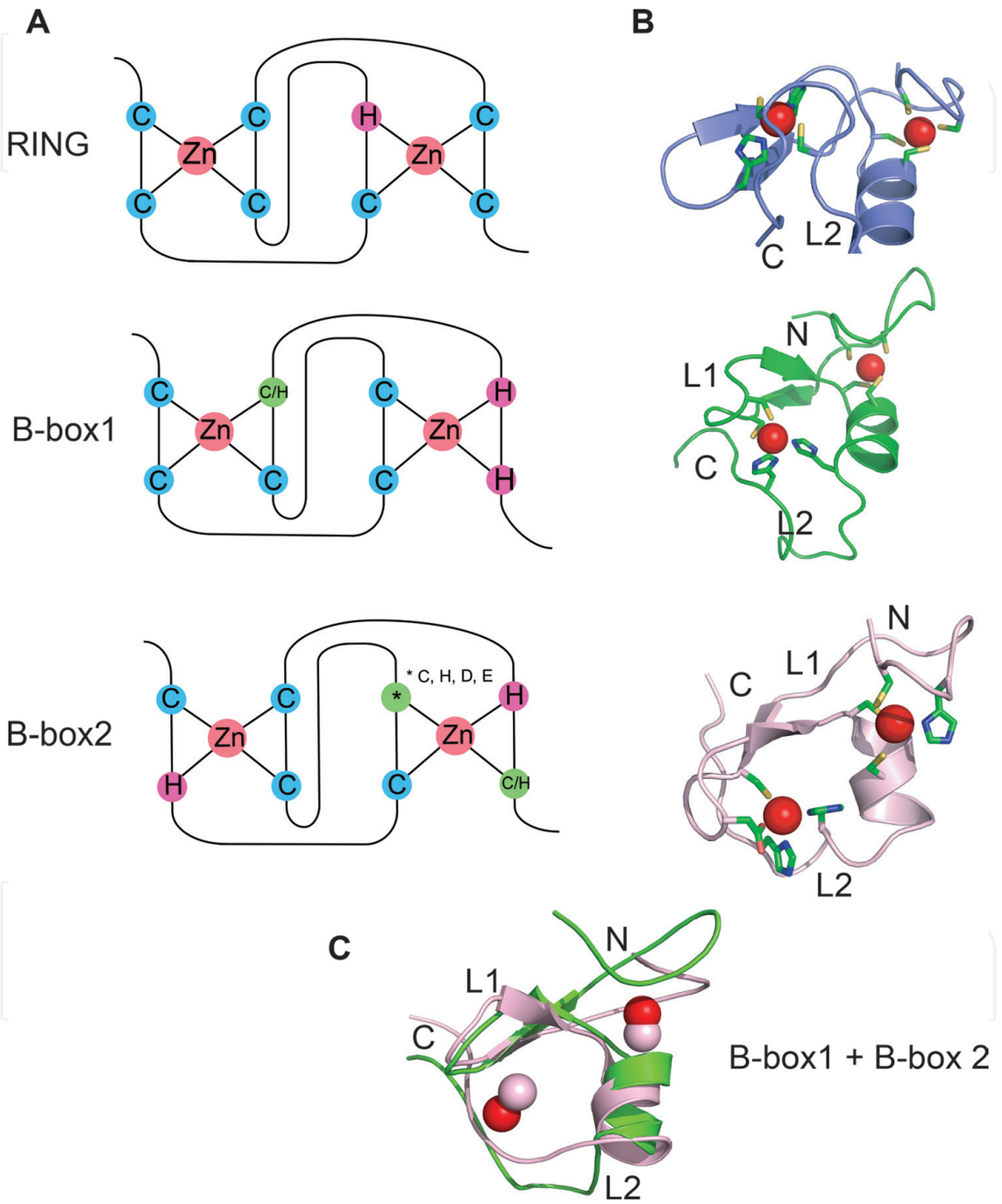

Figure 1.
Zinc-binding and structure of RING and B-box domains. A. Cross-brace zinc-binding mechanism by Cys/His-rich sequences of RING and B-box domains. Unlike other zinc-finger domains in which the zinc ion is coordinated by Cys and His ligands in a sequential manner, the cross-brace mechanism involves coordination by alternating pairs of ligands. The consensus zinc-binding sequences are different for RING and the B-box domains. B. Ribbon drawings of a representative RING (pdb 2hdp) domain and MID1 B-box1 (2ffw) and B-box2 (2dq5) domains. The ββα-canonical RING fold consists of a short α-helix and two loops, both of which contribute ligands to bind the zinc. Loop1 (L1) is usually less mobile than loop2 (L2). The relative locations of the zinc ions (red spheres) are similar among RING domains and the B-box domains. C. The superposition of structures of the B-box1 and B-box2 domains shows that the overall structures are similar. The structure is rendered to smooth out variation of the loop to simplify the image.

regions called B-box domains. The two types of B-box domains play important roles in protein ubiquitination, contributing to substrate targeting, enhancement of ligase activity, and redundancy in ligase activity. This review presents a historical background of the B-box domains commonly found in TRIM proteins, a structural and functional comparison with RING domains, and a summary of recent work demonstrating their role in protein ubiquitination. A brief discussion on the current understanding of RING E3 ligases mechanism of function is presented. We also discuss findings that reveal B-box domains are found in non-TRIM plant proteins without an accompanying RING domain. Finally, we argue that the B-box domains represent a new addition to the RING-class E3 ligases with a more versatile role than RING E3 ligase, namely they bind substrates, regulate E3 ligase activity of the adjacent RING domain, enhance E3 ligase activity of TRIM proteins, and actually function as E3 ligases.

2. Background

B-box and RING domains fall under the category of zinc-finger domains, which are present in a diverse family of proteins that includes transcription factors, ribonucleoproteins, proto-oncoproteins, and E3 ligases [1]. Zinc-finger domains or proteins are characterized as having cysteine and histidine residues arranged in one of several motifs that are relatively conserved in other proteins [2]. The thiol group (S^-) of the cysteine and a nitrogen atom of the histidine imidazole side-chain tetrahedrally bind a zinc ion [2].

Most zinc-finger proteins typically coordinate either a single zinc ion or two zinc ions, depending on the number of cysteine and histidine residues and their position within the sequence [3]. A defining property of zinc-finger domains is that zinc coordination is required to stabilize the tertiary structure. Loss of zinc coordination by a mutation of any of its cysteine or histidine residue results in complete unfolding of the protein structure. Protonation of the cysteine or histidine by decreasing the pH of the protein solution will also result in unfolding. For domains that bind two zinc ions, disruption of coordination of one zinc ion is usually accompanied by the loss of binding of the other zinc ion, causing the domain to become unfolded rather than partially folded with one zinc ion [4, 5].

Zinc-finger domains were identified in the mid-1980s within the *Xenopus* nuclear factor 7 (XNF7), first by Aaron Klug [6–8]. The subgroups of A-box and B-box cysteine/histidine-region domains were first identified a few years later with the *Xenopus* transcription protein TFIIIA ([9]). The A-box domain precedes the B-box region by 20–45 amino acids. Subsequently, other proteins were observed to have a similar A-box motif without an accompanying B-box region. The A-box regions constitute 60–80 amino acids with a zinc-binding consensus sequence of $C\text{-}X_2\text{-}C\text{-}X_{[9–39]}\text{-}C\text{-}X_{[1–3]}\text{-}H\text{-}X_{[2–3]}\text{-}C\text{-}X_2\text{-}C\text{-}X_{[4–48]}\text{-}C\text{-}X_2\text{-}C$. This consensus sequence reveals eight zinc-binding ligands (cysteines) that can coordinate two zinc ions. There are also variations in the zinc ligands as A-box domains are observed to have more than one histidine. By the early 1990s, the A-box domain was renamed *r*eally *i*nteresting *n*ew *g*ene (RING). The uncreative moniker remains for the B-box domain [10].

For the next 2 decades, the number of proteins observed with the RING and B-box domain pairs would have increased, with most belonging to TRIM proteins that are defined by their N-terminal RING, B-box, and coiled-coil (RBCC) domains [11, 12]. This RBCC domain arrangement is conserved and found in all multicellular organisms [12]. In humans, the RBCC domain is observed in a family of over 50 proteins; although few have been characterized in detail, their importance is underscored by

the fact that some are oncoproteins (e.g., PML, RFP, and TIF1a), while others, when mutated, give rise to various congenital abnormalities [13, 14]. Members of this large protein family are found to play regulatory roles in a variety of cellular processes, including sperm vesicle exocytosis and intracellular release of HIV [15, 16]. The RBCC domain arrangement indicates at the very least that TRIM proteins have an overall common function. The RBCC proteins can have quite diverse C-terminal domain arrangements [17].

Interestingly, many TRIM proteins possess three consecutive cysteine/histidine-rich regions, the first being the RING domain. The other two domains are referred to as B-box1 and B-box2 domains ([18]). While the nomenclatures suggest that the two types of B-box domains are homologous, they do not share any discernable sequence similarity with each other or with RING domains. A single B-box domain in TRIM proteins is always of the type 2 form (B-box2), while TRIMs with two have the B-box1 domain preceding the B-box2 domain. The name may have persisted to prevent confusion in distinguishing the presence of the two types of B-box domains in TRIM proteins. The B-box1 domain is slightly larger (50–60 aa) with a zinc-binding consensus sequence of C-X_2-C-$X_{7–12}$C-X_2-C-X_4-C-X_2-[C/H]-$X_{3–4}$-H-$X_{4–9}$-H **[C5(C/H)H2]**. The B-box2 domains are 35–45 and have a consensus sequence of C-X_2-H-$X_{7–9}$-C-X_2-[C/D/E]-X_4-C-X_2-C-$X_{3–6}$-H-$X_{2–4}$-[C/H] **[CHC(C/D/E) C2H(C/H)]** [4]. Comparison of the consensus sequences of RING and the B-box domains reveals two regions in which the number of amino acids between zinc-binding pairs is different. RING domains have the longest length ($X_{[9-39]}$, $X_{[4-48]}$) following by the B-box1 domain ($X_{7–12}$,$X_{4–9}$) and B-box2 domain ($X_{7–9}$, $X_{3–6}$).

3. Description of B-box domain structures

Despite their prevalence and location downstream of RING domains in TRIM proteins, very little was initially done to characterize the structures and functions of B-box domains. We postulate that this might have been so because of difficulties in obtaining sufficient quantities of the B-box domains for structural and functional studies. Indeed, each type of the B-box domain has proven to be quite challenging to express and purify using *E. coli*. The same can be said for RING domains because there are only a few dozen structures solved, and given their uniqueness in defining substrate specificity, structural comparisons would be important to identify differences in mechanism of function and activity. Our experience revealed that the B-box and RING domains tend to form inclusion bodies [19]. In identifying conditions to obtain large quantities of these domains, we have established a purification protocol that not only works for B-box domains but also for other proteins that are prone to forming inclusion bodies [19, 20]. The protocol can extract natively folded proteins from inclusion bodies without refolding. Despite some of these initial challenges, there are now several structures of both types of B-box domains in the protein database.

The first comprehensive structural studies of B-box domains were based on the TRIM18/MID1 protein [13, 21]. Human MID1 is required for proper midline development during embryogenesis ([22–26]). Mutations of MID1, some of which are found within the B-box domains, are associated with X-Linked Opitz G/BBB syndrome (XLOS), a congenital disorder characterized by clefts of the lip and palate, cardiac structural defects, and genital anomalies [14, 27].

The structure of the B-box1 domain (residues Gln87-Pro165) was solved in 2006 by analyzing multidimensional data acquired by nuclear magnetic resonance (NMR) spectroscopy. The B-box1 domain was observed to coordinate two zinc ions in a cross-brace manner with six cysteine and two histidine residues (**Figure 1**) [13]. Residues

Ala115 to Pro165 form the core of the structure, while the preceding 30 amino acids are unstructured and initially included to aid in solubility. The structure consists of a two-turn α-helix that is preceded by a long structured loop consisting of two short β-strands separated by a type-2 β-turn. Two cysteine residues within the first part of the structured lasso-like loop1 coordinate one zinc ion with two other cysteine residues located within the first helical turn of the helix. Two cysteine residues that are part of the β-turn and two histidine residues, one located at the end of the α-helix and the other on the loop2 that follows the helix, coordinate the second zinc ion. The overall structure is very similar to the ββα-canonical RING fold (**Figure 1**).

The structure of MID1 B-box2 domain was solved a year later, using NMR data. In contrast to the MID1 B-box1 domain, the MID1 B-box2 consists of seven classical cysteine and histidine zinc-binding residues, suggesting that only one zinc might be coordinated by four of these residues (**Figure 1**). Sequence alignment of TRIM B-box2 domains reveals that approximately half of B-box2 domains consist of aspartate residues and the other half a cysteine residues in the same location. This observation suggests that Asp must be a highly conserved change [21] that should be performing the same role as the cysteine residue. Indeed, the MID1 B-box2 domain coordinates two zinc ions in a similar cross-brace manner as the B-box1 and RING domains. Two histidine, cysteine, and aspartate residues coordinate one zinc ion. The carboxylate oxygen of this conserved aspartate side chain participates in zinc coordination. The aspartate residue forms the necessary zinc-knuckle conformation with a cysteine residue two positions away (CxxD) to tetrahedrally coordinate the zinc ion [2, 21]. Although carboxylate groups are involved in binding catalytic zinc ions, for example, carbonic anhydrase [28–30], or other non-structural metals, this was the first demonstration in a zinc-finger protein. The B-box2 domain adopts a two-turn α-helix, two short β-strands separated by a type-2 β-turn, and two structured loops adjacent to the helix. Despite a lack of sequence similarity, the structures of the two types of B-box domains are remarkably similar (**Figure 1C**). The positions of the two zinc ions are in similar locations, namely near the N-terminus of the helix and to the bottom left of the helix (given the specific orientation shown in **Figure 1B**). Importantly, the mechanism of zinc coordination (cross-brace) and the ββα-fold are comparable to those of RING domains.

Structures of the B-box1 domain from TRIM19 and the B-box2 domain from TRIM1/MID2, TRIM5α, TRIM21, TRIM29, TRIM39, TRIM41, TRIM54, and TRIM63/MuRF1 have been solved. All the B-box domain structures are similar. Consequently, we conclude that MID1 consists of three consecutive domains with RING folds. Thus, the TRIM protein family must represent a new class of E3 RING-type ligase, consisting of two or three consecutive RING folds.

To identify a possible role of the two adjacent B-box domains, the structure of both was determined in their native tandem form (res A110-E214). The two B-box domains maintained their original structures and pack against each other with the interface formed by residues located on the structured loop-1 near the two antiparallel β-strands. The surface area of the interface is 188 $Å^2$ (17% of the total surface). Interestingly, the tandem globular structure is very reminiscent of the intermolecular association observed for heterodimeric RING structures, such as the BARD1 and BRCA1 domains (12) and the polycomb group protein (Bmi-1) and Ring1B polycomb group (14), and the homodimeric RINGs, such as HDM2 [31], RNF4 and 8 [32, 33], and cIAP2 [34]. The TRIM19 B-box1 and TRIM54 B-box2 domains were solved as symmetric dimers by X-ray crystallography. The structures of RING dimers reveal the domains interacting via residues located on and near loop-1. The BRCA1-BARD and RNF8 dimers also include adjacent structures, such as helical dimers. The area of the interface of the hetero- and homo-RING dimers is approximately 150–200 $Å^2$, similar to that observed for the MID1 B-box1,2 heterodimer. In spite of their

interactions, it appears that in the case of MID1, unfolding of the B-box1 structure, via a mutation of one its zinc-binding residues, had little effects on the structure and stability of the B-box2 domain [4]. This observation suggests the possibility that each B-box domain could function independently or have redundant E3 ligase function.

The structures of two B-box domains were solved in complex with another TRIM domain. The TRIM5α B-box2 was crystallized with its coiled-coil domain, which contributes to oligomerization. Binding studies using NMR and dynamic light scattering using a TRIM5α proteins with a native and mutant B-box2 domain reveal that the B-box2 domain contribute to higher order self-association [35]. Given that the B-box2 domain is required for substrate ubiquitination, self-association may contribute to enhanced E3 ligase activity and substrate targeting [36, 37] of the native TRIM5α. The B-box domains of TRIM27 are also determined to be crucial for multimerization by possibly helping to orient the coiled-coil domain in a way that maintained the multimer interaction [38]. In contrast, the B-box2 domain of TRIM21 was crystallized with the N-terminal RING domain, and the structure reveals that the B-box2 domain interacts with the RING domain on a surface that is important for RING-E2 interaction. In this case, the structure suggests that TRIM21 B-box2 may have an autoinhibitory effect, although further studies are required. It is possible that the structures of these complexes may be affected by protein packing within the crystal lattice. More work needs to be done to understand the mechanism of function of these B-box domains, which we postulate are now key players in the ubiquitination field.

4. A brief description of ubiquitination and the role of E3 ligases

In order to appreciate the function of RING and B-box domains, a brief summary of protein ubiquitination is provided. All living organisms employ a fairly common mechanism to recycle proteins so as to regulate protein function, proteostasis, and cell cycle. Eukaryotic cells employ protein ubiquitination, a post-translational modification using the highly stable 76-amino acid ubiquitin protein (Ub) [39–41]. Bacteria use prokaryotic ubiquitin-like protein (Pup) in an analogous manner [42]. Polyubiquitinated proteins, usually with a chain of at least four-linked Ub, are targeted to the proteasome where they are proteolytically cleaved into peptides [39, 40, 43]. Ubiquitin chains can form via any of its seven lysine residues or combinations of the seven; homogenous chain links, example K48, promote protein degradation [44–46], but some have signaling functions [47, 48]. The Ubs are cleaved by deubiquitinating enzymes (DUBS) and recycled [49]. Although mono- and diubiquitinated proteins are directed to the proteasome [50], there is evidence that this level of modification serves a signaling role, in which modified proteins can have their functions and cellular location altered. The monoubiquitination of cytosolic proteins results in translocation to the nucleus to participate in DNA repair, transcription regulation, and inflammatory response [39, 51–54].

Ubiquitination involves three classes of enzymes. First, the E1-activating enzyme (E1) catalyzes the adenylation of the C-terminal glycine of Ub (Ub~AMP). This phosphoester bond undergoes a nucleophilic attack by the sulfhydryl group of the active site cysteine residue on the E1. In the next step, the Ub is transferred to an active site cysteine residue on a family of E2 conjugating enzymes (E2) to form an activated thioester-linked E2~Ub complex [39, 43]. Typically most types of E2 enzymes require the concerted action of an E3 ligase (E3) to target and facilitate substrate ubiquitination [40, 43]. There are several classes of E3 ligases: the homologous to the E6-AP Carboxyl Terminus (HECT), RING-InBetweenRing-RING families, and RING class [39–41, 50, 55]. The HECT and RING-IBR-RING families

accept the Ub via a trans-thiolation reaction from the E2 before transferring it to the target protein. The RING E3 ligase, which includes the Skp-Cullin-F-box (SCF) complex, U-box, and now the B-box, represents the overwhelming majority of E3 ligases. While the mechanism is unclear, the RING-type ligase binds and places in close proximity to the target protein and the E2 enzyme. With the SCF complex, the RING domain (aka RBX) recruits the E2~Ub, while another SCF subunit (usually, the F-box) binds the substrate. In the last reaction, the E2~Ub thioester bond undergoes a nucleophilic attack (thiolysis) by a lysine residue of the target protein whereby the side-chain amino group forms an isopeptide bond with the C-terminal carboxylate group of Ub. Subsequent Ubs can be attached to other lysine residues of the substrate, but more commonly observed to form a polyubiquitin chain with linkages to one of seven lysine residues of the Ub [39, 40, 43]. Chains can be formed with Lys 6, 11, 27, 29, 33 and the N-terminus (M1) amino group, but the two more common reported linkages involve Lys48 and Lys63 [44]. We argue that the level or amount of polyubiquitination or Ub processivity can be an assessment of the level of E3 ligase activity by a RING protein.

Typically, confirmation that a RING domain/protein possesses E3 ligase activity is accomplished by performing *in vitro* autoubiquitination assays consisting of all the protein components except the substrate. In most cases, the protein substrate is unknown. In these reactions, the proximity of the RING domain with the activated

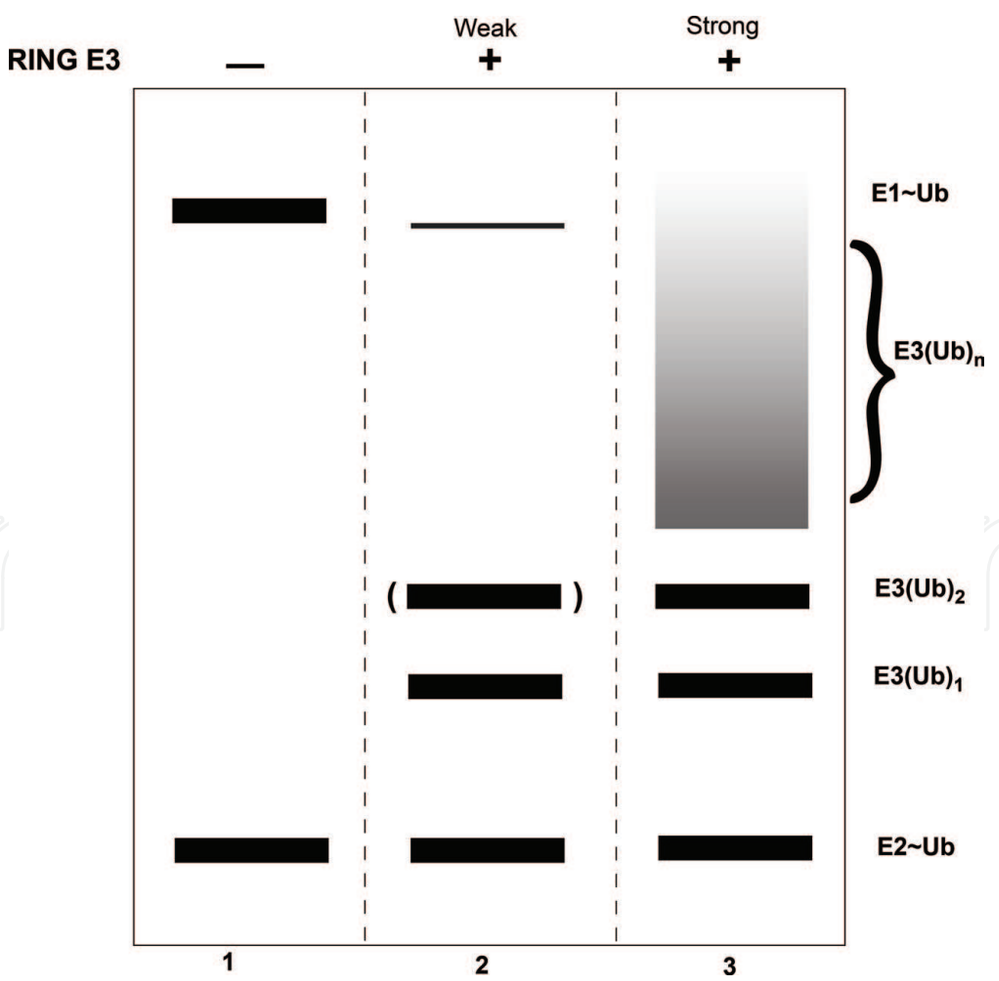

Figure 2.
Expected RING E3 ligase results. Cartoon representation of a Western blot image showing autoubiquitination results in no E3 ligase (ln1), a RING-type E3 with "weak" E3 ligase activity forming mono- and sometimes (di-ubiquitinated) products, and a RING E3 with stronger activities (ln3). The smearing represents polyubiquitinated products with various amounts of Ub on the protein. Substrate ubiquitination will usually mirror the results shown.

E2~Ub complex promotes auto- or self-ubiquitination, suggesting that a RING protein is a substrate of its own E3 ligase activity. How autoubiquitination affects substrate ubiquitination is a subject of intense studies. A protein mixture with E1, E2, E3, Ub, and ATP is incubated, and ubiquitinated E3 is probed by Western blot with an antibody against the Ub or the E3 ligase (**Figure 2**). Protein bands corresponding to a RING domain with covalently attached Ubs typically indicate E3 ligase activity [39, 40]. The level of E3 ligase activity can be estimated by the rate and amount of autoubiquitinated products observed as a function of assay time [56, 57]. Western blot images of various levels of polyubiquitinated products are often indicated with a smearing of high molecular weight products, while a less active enzyme is indicated by less smearing or the presence of mono- or di-ubiquitinated products (**Figure 2**). However, it should be noted that even though an E3 ligase catalyzes mono- and di-ubiquitination does not mean it is a "weak" ligase; it is wholly possible that it has evolved to function at this level, and as noted, mono- and di-ubiquitination may serve as signaling events [54, 58]. There are lots of questions about how and why RING domains exhibit different levels of substrate ubiquitination.

5. Possible mechanism of action of RING E3 ligases

As noted, the mechanism of function of RING E3 ligases is unclear, but considerable progress has been made to provide insights. The structures of several E2-RING complexes reveal that the RING domain is positioned ~15 Å from the active site and the thioester linkage between the E2 and Ub [59, 60]. Based on these structures, it is unclear how the RING domain affects reactivity or electrophilicity on the E2~Ub linkage. To gain insights on the role of RING E3 ligases, Klevit and co-workers [61, 62] used molecular dynamic and NMR studies to show that the bound RING E3 ligase promoted a "closed" E2~Ub conformation, whereby the Ub populates one interaction mode with slightly greater frequency. In the absence of a bound RING domain, the covalently attached Ub is highly mobile and does not favor any specific surface of E2 to interact [62]. There is no fixed or stable structure between the Ub and E2 proteins. Promoting the positioning of the Ub to the "back" surface of the E2 reduces steric hindrance for nucleophilic attack by the incoming lysine residue. In addition, key amino acid interactions at the E2-RING binding interface appear to contribute to the activation of the thioester bond. For example, residue Gln92 of UbcH5 (Ube2D1), which is located on a helical turn adjacent to active site Cys-85, forms a hydrogen bond with an arginine or lysine residue on loop-2 of the RING domain. Disruption of this interaction through mutation of Gln92 or the arginine severely disrupts the rate of Ub transfer [62, 63]. In fact, the rate of thiolysis with free lysine, as the substrate, for either mutant is comparable to that of E2~Ub without a bound RING domain. Thus, it is generally accepted, given what is known from the various E2-RING interaction studies that the RING domain contributes allosterically to electrophilicity of the thioester bond.

6. The B-box domains are new members of the RING E3 ligase

Given that the B-box domains have similar RING folds, it was postulated to function similarly. First confirmation that the B-box domains possess E3 ligase activity was demonstrated with MID1 [64]. The *in vitro* assays revealed that each type of B-box domain exhibited weak E3 ligase activity. Mono-ubiquitinated products were observed. Varying degrees of RING E3 ligases activities have

been reported in the literature, but no explanation has been provided; for some instances, RING hetero- or homodimerization is necessary. Despite the similar overall fold of RING and B-box domains, we postulate that subtle amino acid differences and structural variations between RING folds may contribute to differences in the levels of activities. Furthermore, it is possible that the cohort E2 enzyme used may provide a specific level of activity [59]. Most RING E3 ligases are confirmed using the common $UbcH5_{a–c}$ (Ube2D1–3) E2 family. For MID1 B-box domains, mono-ubiquitination activities were observed with at least 12 different E2 enzymes [64]. As noted above, it is possible that the level of activities of the B-box domains may be physiological and evolutionarily determined. Below, the structures of RING and B-box domains are compared to provide rationales for the differences in activities.

Intriguingly, the tandem B-box domains also exhibit weak E3 ligase activity, with no greater level of autoubiquitination activity than that observed with the B-box1 domain [64]. This is in contrast to hetero- and homodimeric RING dimers, which exhibited greater activity than the mono form [32, 33, 65]. The BRCA1-BARD1 complex, in which BRCA1 (breast cancer 1) heterodimerizes with BARD1 (BRCA1-associated RING domain), exhibited enhanced activities compared to BRCA1 alone; BARD1 does not exhibit ligase activity [65–68]. Enhancements of activities were also observed for MDM2/HDX [69], RNF4 [33], inhibitor of apoptosis (IAP) proteins [70], BMI1-RING1 [71], and membrane-associated RING-CH family of E3 ubiquitin ligases (MARCH1) RING dimers [72], to name a few. The mechanism of E3 ligase enhancement by RING dimers or the lack of enhancement by the MID1 B-box domains is unclear. However, there are several publications that proposed rationales of the role of RING dimers [32, 73], but they will not be discussed here.

Despite the MID1 B-box domains not showing strong ligase activity, studies with TRIM16 revealed that its B-box domains exhibited greater level of activities. There were substantial amount of polyubiquitinated products, as demonstrated by the intensity of the smearing observed by Western blot analysis [74]. Both *in vivo* and *in vitro* ubiquitination assays with constructs containing B-box1 and B-box2 domain deletions resulted in the loss of the polyubiquitin smearing. TRIM16 is a pseudo-TRIM that lacks a RING domain; it is possible that TRIM16 B-box1 and 2 domains have evolved to possess increased activity in light of the missing RING domain. However, results from TRIM16 indicate that TRIM16 can dimerize with other TRIM proteins, and it may be that these interactions contribute to *in vivo* increased activity. This observation suggests a more intricate mechanism of action for TRIM proteins, namely that they can homo- and heterodimerize, and this can affect the levels of E3 ligase activity. Furthermore, recent *in vitro* and *in vivo* studies with TRIM27/rtf, a protein with RING–B-box2-CC domain, showed that the B-box2 domain, and not the RING domain, is responsible for substrate binding and ubiquitination [75].

7. Tandem RING and B-box domains are more active: could it be E4 ligases?

To understand the role of the B-box domains in the context of being adjacent to the RING domains, as they are commonly found in TRIM proteins, autoubiquitination assays were performed with the MID1 RING domain in tandem with B-box1 (RING-B-box1 (RB1)) and both B-box domains (RING–B-box1–B-box2 (RB1B2)). The goal was to determine whether each domain functions

independently or if they have synergistic contribution to justify a possible evolutional reason for their presence in tandem. In the case of RB1, greater amount of polyubiquitinated products were observed compared with the results of the ubiquitination assay with the RING domain alone [14, 64, 76]. The rate of product formation was qualitatively faster. Whereas polyubiquitinated products were observed with the MID1 RING domain after 120 minutes, polyubiquitinated products were observed within the first 5–10 minutes of the assay with RB1. Similarly, the MID1 RB1B2 protein construct exhibited comparably rapid ligase activity as the RB1 domain construct. Within experimental error, it was difficult to determine whether there was greater or lesser amount of polyubiquitinated products. Therefore, it was difficult to identify the contribution of the B-box2 domain within the RB1B2 construct.

To probe whether the B-box2 domain contributes to ligase activity as part of the RB1B2 construct, a C142S mutation was introduced within the B-box1 domain (RB1*B2). Cysteine-142 coordinates one of the two zinc ions, and its mutations to serine resulted in the loss of coordination of both zinc ions and unfolding of the B-box1 domain [4]. By Western blot, the E3 ligase activity of the RB1*B2 protein construct was indistinguishable from the RB1B2 construct, indicating that the B-box2 domain can compensate for the loss of function of the B-box1 domain. To confirm that the B-box2 domain has the same enhancing role as the B-box1 domain, an RB1* protein construct was designed, and the activity was observed to be similar to that of just the RING domain [64].

These observations indicate that the B-box domains, by some unknown mechanism, appear to enhance the E3 ligase functionality of the adjacent RING domain. It is wholly possible that the enhancement observed could be that both RING and the B-box domains have gained E3 ligase activities, there is some synergy in activities, or that the B-box domains may function as E4 ligases [77–80]. E4 ligases are domains with a RING fold that enhance the ligase activity of RING E3 ligases. Possible examples of E4-enhancing ligases are the BARD1 and HDMX RING domains. In the mid-2000s, the U-box domain, which adopts a similar ββα-RING fold but without the coordination of zinc ions ([81]), was initially shown to play an E4-enhancing role for RING E3 domains [82]. Subsequently, it was concluded that U-box domains can function as E3 ligases and now represent a new member of the RING-type E3 ligases with a similar mechanism of action as RING domains [61, 83, 84].

The function of the B-box domains of TRIM5α, TRIM25, and TRIM32 is also studied [85, 86]. TRIM5α possesses anti-viral/anti-HIV activities [56, 87]. TRIM25 plays a crucial anti-viral role by ubiquitinating the N-terminal caspase activation and recruitment domains (CARDs) of the recognition receptor RIG-I [86, 88]. Mutations of TRIM32 are associated with limb-girdle muscular dystrophy type 2H. TRIM25 consists of RING, B-box1, and B-box2, while TRIM5α and TRIM32 have the RING and B-box2 domains. TRIM25 possesses both Ub and interferon-stimulated gene 15 (ISG15)-E3 ligase activities [86, 88]. ISGylation serves more of a signaling role, as ISG15-modified proteins have altered functions [89]. For these proteins, the B-box domains are required for enhanced activities. Using thiolysis assays (nucleophilic attack on the thioester of charged E2~Ub by lysine), the role of the B-box domains was assessed for TRIM25 and 32 [86]. The constructs with the RING domain alone marginally activated the reaction, but those including the B-box domains significantly accelerated thiolysis.

In the case of TRIM5α, autoubiquitination assays with monomeric RING alone did not produce polyubiquitinated products/chains. In contrast, the RB2 and RB2CC protein constructs showed considerable increases in activities, which were

attributed to the presence of the B-box domain. As control, the RB2*CC protein construct with a destabilizing B-box2 mutant resulted similar levels of ubiquitination products as just with the RING domain [36].

In summary, the results from the RB1, RB1B2, RB1*B2, and RB1* autoubiquitination assays of MID1/TRIM18, TRIM25, and TRIM32 suggest that TRIM proteins with RING and two B-box domains have some redundancies in the enhancement role of the B-box domains. Furthermore, given that the RING-less TRIM16 tandem B-box possesses strong ligase activity adds to the support that the B-box domains can possibly have dual roles, functioning as E3 and E4 ligases.

8. The B-box domains are required for substrate polyubiquitination

While the *in vitro* assays confirmed E3 ligase activities of B-box domains and their possible role in E3 ligase enhancement, it is important to resolve whether observations of autoubiquitination activities translate to substrate ubiquitination. In the case of MID1, there are three known substrates: the catalytic subunit of protein phosphatase 2A (PP2Ac) [14, 90], alpha4 [76], and the fused kinase (FK) [91]. PP2Ac is part of a heterotrimeric PP2A complex consisting of the scaffolding subunit PP2Aa (PR65) and one of several regulatory subunits (PP2Ab) that defines cellular location and substrate specificity. PP2A functions as a master switch to control metabolism (review [92]), cell cycle progression (via cdc2 kinase activation), DNA replication, transcription and translation, cell proliferation, cytoskeleton dynamics and cell mobility, and apoptosis [93–100]. PP2A is considered a tumor suppressor, deactivating oncogenic MEK1 and ERK within the RAS-RAF-MEK-ERK/MAP kinase cascade [92, 96, 101]. The alpha4 protein [102–106] regulates PP2A within the target of rapamycin (TOR) signaling pathway that controls transcription, protein synthesis, and cell cycle progression in response to nutrients and extracellular stimuli [104, 105, 107–112]. Alpha4 binds the PP2Ac and induces a conformational change that keeps PP2Ac in an inactive conformation until needed [14, 113, 114]. The fused kinase is a key regulator within the Sonic Hedgehog pathway important for cell polarization and body symmetry [115]. Specifically, FK is shown to activate Hh- and Ci-dependent transcriptional activation in *Drosophila* Schneider 2 cells.

For all three substrates, full-length MID1 was shown to catalyze their polyubiquitination [14, 76, 91]. The role of the B-box domains for substrate ubiquitination was demonstrated for PP2Ac and alpha4 [14, 76]. With just the RING domain, a weak band was observed on the Western blot, indicating low amount of monoubiquitinated products [14, 76]. In contrast, polyubiquitinated products were observed with the RB1 and RB1B2 protein constructs. The results of the assays with the RB1* (C141S) protein construct yielded monoubiquitinated PP2Ac and alpha4 products, confirming the B-box1 domain is important for substrate targeting and polyubiquitination. The RB1*B2 protein construct catalyzed the polyubiquitination of PP2Ac but not alpha4. These results indicate a few things: the B-box binding of protein substrates is a critical role for polyubiquitination, the B-box2 domain can compensate for the unfolded B-box1 domain, and the B-box2 domain can contribute to some B-box1 redundancies in MID1's overall E3 ligase activity. The levels of ubiquitination of PP2Ac and alpha4 parallel the results observed with the autoubiquitination assays, confirming the various roles of the B-box domains. Similar results were observed with TRIM5α TRIM25, TRIM19/PML, and TRIM63.

TRIM5α targets the HIV capsid protein. Deletion of the B-box2 domain affected oligomerization state, E3 ligase activity, and substrate ubiquitination [35, 37, 56].

TRIM25 targets RIG-I. *In vivo* studies with HEK293T cells were performed with wild-type TRIM25 and with the B-box domains deleted individually and as a pair. Protein constructs consisting of either B-box domain yielded polyubiquitinated RIG-I products, supporting key observations with the MID1 protein [88]. TRIM19/PML is a tumor suppressor protein that is associated with a wide variety of cancers. TRIM19 is shown to function as a small ubiquitin-like modifier (SUMO) protein E3 ligase targeting MDM2, which functions as a regulator of protein p53. Sumoylation affects protein stability and cellular localization and sometimes serves as precursor to prime the protein substrate for ubiquitination. The results of *in vivo* and *in vitro* sumoylation assays revealed that the B-box domains are required for MDM2 sumoylation. Similarly, mutations destabilizing of the B-box2 domain of TRIM28/KAP-1 completely eliminated KRAB domain binding, demonstrating that the B-box2 domain is important for binding and targeting of the KRAB [116]. TRIM63/MuRF1 is found in striated muscle and observed to be upregulated with muscle atrophy. The muscle-type creatine kinase (M-CK) is one substrate of TRIM63 and co-precipitated with TRIM63 only with an intact B-box2 domain [117]. Ubiquitination of M-CK required the B-box2 domain. Furthermore, the B-box2 domain also contributed to the overall oligomeric TRIM63 structure [118]. It is unclear whether B-box2 domain dimerization or oligomerization is a universal observation or protein specific. In the case of MID1, there is no evidence that the B-box2 domain is dimer (unpublished data).

Finally and importantly, studies with TRIM27/rfp revealed a central role of the B-box domain in substrate binding and ubiquitination and subsequent degradation [75]. This report was the first demonstration that E3 ligase activity of TRIM27, a TRIM protein with a RING domain, is conferred to the B-box domain instead [75]. This unique finding has not, to our knowledge, been observed with any other TRIM proteins containing both RING and B-box domains.

9. MID1 B-box1 domain E3 ligase activity can be enhanced

As noted, we postulate that RING E3 ligases that exhibit different levels of *in vitro* activities might be due to evolutionary determinants that include subtle differences in amino acids and structures between the RING E3 ligases (**Figures 3** and **4**). This idea is supported with the MID1 B-box1 domain in which an XLOS-specific mutation, P151L, yielded a B-box1 domain with greater E3 ligase activity [119]. Furthermore, the $RB1^{+}$(P151L) protein construct possesses greater activity than the wild-type RB1 protein construct. NMR data revealed that the B-box1 P151L mutant is folded. This proline is conserved among many TRIM B-box1 domains, and it may be a key determinant for the weaker E3 ligase activity observed. Residue P151 is located at the end of the helix and beginning of loop2. Residues of loop2 participate in E2-RING interactions, specifically allowing an arginine or lysine residue to hydrogen bond with E2-Gln92, necessary for activation of the thioester bond in the case of Ube2D1. For the MID1 B-box1 domain, the rigidity of the backbone property of proline-151 positions loop2 differently, which prevents key loop2-E2 interactions (**Figure 4A**) [119]. The P151L mutation is most likely repositioned loop2 for more favorable interactions with the E2 (Ube2D1) enzyme used in the assay. In the case of TRIM16, there is no proline in the corresponding location, and this maybe the reason why it exhibits greater E3 ligase activity than the MID1 B-box1 domain. The lower activity of the B-box2 domain cannot be explained because there is no proline residue to reorient loop2. However, compared to the RING consensus sequence,

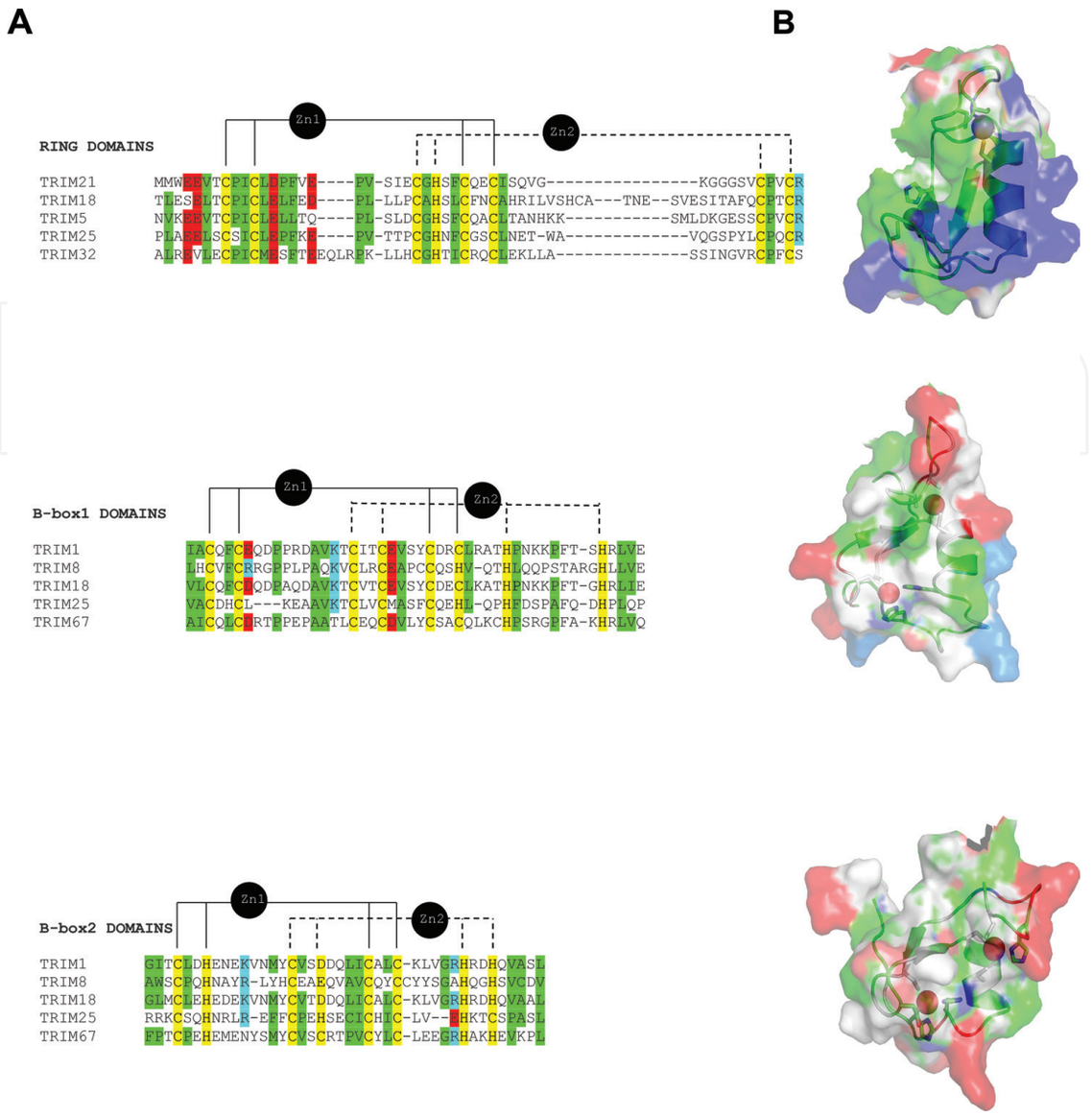

Figure 3.
RING and B-box domain comparison. A. Sequence alignments of five representative RING domains and five B-box1 and B-box2 domains from TRIM proteins. The amino acids involved the two zinc ions are identified in yellow and lines above connecting the corresponding residues consistent with the cross-brace mechanism. Some more conserved hydrophobic residues are colored green, while the acidic and basic residues are colored red and cyan. B. Surface representation of HDM2 RING and the MID1 B-box1 and B-box2 domains displaying the surface in same relative orientation for which the E2 enzyme interacts. Hydrophobic (green), basic (K, R, blue), acid (E, D, red), and uncharged polar (white) regions are shown. The pattern of amino acid type distribution is similar for many of the RING and solved B-box1 and B-box2 structures.

loop2 of MID1 B-box2 and several B-box2 domains are significantly shorter, and this could limit optimal interactions with the E2 enzyme.

Intriguingly, even though the P151L mutant B-box1 possesses greater activity, substrate ubiquitination assays revealed that the mutation disrupts binding and targeting of the alpha4 protein. This observation strongly supports our hypothesis that RING E3 ligase activities may be a compromise between level of activity and substrate binding, as defined evolutionarily. In unpublished work, we have identified several specific amino acids in RING domains that are important for RING–E2 interaction but that are not present in B-box1 domains. Introduction of these amino acids into the MID1 B-box1 domain resulted in significant increases in auto-ubiquitination activity, including polyubiquitination (unpublished).

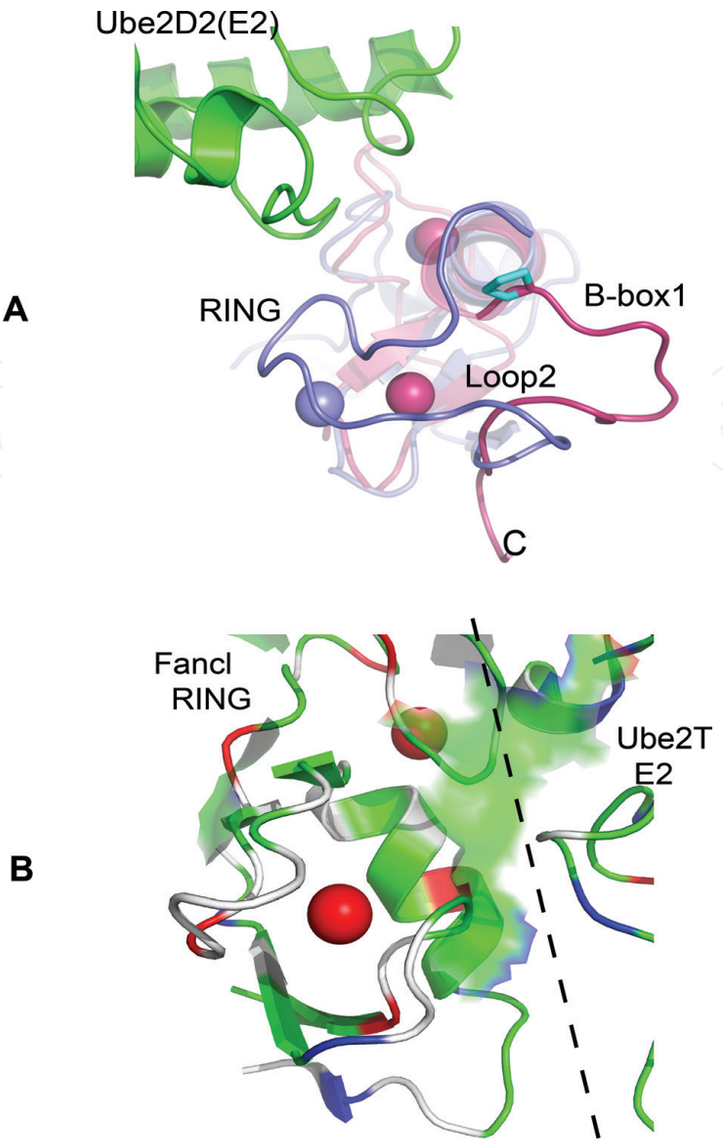

Figure 4.
*E2-E3 interactions. A. Ribbon representation of the UbcH5/Ube2D1(green)-cCbl RING (purple) complex with the structure of the B-box1 domain (red) superimposed onto the RING domain. In this orientation, loop2 is positioned away from the E2-RING binding interface. Residues of RING loop2 make important E2-binding interaction; similar interactions are not observed with the B-box1 domain. B. Close-up to the E2-binding surface of the fancl RING domain; the dashed line separates the surface of the RING and E2 Ube2T (most of which is not shown for clarity). Consistent with several E2-RING complexes, the E2 enzymes bind on a fairly large hydrophobic surface on one side of the RING domain (***Figure 1***) with specific electrostatic and hydrogen bonding interactions. Green = hydrophobic residues, blue = basic, red = electrostatic, white = uncharged polar, and red-sphere = zinc ions.*

10. Structural comparison of RING and B-box domains

In light of our findings that the E3 ligase activity of the MID1 B-box1 domain can be enhanced, we examine the structures of RING and B-box domains to understand if there may be additional features that can rationalize the difference in activities of MID1 RING and B-box domains. While it is not feasible to provide detailed analyses of all the differences between the various RING and B-box domain structures, we make general qualitative comparisons. As noted, the overall structures of RING and B-box domains are similar (**Figure 1**). In the case of the MID1 B-box domain, the position and size of loop2 may contribute to their decreased E3 ligase activities (**Figure 4A**). The sequences of a few RING and B-box domain are aligned, and distributions of amino acid types on the E2-binding surface on the RING and B-box domains are depicted for comparison (**Figures 3** and **4**). There are some key differences in amino

acids between the RING and B-box domains that may also contribute to differences in the level of activity. However, those will not be discussed in detail here. The exact mechanism of E2-Bbox binding has not been characterized, and therefore, for the following discussion, we make the assumption that the B-box domains interact with the E2 enzyme in a similar manner as RING domains. The different types of residues (hydrophobic [green], acidic [red], basic [blue], and uncharged polar [gray]) are displayed. We used the HDM2 and human Fanconi anemia (fancl) RING domains [31, 120]. On the E2-binding surfaces, both RING domains (**Figures 3** and **4**) show predominantly hydrophobic residues. With the structure of the HDM2 RING domain (PDB 2hdp), there is also a large adjacent basic patch on the outer surface of the helix, but its role in E2 binding is not clear. There is also a basic residue on loop2 and a small acidic patch toward the top of the structure, and these participate in Ube2D2 E2 binding. For the fancl RING domain, the large hydrophobic patch is located in same region, but there are no large charged surfaces. Evaluation of fancl-RING-Ube2T complex (PDB 4ccg) reveals that the E2 enzyme interacts predominantly with the hydrophobic region of the RING domain (**Figure 4B**) [120]. Structures of other RING-E2 complexes reveal similar types of interactions.

In contrast, the hydrophobic patches on the MID1 B-box1 and B-box2 structures are smaller and not as contiguous as those observed with the HDM2 and Fancl RING structures (**Figure 3**). There are more charged residues on the surface. The PML/TRIM19 B-box1 domain (PDB 2mvw) [121] has more polar residues distributed instead of hydrophobic residues. Two smaller hydrophobic patches are observed on opposite sides. The distribution of residues for the TRIM5α B-box2 domain (PDB 2ecv) is very similar to that of the MID1 B-box1 structure. These comparisons reveal that there are differences in amino acid types at the canonical E2-binding site that might influence the mechanism of interactions between RING and B-box domains with their cognate E2 enzymes and hence the level of activity.

Interestingly, the structure of fancl RING domain with Ube2T E2 reveals that Ube2T does not have a corresponding Gln92 residue to form a hydrogen bond with a basic residue on loop2 of the RING domain, which is present is several RING and U-box domains. Instead, the complementary positions consist of hydrophobic residues, suggesting that allosteric effects of RING binding might be transmitted via hydrophobic interactions. In contrast, the HDM2 RING domain has an arginine that can form a hydrogen bond with Gln92 of the Ube2D2/UbcH5 E2 enzyme. This interaction is important for allosteric effects to influence cleavage of the thioester bond. It is possible that these subtle differences in binding mechanisms might provide a rationale for differences in the level of E3 ligase activities observed for RING-type E3 ligases. Differences in activity may also be due to mismatch in cognate E2-RING partners with *in vitro* ubiquitination assays. For example, some RING and B-box E3 ligases may possess sumoylation and ISGylation activities and, therefore, prefer different E2 conjugating enzymes.

11. B-box2 domain may additionally possess a regulatory role

In addition to the above noted roles of the B-box domains, it has been suggested that the presence of MID1 B-box2 domain impacts the binding efficiency of the B-box1 domain to alpha4. Binding studies with the MID1 and alpha4 proteins revealed tightest binding with a RB1 construct and reduction in binding with RB1B2 and larger MID1 constructs [90]. The apparent reduction in RB1-alpha4 binding may due to the B-box2 domain binding in an overlapping site with alpha4 [122]. Interestingly, we saw a reduction of the band intensities of the polyubiquitinated products of both auto- and alpha4-ubiquitinations with RB1B2 [76], indicating that B-box2 domain is

regulating the alpha4 interaction. On the other hand, it is possible that the decrease is due to the two B-box domains affecting the RB1B2 interaction with the E2 enzyme.

Another TRIM protein for which the B-box2 domain has been ascribed a possible regulatory function is TRIM21, which is involved in immune signaling and is found in almost all cell types and tissues in mammals. In contrast to MID1, the TRIM21 RING domain exhibited greater ligase activity than the RB2 protein construct [123]. The result is confirmed by the E2~Ub thiolysis assays: the rate of Ub discharge was greater with the RING than with the RB2 domain construct. NMR experiments confirmed that the RING and B-box2 domains interact via the surface important for self-oligomerization [124].

12. The role of B-box domains in RINGless-TRIM proteins

There are currently six characterized human TRIM proteins that lack the N-terminal RING domain: TRIM14, TRIM16 (EBBP), TRIM20 (PYRIN/MEFV), TRIM29 (ATDC), TRIM44 (DIPB), and TRIM66. RINGless TRIM proteins are found in *Drosophila melanogaster (*Brat, Wech/Dappled*)* and *C. elegans* (LIN-41), but the functions of these proteins are not characterized. A detailed list of all current TRIM, RINGless TRIM, and BBX proteins are shown in **Figure 5**.

TRIM16 is a transcriptional regulator involved in regulating neuroblastoma cell growth, migration, and tumorigenicity [74]. Apparently, it can function as E3 ligase via both homodimerization and heterodimerization with TRIM18, TRIM19, and TRIM24 [125]. The tandem B-box domains are capable of very weak homodimerizing interactions in the absence of the coiled-coil domain. *In vitro* ubiquitination assay with TRIM16 consisting of domain deletions confirmed that the B-box domains confer E3 ligase activity [74]. TRIM20 is involved in innate immune response and is associated with the autoinflammatory disorder familial Mediterranean fever (FMF), characterized by pyogenic arthritis and pyoderma gangrenosum [126]. For TRIM20,

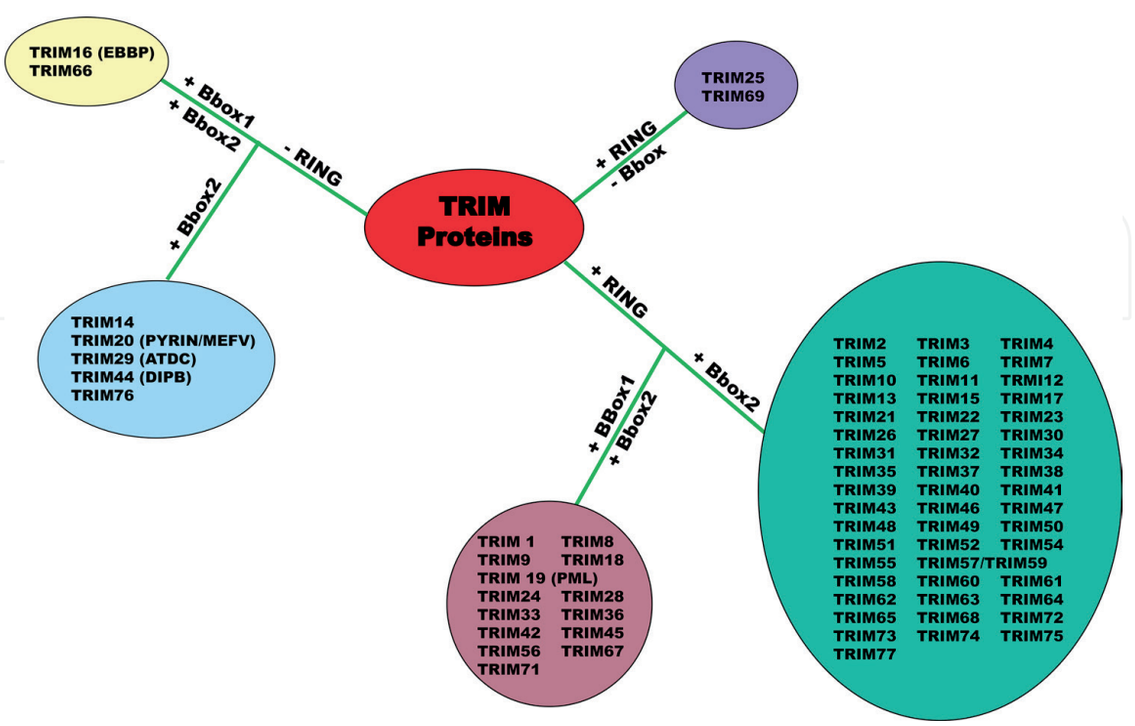

Figure 5.
B-box containing TRIM proteins. Protein family distribution of all currently known TRIM and RINGless TRIM proteins grouped by the presence of the B-box1 and B-box2 domains. Pseudo-TRIM16 and 66 lack a RING domain, and pseudo-TRIM 25 and 69 lack the B-box domains.

a pyrin domain (PYD) that belongs to the death domain family associated with apoptosis and inflammatory responses is in place of the RING domain. TRIM20 targets the proline/serine/threonine phosphatase-interacting protein 1 (PSTPIP1) by interactions via the B-box domain. The binding causes the B-box domain to unmask the PYD domain, allowing it to interact with downstream binding partners that are important for regulating inflammation. Deletion of the B-box domain resulted in constitutively active PYD and TRIM20 [127].

Very little is known about the other RINGless-TRIM proteins (**Figures 5** and **6**). TRIM29 is an oncogene that regulates p53 and is overexpressed in many different cancers including breast, lung, bladder, and pancreatic [128, 129]. It can form both homodimers and heterodimers with TRIM1/MID2, TRIM11, TRIM23, and TRIM27 [125]. Despite the lack of the RING domain, *in vivo* studies showed that TRIM27 maintained E3 ligase activities in response to viral and bacterial infections [130]. E3 ligase activity of TRIM29 was confirmed through its single B-box2 domain [131]. TRIM44 is involved in antiviral and immune response regulation, with some functions linking it to cancer. It can form a heterodimer with TRIM8, TRIM17, TRIM27, and TRIM69. Instead of a RING domain, TRIM44 contains a zinc-finger ubiquitin protease domain (UBP) that functions as a deubiquitinase [125]. Interestingly, TRIM44 is the only known deubiquitinase among the TRIM protein family [132, 133]. TRIM14 is involved in antiviral innate immune response [134, 135]. TRIM66 is correlated with the proliferation, invasion, and migration of non-small cell lung cancer [136]. TRIM66 possesses a PHD zinc-finger domain that preceded the B-box domains. The PHD domain is shown to coordinate two zinc ions and adopts RING fold, but its function as an E4 ligase is not clear.

While the mechanism of action is not clear, it is possible that RINGless-TRIM proteins function through homo- or heterodimerization via at least one of its B-box domains. Self-association/oligomerization through the B-box domains is shown to contribute to E3 ligase activity possible via an apparent localized concentration effect.

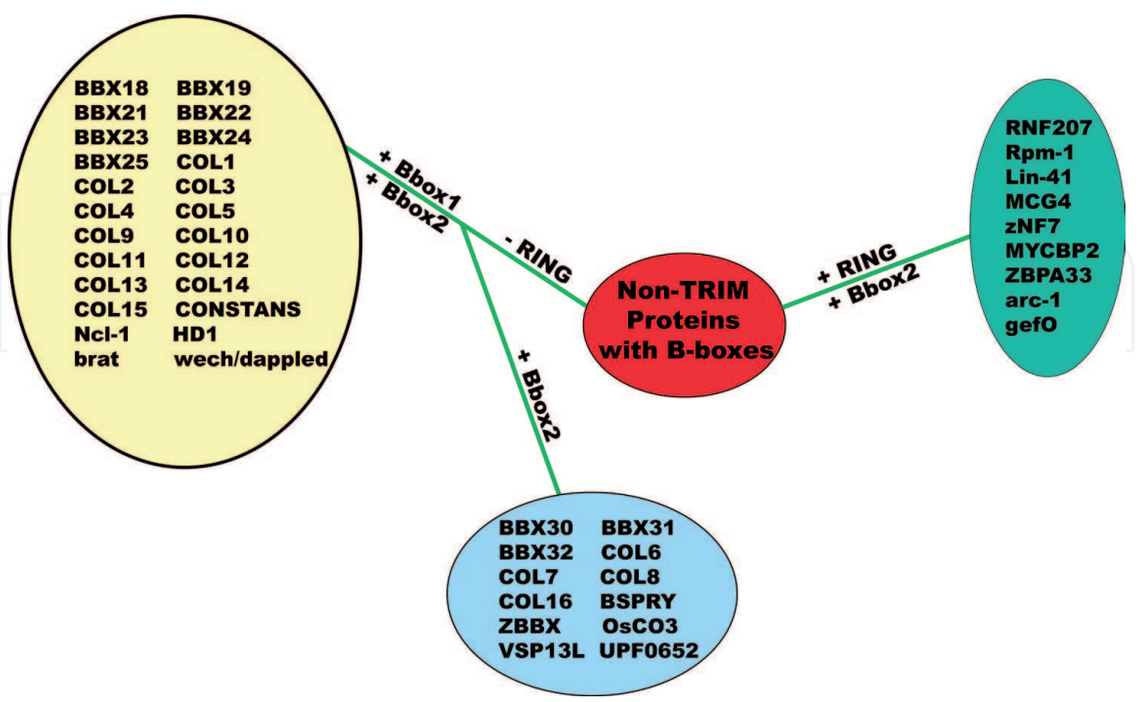

Figure 6.
B-box containing non-TRIM proteins. Protein family distribution of all currently known non-TRIM proteins arranged by the presence of the B-box1 and B-box2 domains. These proteins are generally termed BBX (B-box) and COL (Constans-like) and are found in plants. There are nine non-BBX non-TRIM proteins that contain RING and B-box domains but lack the coiled-coil domain required for TRIM definition; these are not found in plants.

13. B-box domains are found in plants

While the majority of B-box domains are found in mammals, recent publications have identified 32 B-box proteins (known as BBX proteins) in *Arabidopsis*, rice, and more than a dozen other plants species [137]. These non-TRIM proteins do not possess a RING domain, and the B-box domains are usually found at the N-terminus (**Figure 6**). The conservation of BBX proteins in multiple plant species suggests that these proteins play important roles in plant physiology similar to TRIM proteins in mammals [138]. The parallels of TRIM and BBX proteins regarding the B-box domains conferring E3 ligase activity suggest conservation of function across multiple kingdoms. Like TRIM proteins, there is a mixture of BBX proteins that consists of either a single or tandem B-box domains. Of the 32 BBX proteins, 21 of them have tandem B-box domains, in the same order observed in RING-less TRIM proteins. In rice (*Oryza sativa*), more than half of its 30 known BBX proteins contain two B-box domains [139]. The BBX proteins do not contain a coiled-coil domain but shown to interact with proteins that contain a coiled-coil domain to create the TRIM equivalent of a RBCC motif (see review [140]). Studies of BBX proteins containing tandem B-box domains revealed that only one of the B-box domains is essential for maintaining biological activity in plants, supporting redundancy observed with several TRIM proteins [141].

Defining specific functions to the B-box domains of BBX proteins is lacking, probably because of their recent realization in plants. Without specifics, a large number of these BBX proteins are postulated to be involved in the ubiquitination pathway. Several BBX proteins are shown to interact with an *Arabidopsis* RING E3 ligase called *co*nstitutive *p*hotomorphogenic-1 (COP1) through interactions with the B-box domain, during the dark cycle of plants (see review [140]), but it is unclear whether this association is as an E4 ligase to COP1, as a substrate or something else. Similar to TRIM B-box domains, the BBX B-box domains play important roles in protein-protein interactions, whether directly or indirectly [140]. They can form heterodimers and facilitate transcriptional regulation. For example, BBX21, BBX22, BBX24, and BBX25 interact with a COP1-associated protein called HY5. The BBX21 and BBX22 activated HY5 [142], while BBX24 and BBX25 repressed its activity [143, 144].

14. Summary

While it is clear that there is still much to be learned about TRIM proteins and their E3 ligase activity, there has been a great push toward better understanding of the role of the B-box domains over the past decade. Although the RING domain has received much of the spotlight, it is now clear that the B-box domains are integral for substrate binding/targeting, protein ubiquitination, and enhancement or activation of the ligase activity of TRIM and BBX protein families. B-box domains have diverse roles that include protein-protein interactions, substrate ubiquitination and sumoylation, and transcriptional regulation. What is not clear is whether B-box domain really served as E4-enhancing ligase to enhance the ligase activity of the RING domains, as observed in dimer RING E3 ligases, or whether they synergistically gain activity alongside the RING domains. Their role in contributing to oligomerization for some TRIM and BBX proteins to account for enhanced E3 ligase activity may be due to an apparent increase in the localized concentration of the TRIM or BBX protein. However, more studies are needed. Progress will require multifaceted approaches involving structure determination, protein-protein binding studies, and functional assays. Nonetheless, we hope that sufficient evidence have been provided, including those of TRIM18/MID1 and TRIM27, demonstrating

that B-box domains are and should be considered E3 ligases and not as a supporting player to RING domains.

Acknowledgements

The author thanks Dr. Katharine Wright for her work in the lab and for contributing ideas for this review, and Ms. Jessica Webb for her help and contributions with the figures. This work was supported in part by a National Science Foundation grant (MAM, 1808391).

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