RESEARCH LETTER

TRAF trimers form immune signalling networks *via* RING domain dimerization

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(Received 11 August 2022, revised 5 October 2022, accepted 7 October 2022, available online 25 November 2022)

doi:10.1002/1873-3468.14530

Edited by Kazuhiro Iwai

For many inflammatory cytokines, the response elicited is dependent on the recruitment of the tumour necrosis factor receptor-associated factor (TRAF) family of adaptor proteins. All TRAF proteins have a trimeric C-terminal TRAF domain, while at the N-terminus most TRAFs have a RING domain that forms dimers. The symmetry mismatch of the N- and C-terminal halves of TRAF proteins means that when receptors cluster, it is presumed that RING dimers connect TRAF trimers to form a network. Here, using purified TRAF6 proteins, we provide direct evidence in support of this model, and we show that TRAF6 trimers bind Lys63-linked ubiquitin chains to promote their processive assembly. This study provides critical evidence in support of TRAF trimers as key players in signalling.

Keywords: E3 ligase; immune signalling; oligomerisation; signalling networks; ubiquitin

Tumour necrosis factor (TNF) receptor-associated factors (TRAFs) are a family of proteins that play a crucial role in immune signalling [1,2]. TRAFs have been reported to associate with several receptor families such as the TNF superfamily, Toll-like receptor (TLR), RIG-I like receptors (RLR's), NOD-like receptor (NLR) and cytokine receptors to regulate signalling [1]. Assembly of scaffolding ubiquitin chains is a common feature of these pathways, and TRAFs are widely thought to play a role in regulating their formation [3,4]. Given the importance of TRAFs in immune signalling, it is not surprising that disruption of TRAF function has been linked to the development of diseases, including cancer and inflammatory disorders [2,5,6]. For example, overexpression of TRAF6 is associated with tumour formation and poor outcomes for gastric cancer and glioblastoma patients [7,8], while inhibition of TRAF6 was recently shown to offer potential in the treatment of colorectal cancer [9]. In mice, deletion of TRAF3 results in death soon after birth [10]. Meanwhile, because TRAF3 normally helps restrain B-cell receptor signalling [11], its loss from B-cells leads to the development of lymphoma [12].

All TRAF proteins are characterised by the presence of both TRAF-C and coiled-coil (CC) domains at their C-termini (Fig. 1A). These domains self-associate to form stable trimers [13–15]. The trimeric TRAF-C domain interacts with the cytoplasmic domain of several immune receptors and is important for receptor-mediated signalling [16]. At their N-termini, most TRAFs have a RING domain and several Zn-finger (ZF) domains (Fig. 1A). The RING domains can dimerise, but the dimer is not highly stable, and structures of the TRAF RING domain in its monomeric

Abbreviations
CC, coiled coil; FADD, FAS-associated death domain; GST, glutathione-*S*-transferase; NLR, NOD-like receptor; RING, really interesting new gene; RLR, RIG-I like receptors; TIRAP, TIR domain-containing protein; TLR, Toll-like receptor; TNF, tumour necrosis factor; TRADD, TNF receptor-associated death domain; TRAF, TNF receptor-associated factor; TRIM, tripartite motif; ZF, Zn-finger.

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Fig. 1. TRAFs and networks. (A) Schematic showing the domain organisation of TRAFs. The TRAF6 RING homodimer is shown on the left (PDB: 5VO0). One protomer in the dimer is shown as a surface, while the other is shown as a ribbon. The Zn ions are shown as red spheres. On the right, the TRAF3 TRAF-C and coiled-coil homotrimer (PDB: 1RF3) is shown. One protomer, at the rear, is shown as a surface, while two protomers in the trimer are shown as ribbons. The interacting receptor peptides are highlighted in blue. RING, really interesting new gene; ZFs, Zn-finger domains; CC, coiled coil domain; TRAF-C, tumour necrosis factor receptor-associated factor C-terminal domain. (B) A model suggesting how TRAF RING dimers may act as bridges to stabilise TRAF networks. (C) Schematic representation of the TRAF6 constructs used in this study with the residue boundaries indicated.

and dimeric forms have been reported [17,18]. RING domains are commonly associated with ubiquitin transfer, and it is generally assumed that TRAF proteins are E3 ubiquitin ligases, although this has only been well established for TRAF6 [18,19].

Assembly of ubiquitin chains relies on a cascade of proteins that activate and conjugate ubiquitin to an E2-ubiquitin-conjugating enzyme. Once loaded onto the E2, it is the E2~Ub conjugate that interacts with RING E3s, before ubiquitin is transferred to a substrate [20]. A number of RING E3 ligases form dimers and this regulates ubiquitin ligase activity because the E2~Ub conjugate must interact with both protomers in the dimer to be activated. This is also true for TRAF6, where the RING domain of one protomer interacts with the E2 of the conjugate, while ubiquitin interacts with ZF1 from the other protomer [19,21]. Thus, RING dimerization regulates ubiquitin transfer by TRAF6 *in vitro*, and it is assumed that it has the potential to regulate the E3 ligase activity of

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TRAF6 in cells. When activated, TRAF6 assembles Lys63-linked ubiquitin chains that are thought to help stabilise the formation of immune signalling platforms [22]. The importance of the interaction between TRAF6 and Ubc13, an E2 that is dedicated to the assembly of Lys63 chains, is emphasised by several recent studies that have shown that when Ubc13 levels are diminished due to suppressed expression [23], deamidation [24] or sequestration [25], TRAF6-dependent processes are disrupted. Despite their importance for sustained signalling, the mechanism by which Lys63 chains are assembled by TRAF6 remains poorly understood.

Changes in cell response are often triggered when ligands bind to cell surface receptors [26,27]. Ligands are often multi-valent, and binding provokes oligomerisation of the receptor, followed by receptor clustering [28]. The intracellular domains of the clustered receptors then recruit adaptors proteins, such as the TRAFs, TRADD, FADD or TIRAP [29,30]. It is thought that the initial interactions are transient, but as the signal is sustained, the receptor complex is stabilised by additional interactions resulting in the formation of a stable signalling platform [26,28]. In the case of TRAFs, it is assumed that they are recruited to trivalent receptors either by direct interaction of the trimeric TRAF-C domain with trimeric receptors, or *via* other adaptors, such as TRADD. Then, upon a sustained signal, receptor clustering brings several TRAF trimers into close proximity that can be bridged by RING dimerisation [31]. In this way, RING dimers can connect the trimeric TRAF networks to form a stable signalling platform (Fig. 1B).

To better understand how TRAF signalling complexes form, using purified recombinant TRAF6 proteins (Fig. 1C), we show that RING dimerisation underpins the formation of higher-order TRAF6 oligomers. We also show that TRAF6 trimers enhance the E3 ligase activity so that the processive assembly of long Lys63-linked ubiquitin chains is favoured. Together, this study suggests a mechanism by which receptor activation can enhance ubiquitin chain assembly, and influence cellular signalling.

Materials and methods

Plasmids and cloning

Homo sapiens TRAF6 RING-ZnF-Coiled-Coil (TRAF6 RZCC; residues 50–328) was cloned into an N-terminal glutathione-*S*-transferase (GST)-His_6 tag expression vector of the NKI LIC Suite [32], while TRAF6 RING-ZnF_{1-3} (TRAF6 RZ3; residues 50–211) was cloned into pGEX-6P3 vector with an N-terminal GST tag [19]. Following removal of the GST tag, the proteins retained five residues from the linker.

Purification of proteins

All proteins were expressed in *Escherichia coli* BL21 or BL21-star (DE3) cells (Novagen, Sigma-Aldrich, St. Louis, MO, USA). E1, Ubc13, Uev1a, wild-type ubiquitin, ubiquitin variants, fluorescently labelled ubiquitin and Ubc13~Ub^{K63R} thioester conjugate were purified as described previously [19]. The TRAF6 RZ3 protein was also purified as described in Middleton et al. [19]. Cultures of *E. coli* cells transformed with the TRAF6 RZ3 construct were grown to $O.D._{600}$ ~ 0.6, then 0.2 mM IPTG and 0.1 mM $ZnCl_2$ were added before incubation overnight at 18 °C. Following cell lysis, the clarified lysate was subjected to affinity, ion-exchange and size-exclusion chromatography. The TRAF6 RZCC protein was expressed with an N-terminal GST-His_6 tag as for the RZ3 protein. Cells were lysed using an emulsiflex in buffer containing 50 mM Tris–HCl (pH 8.5) and 300 mM NaCl, and the clarified lysate was bound to glutathione sepharose resin (Cytiva, Uppsala, Sweden) at 4 °C for an hour with constant rotation. The GST-His_6 tag was cleaved by overnight incubation with 3C protease. Following cleavage, the RZCC protein was diluted 10-fold in 50 mM Tris pH 8.5 to reduce the salt concentration. The protein was then subjected to ion-exchange chromatography using a 1 mL MonoQ column (Cytiva), and eluted using a linear gradient of 0–1 M NaCl. The recovered protein was then exchanged into 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl. The RING dimer interface mutant (F118A) forms of both RZ3 and RZCC proteins were purified in a similar manner. The proteins used for SEC-MALS experiments were further purified by size-exclusion chromatography using a Superose 6 10/300 GL Increase column (Cytiva) for wild-type RZCC and a HiLoad Superdex 200 column (Cytiva) for the dimer interface mutant (F118A) pre-equilibrated with 20 mM Tris–HCl pH 7.5, 150 mM NaCl.

Preparation of poly-ubiquitin chains

Lys63-linked polyubiquitin chains were formed following incubation of 0.1 μM of E1, 6 μM of Ubc13 and Uev1a, and 50 μM of wild-type ubiquitin at 37 °C for 60 min in a buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM DTT, 2 mM ATP, 5 mM $MgCl_2$ and 0.1 M NaCl. The reaction was stopped by the addition of 1 mM EDTA and then diluted five-fold in 50 mM ammonium acetate (pH 4.5) and 1 mM EDTA. Ubiquitin chains were separated using a MonoS column (Cytiva) over a 100 CV linear 1 M NaCl gradient. Following SDS/PAGE analysis of the fractions, the fractions were pooled and further purified by size-exclusion chromatography in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl. Di- and tri-polyubiquitin chains were

purified on a HiLoad Superdex 75 (Cytiva) column, while tetra- to hepta-polyubiquitin chains were purified using a HiLoad Superdex 200 (Cytiva) columns. Purified chains were concentrated to ~ 2 mg·mL^{-1} for assays. Lys48-linked chains were prepared in a similar manner except Ubc13 was replaced with 6 µM Ube2K and the E3, RNF125 was included. Tetra Met1-linked chains were expressed without a tag and purified using ion exchange as described above.

Analytical SEC and SEC-MALS

The elution profile of wild-type and mutant TRAF6 RZCC proteins was evaluated by injecting 100 µL each of the purified proteins (at 50 µM) over a Superose 6 10/300 GL Increase column (Cytiva), pre-equilibrated in 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, at a flow rate of 0.5 mL·min^{-1}. The recovered fractions were analysed by SDS/PAGE.

For determination of the oligomeric status of the wild-type and F118A mutant TRAF6 RZCC proteins, multiangle light scattering (MALS; Dawn 8+; Wyatt Technology, Sanat Barbara, CA, USA) coupled to a Superose 6 10/300 GL Increase column (Cytiva) was used. One hundred microlitre each of the purified proteins (at 70 µM) was loaded onto the column pre-equilibrated in 20 mM Tris–HCl (pH 7.5), 150 mM NaCl and 2 mM DTT. Data were analysed using ASTRA version 5.3.4 software (Wyatt Technology).

Cross-linking

The carboxyl-/amine-crosslinking was performed using EDC (22980; Thermo Fisher Scientific, Waltham, MA, USA), a zero-length cross-linker that can crosslink acidic and basic residues that are in close proximity. The crosslinking reaction was set up by mixing 35 µM of protein (TRAF6 RZCC WT or F118A) with 6250 µM of EDC at a ratio of 1 : 180 (protein : EDC) in the crosslinking buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl and 2 mM DTT. The proteins were allowed to react at room temperature, and the samples were collected after 3 and 18 h. The crosslinked reaction products were mixed with the sample buffer containing reducing agent and resolved on 12% Bis-Tris (BOLT) gels (Thermo Fisher).

Ubiquitylation assays

Multi-turnover and discharge assays were performed as described previously [19,33]. Briefly, for the multi-turnover assays, 0.1 µM of E1, 6 µM of Ubc13 and Uev1a, 50 µM of wild-type ubiquitin and 4 µM of purified TRAF proteins were mixed with buffer containing 20 mM Tris–HCl (pH 7.5), 2 mM DTT, 2 mM ATP, 5 mM $MgCl_2$ and 0.1 M NaCl, and incubated at 37 °C for the indicated time points. The reactions were spiked with 10% fluorescently labelled wild-type ubiquitin.

For the discharge assay, 10 µM of purified Ubc13~Ub^{K63R} thioester conjugate, 15 µM Uev1A, 50 µM ubiquitin D77 (a ubiquitin variant that has an additional Asp at its C-terminus that prevents charging by the E1) and 4 µM TRAF proteins were mixed and incubated at 20 °C for the indicated time points. The reactions were stopped using a non-reducing SDS/PAGE loading dye and resolved using SDS/PAGE. The gels were imaged using IMAGE STUDIO LITE (LI-COR Biosciences, Lincoln, NE, USA).

For the single-turnover assays, Ubc13~Ub^{K0} thioester conjugates were used. In the K0-ubiquitin variant, all the Lys residues have been mutated so that it cannot form chains. Similar to discharge assays, 10 µM of purified Ubc13~Ub^{K0} thioester conjugate, 15 µM Uev1A and 4 µM TRAF proteins were mixed with 50 µM polyubiquitin chains of variable lengths (di-, tetra- and hepta-). The reaction was incubated at 20 °C for the indicated time points and the reactions were stopped using a reducing SDS/PAGE loading dye. The gels were resolved and imaged as mentioned above.

For quantification of activity, Cy3-labelled ubiquitin was used in assays and the intensity of fluorescence from the Cy3 labelled bands was measured using the IMAGE STUDIO LITE software from LI-COR. The intensity values (obtained were plotted using PRISM (GraphPad Software, San Diego, CA, USA).

GST pulldown assays

To assess the binding of TRAF6 RZ3 and RZCC proteins to ubiquitin chains, GST-fused TRAF6 proteins were incubated with Lys63-linked or Lys48-linked polyubiquitin chains or tetra Met1-linked chains in buffer containing: 20 mM Tris–HCl (pH 8.5), 150 mM NaCl, 0.2% TWEEN 20 and 2 mM DTT, at 4 °C for 1 h. Subsequently, the resin was washed three times with the same buffer before being mixed with SDS-loading dye and separated using 10–20% gradient SDS/PAGE gels. Following transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA; #1620112) using the wet transfer method, the membrane was blocked with 5% skimmed milk for 1 h at room temperature. Ubiquitin chains were detected using an anti-Ubiquitin antibody (Santa Cruz Biotechnology, Dallas, TX, USA; #sc-8017) overnight at 4 °C. The membrane was washed, developed using HRP-conjugated secondary antibody (Abcam, Cambridge, UK; #ab6728) and visualised using IMAGE STUDIO LITE (LI-COR Biosciences).

Results

RING dimers promote the formation of a higher-order network

To investigate the higher-order association of TRAFs, as well as the E3 ligase activity of the RING domains,

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we utilised two different TRAF6 proteins (Fig. 1C). We predicted that one variant could form trimers as it included the RING domain, all five Zn-Fingers and the coiled-coil domain (referred to as RZCC). The second protein included just the RING domain and the first three Zn-Finger domains (referred to as RZ3). We have previously shown that RZ3 forms RING domain-mediated dimers at high concentration and that it efficiently assembles ubiquitin chains [19]. We also prepared mutant forms of both proteins in which Phe118 at the RING dimer interface was mutated to Ala (F118A) (Fig. S1a). This mutation is known to prevent RING dimerisation [18,19]. All proteins were purified to homogeneity (Fig. S1b).

Initially, we characterised the oligomeric state of the two RZCC proteins. Analysis by analytical size-exclusion chromatography (SEC) showed that while the RZCC-F118A protein ($RZCC^{F118A}$) eluted as a sharp peak, consistent with the formation of discrete species of uniform mass, the wild-type protein ($RZCC^{WT}$) eluted as a broad peak, with the majority eluting much earlier than the mutant form (Fig. 2A). Because the ability of the RING domains to dimerise differs between the two proteins, this suggested that RING dimerisation promoted self-association of TRAF6 RZCC. Trimeric RZCC TRAF6 has a calculated mass of 90 kDa, and to determine if trimers or higher molecular weight complexes formed, we analysed 70 μM samples of both proteins using SEC-MALS. As expected, the mass of $RZCC^{F118A}$ was consistent with the formation of a trimer (Fig. S1c), whereas $RZCC^{WT}$ formed a high molecular weight complex, with the mass suggesting it was in equilibrium between a monomeric trimer and a dimeric trimer. Next, self-association was analysed using native PAGE. Although both proteins migrated anomalously, presumably because the native charge-to-mass ratio is preserved, for $RZCC^{WT}$ we observed a high molecular weight smear, with the intensity of the smear increasing as the protein concentration increased from 0.5 to 4 μM (Fig. 2B; left panel). In contrast, for $RZCC^{F118A}$ no smearing was observed, even at 4 μM (Fig. 2B; right panel). This suggested that the trimeric RZCC was self-associated at higher concentrations, but this relied on the integrity of the RING dimer interface. The importance of RING dimers for the association of the

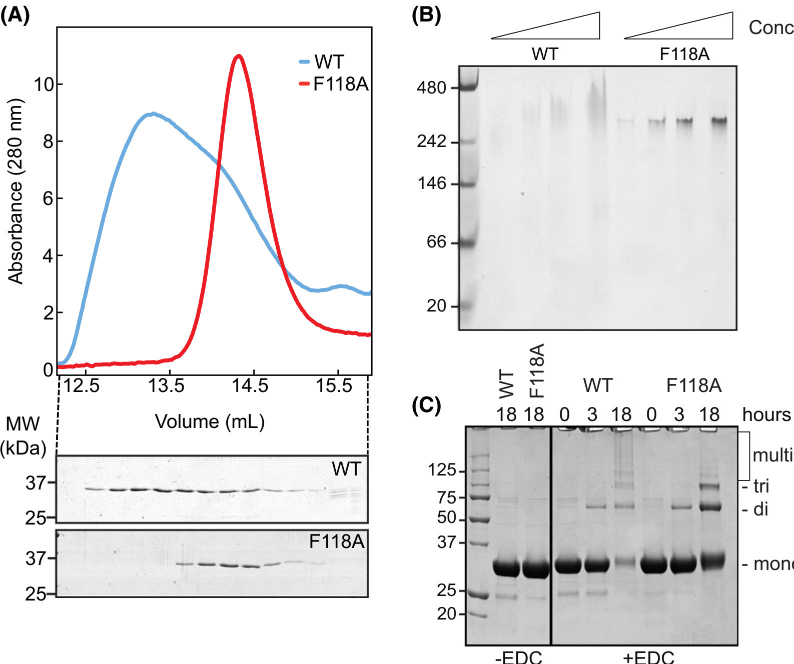

Fig. 2. The TRAF RING dimer stabilises higher order networks. (A) SEC analysis comparing elution profiles of wild-type and the RING dimer mutant (F118A) of TRAF6 RZCC. The same fractions for both proteins were resolved by 16% SDS/PAGE gels (bottom panel). (B) Native PAGE gel showing oligomerisation of TRAF6 wild-type and dimer interface mutant (F118A) RZCC proteins. The concentration of protein loaded was 0.5, 1.0, 2.0 and 4.0 μM. (C) Analysis of WT and F118A RZCC following cross-linking with EDC. Samples were mixed with SDS/PAGE loading buffer and separated on a 12% Bis Tris (BOLT) gel. All gels were stained with Coomassie Blue and the molecular weight standards are indicated.

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RZCC proteins was also apparent in cross-linking experiments (Fig. 2C). When the TRAF6 RZCC proteins were incubated with EDC, a zero-length crosslinker that links acidic and basic residues which are in very close proximity, extensive crosslinking was observed for $RZCC^{WT}$, but not for $RZCC^{F118A}$. Notably, after 18 h, little monomeric $RZCC^{WT}$ remained, with most protein converted to high molecular weight complexes, whereas the crosslinking was much less efficient for $RZCC^{F118A}$, and dimers and trimers dominated.

Together these experiments support a model whereby TRAF6 trimers can form an extended higher-order network that relies on RING dimerisation.

The E3 ligase activity of trimeric TRAF6 is increased

RING dimerisation is required for ubiquitin transfer by TRAF6 and we next compared the activity of trimeric RZCC and RZ3. Initially, we assessed activity using discharge assays that measure the ability of the TRAF6 RING dimer to stabilise the activated conformation of the Ubc13~Ub conjugate and promote the formation of di-ubiquitin. In this assay, the activity of trimeric RZCC was increased compared to RZ3 (Fig. 3A,B; Fig. S2a), presumably because RING dimerisation was favoured in the trimeric protein. Indeed when activity was assessed using chain building assays, trimeric TRAF6 not only had an increased ability to promote ubiquitin transfer, but the formation of high molecular weight chains was favoured for $RZCC^{WT}$ (Fig. 3C,D; Fig. S2b). As expected, trimeric TRAF6 did not overcome the deleterious effect of disrupting the RING dimer, with the activity of both F118A mutants similar to that observed when no E3 was added. However, the increased formation of extended chains by trimeric $RZCC^{WT}$, even in the presence of a considerable excess of free ubiquitin, suggested that TRAF6 preferentially transferred ubiquitin to ubiquitin polymers.

Trimeric TRAF6 binds and preferentially modifies Lys63-linked ubiquitin chains

Previously the coiled-coil domain of TRAF6 has been reported to increase ubiquitin transfer [34]. Xia and coworkers suggested that this was because the coiled-coil domain interacted with the Ubc13~Ub conjugate. While recruitment of the conjugate would be expected to increase activity and may contribute to the increased activity of the RZCC protein compared to RZ3 (Fig. 3C,D), it is unclear how this interaction would preferentially promote the formation of long ubiquitin chains. In general, the processive assembly of ubiquitin chains is thought to be favoured by interaction of the E3 with the substrate [35], with this aspect being most well understood for the anaphase-promoting complex (APC/C) [36,37]. In the case of TRAF6, we hypothesised that Lys63-linked ubiquitin chains might interact with the RZCC protein. To evaluate this, we prepared Lys63-linked ubiquitin chains and assessed the ability of the GST-fused RZCC and RZ3 forms of TRAF6 to bind them using a pulldown assay. Consistent with its ability to efficiently assemble long chains, TRAF6 RZCC pulled down ubiquitin chains, especially high molecular weight species. In contrast, RZ3 had a limited ability to pulldown the chains (Fig. 4A; Fig. S2c). The interaction between TRAF6 and ubiquitin chains appears to be specific for Lys63 chains, as no interaction with Lys48 or linear Met1-linked chains was detected (Fig. 4B).

To directly determine if RZCC had an enhanced ability to extend long Lys63-linked ubiquitin chains compared to RZ3, we purified unlabelled Lys63-linked chains of defined lengths (Fig. S2d) and then used these as substrates in activity assays with TRAF6 proteins, Ubc13, Uev1A and labelled K0-ubiquitin. In this assay, activity was monitored by following the addition of a single ubiquitin molecule to the unlabelled ubiquitin chains (substrate) (Fig. 4C). Consistent with the binding data (Fig. 4A), RZ3 had a comparable ability to add ubiquitin to all substrates, irrespective of length (Fig. 4D,E; Fig. S2e). In contrast, not only did RZCC have an increased ability to modify chains of all lengths, but activity was enhanced for longer chains (Fig. 4F,G; Fig. S2f). The preferential extension of long chains by the RZCC protein was most obvious in assays that included both unlabelled short (di-ubiquitin) and long (hepta-ubiquitin) chains (Fig. 4F; Fig. S2f). Notably, while RZ3 modified the two substrates to a similar extent, modification of Ub_7 by RZCC was considerably increased compared to Ub_2. Together, these data show that the trimeric form of TRAF6 binds and preferentially extends ubiquitin chains.

Discussion

TRAF proteins are well established as adaptor proteins that regulate multiple immune signalling pathways. For TRAFs, the interaction of the TRAF-C domain with the cytoplasmic domains of trimeric receptors is viewed as a classical nucleation event, whereas RING dimerisation is considered to be the stabilising interaction. Despite this widely held view of

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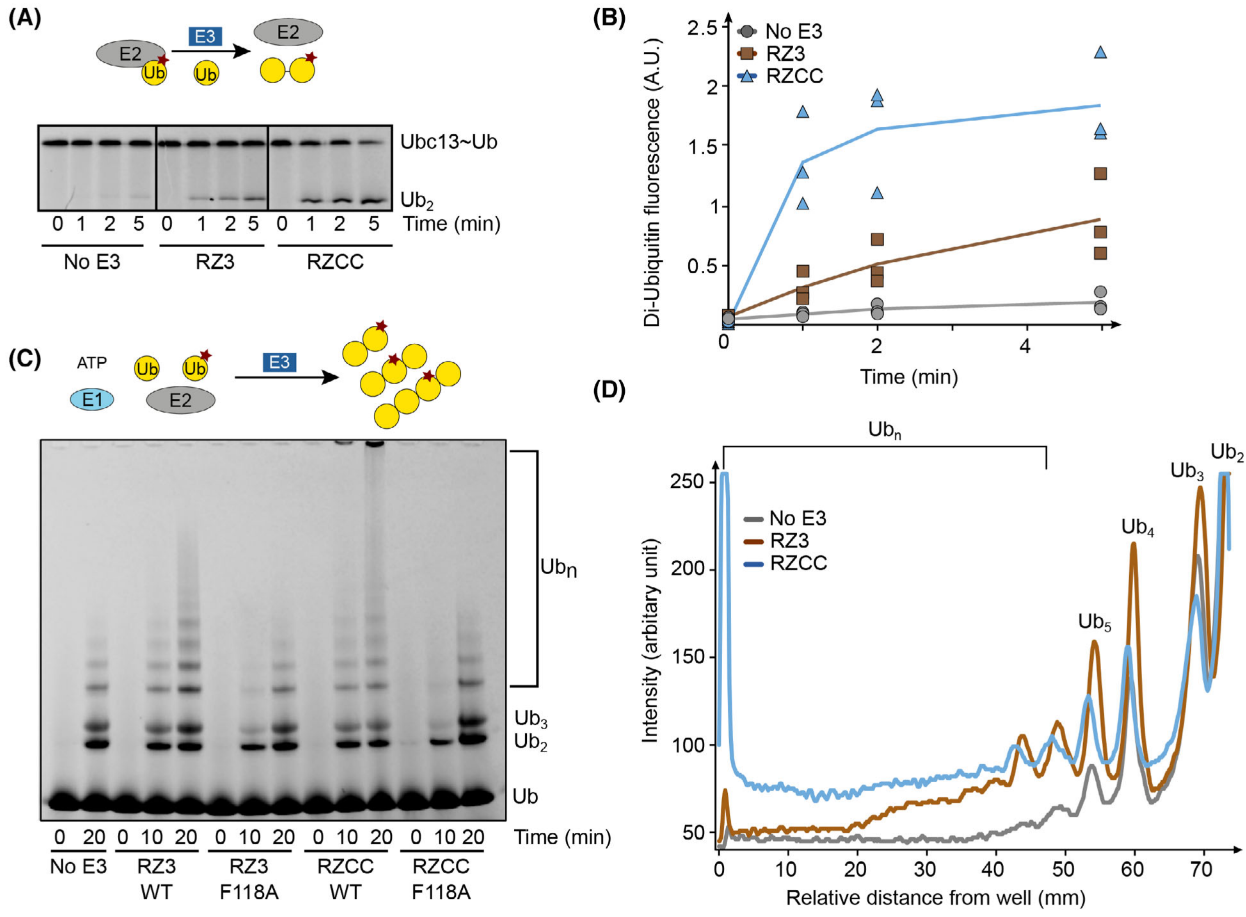

Fig. 3. The E3 ligase activity of dimeric and trimeric TRAF6. (A) Schematic showing discharge assay, labelled ubiquitin is indicated with a red star. Discharge assay comparing the ability of TRAF6 RZ3 and RZCC to promote the formation of di-ubiquitin at the indicated times. (B) Quantification of di-ubiquitin formation in panel (A). Experiments were performed in triplicate and all measurements are shown. (C) Schematic showing a multi-turnover assay, labelled ubiquitin is indicated with a red star. Multi-turnover assays comparing the ability of TRAF6 RZ3 and RZCC proteins to assemble ubiquitin chains. (D) To enable comparison of WT RZ3 and RZCC proteins a line scan plot showing the formation of chain at 20 min in panel (C) is shown. Note the higher molecular weight chains were not fully resolved from each other. In both discharge and multi-turnover assays ubiquitin was labelled with Cy3 and all gels were imaged and quantified using IMAGE STUDIO LITE (LI-COR Biosciences).

TRAF networks, which is based on the symmetry mismatch between their N- and C-terminal halves, there is little data to directly support this notion. Here we show, for the first time using purified recombinant proteins, that higher-order TRAF network formation is dependent on RING dimerisation (Fig. 2). Notably, when RING dimerisation was prevented by mutation of the RING dimer interface, trimeric TRAF6 did not associate to form higher-order complexes. This data is consistent with previous models that suggest activation of many signalling pathways relies on receptor clustering because they bring together multiple trimeric TRAFs [28]. It is presumed that the symmetry mismatch of the trimeric TRAF-C/coiled-coil domains and the dimeric RING domain is exacerbated in the clusters, favouring RING dimerisation between different trimers. As a result, RING dimerisation serves as a platform to link two trimeric TRAFs and stabilise cluster formation (Fig. 1B).

The synthesis of Lys63-linked ubiquitin chains by TRAF6 is widely recognised as having essential roles in the activation of numerous signalling pathways [38–40]. TRAF6 RING dimers promote the formation of Lys63-linked chains because stabilisation of the Ubc13~Ub conjugate in the active conformation requires interaction of Ubc13 with the RING domain of one protomer as well as contacts between the linked ubiquitin molecule and ZF1 of the other protomer [18,19]. However, activity is significantly enhanced by the presence of the coiled-coil domain [34], and here

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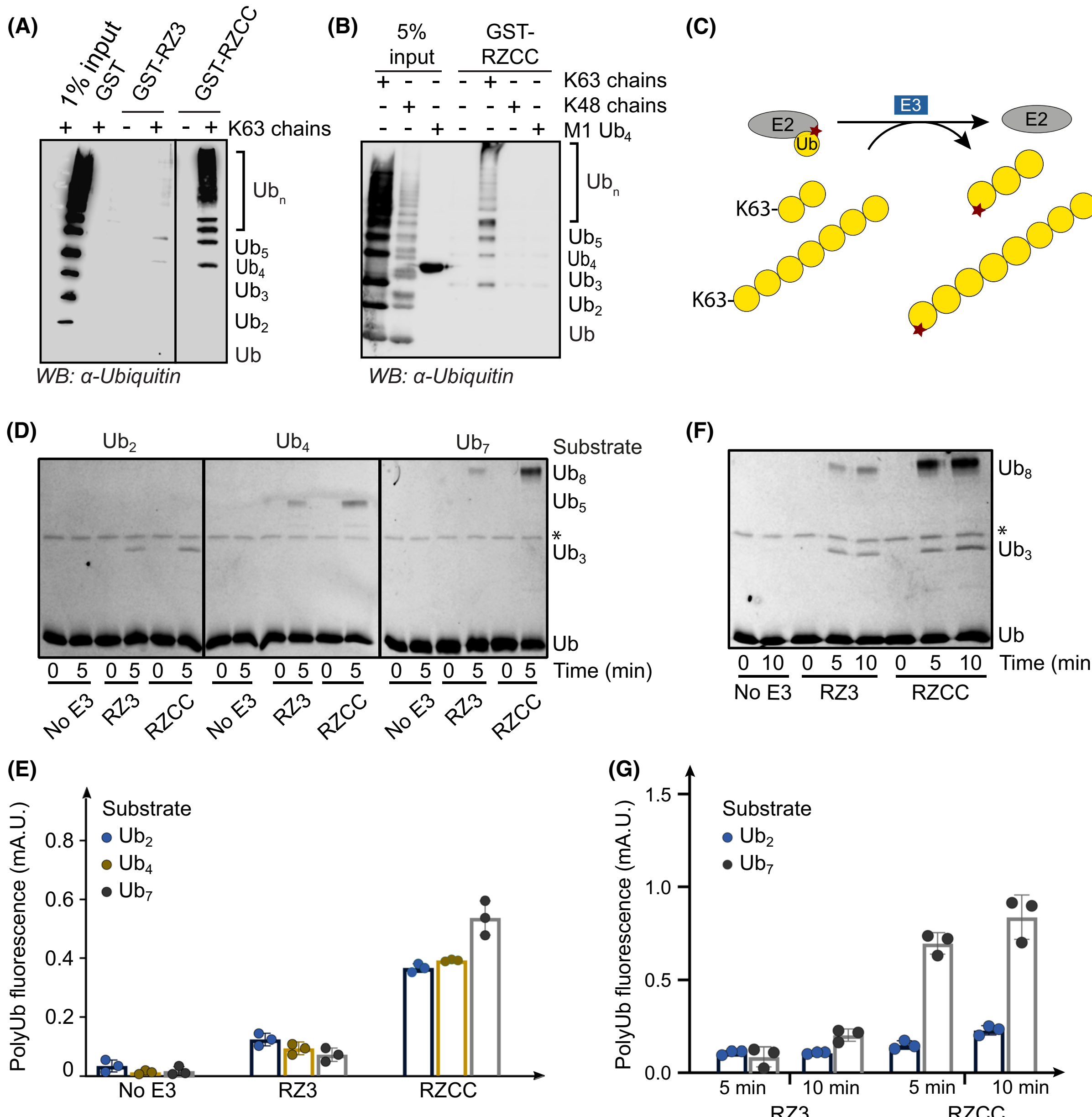

Fig. 4. Trimeric TRAF6 preferentially extends long ubiquitin chains. (A) The indicated GST-fusion proteins were mixed with Lys63-linked ubiquitin chains (K63 chains), washed and the recovered proteins were separated by SDS/PAGE. Ubiquitin chains were detected using an anti-Ubiquitin antibody. (B) The ability of GST-RZCC to bind ubiquitin chains with different linkages was assessed in a similar manner. (C) Schematic showing the assay system used to assess transfer of labelled ubiquitin to purified unlabelled ubiquitin chains of distinct lengths. Labelled ubiquitin is indicated with a red star. (D) Single-turnover assay using unlabelled Lys63-linked polyubiquitin chains (Ub_2, Ub_4 and Ub_7) as the substrate and Cy3-labelled K0-ubiquitin. The gel was imaged using IMAGE STUDIO LITE (LI-COR Biosciences). * indicates an unidentified band. (E) Quantification of the addition of K0-ubiquitin to the unlabelled chains is shown. Experiments were performed in triplicate and all measurements are shown. (F) A similar assay to that shown in panel (D) except an equimolar amount of di- (Ub_2) and hepta- (Ub_7) chains were included in each reaction. * indicates an unidentified band. (G) The addition of ubiquitin to di- and hepta-ubiquitin substrates at each time point is shown for both TRAF6 RZ3 and RZCC. Experiments were performed in triplicate and all measurements are shown.

we see both increased ubiquitin discharge and ubiquitin chain formation by trimeric TRAF6 (Fig. 3A,C). While it is anticipated that trimeric TRAF6 favours RING dimerisation and this would increase activity, the molecular basis for increased ubiquitin chain assembly by trimeric TRAF6 is not as well

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understood. Here, we suggest that the presence of several zinc fingers and the coiled-coil domains in trimeric TRAF6 promotes binding to Lys63-linked ubiquitin chains, especially to long poly-ubiquitin chains, and this promotes assembly of long chains. Some zinc finger domains are known to bind ubiquitin [41], and it is possible that multiple weak interactions occur between ubiquitin(s) and the zinc finger domains of TRAF6. These interactions are likely to be augmented when multiple trimers are present in the signalling complex, thus facilitating the processive assembly of ubiquitin chains by TRAF6 trimers. Thus, we propose that substrate recruitment by the trimeric TRAF6 proteins favours the extension of long ubiquitin chains. Further studies will be required, but it seems likely that both stabilisation of the RING dimer by trimeric TRAF6 and the presence of additional domains in TRAF6 could play important roles in enhancing activity.

The TRIM tripartite motif containing (TRIM) family of E3 ligases, which also build Lys63-linked ubiquitin chains and regulate immune signalling [42], rely on RING dimerisation for the assembly of ubiquitin chains [43]. Like TRAFs, TRIM proteins are thought to self-associate and form higher-order networks because, as well as RING dimerisation, the coiled-coil and B-Box domains trimerise. One of the best studied TRIM proteins is TRIM5, which binds to viral capsid proteins and synthesises Lys63-linked ubiquitin chains that activate immune signalling [44–47]. In TRIM5 the B-Box domains trimerise [48], while RING dimers are required for efficient ubiquitin chain assembly [47]. In the absence of a substrate, the RING domain of TRIM5 is monomeric and ubiquitin transfer is restricted. However, when TRIM5 binds to capsid proteins, a hexagonal lattice forms, with RING dimerisation critical for lattice formation [45,49]. Thus, substrate binding and high-order assembly of TRIM5 are coupled to the activation of ubiquitin transfer and downstream signalling [49]. As seen for TRIM5, our results suggest that the higher-order assembly of TRAF6 also regulates its E3 ligase activity. While several studies have suggested that for TRAF network formation (Fig. 5) the initiating event is recruitment to receptor complexes, this is something that needs to be investigated further. The extent to which networks form also remains uncertain, and it seems likely that at lower concentrations, the formation of intratrimer dimers (as illustrated in Fig. 1B, right) may limit network formation.

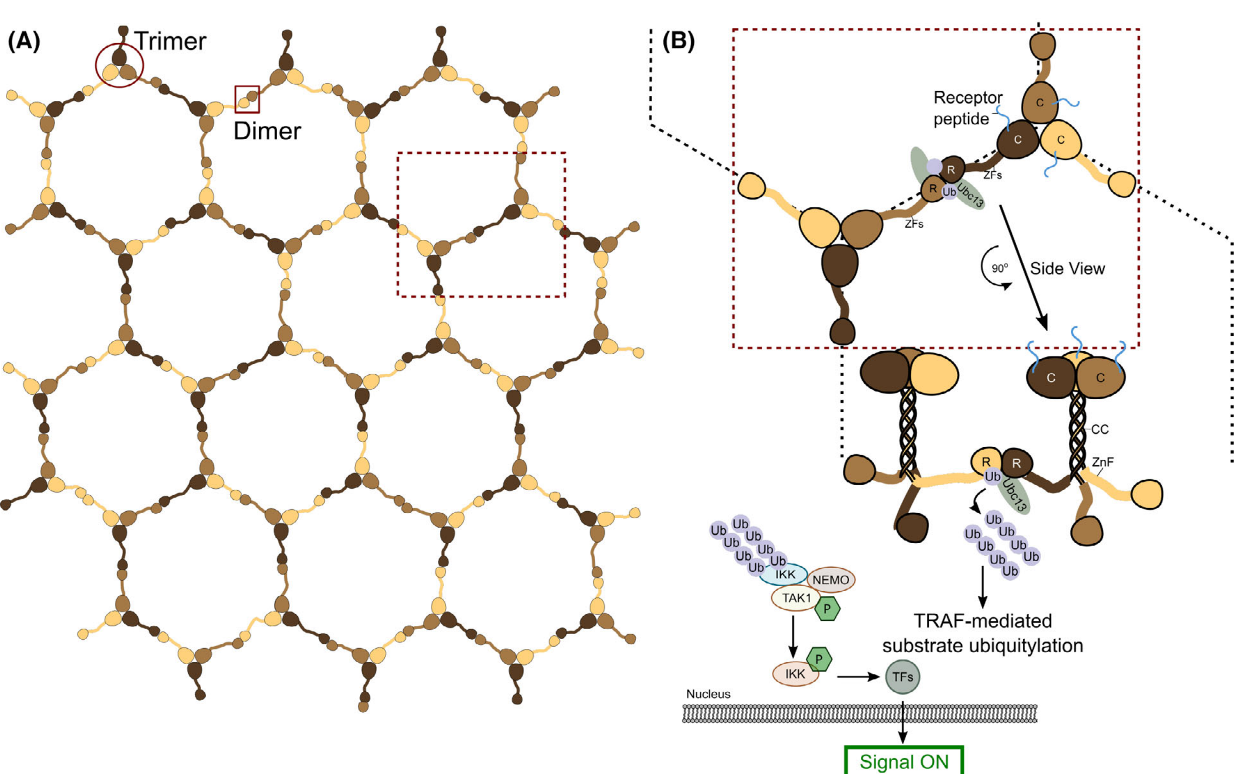

Fig. 5. Model of a TRAF network. (A) Potential TRAF honeycomb network formed by RING dimers. The trimers and dimers within the network are highlighted. (B) Close up of the network highlighting the site of polyubiquitin chain assembly by TRAFs. C, TRAF-C domain; R, RING domain; Ub, ubiquitin.

For TRAFs, it seems likely that more complex networks may also form because many signalling clusters contain several different TRAF proteins [50–52]. It is generally presumed that trimeric receptors recruit TRAF homotrimers and that multiple TRAFs are present because the binding sites on the receptors are promiscuous, with different TRAF homotrimers bound to receptor trimers [13]. However, TRAF1 and TRAF2 are both well-established members of the TNFR complex [53], and stable heterotrimers that include one TRAF1 molecule and two TRAF2 molecules form [54]. This raises the possibility that other TRAF heterotrimers form and are recruited to receptors. Adding further complexity, our recent studies have established that TRAF RING domains can form stable heterodimers, and at least some pairs that include one copy of TRAF6 are active E3 ligases [19,33]. Together these studies suggest that both homotypic and heterotypic interactions may facilitate the formation of TRAF complexes (Fig. 5A). Furthermore, it seems possible that heteromeric TRAF complexes could modulate downstream signalling by regulating the extent of network assembly and ubiquitin chain formation (Fig. 5B).

Many signalling pathways that depend on TRAFs are important therapeutic targets, with antibody-based activation of multiple members of the TNF superfamily a cornerstone of immune-oncology. Recent studies suggest there is potential to disrupt signalling by manipulating cluster formation by either targeting upstream events [55], or TRAF recruitment [56], highlighting the importance of deciphering how TRAF networks form, and are stabilised. While our study provides a framework for considering how these scaffolds regulate signal transduction, there is much that remains to be understood about how TRAF networks assemble and regulate cellular processes. In particular, it will be important to understand whether all TRAF proteins form networks and whether the profile of TRAF proteins recruited determines the extent to which clusters form.

Acknowledgements

The authors thank Adam Middleton and members of the Day laboratory for helpful discussions. The project was supported by a funding from the Health Research Council of New Zealand. Open access publishing facilitated by University of Otago, as part of the Wiley - University of Otago agreement via the Council of Australian University Librarians.

Author contributions

AD, MF, PP and JZ completed the assays and biochemical experiments. All authors analysed data. CLD supervised this study and wrote the manuscript together with AD.

Data accessibility

The data that support the findings in this study are available in figures or supplemental material associated with this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Recombinant TRAF6 proteins.

Fig. S2. Ubiquitiylation assays.

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