The TRAF6 RING finger domain mediates physical interaction with Ubc13

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Abstract Tumor necrosis factor receptor associated factor 6 (TRAF6) is an important signaling molecule involved in a diverse array of physiological processes. It has been proposed that TRAF6, a RING finger-containing protein, acts as a ubiquitin ligase (E3) and a target for Lys-63 linked polyubiquitination mediated by Ubc13–Uev, a ubiquitin conjugating (E2) complex. However, the physical interaction between TRAF6 and this E2 complex has not been reported. We used the yeast two-hybrid assay to demonstrate that TRAF6 indeed interacts with the E2 complex through its direct binding to Ubc13. Either a single Cys-to-Ser substitution within the TRAF6 RING finger domain or an amino acid substitution on the Ubc13 surface, that is predicted to interact with RING finger proteins, is able to abolish the interaction. In addition, we found that TRAF6 can interact with itself and this self-interaction domain is mapped to the N-terminus containing the RING finger motif. Based on this study and our previous Ubc13–Uev structural analysis, the interface of Ubc13-TRAF6 RING finger can be predicted.

Keywords: Ubiquitination; E2/Ubc; E3/Ubl; RING finger; Yeast two-hybrid assay; Protein–protein interaction

1. Introduction

Tumor necrosis factor receptor associated factor 6 (TRAF6) plays a crucial role in various cellular processes including adaptive and innate immunity, inflammation, bone metabolism as well as the development of several tissues including lymph nodes, mammary glands, skin and the central nervous system (for a recent review, see [1]). Transgenic mice defective in TRAF6 exhibit exencephaly and deregulated apoptosis within the developing central nervous system as well as severe osteopetrosis and have impaired osteoclast function [2,3]. It was recently found [4] that TRAF6 is a signal transducer in the NF-κB pathway and activates IκB kinase (IKK). This kinase is able to phosphorylate IκB and cause its rapid degradation by the ubiquitin (Ub)-proteasome pathway. TRAF6 is also required for TAK1-mediated phosphorylation of MKK6 in the JNK-p38 kinase pathway [5]. The above two TRAF6 activities require a heterodimeric Ub-conjugating enzyme (Ubc or E2) composed of Ubc13 and a Ubc variant (Uev), Uev1A or Mms2. This Ubc13–Uev complex is known to catalyze the synthesis of unique poly-Ub chains linked through Lys-63 [6], an activity that is required for both IKK and MKK6 phosphorylation by the TAK1 kinase complex [4,5]. TRAF6 contains five zinc finger repeats and a RING finger motif [7], suggesting that it may act as a Ub ligase (Ubl or E3). Interestingly, TRAF6 is also the target of Ubc13–Uev mediated Lys-63 ubiquitination [5]. If TRAF6 indeed functions as an E3, one would expect direct physical interaction between TRAF6 and its cognate E2. Despite repeated attempts, however, evidence for the physical interaction between TRAF6 and Ubc13–Uev has not been obtained through in vitro studies (J. Chen, personal communication and our own observations). To address whether TRAF6 interacts with Ubc13–Uev through a direct interaction with either Ubc13 or Uev, we performed the yeast two-hybrid assay and demonstrated a direct interaction between TRAF6 and Ubc13. Furthermore, deletion and site-directed mutagenesis mapped the self- and Ubc13-interaction region of TRAF6 to its N-terminus, which contains the RING finger motif.

2. Materials and methods

2.1. Yeast strains and cell culture

Saccharomyces cerevisiae haploid strains used in this study include Y190 (*MATa gal4 gal80 his3 trp1 ade2-101 ura3 leu2 URA3::*P_{GAL1}*-lacZ LYS2::*P_{GAL1}*-HIS3*, a gift from Dr. D. Gietz, University of Manitoba, Winnipeg, Canada) and PJ69-4a (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ* P_{GAL2}*-ADE2 LYS2::*P_{GAL1}*-HIS3 met2::*P_{GAL7}*-lacZ*, a gift from Dr. P. James, University of Wisconsin at Madison, USA). Yeast cells were cultured at 30 °C either in a complete YPD medium or in a synthetic dextrose (SD) medium supplemented with necessary nutrients as recommended [8]. To make plates, 2% agar was added to either YPD or SD medium prior to autoclaving.

Yeast cells were transformed using a dimethyl sulfoxide enhanced LiAc method as described [9].

2.2. Plasmids

The full-length mouse *TRAF6* (mTRAF6) cDNA was obtained from Dr. J. Chen (University of Texas Southwestern Medical Center, Dallas, USA) and the full-length human *TRAF6* (hTRAF6) cDNA was obtained from Dr. V.M. Dixit (Genentech, Inc., San Francisco, USA). Both *TRAF6* genes were cloned as *Eco*RI–*Sal*I fragments into pGBT9 and pGAD424 [10] to form pGBT-mTRAF6, pGAD-mTRAF6, pGBT-hTRAF6 and pGAD-hTRAF6. To determine domain(s) required for TRAF6 self-interaction and the interaction with

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Abbreviations: β-gal, β-galactosidase; IKK, IκB kinase; TRAF, tumor necrosis factor receptor associated factor; hTRAF6, human TRAF6; mTRAF6, mouse TRAF6; Ub, ubiquitin; Ubc or E2, ubiquitin conjugating enzyme; Ubl or E3, ubiquitin ligase; Uev, Ubc variant; 3-AT, 1,2,4-amino triazole

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Ubc13, N-terminal and C-terminal deletion constructs were made as follows. Plasmid pGAD-mTRAF6N containing coding sequences for the N-terminal 279 amino acids of mTRAF6 was made by *Hpa*I and *Bgl*II digestion of pGAD-mTRAF6. The *Bgl*II cohesive end was filled using the Klenow fragment of *Escherichia coli* DNA polymerase I and the resulting DNA was self-ligated. To create pGBT-mTRAF6C containing coding sequences for amino acids 293–530 of mTRAF6, pGBT-mTRAF6 was digested with *Eco*RI and *Sma*I. The *Eco*RI cohesive end was filled using Klenow and the resulting DNA was self-ligated in the presence of an 8-bp *Bgl*II linker to restore the reading frame of the fusion gene.

The human *UBC13* open reading frame was PCR amplified as an *Eco*RI–*Sal*I fragment and cloned into pGBT9 and pGAD424 to form pGBT-hUBC13 and pGAD-hUBC13, respectively. *hMMS2* and *UEV1A* cDNAs were isolated as previously reported [11]. Their open reading frames were PCR amplified as *Bam*HI fragments and cloned into pGAD424 in a correct orientation to form pGAD-hMMS2 and pGAD-UEV1A, respectively. The coding regions of *hMMS2* and *UEV1A* without stop codons were PCR amplified as *Bam*HI–*Sal*I fragments and cloned into pG4BD-1 (received from Dr. R.B. Brazas, University of California, San Francisco, USA) to form phMMS2-G4BD and pUEV1A-G4BD, respectively, as C-terminal fusions to $Gal4_{BD}$.

Yeast two-hybrid plasmids containing a site-specific mutation within *mTRAF6* that results in the C70S substitution and within *hUBC13* that results in the M64A substitution were constructed by a mega-primer approach [12].

2.3. Yeast two-hybrid analyses

The yeast two-hybrid strain Y190 was co-transformed with different combinations of pGBT and pGAD-based plasmids. The co-transformed colonies were initially selected on SD-Trp-Leu. For each transformation, at least five independent colonies were replica plated onto SD-Trp-Leu plates to assess β-galactosidase (β-gal) activity by a filter assay as previously described [13] and onto SD-Trp-Leu-His with various concentrations of 1,2,4-amino triazole (3-AT) to test the activation of the P_{GAL1}-*HIS3* gene.

The yeast two-hybrid strain PJ69-4a [14] was co-transformed with different combinations of $Gal4_{BD}$ and $Gal4_{AD}$ constructs. The co-transformed colonies were initially selected on SD-Trp-Leu. For each transformation, at least five independent colonies were replica plated onto SD-Trp-Leu-His+3-AT to test the activation of the P_{GAL1}-*HIS3* gene and onto SD-Leu-Trp-Ade plates to test the activation of P_{GAL2}-*ADE2*.

2.4. Quantitative β-gal assay

The β-gal assay was performed as previously described [15] to quantitatively measure the strength of yeast two-hybrid interactions. Briefly, 0.5 ml of overnight culture of Y190 double transformants was used to inoculate 2.5 ml of fresh selective medium. When the cell density reached an $OD_{600\,nm}$ of approximately 0.7–1.0, 1 ml of culture was used to determine absorbance (cell density) at $OD_{600\,nm}$. Cells from the other 2 ml of culture were collected by centrifugation and used for the β-gal assay. β-Gal activity was standardized by subtracting the β-gal activity found in the co-transformants containing vectors pGAD424 and pGBT9. β-Gal activity is expressed in Miller Units (MU) [16]. Results shown are the average of at least three independent experiments.

2.5. Western-blot analysis

Y190 cells transformed with two-hybrid constructs were grown in 10 ml SD-Trp or SD-Leu liquid media at 30 °C overnight, subcultured in the corresponding fresh medium and allowed to grow at 30 °C until a cell density of $OD_{600\,nm}$ of 0.8 was reached. Yeast cells were harvested by centrifugation at 3000 × *g* for 10 min at 4 °C. Yeast crude cell extracts were made with the YeastBuster protein extraction reagent (Novagen, Darmstadt, Germany) following the manufacturer's instructions. The yeast cell extract was run on a 10% SDS–PAGE gel and then transferred to a PVDF membrane (polyvinylidene difluoride, Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated in a blocking solution (PBS, 3% non-fat milk) at room temperature for 1 h. Anti-$Gal4_{BD}$ or anti-$Gal4_{AD}$ antibodies (Santa Cruz Biotechnology, CA, USA) were diluted 1:500 in 10 ml PBST (PBS, 0.05% Tween (v/v), 1% non-fat milk) and the PBST solution was incubated with the membranes at 4 °C overnight. The secondary antibody, anti-mouse IgG conjugated with HRP, was used at a 1:5000 dilution. The Western Lightning Chemiluminescence Reagent (Perkin–Elmer Life Science, Boston, MA, USA) was used as the substrate for detection and the membrane was exposed to X-ray film for visualization.

3. Results and discussion

Since biochemical and immunological methods were unable to detect physical interactions between TRAF6 and Ubc13–Uev, we suspected that the binding of these proteins may be transient or unstable during cell extract preparation. Hence, we used the yeast two-hybrid analysis to determine if they indeed interact.

Based on our previous Ubc13–Mms2 structural analysis [17], we predict that TRAF6 may interact with Ubc13, since Ubc13 is a true E2 and, after binding to Mms2, a surface predicted to interact with a RING finger protein is still available. Fig. 1A shows that mTRAF6 indeed interacts with Ubc13 based on a reconstituted transactivation of the P_{GAL1}-*lacZ* reporter in strain Y190. This interaction is dependent on the co-existence of Ubc13 and mTRAF6, as neither construct showed positive results when combining with the vector. The interaction is also independent of the fusion partners, as

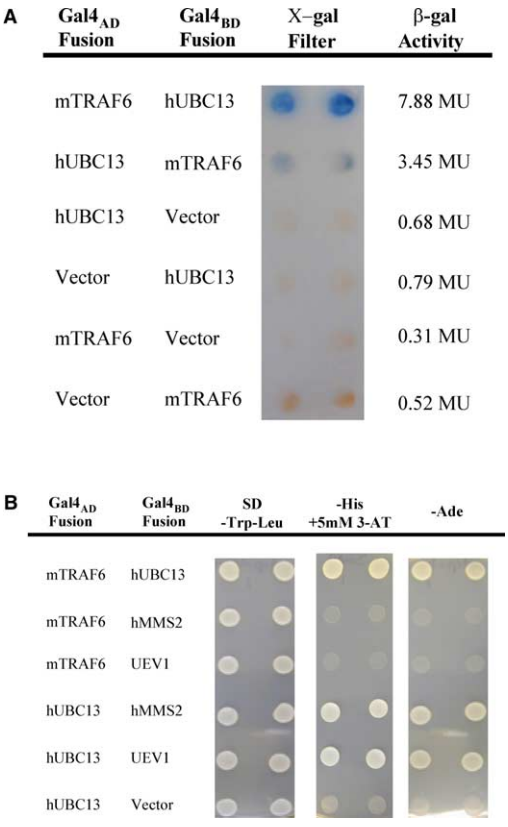

Fig. 1. Interaction of mTRAF6 with Ubc13 in yeast two-hybrid assays. A: mTRAF6-Ubc13 interaction is independent of their fusion partners. Y190 cells, co-transformed with $Gal4_{AD}$ and $Gal4_{BD}$ constructs, were tested by a filter assay (two independent colonies for each transformation) and a quantitative β-gal assay as expressed in MU. B: mTRAF6 interacts with Ubc13 but not with Uev1A or hMms2. PJ69-4a cells, co-transformed with $Gal4_{AD}$ and $Gal4_{BD}$ constructs, were replicated on SD-Trp-Leu-Ade and SD-Trp-Leu-His+5 mM 3-AT plates and the plates were incubated for three days at 30 °C.

mTRAF6 and *UBC13* cloned in both orientations are able to produce positive results. However, a stronger interaction appears to occur when Ubc13 is fused with $Gal4_{BD}$ and mTRAF6 is fused to $Gal4_{AD}$. Quantitative β-gal analysis further confirmed such an interaction. The P_{GAL2}-*ADE2* reporter gene in strain PJ69-4a was reported to be highly stringent [14]. We found that the mTRAF6–Ubc13 interaction is able to confer growth on SD-Ade as well as SD-His+5 mM 3-AT plates (Fig. 1B). Similar experiments also showed interaction between hTRAF6 and Ubc13 (data not shown). Nevertheless, one has to bear in mind that the strength of interaction between TRAF6 and Ubc13 is significantly less than that between hMms2 and Ubc13, as judged by a semi-quantitative X-gal filter assay (see Fig. 3C), as well as in vitro protein–protein interaction assays showing that while hMms2 and Ubc13 are able to form a stable complex under various conditions [18], we were unable to detect such a physical interaction using purified hUbc13 and mTRAF6 (data not shown).

The positive identification of a TRAF6–Ubc13 interaction does not rule out the possibility that another partner of the Ubc13–Uev complex is involved in direct contact with TRAF6, since Uev is a structural homolog [11] of Ubc13, can potentially bind the TRAF6 RING finger, and is absolutely required for TRAF6- and Ubc13-mediated ubiquitination [4,5]. It also does not rule out the possibility that the observed TRAF6–Ubc13 interaction in the yeast two-hybrid assay is mediated by a Uev in host cells, since Mms2 in yeast cells is able to form a complex with mammalian Ubc13 to allow it to functionally complement the yeast *ubc13* null mutant [19]. There are two human Uev homologs, hMms2 and Uev1, that share >90% amino acid sequence identity in their core domains and both are able to functionally complement the yeast *mms2* null mutant [11]. We found that while both Uev1 and hMms2 are able to form complexes with Ubc13, neither can directly interact with TRAF6 in the yeast two-hybrid assay (Fig. 1B). This result further supports the direct interaction between TRAF6 and Ubc13.

TRAF6 contains several predicted functional domains (Fig. 2A), including an N-terminal RING finger, five zinc-finger repeats embedded in a CART domain, a mushroom-like TRAF domain consisting of coiled-coil TRAF-N (“stalk”) and TRAF-C (“head”) [20]. In order to determine which domain(s) of TRAF6 is required for its interaction with Ubc13, we tested different mTRAF6 deletion constructs for their ability to interact with Ubc13 in the yeast two-hybrid assay. Deletion of the C-terminal half of TRAF6 containing TRAF domains does not affect interaction with Ubc13, whereas

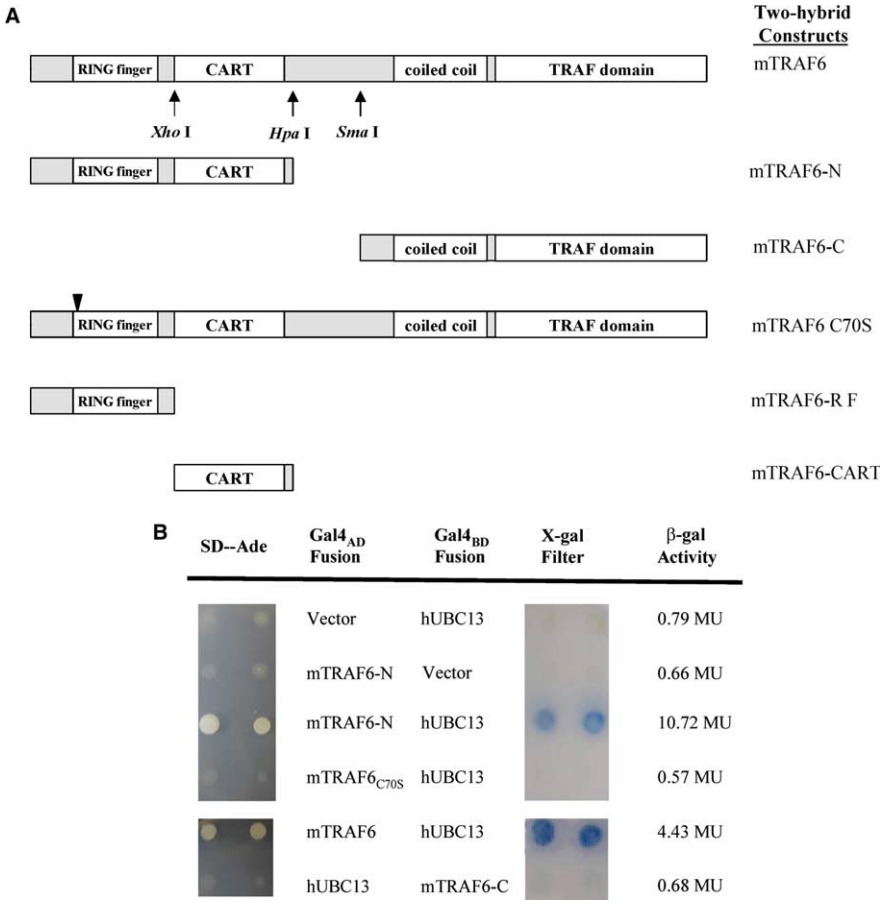

Fig. 2. Predicted mTRAF6 domains and domain deletion/mutagenesis analysis. A: A schematic diagram of mTRAF6 functional domains and deletion/mutation constructs. A few restriction sites used for deletion analysis are marked with arrows. The arrowhead points to the position of C70S mutation within the RING finger domain. B: Deletion and mutagenesis analysis of mTRAF6 in a yeast two-hybrid assay. Both Y190 and PJ69-4a were co-transformed and the transformants were analyzed by various assays as described in Fig. 1 legend.

N-terminal deletion removing the RING finger and CART domains completely abolishes Ubc13 interaction (Fig. 2B). Further deletion of the CART/zinc fingers or the RING finger also abolished the interaction of TRAF6 with Ubc13 (data not shown), indicating that the N-terminal RING and zinc fingers are required for the interaction. However, one cannot rule out the possibility that deletion of CART or the RING finger motif affects its proper folding or destabilizes the protein. To address this question, we specifically asked if the RING finger motif of TRAF6 is absolutely required for the interaction with Ubc13, since many RING finger proteins are found to function as an E3 [21,22] and their RING fingers appear to be required for the specific E2–E3 interaction [23]. If TRAF6 indeed functions as an E3 for Lys-63 mediated polyubiquitination, one would predict that its RING finger motif is essential for binding to Ubc13. It is known that the N-terminal Cys residue within the RING finger is essential for metal binding and function [24]. When the corresponding C70 is mutated to a conserved residue Ser, mTRAF6 was no longer able to interact with Ubc13 (Fig. 2B). The lack of interaction is not due to reduced expression of the mutant *TRAF6* gene or the unstable mutant protein in the yeast cells, as judged by Western-blot analysis (data not shown).

In order to further confirm the direct interaction between TRAF6 and Ubc13, we sought to identify the Ubc13 surface region essential for TRAF6 binding. The crystal structure of an E2 (UbcH7)-E3 (c-Cbl) complex has been reported [23] and c-Cbl contains a RING finger motif essential for the complex formation. By superimposing and subsequently replacing Ubc13 [17] for UbcH7 in the UbcH7-Cbl crystal structure (Fig. 3A), we identified residues of the E2–E3 interface that are conserved between Ubc13 and UbcH7 (Figs. 3A and B, red). The sequence alignment of UbcH7 with Ubc13 from various sources (Fig. 3B) indicates that within this interaction region, the hUbc13 M64 residue is not conserved with UbcH7; however, it is invariable among all Ubc13 homologs (Figs. 3A and B, yellow). The M64A single amino-acid substitution in Ubc13 completely abolishes its interaction with TRAF6 but does not have apparent effects on its interaction with hMms2 (Fig. 3C). It was noticed that the corresponding M64A substitution in yeast Ubc13 also abolished interaction with Rad5 [25], a RING-finger protein involved in error-free DNA postreplication repair [26] through polyubiquitination of PCNA [27]. Taken together, the deletion and site-specific mutagenesis results strongly support our structural model proposed in Fig. 3A.

To ask if the observed physical interaction between TRAF6 and hUbc13 has biological relevance, the small interference RNA (siRNA) technology was applied to suppress endogenous *hUBC13* expression and it was found that siRNA against *hUBC13* reduced *TRAF6*-mediated NF-κB activation in cultured human embryonic kidney 293T cells [28].

It has been shown that the family of TRAF proteins undergoes self-association [29]. However, evidence for the TRAF6 self-interaction has not been reported. The yeast two-hybrid analysis is ideal to study protein self-interactions. As shown in Fig. 4, full-length TRAF6 is able to interact with itself and the N-terminus appears to be sufficient for this interaction. Deletion of the RING finger domain or the C70S substitution within the RING finger motif abolishes the interaction, suggesting that the RING finger motif is required for homo-oligomerization.

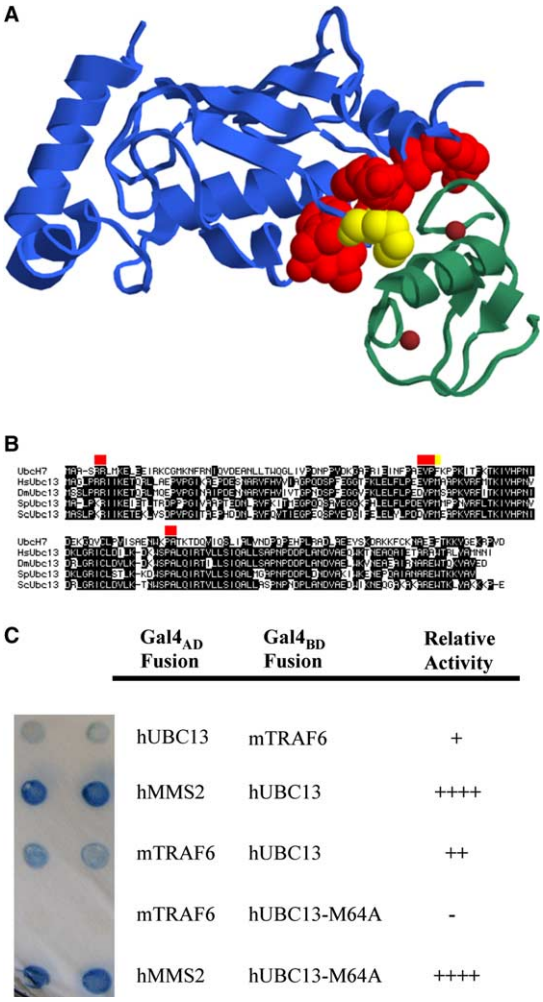

Fig. 3. Identification of amino acid residues important for Ubc13 to interact with a RING-finger motif. A: Predicted Ubc13 surface that interacts with a RING-finger motif. The Ubc13 structure ([17], blue) is oriented after UbcH7 based on the structure of UbcH7-c-Cbl [23]. Only the RING-finger motif of c-Cbl (green) is shown, with zinc atoms shown in maroon. Ubc13 residues shown in space-fill are those that fall in proximity to the interface between Ubc13 and the RING finger. Red residues are those conserved between UbcH7 and Ubc13, whereas the M64 residue (in yellow) is unique to Ubc13. The corresponding F63 in UbcH7 was thought to be important to provide specificity for the E3 [23]. B: Amino acid sequence alignment of Ubc13 from human (Hs), *Drosophila* (Dm), *Schizosaccharomyces pombe* (Sp) and *Saccharomyces cerevisiae* (Sc) with UbcH7. Residues indicated by red and yellow boxes correspond to those shown in A. Note that the Met residue in yellow is identical among all Ubc13 but differs from UbcH7. C: The Ubc13 M64A substitution abolishes its interaction with mTRAF6. Y190 cells, co-transformed with $Gal4_{AD}$ and $Gal4_{BD}$ construct, were replicated on SD-Trp-Leu plates and independent colonies were tested by a filter assay. Two representative colonies from each treatment are shown after 36-h incubation at 30 °C. Relative β-gal activity is measured by the time required to develop blue color and scored as follows: ++++, within 0.5 h; +++, 0.5–1.5 h; ++, 1.5–3 h; +, 3–5 h. Colonies remaining colorless after 12 h were considered negative.

In summary, we have demonstrated a direct interaction between TRAF6 and Ubc13 using the yeast two-hybrid assay. Since such an interaction cannot be readily detected by other in vitro methods, we propose that the association is probably transient, which is in contrast to the stable Ubc13–Uev complex [18]. We also demonstrated that two mammalian Uevs,

X-gal Filter	$Gal4_{AD}$ Fusion	$Gal4_{BD}$ Fusion	β-gal Activity
	mTRAF6	mTRAF6	4.43 MU
	mTRAF6	Vector	0.31 MU
	Vector	mTRAF6	0.52 MU
	mTRAF6-N	Vector	0.66 MU
	mTRAF6-N	mTRAF6	6.40 MU
	mTRAF6	mTRAF6-C	0.59 MU
	$mTRAF6_{C70S}$	mTRAF6	1.04 MU

Fig. 4. The N-terminal region and RING finger are required for mTRAF6 self-interaction. Y190 cells co-transformed with a full-length TRAF6 and various TRAF6 deletion constructs were tested by a filter assay and a quantitative β-gal assay as expressed in MU.

hMms2 and Uev1A, do not directly interact with TRAF6 and are dispensable for the TRAF6–Ubc13 interaction. However, the E2 complex consisting of Ubc13 and a Uev is required for Lys-63 polyubiquitination and it is most likely that TRAF6 will interact with the E2 complex rather than Ubc13 alone in vivo. TRAF6 may serve as an E3 or as a target for Ubc13-mediated Lys-63 chain assembly. While the latter has been demonstrated experimentally, evidence to support its E3 role has not been reported. We demonstrated that the amino terminus of TRAF6 containing the RING finger and zinc finger domains is essential and sufficient for this interaction. The essential role of the TRAF6 RING finger motif is further confirmed by the lack of Ubc13 interaction after a single conserved amino acid substitution within TRAF6, which is consistent with the notion that most RING finger proteins are indeed E3s.

We were also able to demonstrate that, like other TRAF proteins, TRAF6 is able to interact with itself. Interestingly, the amino terminus containing the RING finger and zinc finger domains was also essential and sufficient for this self-association. It is unclear whether the self-association of TRAF6 is a prerequisite for its interaction with Ubc13 and if so, what is the cellular role(s) of this homodimer (or homo-oligomer) formation.

Due to the lack of a stable interaction between TRAF6 and Ubc13–Uev, conventional studies to investigate the structure–function relationship of this important E2–E3 complex are not feasible. The yeast two-hybrid analysis as demonstrated in this report provides an alternative method to conduct such investigations. Furthermore, recent studies suggest that in addition to TRAF6, other RING-finger proteins, including TRAF2 [30,31] and Chfr [32], as well as non-RING-finger proteins, such as Bcl10 and paracaspase [28], also function in Ubc13-mediated Lys-63 polyubiquitination. Hence, the yeast two-hybrid assay will be a useful tool in determining physical interactions between the above proteins and Ubc13–Uev, as well as in screening for additional Ubc13–Uev interacting proteins.

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