

# Functional interactions between ubiquitin E2 enzymes and TRIM proteins

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The TRIM (tripartite motif) family of proteins is characterized by the presence of the tripartite motif module, composed of a RING domain, one or two B-box domains and a coiled-coil region. TRIM proteins are involved in many cellular processes and represent the largest subfamily of RING-containing putative ubiquitin E3 ligases. Whereas their role as E3 ubiquitin ligases has been presumed, and in several cases established, little is known about their specific interactions with the ubiquitin-conjugating E2 enzymes or UBE2s. In the present paper, we report a thorough screening of interactions between the TRIM and UBE2 families. We found a general preference of the TRIM proteins for the D and E classes of UBE2 enzymes, but we also revealed very specific interactions between TRIM9 and UBE2G2, and TRIM32

and UBE2V1/2. Furthermore, we demonstrated that the TRIM E3 activity is only manifest with the UBE2 with which they interact. For most specific interactions, we could also observe subcellular co-localization of the TRIM involved and its cognate UBE2 enzyme, suggesting that the specific selection of TRIM–UBE2 pairs has physiological relevance. Our findings represent the basis for future studies on the specific reactions catalysed by the TRIM E3 ligases to determine the fate of their targets.

**Key words:** RING domain, tripartite motif protein (TRIM protein), ubiquitin-conjugating E2 enzyme, ubiquitin E3 ligase, ubiquitylation.

## INTRODUCTION

The proteins that belong to the TRIM (tripartite motif) family, also known as the RBCC (RING finger, B-box and coiled-coil) family, are defined by the presence of a three-domain module composed of a RING finger, one or two B-box domains and a coiled-coil region [1]. The tripartite motif is invariably present at the N-terminus of these proteins and is associated with several different C-terminal domains [1,2]. In humans, the TRIM family has 68 members and they are involved in many cellular processes such as apoptosis, cell-cycle regulation and innate cellular response to retroviral infections [3–5]. Alteration of TRIM proteins results in a range of pathological conditions, including tumour growth and progression, Mendelian genetic disorders, and immunological diseases [1,3]. Given the presence of the RING domain, all TRIM proteins are very likely to be involved in the pathway of ubiquitin modification [3,6].

Ubiquitylation is a form of post-translational modification in eukaryotes in which ubiquitin, a highly evolutionarily conserved 76-residue polypeptide, is linked to target proteins [7]. Ubiquitylation occurs through the sequential actions of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2 or UBE2) and a ubiquitin ligase (E3). Ubiquitylation is initiated by the formation of a thiol-ester linkage between the C-terminus of ubiquitin and the active-site cysteine residue of the E1. Ubiquitin is then transferred to the E2, again through a thiol-ester linkage. The third step is dependent on the nature of the E3 ubiquitin ligase: RING-, PHD- and U-box-type E3 enzymes act as scaffolds that bring the ubiquitin-charged E2 enzyme and the substrate into close proximity allowing ubiquitin transfer, whereas HECT (homologous with E6-associated protein C-terminus)-type E3 enzymes form a thiol-

ester linkage with ubiquitin before its transfer to the substrate [8]. TRIM proteins represent the largest subfamily of the RING domain putative E3 ligases [3]. Indeed, previous studies have demonstrated the E3 function for some TRIM family members, e.g. TRIM23/ARD1, TRIM11, TRIM18/Mid1, TRIM21/Ro52, TRIM25/Efp and TRIM32 [9–14].

During the ubiquitylation process, target protein specificity is provided by the E3 enzyme, whereas the E2 and E3 combination determines the topology and length of the ubiquitin chains to be conjugated to the substrate [15]. Whereas the human genome presents approx. 600 E3 coding genes, 35–40 genes encode putative E2 proteins, some of which are only defined by homology with well-characterized E2 enzymes. The E2 enzymes are constituted by a 150-residue UBC (ubiquitin-conjugating core) domain and, depending on the presence of N-terminal or C-terminal tails, or both, they are grouped into four different classes [16]. Apart from this structural classification, many efforts in recent years have been addressing the key role of these enzymes in governing the type and processivity of the assembled ubiquitin chains, one of the most important features of the ubiquitylation process as it determines the fate of the modified substrate [17].

However, even if it is clear that E2 and E3 proteins work together to generate different forms of substrate modification including mono-, multi- and poly-ubiquitin chains of up to seven different linkage types, much has still to be uncovered about specific E2–E3 interactions and how this is achieved and regulated. Recently, Markson et al. [18] and van Wijk et al. [19] addressed this issue by defining networks of E2–RING interactions. Although included in the initial panels, the specific activities of the TRIM proteins were not addressed in these studies. In the case of the TRIM proteins, even when the E3 activity is assessed, little is known

Abbreviations used: AD, activation domain; DAPI, 4',6-diamidino-2-phenylindole; DBD, DNA-binding domain; ERAD, endoplasmic reticulum-associated degradation; GFP, green fluorescent protein; HA, haemagglutinin; HEK, human embryonic kidney; MBP, maltose-binding protein; NP40, Nonidet P40; SNARE, soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor; TRIM, tripartite motif; UBC, ubiquitin-conjugating core; UBE2, ubiquitin-conjugating E2 enzyme; UIP48, UBCM4-interacting protein 48.

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about the specific UBE2 partner usage. We therefore sought to functionally define the specific E3–E2 interactions focusing on the TRIM family. In the present paper, we report that almost all TRIM family members interact directly with several E2s with precise specificity and that this specificity is translated into their ubiquitin ligase function.

## EXPERIMENTAL

### Constructs

The cDNAs of 21 of the TRIM proteins used in the present study were already available in our laboratory in several vectors [1]. The cDNAs encoding the full-length ORF (open reading frame) of UBE2s (see Supplementary Table S1 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>), UIP48 (UBCM4-interacting protein 48) [20] and TRIM15, 16, 17, 37, 39, 40, 42, 43, 44, 45, 46, 48 and 52, were amplified by PCR with PfuI polymerase (Promega) using appropriate primers from HeLa or HEK (human embryonic kidney)-293T cell cDNA. The cDNAs were cloned in the two-hybrid vectors (pEG202 and pJG4-5) and in HA (haemagglutinin)-pcDNA3 and Myc/GFP (green fluorescent protein)-pcDNA3 vectors.

### Two-hybrid analysis

Binary two-hybrid screening was performed as described in [21]. Briefly, the bait plasmids (pEG202) express the cDNA fused directionally to the first 202 residues of LexA under the control of the constitutive *ADH* (alcohol dehydrogenase) promoter. Prey plasmids (pJG4-5) express the cDNA fused to the B42 activation domain, the SV40T (simian virus 40 large T-antigen) nuclear localization signal and an HA tag under the control of the inducible GAL1 promoter. The expression of UBE2 enzymes in the two-hybrid vectors was checked by immunoblotting using anti-HA and anti-LexA antibodies (results not shown). The EGY42/EGY48 diploid strain was generated by mating for every pairwise combination. A *lacZ* fusion with six LexA operators in the pSH18-34 vector and a genome-integrated *LEU2* fusion with four LexA operators were used as reporters. The expression of the two reporters was used to establish the interaction, by blue-turning colonies and growth in the absence of leucine. The arbitrary score shown in Figure 1 was calculated as the fraction of detected interactions on the number of the experiments (at least seven performed for each pair tested) multiplied by 0.5 if the interaction was observed with only one reporter gene.

### Cell culture and immunoblotting

HEK-293T cells were grown in Dulbecco's modified Eagle's medium with 10% (v/v) FBS (fetal bovine serum) (Invitrogen), 100 µg/ml streptomycin and 100 units/ml penicillin in a 5% CO<sub>2</sub> atmosphere at 37°C. Cells were transfected using the calcium phosphate precipitation method [22]. Proteins were separated by SDS/PAGE (10% gels) and transferred on to a PVDF membrane (Millipore). After blocking with 5% (w/v) skimmed milk powder in 20 mM Tris/HCl (pH 7.4) and 150 mM NaCl containing 1% Tween 20, the membranes were probed with the primary antibodies at the dilutions indicated: mouse anti-HA antibody (1:1000; Roche), rabbit anti-LexA (1:1000; Sigma), mouse anti-Myc antibody (1:1000; Santa Cruz Biotechnology), sheep anti-MBP (maltose-binding protein) (1:1000; Santa Cruz Biotechnology), or rabbit anti-ubiquitin (1:5000; Bethyl Laboratory). Blots were washed

three times with Tris-buffered saline/0.1% Tween 20 and incubated with horseradish peroxidase-conjugated anti-(mouse IgG) antibody (1:3000; GE Healthcare), anti-(rabbit IgG) antibody (1:3000; GE Healthcare) or anti-(sheep IgG) antibody (1:3000; GE Healthcare), then developed with an ECL (enhanced chemiluminescence) detection system (BioFX Laboratories).

### Immunofluorescence

HeLa cells were seeded on glass coverslips in 12-multiwell plates 1 day before transfection with Lipofectamine™ 2000 (Invitrogen) to obtain a low expression level. At 24 h after transfection, the coverslips were washed in ice-cold PBS and cells were fixed with 4% (w/v) paraformaldehyde in PBS for 5 min. Cells were then permeabilized in 0.5% Triton X-100 in PBS for 10 min. Non-specific binding sites were blocked by incubating with 5% (v/v) BSA, 0.1% Tween in PBS for 1 h. Coverslips were then incubated with anti-HA monoclonal antibody (1:500; Roche) for 2 h. After washing, coverslips were incubated with Cy3 (indocarbocyanine)-conjugated anti-mouse secondary antibody (1:100; Jackson Laboratories). Slides were mounted using Vectashield with DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories); images were acquired on a Leica DM2500 microscope and processed with the Leica Application Suite V3 software and on Nikon confocal D-Eclipse C1 imaging microscope with Nikon software and processed either as greyscale or dual-colour TIFF images in Adobe Photoshop.

### Protein expression and purification

TRIM proteins were cloned in the prokaryotic expression vector pMAL-c2x (New England Biolabs) and expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen). For each TRIM protein, when at a  $D_{600}$  of 0.8, 1 litre of cell culture was induced with 150 µM IPTG (isopropyl β-D-thiogalactopyranoside) and grown overnight at 24°C. Then, cells were harvested and flash-frozen. A 50 ml volume of lysis buffer containing 50 mM Tris/HCl (pH 7.5), 500 mM NaCl and 0.5 mM 2-mercaptoethanol was added for every 1 litre of culture. After sonication, cell lysates were cleared at 11 000 rev./min for 30 min at 4°C using a Beckman JA-12 rotor, and proteins were purified using amylose-bound chromatography. The correct production of the MBP–TRIM protein was checked by SDS/PAGE (10% gels) upon Coomassie Blue staining.

### MBP pull-down assays

MBP–TRIM proteins were immobilized on amylose resin (New England Biolabs) and incubated with approx. 5 µg of HEK-293T crude extract transiently transfected with Myc/GFP–UBE2 enzymes for 4 h at 4°C in lysis buffer (20 mM Tris/HCl, pH 7.5, 20% glycerol, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1.5 mM PMSF and 1 mg/ml each of aprotinin, leupeptin and pepstatin). The resin was washed three times with lysis buffer; bound proteins were separated by SDS/PAGE (10% gels), and UBE2 enzymes were visualized by immunoblotting using the anti-Myc antibody. MBP–TRIM proteins were visualized by Coomassie Blue staining.

### In vitro ubiquitylation assays

The *in vitro* ubiquitylation assays were carried out in a volume of 15 µl containing 34 nM Uba1, 0.8 µM UBE2, 35 µM ubiquitin, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 150 mM NaCl, 0.5 mM TCEP



[tris-(2-carboxyethyl)phosphine], 0.1% NP40 (Nonidet P40) and 2.2  $\mu$ M MBP-TRIM protein in 50 mM Tris/HCl (pH 7.5). MBP was used at 2  $\mu$ M. After incubation at 37°C for 2 h, the reactions were terminated by the addition of Laemmli sample buffer and analysed by SDS/PAGE (10% gels). Immunoblots using anti-ubiquitin and anti-MBP antibodies were performed.

### ***In vivo* ubiquitylation assays**

HEK-293T cells were co-transfected with HA-ubiquitin and Myc/GFP-TRIM proteins. When applicable, 42 h after transfection, the culture medium was replaced with fresh medium containing MG132 proteasome inhibitor (Sigma) at a final concentration of 20  $\mu$ M and the cells were cultured further for 6 h. The total cell lysate was prepared in RIPA buffer (50 mM Tris/HCl, pH 8, 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, 1.5 mM PMSF and 1 mg/ml each of aprotinin, leupeptin and pepstatin). DNA in the sample was sheared with a 22-gauge needle, and the lysate was centrifuged at 13 000 rev./min for 30 min at 4°C using a Sigma 12024H rotor. The supernatant was then incubated with 1  $\mu$ g of anti-Myc antibody (9E10; Santa Cruz Biotechnology) overnight at 4°C. After adding Protein A-Sepharose beads (Sigma), complexes were washed with lysis buffer. Immunoprecipitates were separated by SDS/PAGE (10% gels) and analysed by immunoblotting.

## **RESULTS**

### **The TRIM family members interact with UBE2 enzymes**

A requirement for a RING finger protein to act as ubiquitin E3 ligase is the direct interaction with a UBE2 enzyme [6]. To establish whether TRIM proteins could act as RING E3s, we tested the interaction between 42 TRIM proteins (<http://www.trimbase.tigem.it>) against 26 of the best studied UBE2 enzymes (see Supplementary Table S1) in a yeast two-hybrid system (Figure 1A). Each full-length protein was tested both as LexA-DBD (DNA-binding domain) fusion and B42-AD (activation domain) fusion, with only a couple of exceptions. This analysis revealed more than 100 interactions and demonstrated that the majority of TRIM proteins tested interact with one or more UBE2 enzymes (Figure 1A). Numerous interactions were observed with the D (D1-D4) and E (E1-E3) families of UBE2 enzymes, whereas several TRIM proteins also showed binding to UBE2N. Two peculiar interactions were also observed: (i) an exclusive binding between TRIM9 and UBE2G2; and (ii) the interaction of TRIM32, besides the D and E classes and UBE2N, with UBE2V1 and V2 (Figure 1A). The UBE2 enzymes and the TRIM proteins were properly expressed in yeast (results not shown) [1] and the lack of reporter activation mainly underlies real UBE2 selection. Inclusion of UIP48, a RING finger-containing protein able to interact with L3 and L6, but not with the D and E families, demonstrated the specificity of the observed interactions [20] (Figure 1A). In some cases, lack of an interaction may represent an intrinsic deficiency in the yeast two-hybrid experiments.

Within the group of proteins tested, five are 'unorthodox' TRIM members that lack the RING domain [4]. Consistent with the role of this domain in mediating UBE2 binding, these five TRIM proteins did not show any interactions with E2 enzymes (Figure 1A). To confirm further the involvement of the RING domain, we tested single-domain-deletion mutants of TRIM proteins that bind UBE2s in their full-length form, TRIM18 and TRIM32. We found that the RING domain was necessary

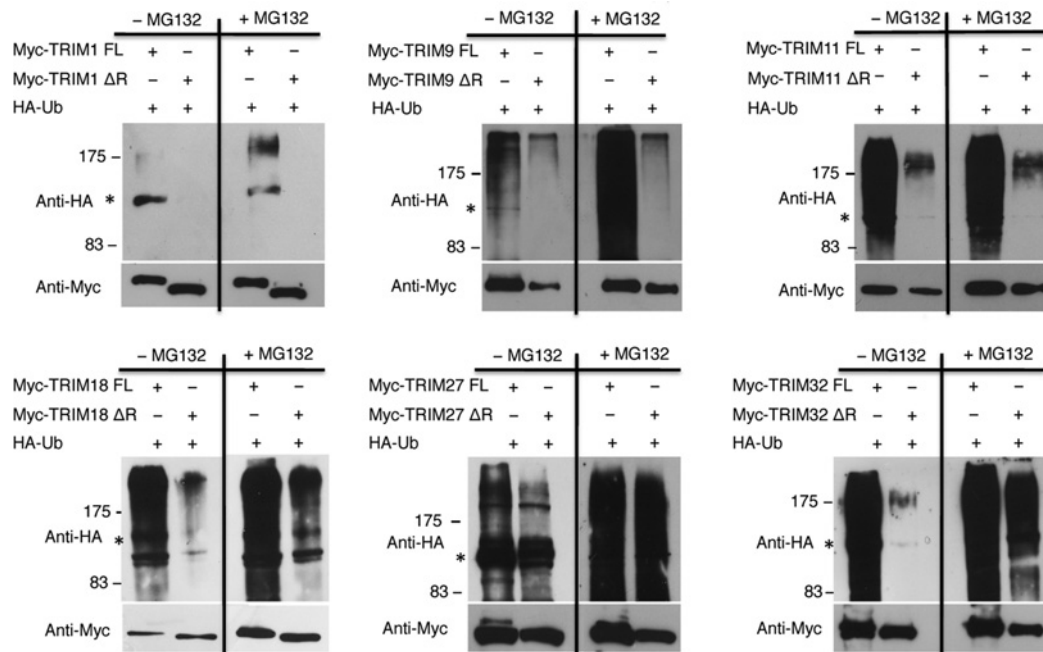
and sufficient for UBE2 binding. This analysis also demonstrated that the B-box domains, although structurally similar to the RING domain [23,24], were not intimately involved in the basic TRIM/E2 interaction (Figures 1B and 1C).

Taken together, these results indicate that the majority of TRIM proteins interact with selected UBE2 enzymes through their RING domain.

### **Ubiquitin E3 ligase activity of TRIM proteins *in vivo***

To determine whether interaction with UBE2 enzymes reflects the ability of TRIM proteins to act as E3 ligases in mammalian cells, we performed *in vivo* ubiquitylation assays using a selection of six TRIM proteins both as full-length and as UBE2-binding-incompetent RING-deleted forms. TRIM1, 9, 11, 18, 27 and 32 were selected as representatives of the different specificities in UBE2 binding. Some of the selected TRIM proteins show very specific binding, whereas others are more promiscuous in the UBE2 interaction and overall they account for the entire spectrum of observed interactions. The same TRIM proteins were used to validate the specific UBE2 interactions and their functional relevance (see below). Each of the Myc/GFP-tagged wild-type TRIM proteins and their RING deletion mutants were co-transfected in HEK-293T cells together with HA-tagged ubiquitin. TRIM proteins were immunoprecipitated with the anti-Myc antibody and analysed by Western blotting using either an antibody against the HA tag to detect ubiquitylated proteins (Figure 2, upper panels) or an antibody against the Myc tag to check for total immunoprecipitated Myc/GFP-TRIM proteins (Figure 2, lower panels). Immunoblotting using anti-HA showed a high-molecular-mass smear for each TRIM protein tested, suggesting that they are self-polyubiquitylated and/or that the proteins co-immunoprecipitating with them are polyubiquitylated in mammalian cells (Figure 2, upper left panels). In contrast, the ubiquitylation observed in the presence of the corresponding RING-deleted mutant was dramatically reduced, indicating that the high-molecular-mass species were generated mainly through the TRIM protein E3 activity (Figure 2, upper left panels). The residual faint ubiquitylation observed corresponding to the RING-deleted TRIM protein might be due to the activity of the endogenous TRIM protein or of other E3 ligases. This still undefined endogenous E3 activity appears to be more relevant in the case of TRIM27, where, even in the presence of the RING-deleted form, comparable polyubiquitylation was observed. In some cases, a more abundant band could be observed within the smear in the anti-HA immunoblot. This was observed to different extents in the different experiments and, considering the apparent molecular mass, this band might represent either mono- or di-ubiquitylated forms of the TRIM protein itself or modified co-immunoprecipitated partner. HEK-293T cells transfected with HA-ubiquitin, either alone or with Myc/GFP empty vector, produced no ubiquitylated species upon anti-Myc immunoprecipitation (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>).

To determine whether the *in vivo* ubiquitylation observed was mainly associated with proteasome-mediated degradation, the experiments described above were also performed in the presence of the proteasomal inhibitor MG132. Treatment with MG132 increased both the ubiquitylation (Figure 2, upper right panels) and the total amount of the TRIM proteins tested (Figure 2, lower right panels). Interestingly, this treatment also increased decisively the ubiquitylation of TRIM27 and TRIM32 RING deletion mutants, suggesting that other E3 ligases are implicated in their ubiquitylation.



**Figure 2** E3 ligase activity of TRIM proteins in mammalian cells

*In vivo* ubiquitylation assay of Myc/GFP (Myc)-tagged TRIM1, TRIM9, TRIM11, TRIM18, TRIM27 and TRIM32 full-length (FL) and RING-deleted ( $\Delta$ R) forms in HEK-293T cells in the presence of HA-ubiquitin (HA-Ub) as indicated. An immunoblot of the anti-Myc immunoprecipitates using anti-HA antibody to detect ubiquitylated proteins (upper panels) and anti-Myc antibody to detect TRIM proteins (lower panels) is shown. Asterisks indicate the predominant band(s) observed in the anti-HA immunoblots (see the text). Molecular masses are indicated in kDa. Where indicated, cells were treated with the MG132 proteasomal inhibitor.

Thus these results indicate that the TRIM proteins tested act as RING-dependent ubiquitin E3 ligases.

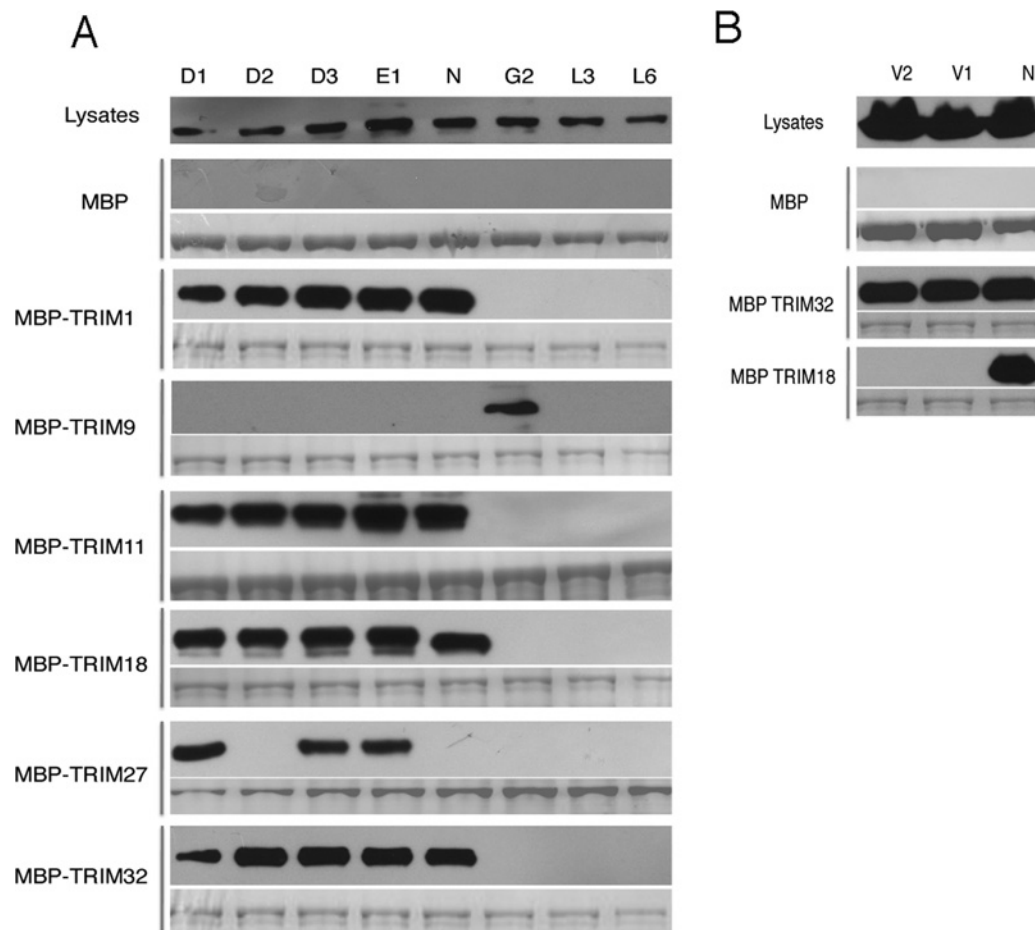
### TRIM proteins bind to UBE2 enzymes *in vitro*

To establish whether the UBE2-binding specificity is relevant in the TRIM ligase activity, we first confirmed the specific TRIM-UBE2 association using an MBP pull-down approach on a selection of TRIM and UBE2 proteins representative of the entire spectrum of interactions observed in the two-hybrid screening. In this assay, we chose TRIM1, 9, 11, 18, 27 and 32 for which we also tested the *in vivo* E3 ligase activity, and eight UBE2 enzymes (D1, D2, D3, E1, N, G2, L3 and L6). MBP-fused TRIM proteins or control MBP were expressed in *E. coli* and purified on amylose beads. MBP-fusions immobilized on the beads were incubated with HEK-293T crude lysates transiently expressing Myc/GFP-UBE2 proteins, and specific binding was revealed by immunoblotting (Figure 3A). UBE2D1, D2, D3, E1 and N were captured by TRIM1, 11, 18 and 32, confirming the interactions in yeast. Interestingly, TRIM27 binds only UBE2D1 and D3, but not the highly similar D2 which also showed a very weak interaction in yeast. TRIM9 only bound UBE2G2, confirming this highly unique interaction. As expected, no binding could be observed with UBE2L3 and L6, reflecting the specific nature of TRIM-UBE2 interactions (Figure 3A). MBP-TRIM32 and MBP-TRIM18 were also incubated with lysates of HEK-293T cells transiently transfected with UBE2N, V1 and V2. As observed already in yeast, TRIM32 also bound UBE2V1 and V2 in addition to UBE2N. As expected, TRIM18 bound UBE2N, but was unable to bind the UBE2V enzymes (Figure 3B).

Thus the specific interaction of the selected TRIM proteins with defined UBE2 enzymes observed in yeast was validated by *in vitro* binding analysis.

### TRIM proteins act as E3 *in vitro* in the presence of their cognate UBE2 enzymes

To establish that selective UBE2 binding by TRIM proteins is translated into functional ubiquitin ligase activity, we performed *in vitro* ubiquitylation assays. We tested the ability of the selected TRIM proteins to catalyse polyubiquitylation *in vitro* by means of the different UBE2 enzymes with which they interact. The above six MBP-TRIM proteins were incubated with ATP, ubiquitin and recombinant E1 and E2 enzymes. Immunoblot analysis of the reaction products using antibodies against ubiquitin revealed the presence of high-molecular-mass polyubiquitylated species (Figure 4). Polyubiquitylation was observed only when the recombinant MBP-TRIM protein was added to the reaction mixture (Figure 4A and see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>). As the MBP-TRIM fusions are the only possible E3 enzymes in the reactions, these results prove further that these TRIM proteins function as E3 enzymes (Figure 4). The reactions were also analysed with antibodies against the MBP portion of the fusion protein, which showed that, in the majority of the cases, polyubiquitylated species are mainly represented by the MBP-TRIM proteins themselves and thus, in the absence of a specific substrate, auto-ubiquitylation is the predominant reaction catalysed (see Supplementary Figure S3 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>). Concomitantly, we also addressed the specific UBE2 usage in these



**Figure 3** MBP pull-down assay confirms specific UBE2 and TRIM protein interactions

(A) MBP pull-down analysis of eight MycGFP-UBE2 enzymes transiently expressed in HEK-293T cells (D1, D2, D3, E1, N, G2, L3 and L6 as indicated) with six MBP-TRIM proteins (TRIM1, 9, 11, 18, 27 and 32). MBP was used as control. An immunoblot with anti-Myc antibody of the input lysates is shown in the top panel (Lysates). For each MBP-TRIM protein, the anti-Myc immunoblot and the Coomassie Blue staining of the gel are shown. (B) MBP pull-down analysis of three MycGFP-UBE2 transiently expressed in HEK-293T cells (N, V1 and V2 as indicated) with MBP-TRIM32 and MBP-TRIM18. MBP was used as control. Other details are as in (A).

reactions. The *in vitro* ubiquitylation assays were carried out in the presence of one of the following recombinant enzymes as the sole UBE2: D1, D2, D3, E1, N, J2, G2, L3 or L6. Figure 4(A) shows that incubation of MBP-TRIM1, 11, 18 and 32 in the presence of UBE2D1, D2, D3, E1 or N resulted in polyubiquitylation recapitulating the binding specificity observed in yeast and in MBP pull-down assays. Moreover, MBP-TRIM11 acted as E3 also in the presence of UBE2J2 that was not present in our original two-hybrid panel. As expected from the binding results, MBP-TRIM27 displayed specific E3 activity in the presence of UBE2D1 and D3, but not with the non-interacting D2. The remarkable binding specificity of MBP-TRIM9 for UBE2G2 was also manifest in the functional assay where E3 ligase activity was only observed when TRIM9 was incubated in the presence of UBE2G2. Consistently with the above results, the incubation of MBP-TRIM proteins in the reaction mixture containing UBE2L3 and L6 did not result in the formation of any polyubiquitylated species (Figure 4A).

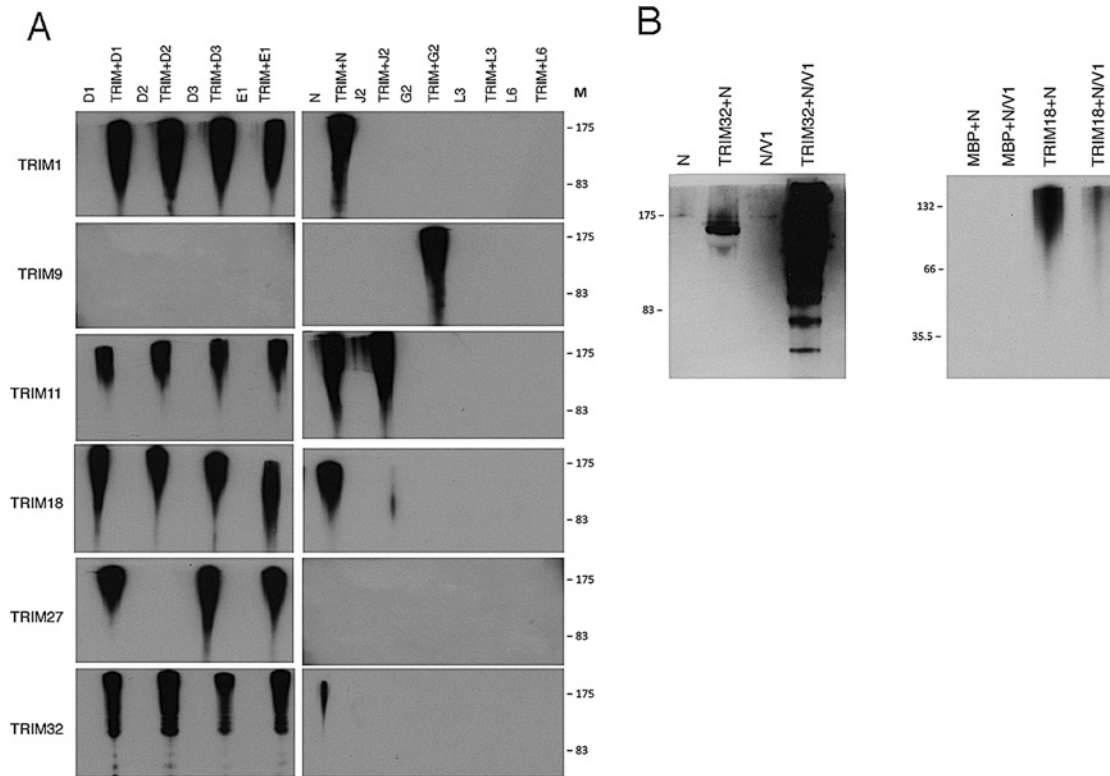
As both yeast two-hybrid and MBP pull-down assays highlighted the interaction between TRIM32 and UBE2V, we tested TRIM32 polyubiquitylation in the presence of ATP, recombinant E1 enzyme, ubiquitin, recombinant UBE2N and V1 proteins. It is known that V1 is a non-autonomous UBE2

that lacks the catalytic cysteine and can only act in co-operation with UBE2N [25]. Consistently, TRIM32 was a more efficient E3 in the presence of both UBE2N and V1 than with UBE2N alone (Figure 4B). TRIM18 used as negative control only showed polyubiquitylation in the presence of UBE2N (Figure 4B, right-hand panel).

Taken together, these results indicate that TRIM proteins act as E3 ligases co-operating with the ubiquitylation machinery in a very specific manner that recapitulates the observed specific TRIM-UBE2 interactions.

#### Specific TRIM-UBE2 co-localization *in vivo*

How the selection and usage of UBE2 enzymes is achieved and what the consequences on TRIM activity are within the cellular context are more complicated issues to address. *In vivo* selection may depend on a variety of parameters including the spatial accessibility of the E2 enzyme. To address this issue, the subcellular distribution of the TRIM proteins and their interacting UBE2 enzymes was investigated by immunofluorescence after transfection of GFP- and HA-tagged constructs in HeLa cells. As reported above, TRIM1, 9, 11, 18, 27 and 32 were mainly localized in the cytoplasm of HeLa cells either in filamentous



**Figure 4** TRIM proteins act as ubiquitin E3 ligases *in vitro* using the UBE2-interacting enzymes

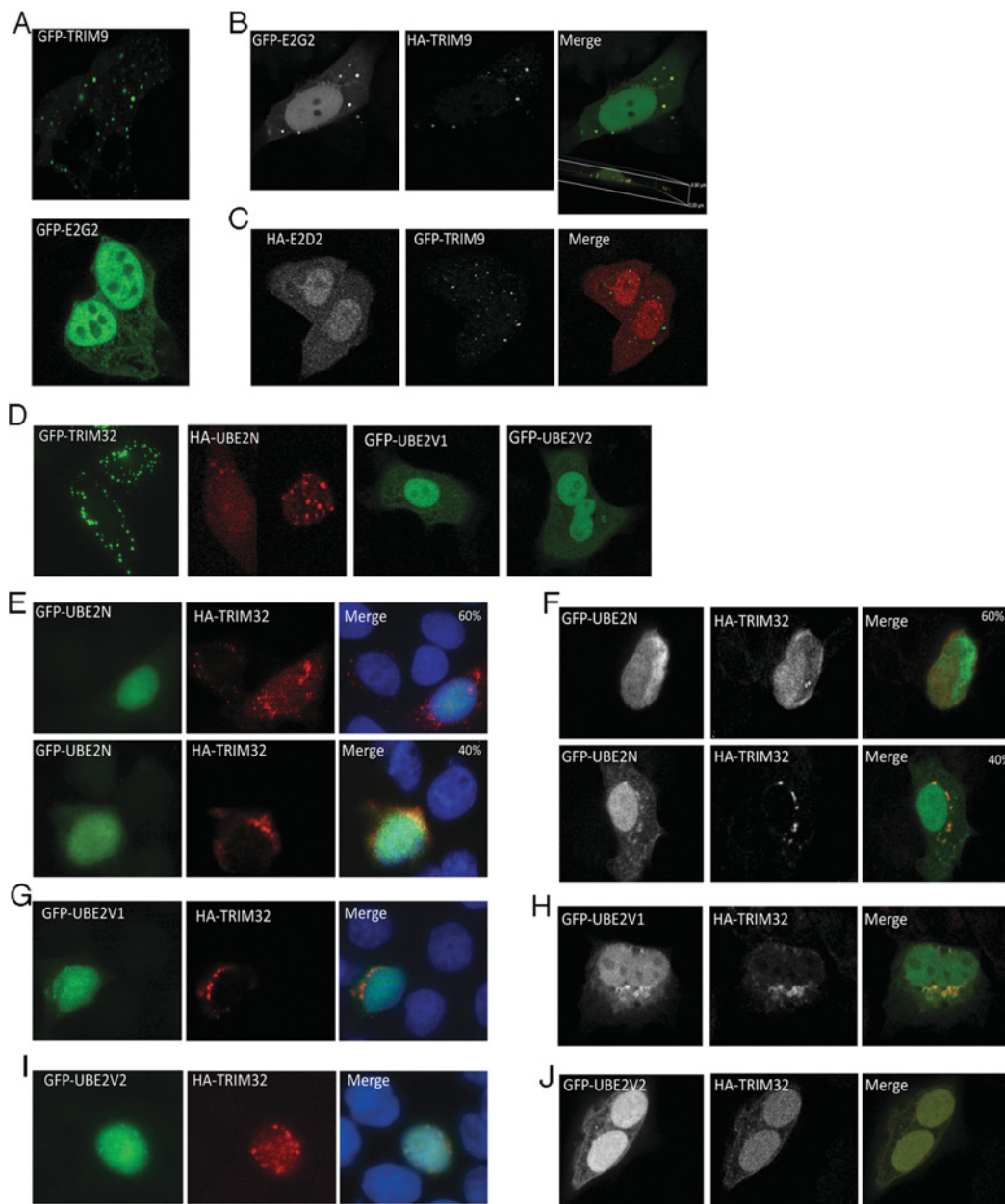
(A) MBP-TRIM proteins (TRIM1, 9, 11, 18, 27 and 32) were tested for E3 ligase activity in *in vitro* ubiquitylation assays in the presence of the UBE2 enzymes indicated (TRIM+D1, D2, D3, E1, N, G2, J2, L3 and L6). As a control, the assay was performed without the TRIM protein (lanes with only UBE2 indicated). An immunoblot with anti-ubiquitin antibody to detect the ubiquitylated species is shown. M, molecular mass (in kDa). (B) *In vitro* ubiquitylation assay using UBE2N and V1, in the combination indicated, in the presence of MBP-TRIM32. As controls, the assay was performed without the TRIM protein, with MBP and with MBP-TRIM18 as indicated. Immunoblots with anti-ubiquitin antibody are shown. Molecular masses are indicate in kDa.

or speckled structures [1]. UBE2D1, D2, D3, G2, N, V1 and V2 were distributed diffusely throughout both the nucleus and cytoplasm (Figure 5 and results not shown). The only exception was UBE2E1, which is a strictly nuclear protein [26]. When co-transfected with TRIM proteins, the UBE2 enzymes generally maintained their distribution and were not apparently enriched in the defined TRIM cellular structures, consistent with the fact that E2 enzymes are likely to be shared by many E3 ligases (results not shown).

However, an exception was the UBE2G2 and TRIM9 partnership. As shown previously [1], TRIM9 is present in cytoplasmic speckles, whereas GFP-UBE2G2 is diffusely distributed throughout both the nucleus and the cytoplasm (Figure 5A). When co-transfected with TRIM9 in HeLa cells, a fraction of UBE2G2 was clearly recruited into TRIM9 cytoplasmic speckles as also demonstrated by a collection of multiple focal planes (*z*-stack) (Figure 5B). Consistent with TRIM-UBE2 interaction results, when HeLa cells were co-transfected with HA-TRIM9 and GFP-UBE2D2, the latter maintained its diffuse distribution in the cell and was not recruited by exogenous TRIM9, supporting the specific effect on UBE2G2 (Figure 5C). To determine whether other TRIM proteins could exert the same effect on UBE2G2, we also transfected HeLa cells with GFP-UBE2G2 and HA-tagged TRIM1, 11, 18, 27 or 32. UBE2G2 was not recruited by any of the above TRIM proteins into any defined structures, supporting further the specific interaction with TRIM9. Confirmation of this unique specificity was provided by TRIM27, which, like TRIM9, formed cytoplasmic speckles, but was unable to recruit

UBE2G2 to these structures (see Supplementary Figure S4 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>).

Moreover, we observed that TRIM32 changed its own localization when co-transfected with either UBE2N or UBE2V2 proteins. In agreement with the literature [1], TRIM32 was localized in cytoplasmic perinuclear speckles, whereas the UBE2N and UBE2V proteins were distributed diffusely throughout the cytoplasm and nucleus (Figure 5D). In 60% of HeLa cells co-transfected with GFP-UBE2N and HA-TRIM32, UBE2N relocated TRIM32 from the cytoplasm to the nucleus (Figures 5E and 5F, upper panels). In 40% of the cells co-transfected with GFP-UBE2N and HA-TRIM32, we observed TRIM32 localized in well-defined speckles around the nucleus. In these cells, UBE2N was distributed diffusely throughout the nucleus and the cytoplasm, although a fraction of UBE2N appeared to co-localize with the cytoplasmic accumulations of TRIM32 (Figures 5E and 5F, lower panels). The specificity of this reciprocal localization was highlighted by the observation that HeLa cells co-transfected with the interacting pair GFP-UBE2N and HA-TRIM18 did not mutually relocate (see Supplementary Figure S5 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>). When we transfected HeLa cells with HA-TRIM32 and GFP-UBE2V1, the latter was partially recruited into TRIM32 speckles around the nucleus (Figures 5G and 5H). Curiously, although few cells were co-transfected with HA-TRIM32 and GFP-UBE2V2 (approx. 30%), we found that UBE2V2 relocated TRIM32 from the cytoplasm to the nucleus (Figures 5I and 5J). These results were confirmed in co-transfection experiments using



**Figure 5** TRIM-UBE2 co-localization in mammalian cells

(A–C) TRIM9 co-localizes with UBE2G2 in cytoplasmic speckles. (A) Immunofluorescence of HeLa cells transfected with either GFP-TRIM9 (upper panel) or GFP-UBE2G2 (lower panel). (B) Immunofluorescence of HeLa cells co-transfected with GFP-UBE2G2 (left-hand panel) and HA-TRIM9 (middle panel). The right-hand panel represents the overlay of left-hand and middle images. The inset shows a lateral view of the above cell as resulting from a z-stack collection of confocal images. (C) Immunofluorescence of HeLa cells co-transfected with HA-UBE2D2 (left-hand panel) and GFP-TRIM9 (middle panel). The right-hand panel represents the overlay of left-hand and middle images. (D–J) TRIM32 co-localizes with UBE2N, V1 and V2 proteins. (D) Immunofluorescence of single HeLa cell transfections with GFP-TRIM32, HA-UBE2N, GFP-UBE2V1 or GFP-UBE2V2. (E) Immunofluorescence of HeLa cells co-transfected with GFP-UBE2N (left-hand panels) and HA-TRIM32 (middle panels). The right-hand panels represent the overlay of left-hand and middle images counterstained with DAPI. Two behaviours of TRIM32 in the presence of UBE2N are represented and their percentage shown (60% in the upper panels; 40% in the lower panels). (F) Confocal microscopy images of experiments as in (E). (G) Immunofluorescence of HeLa cells co-transfected with GFP-UBE2V1 (left-hand panel) and HA-TRIM32 (middle panel). The right-hand panel represents the overlay of left-hand and middle images counterstained with DAPI. (H) Confocal microscopy images of experiments as in (G). (I) Immunofluorescence of HeLa cells co-transfected with HA-TRIM32 and GFP-UBE2V2 as in (G). (J) Confocal microscopy images of experiments as in (I).

the reciprocally tagged constructs (see Supplementary Figure S6 at <http://www.BiochemJ.org/bj/434/bj4340309add.htm>). Thus immunofluorescence experiments suggest that interactions between E2 and TRIM pairs also occur *in vivo*.

Taken together, our results indicate that the TRIM proteins act as E3 ubiquitin ligases *in vitro* and *in vivo*, selectively recruiting UBE2 enzymes for their activity.

## DISCUSSION

In the present paper, we report a thorough analysis of the specific interactions between TRIM family members and UBE2 enzymes. We found that the majority of the TRIM proteins tested interact with UBE2 enzymes with a defined specificity that is maintained in their ability to act as E3 ubiquitin ligases.

A direct interaction between UBE2 and E3 enzymes is required for the ubiquitin ligase reaction; the majority of the TRIM proteins fulfil this rule and we detected more than 100 interactions between UBE2 and TRIM proteins. As already observed with other ligases, in most cases TRIM proteins interact with more than one UBE2 enzyme and vice versa [6]. The lack of UBE2 binding observed with both the naturally occurring and the experimentally deleted RING-less TRIM proteins reinforces the concept that this is the domain offering the surface for direct interaction [27]. Our results also suggest that the B-boxes are not crucial determinants of this interaction. B-box1 and B-box2 are zinc-binding domains found within the tripartite module [4] that assemble to form RING-like structures [23,24]. Given this resemblance, it has often been speculated that the B-boxes might also interact with E2 enzymes. Whether the B-boxes participate in the interaction with the ubiquitylation machinery is a fascinating issue to be addressed also in the light of the success of the tripartite motif in evolution [4].

As a general rule, the TRIM proteins interact preferentially with the common D and E classes, but not with the L class, of UBE2. This is consistent with previous findings showing a preference of the RING domain for either of the two classes [20]. Several TRIM proteins also interact with UBE2N, and, in addition, we revealed important exceptions: the highly specific interaction between TRIM9 and UBE2G2 and the additional interactions observed between TRIM32 and UBE2V1 and V2. The preference shown by the TRIM proteins for subclasses D and E, which are evolutionarily very close [28], reflects their RING domain sequence similarity and the conservation of residues reported to be crucial for E2 interaction [4,29]. The use of the two-hybrid system as an initial screen to identify TRIM–UBE2 interactions turned out to be sensitive enough to discriminate between the different UBE2 enzymes bound by the same TRIM protein. Previous studies utilized this technique to identify specific interactions between RING E3 ligases and UBE2 enzymes, although such interactions are by nature transient and of low affinity [20,30,31]. In particular, Christensen et al. [30] confirmed the specificity detected with the two-hybrid analysis with NMR studies. In our two-hybrid experiment, both families were analysed using the full-length clones and the two directions of the two-hybrid system were investigated; moreover, two different reporter genes were detected in order to increase the reproducibility and confidence in the results. Confirmation of interactions by MBP pull-down and functionally by means of ubiquitylation assays reinforces the appropriateness of the two-hybrid assay in this context. In many cases, undetectable reporter gene expression indicates a lack of interactions, although we cannot exclude that physiologically relevant interactions might be lost in the two-hybrid analysis, especially when a particular conformation and/or the requirement of a mammalian co-factor are needed. As mentioned above, systematic analyses of RING E3 and UBE2 interactions have been reported recently [18,19]. In both of these studies, several TRIM proteins were included in the initial two-hybrid screening. In most cases, the results are consistent with ours, although no specific validation of TRIM proteins was addressed.

We confirmed that the selected TRIM proteins behave *in vivo* as RING-dependent ubiquitin ligases with a major involvement in proteasome-mediated degradation. We also assessed the *in vitro* ubiquitin E3 activity of TRIM1, 9, 11, 18, 27 and 32 in the presence of the specific UBE2 enzyme(s) with which they interact. The binding specificity between TRIM proteins and UBE2 enzymes is perfectly translated in their ability to function as E3 ligases. TRIM11 and 18 *in vivo* activity and the identification of the substrates was reported [10,12,32,33]. We now provide direct proof of their ubiquitin ligase activity *in vitro* and of the

identity of the UBE2 enzymes they use for this function: D1–D3, E1, N and, in the case of TRIM11, also J2, but not L3 and L6. Moreover, we assessed for the first time *in vivo* and *in vitro* E3 activity for TRIM1 and its preference to use the D1–D3, E1 and N classes of UBE2 to exert this function. In the case of TRIM27, despite many biochemical findings of it being a transcriptional and signalling regulator [34,35], few results on the E3 activity are available. Interestingly, in contrast with a recent report [36], we could observe the inability to interact and function with D2.

One of the most specific functional interactions that we assessed is between TRIM9 and UBE2G2. In the case of UBE2G2, structural and biochemical studies demonstrated that, for its specific interaction with the gp78 E3 ligase, an additional domain is required [37,38]. We still do not know whether other regions of the TRIM9 protein are implicated in this specific interaction. However, its RING sequence is peculiar as it presents an extended second loop when compared with the class D- and E-interacting TRIM proteins [4]. We could observe that overexpression of TRIM9 within the cells induces a relocalization of UBE2G2 that is recruited in a specific manner in the TRIM9 cytoplasmic bodies. TRIM9 is a neuron-specific component of a SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) complex associated with synaptic vesicle release control [39]. UBE2G2 is one of the two E2 enzymes involved in ERAD (endoplasmic reticulum-associated degradation) [40]. It is tempting to speculate that TRIM9 might co-operate with UBE2G2 in the ERAD control of membrane-associated synaptic SNARE proteins destined to the secretory pathway.

TRIM32 ubiquitin E3 activity has been already reported on several physiological substrates [41–44]. Consistent with our results, this activity requires the presence of the D and E classes of UBE2 enzymes [41,44]. We added activity with UBE2N to these results. Besides binding with the D, E and N types, we found that TRIM32 interacts in a very specific manner with UBE2V1 and V2 that are catalytically dependent on the UBE2N [25,45]. Indeed, we found that TRIM32 is a more efficient E3 ligase in the presence of UBE2N/V1 than with UBE2N alone. Interestingly, so far, no direct interaction has been reported between an E3 ligase and either UBE2V1 or V2. Our results clearly show that not all of the TRIM proteins observed to bind UBE2N also interact with UBE2V1 and V2, stressing the peculiar ability of TRIM32 in these interactions. Our immunofluorescence experiments show that TRIM32 and the aforementioned UBE2 enzymes change their reciprocal localization within HeLa cells, suggesting further that, *in vivo* also, TRIM32 may take advantage of the use of UBE2V1 and V2 for its activity. Noteworthy, the heterodimer UBE2N/V1 is specifically involved in the NF- $\kappa$ B (nuclear factor  $\kappa$ B) pathway, the same in which TRIM32 also participates through its ability to control PIASy [protein inhibitor of activated STAT (signal transducer and activator of transcription) Y] degradation [44,46].

Our results exclude the usage of L3 and L6; however, ubiquitin E3 activity using L6 has been reported for TRIM25 [11]. TRIM25 is also implicated in ISGylation, an ubiquitin-like modification, which uses L6 as UBE2 enzyme [47]. It is possible that in this case the involvement of a different UBE2 is linked to the double nature of TRIM25 and opens the potential implication of other TRIM proteins in ubiquitin-like modifications [48]. Conclusively assessing the *in vivo* role of the TRIM proteins, however, will require further efforts. Interaction with more than one UBE2 enzyme may underline their consecutive usage, especially when D and E classes are concerned, and the formation of different chains of specific linkage. The complexity of the ubiquitylation machinery in the case of the TRIM members may be complicated further by their ability not only to homointeract, but also to heterointeract; in both cases potentially offering more than one

moiety for UBE2 binding. The combination of these features in association with the control of several substrates may underscore pleiotropic effects of the TRIM protein E3 ligases.

## AUTHOR CONTRIBUTION

Luisa Napolitano contributed to the experimental design, carried out the experiments, analysed the results and helped with the drafting of the paper; Ellis Jaffray contributed to the *in vitro* assays; Ronald Hay contributed to the design of the research and the writing of the paper; Germana Meroni conceived and designed the study, supervised the project and wrote the paper with input from all of the authors.

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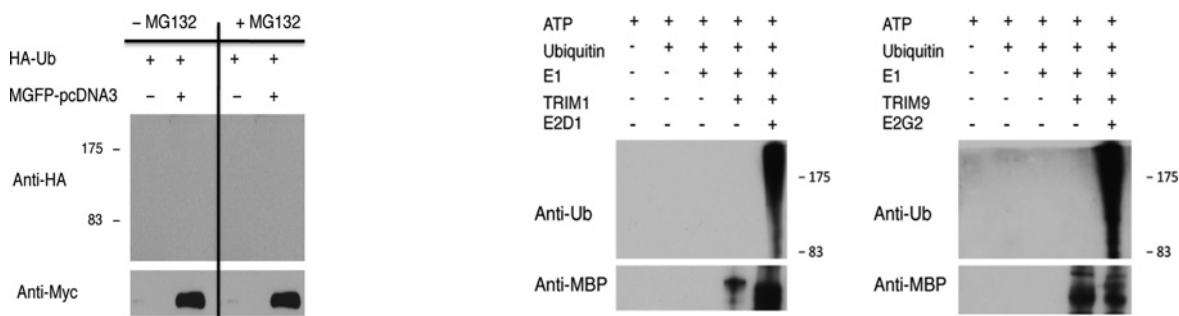
Published as BJ Immediate Publication 8 December 2010, doi:10.1042/BJ20101487

## SUPPLEMENTARY ONLINE DATA

# Functional interactions between ubiquitin E2 enzymes and TRIM proteins

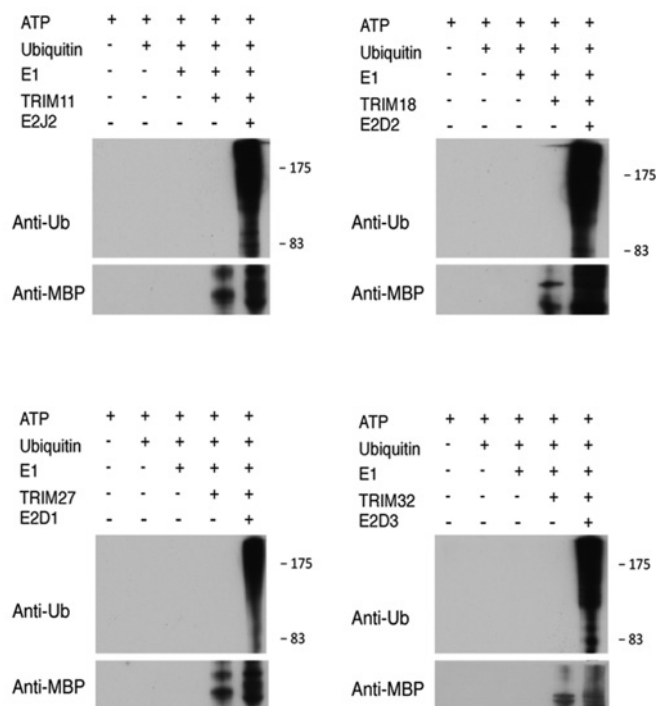
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**Figure S1** Controls for the *in vivo* ubiquitination assay

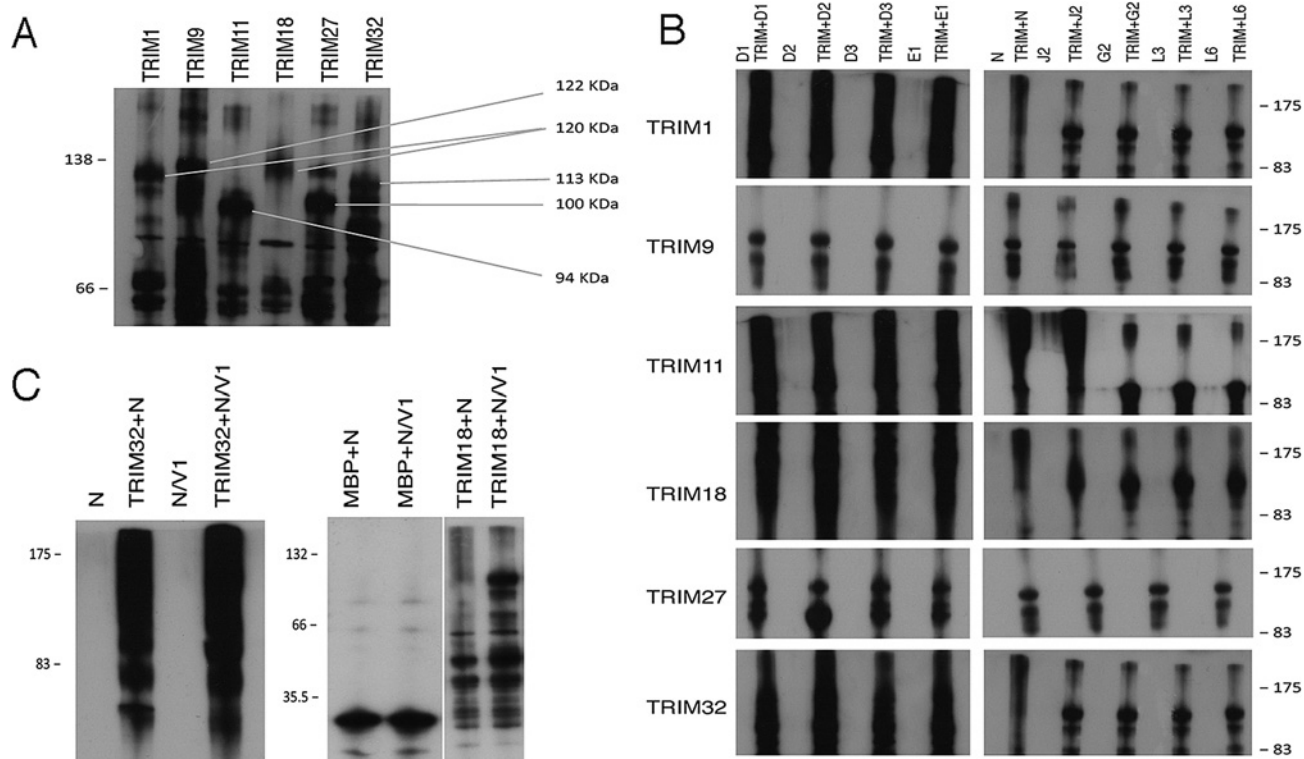
HEK-293T cells transfected with HA-ubiquitin (HA-Ub) either alone or together with Myc/GFP-pcDNA3 (MGFP-pcDNA3) vector were immunoprecipitated with anti-Myc antibody. Where indicated, 42 h after transfection, the cells were treated with the proteasomal inhibitor MG132 for 6 h. Immunoblotting with anti-HA antibody did not reveal any ubiquitinated species (upper panel). Molecular masses are indicated in kDa.



**Figure S2** Controls for the *in vitro* ubiquitination assay

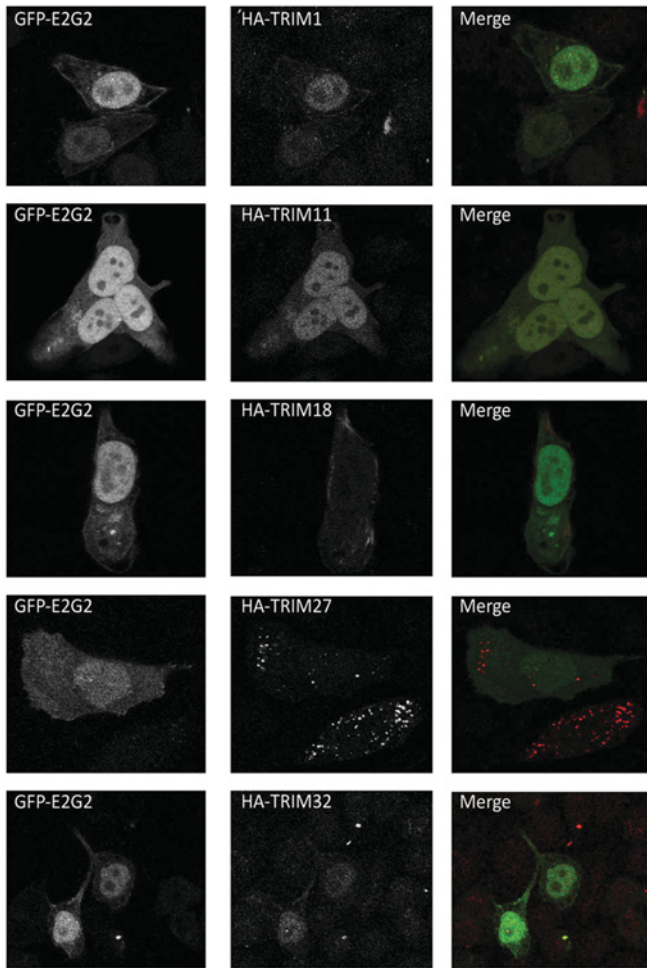
To confirm that TRIM proteins are the essential E3 enzymes in our ubiquitination reactions, for each TRIM protein tested, we set up incomplete mixtures containing different combinations of the reagents (ATP, ubiquitin, E1 enzyme, E2 enzyme and TRIM protein) as indicated. The reactions were analysed by immunoblotting using anti-ubiquitin (Anti-Ub) (upper panel) and anti-MBP (lower panel) antibodies. This confirms that ubiquitin, E1 enzyme, UBE2 enzyme and MBP-TRIM protein are all required for *in vitro* ubiquitination reaction. Consistently, lack of TRIM proteins, as well as of any other of the above reagents did not result in the detection of polyubiquitination smear (left-hand four lanes). As a positive control, ubiquitination was observed with the incubation of MBP-TRIM proteins with the complete mixture (right-hand lanes). Molecular masses are indicated in kDa.

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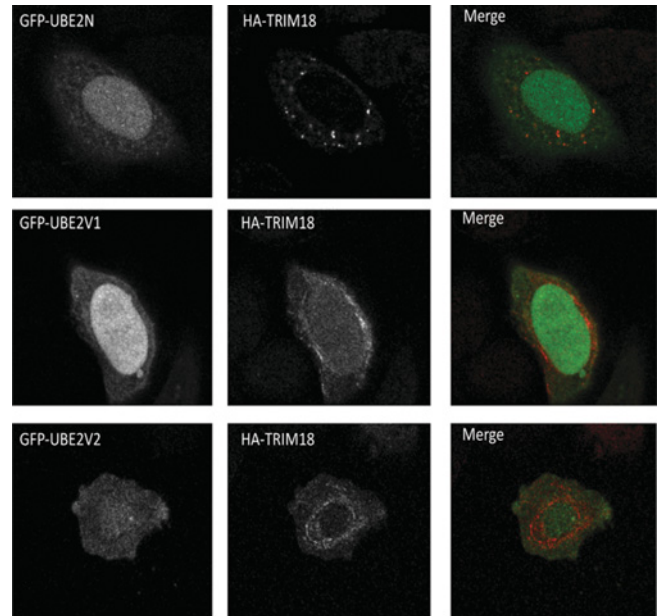
**Figure S3 Immunoblots using anti-MBP antibody**

**(A)** Anti-MBP immunoblot of the purified MBP-TRIM fusion proteins. The full-length MBP-TRIM proteins and their molecular masses (in kDa) are indicated. **(B)** Anti-MBP immunoblot of the *in vitro* ubiquitylation reactions described in the text and revealing the extent of self-ubiquitylation. As shown in **(A)**, the anti-MBP highlights high-molecular-mass species that are nevertheless increased when polyubiquitylation occurs. The results with MBP-TRIM1, MBP-TRIM11, MBP-TRIM18 and MBP-TRIM32 matched perfectly with what was observed with the anti-ubiquitin antibody (Figure 4 of the main text), indicating that self-ubiquitylation is mainly occurring. This is not the case with MBP-TRIM9 and MBP-TRIM27, where we detected reduced self-polyubiquitylation. **(C)** Anti-MBP immunoblot of the ubiquitylation reactions of TRIM32 with the UBE2N/V1 complex.



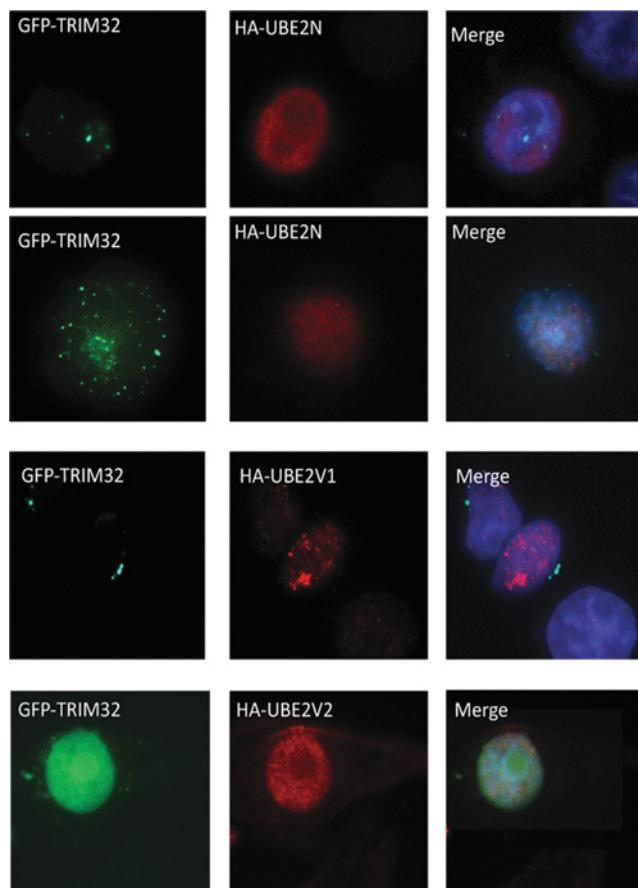
**Figure S4 Immunofluorescence of the UBE2G2 enzyme with TRIM1, 11, 18, 27 or 32 in HeLa cells**

To prove that UBE2G2 recruitment by TRIM9 is specific, we repeated the same experiment shown in Figure 5(B) of the main text using the other TRIM proteins as controls. We confirmed that UBE2G2 was never localized in these TRIM-defined subcellular structures.



**Figure S5 Immunofluorescence of TRIM18 with UBE2N, V1, or V2 in HeLa cells**

To prove the specificity of TRIM32 co-localization with the above UBE2 enzymes, we repeated the same experiment shown in Figures 5(E)–5(J) of the main text using TRIM18 as a control. In contrast with TRIM32, TRIM18 maintains its cytoplasmic localization also in the presence of the different UBE2 enzymes.



**Figure S6 TRIM32 co-localises with UBE2N, V1 and V2 proteins**

The same experiments shown in Figures 5(E)–5(J) of the main text were performed using the reciprocally tagged constructs as indicated (GFP–TRIM32 and HA–UBE2N, V1 and V2) confirming the partial relocalization of TRIM32 by UBE2N (upper panels) and UBE2V2 (lower panels).

**Table S1 Ube2 enzymes used in the present study**

Official symbol	Other alias(es)	Gene identifier
UBE2A	HHR6A, RAD6A, UBC2	7319
UBE2B	HR6B, UBC2, RAD6B, HHR6B, E2–17KDa	7320
UBE2C	RP3-447F3.1, UBCH10, dJ447F3.2	11065
UBE2D1	E2(17)KB1, SFT, UBC4/5, UBCH5, UBCH5A	7321
UBE2D2	E2(17)KB2, PUBC1, UBC4, UBC4/5, UBCH5B	7322
UBE2D3	E2(17)KB3, MGC43926, MGC5416, UBC4/5, UBCH5C	7323
UBE2D4	HBUCE1	51619
UBE2E1	UBCH6	7324
UBE2E2	FLJ25157, UBCH8	7325
UBE2E3	UBCH9, Ub-M2	10477
UBE2F	MGC105540, RGD1307608	363284
UBE2G1	E217K, UBC7, UBE2G	7326
UBE2G2	1110003005Rik, D10Xrf369, UBC7, Ubc7p	22213
UBE2H	E2–20K, UBC8, UBCH, UBCH2	7328
UBE2J1	CGI-76, HSPC153, HSPC205, NCUBE1, Ubc6p	51465
UBE2K	AW492011, D5Ert601e, E2-25k, Hip2, Hyg, Lig	53323
UBE2L3	E2-F1, L-UBC, UBCH7, UbcM4	7332
UBE2L6	MGC40331, RIG-B, UBCH8	9246
UBE2M	UBC-RS2, UBC12, hUbc12	9040
UBE2N	UBC13	541130
UBE2Q1	GTAP, NICE-5, PRO3094, UBE2Q	55585
UBE2R1	cdc34	216150
UBE2S	E2-EPF, E2EPF, EPF5	27338
UBE2T	HSPC150, PIG50	29089
UBE2V1	CROC1, A1256840, 0610011J09Rik, Ube2v1	66589
UBE2V2	MMS2, UEV-2, UEV2	7336