

## Functional interactions between Ubiquitin E2 enzymes and TRIM proteins

Luisa M. Napolitano<sup>\*,†</sup>, Ellis G. Jaffray<sup>‡</sup>, Ronald T. Hay<sup>‡</sup>, Germana Meroni<sup>\*,1</sup>

<sup>\*</sup> Cluster in Biomedicine (CBM), AREA Science Park, Trieste, Italy

<sup>†</sup> Telethon Institute of Genetics and Medicine, Naples, Italy

<sup>‡</sup> Wellcome Trust Centre for Gene Regulation and Expression, College of Life Science, University of Dundee, Dundee, Scotland, UK

<sup>1</sup> Corresponding author:

Germana Meroni

Cluster in Biomedicine (CBM), AREA Science Park,

s.s. 14 km 163.5 Basovizza

34149 Trieste (Italy)

phone +39 040 3757718

fax +39 040 3757710

Email: germana.meroni@cbm.fvg.it

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## ABSTRACT

The TRIM 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. While 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 enzymes or UBE2s. Here, 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-UBE2G2 and TRIM32-UBE2V1/2. Furthermore, we demonstrated that the TRIM E3 activity is only manifest with the UBE2 they interact with. For most specific interactions we could also observe subcellular co-localisation 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 catalyzed by the TRIM E3 ligases to determine the fate of their targets.

## Keywords

TRIM, Ubiquitin conjugating E2 enzymes, Ubiquitin E3 ligases, Ubiquitylation, RING domain

## Abbreviations

TRIM, Tripartite Motif; UBE2, Ubiquitin Conjugating Enzymes; MBP, Maltose Binding Protein; GFP, Green Fluorescent Protein.

## INTRODUCTION

The proteins that belong to the tripartite motif (TRIM) family, also known as the RBCC 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 involved in the pathway of ubiquitin modification [3, 6].

Ubiquitylation is a form of post-translational modification in eukaryotes in which ubiquitin, a highly evolutionary 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 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 E3s act as scaffolds that bring the ubiquitin-charged E2 and the substrate in close proximity allowing ubiquitin transfer, whereas HECT-type E3s 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, 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 approximately 600 E3 coding genes, 35 - 40 genes encode putative E2 proteins, some of which are only defined by homology to well characterized E2 enzymes. The E2 enzymes are constituted by a 150-residue-ubiquitin conjugating core (UBC) domain and, depending on the presence of N-terminal or C-terminal tails, or both, they are grouped in 4 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 E2/E3 specific interactions and how this is achieved and regulated. Recently, Markson et al. and van Wijk et al. 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 [18, 19]. In the case of the TRIM proteins, even when the E3 activity is assessed, little is known about the specific UBE2 partner usage. Therefore, we sought to functionally define the E3/E2 specific interactions focusing on the TRIM family. Here, we report that almost all

TRIM family members directly interact 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 this work were already available in our laboratory in several vectors [1]. The cDNAs encoding the full-length ORF of Ubiquitin Conjugating Enzymes E2 (Supplementary Table 1), UIP48 [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 HEK293T cell cDNA. The cDNAs were cloned in the two-hybrid vectors (pEG202 and pJG4-5) and in HA- and MycGFP-pcDNA3 vectors.

**Two-hybrid analysis.** Binary two-hybrid screening was performed as described [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 promoter. Prey plasmids (pJG4-5) express the cDNA fused to the B42 activation domain, the SV40T 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 immunoblot using anti-HA and anti-LexA antibodies (data not shown). EGY42/EGY48 diploid strain was generated by mating for every pair-wise combination. Six LexA-operators *lacZ* in the pSH18-34 vector and a genome integrated 4 LexA-operators *LEU2* are used as reporters. The expression of two reporters was used to establish the interaction, blue-turning colonies and growth in the absence of Leu. The arbitrary score of Figure 1 was calculated as the fraction of detected interactions on the number of the experiments (at least 7 performed for each pair tested) time 0.5 if the interaction was observed with only one reporter gene.

**Cell culture and Immunoblot.** HEK293T cells were grown in Dulbecco's modified Eagle medium with 10% FBS (Invitrogen), 100 µg/ml of Streptomycin and 100 units/ml of penicillin in a 5% CO<sub>2</sub> atmosphere at 37°C. Cells were transfected by the calcium phosphate precipitation method [22]. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After blocking with 5% skim milk in 20 mM Tris 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), sheep anti-MBP (1:1000, Santa Cruz), or rabbit anti-ubiquitin (1:5000; Bethyl Laboratory). Blots were washed three times with Tris-buffered saline/ Tween 20 0.1% and incubated with either peroxidase-conjugated anti-mouse IgG antibody (1:3000, GE Healthcare), anti-rabbit IgG antibody (1:3000, GE Healthcare) or anti-sheep IgG (1:3000, GE Healthcare), then developed with a chemiluminescence detection system (ECL, BioFX Laboratories).

**Immunofluorescence.** Hela cells were seeded on glass cover slips in 12-multi well plates the day before transfection with Lipofectamine 2000 (Invitrogen) to obtain low expression level. Twenty-four hours after transfection the cover slips were washed in cold PBS and cells were fixed with 4% 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% Bovine Serum Albumine (BSA), 0.1% Tween in PBS for 1 hour. Cover slips were then incubated with anti-HA

monoclonal antibody (1:500, Roche) for 2 hours. After washing, cover slips were incubated with Cy3-conjugated anti-mouse secondary antibody (1:100, Jackson laboratories). Slides were mounted using Vectashield with DAPI (Vector Laboratories); images were acquired on 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 grey scale or dual colour TIFF images in Adobe Photoshop.

**Protein expression and purification.** TRIM proteins were cloned in the prokaryotic expression vectors pMAL-c2x (New England Biolabs) and expressed in *Escherichia coli* Rosetta (DE3) cells (Novagen). For each TRIM protein, when at OD<sub>600</sub> of 0.8, 1L of cell culture was induced with 150  $\mu$ M IPTG and grown overnight at 24°C. Then, cells were harvested and flash-frozen. 50 ml lysis buffer containing 50 mM Tris-HCl (pH7.5), 500 mM NaCl and 0.5 mM  $\beta$ -mercaptoethanol was added for litre of culture. After sonication, cell lysates were cleared using a JA-12 Beckman rotor (10,000g, 30 min, 4°C) and proteins were purified using an amylose-bound chromatography. The correct production of the MBP-TRIM protein was checked on SDS-PAGE upon Coomassie staining.

**MBP pull-down assays.** MBP-TRIM proteins were immobilized on amylose resin (New England Biolabs) and incubated with approximately 5 $\mu$ g of HEK293T crude extract transiently transfected with MycGFP-UBE2 enzymes for 4 hrs at 4°C in Lysis Buffer (20 mM Tris pH 7.5, 20% Glycerol, 50mM NaCl, 5mM EDTA pH8, 0.1% Triton, 1.5mM PMSF and 1 mg/ml aprotinin, leupeptin and pepstatin). The resin was washed three times with Lysis Buffer; bound proteins were separated on SDS-PAGE and UBE2 enzymes visualized with immunoblotting using the anti-Myc antibody. MBP-TRIM proteins were visualized with Coomassie staining.

***In vitro* ubiquitylation assays.** The *in vitro* ubiquitylation assays were carried out in a volume of 15  $\mu$ L containing 34 nM Uba1, 0.8  $\mu$ M E2-conjugating enzyme, 35  $\mu$ M ubiquitin, 5 mM MgCl<sub>2</sub>, 2 mM ATP, 150 mM NaCl, 0.5 mM TCEP, 0.1% NP40 and 2.2  $\mu$ M for each MBP-TRIM protein in 50 mM Tris-HCl pH7.5. MBP protein was used at 2  $\mu$ M. After incubation at 37°C for 2 hrs, the reactions were terminated by the addition of Laemmli sample buffer and analysed by SDS-PAGE. Immunoblots using anti-Ubiquitin and anti-MBP antibodies were performed.

***In vivo* ubiquitylation assays.** HEK293T cells were co-transfected with HA-Ubiquitin and MycGFP-TRIM proteins. When applicable, 42 hrs 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 further cultured 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% DOC, 1% NP-40, 1.5 mM PMSF and 1 mg/ml aprotinin, leupeptin and pepstatin). DNA in the sample was sheared with a 22-gauge needle and the lysate was centrifuged using a Sigma 12024H rotor (15,000g, 30 min, 4°C). The supernatant was then incubated with 1 $\mu$ g anti-Myc antibody (9E10 Santa-Cruz) over-night at 4°C. After adding protein A-Sepharose beads (Sigma), complexes were washed with lysis buffer. Immunoprecipitates were separated on SDS-PAGE and analyzed 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 if TRIM proteins could act as RING E3s, we tested the interaction between 42 TRIM proteins (www.trimbase.tigem.it) against 26 of the best studied UBE2 enzymes (Supplementary Table 1) in a yeast two-hybrid system (Figure 1A). Each full-length protein was tested both as LexA-DBD-fusion and B42-AD-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 (D1-4) and E (E1-3) families of UBE2 enzymes while 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 (data not shown) [1] and 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, 5 are 'unorthodox' TRIM members that lack the RING domain [4]. Consistent with the role of this domain in mediating UBE2 binding, these 5 TRIM proteins did not show any interactions with E2 enzymes (Figure 1A). To further confirm 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 (Figure 1B and 1C).

Taken together, these data 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 6 TRIM proteins both as full-length and as UBE2 binding incompetent RING deleted forms. TRIM1, 9, 11, 18, 27, 32 were selected as representatives of the different specificities in UBE2 binding. Some of the selected TRIM proteins show very specific binding while others are more promiscuous in 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 MycGFP-tagged wild-type TRIM proteins and their RING deletion mutants were co-transfected in HEK293T cells together with HA-tagged ubiquitin. TRIM proteins were immunoprecipitated with the anti-Myc antibody and analyzed 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

MycGFP-TRIM proteins (Figure 2, lower panels). Immunoblotting using anti-HA showed a high molecular weight (HMW) smear for each TRIM proteins tested, suggesting that they are self-polyubiquitylated and/or that proteins co-immunoprecipitating with them are polyubiquitylated in mammalian cells (Figure 2, left upper panels). In contrast, the ubiquitylation observed in the presence of the corresponding RING-deleted mutant was dramatically reduced indicating that the HMW species were generated mainly through the TRIM protein E3 activity (Figure 2, left upper panels). The residual faint ubiquitylation observed in correspondence of 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 at different extent in the different experiments and, considering the apparent molecular weight, this band might represent either mono- or di-ubiquitylated forms of the TRIM protein itself or modified co-immunoprecipitated partner. HEK293T cells transfected with HA-ubiquitin, either alone or with MycGFP-empty vector, produced no ubiquitylated species upon anti-Myc immunoprecipitation (Supplementary Figure 1).

To determine whether the observed *in vivo* ubiquitylation was mainly associated to 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, right upper panels) and the total amount of the TRIM proteins tested (Figure 2, right lower panels). Interestingly, this treatment increased decisively also the ubiquitylation of TRIM27 and TRIM32 RING deletion mutants suggesting that other E3 ligases are implicated in their ubiquitylation.

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 if the UBE2 binding specificity is relevant in the TRIM ligase activity, we first confirmed the specific TRIM-UBE2 association using a maltose binding protein (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, 32, for which we also tested the *in vivo* E3 ligase activity, and 8 UBE2 enzymes (D1, D2, D3, E1, N, G2, L3, 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 HEK293T crude lysates transiently expressing MycGFP-UBE2 proteins and specific binding was revealed by immunoblot (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 HEK293T cells transiently transfected with UBE2N, V1 and V2. As already observed in yeast, TRIM32 also bound UBE2V1 and V2 in addition to UBE2N. As expected TRIM18 bound UBE2N but was unable to bind 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 catalyze poly-ubiquitylation *in vitro* by means of the different UBE2 enzymes they interact with. The above 6 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 HMW polyubiquitylated species (Figure 4). Polyubiquitylation was observed only when the recombinant MBP-TRIM protein was added to the reaction mixture (Figure 4A and Supplementary Figure 2). As the MBP-TRIM fusions are the only possible E3 in the reactions, these results further prove that these TRIM proteins function as E3 enzymes (Figure 4). The reactions were also analyzed with antibodies against the MBP portion of the 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 catalyzed (Supplementary Figure 3). Concomitantly, we also addressed the specific UBE2 usage in these reactions. The *in vitro* ubiquitylation assays were carried out in the presence of one of the following recombinant enzymes as sole UBE2: D1, D2, D3, E1, N, J2, G2, L3, L6. Figure 4A shows that incubation of MBP-TRIM1, 11, 18 and 32 in the presence of either 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 data, 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 data, 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 cooperation 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 panel).

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

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

How selection and usage of UBE2 enzymes is achieved and what are the consequences on TRIM activity within the cellular context is a more complicated issue 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 already reported, TRIM1, 9, 11, 18, 27, and 32 were mainly localised in the cytoplasm of HeLa cells either in filamentous or speckled structures [1]. UBE2D1, D2, D3, G2, N, V1, and V2 were diffusely distributed throughout both nucleus and cytoplasm (Figure 5 and data 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 TRIM defined cellular structures consistent with the fact that E2 enzymes are likely to be shared by many E3 ligases (data not shown).

However, an exception was the UBE2G2 and TRIM9 partnership. As already shown [1], TRIM9 is present in cytoplasmic speckles while 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 data, when HeLa cells were co-transfected with HA-TRIM9 and GFP-UBE2D2, the latter maintained its diffuse distribution in the cell and it was not recruited by exogenous TRIM9, supporting the specific effect on UBE2G2 (Figure 5C). To determine if other TRIM proteins could exert the same effect on UBE2G2, we also transfected HeLa cells with GFP-UBE2G2 and either HA-tagged TRIM1, 11, 18, 27, or 32. UBE2G2 was not recruited by any of the above TRIM proteins into any defined structures further supporting 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 (Supplementary Figure 4).

Moreover, we observed that TRIM32 changed its own localisation when co-transfected with either UBE2N or UBE2V2 proteins. In agreement with the literature [1], TRIM32 was localised in cytoplasmic perinuclear speckles, whereas UBE2N and UBE2V proteins were diffusely distributed 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 (Figure 5E and F, upper panels). In 40% of the cells co-transfected with GFP-UBE2N and HA-TRIM32 we observed TRIM32 localised in well-defined speckles around the nucleus. In these cells UBE2N was diffusely distributed throughout the nucleus and the cytoplasm although a fraction of UBE2N appeared to colocalise with the cytoplasmic accumulations of TRIM32 (Figure 5E and F, lower panels). The specificity of this reciprocal localisation was highlighted by the observation that HeLa cells co-transfected with the interacting pair GFP-UBE2N and HA-TRIM18 did not mutually relocate (Supplementary Figure 5). When we transfected HeLa cells with HA-TRIM32 and GFP-UBE2V1, the latter was partially recruited into TRIM32 speckles around the nucleus (Figure 5G and H). Curiously, although few cells were co-transfected with HA-TRIM32 and GFP-UBE2V2 (approximately 30%), we found that UBE2V2 relocated TRIM32 from the cytoplasm to the nucleus (Figure 5I and J). These data were confirmed in co-transfection experiments using the reciprocally tagged constructs (Supplementary Figure 6). Thus, immunofluorescence experiments suggest that interactions between E2-TRIM pairs also occurs *in vivo*.

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

## DISCUSSION

Here, 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 data 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 light of the success of the tripartite motif in evolution [4].

As a general rule, the TRIM proteins preferentially interact with the common D and E classes but not with the L class of UBE2. This is consistent with previous data showing preference of the RING domain for either of the two classes [20]. Several TRIM proteins interact also 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 among the different UBE2 enzymes bound by the same TRIM protein. Previous works utilized this technique to identify specific interactions between RING E3 ligases and UBE2 enzymes although such interactions are by nature transient and low affinity [20, 30, 31]. In particular, Christensen et al., confirmed the specificity detected with the two-hybrid analysis with NMR studies [30]. In our two-hybrid experiment, both families were analyzed 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 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 in this context. In many of the 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 recently been reported [18, 19]. In both these studies, several TRIM proteins were included in the initial two-hybrid screening. In most cases the data 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) they interact with. The binding specificity between TRIM proteins and UBE2 enzymes is perfectly translated in the 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: D1-3, 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 attitude to use D1-3, E1, N classes of UBE2 to exert this function. In the case of TRIM27, despite many biochemical findings as transcriptional and signalling regulator [34, 35], few data on the E3 activity are available. Interestingly, differently from a recent report, we could observe the inability to interact and function with D2 [36].

One of the most specific functional interaction 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 if other regions of the TRIM9 protein are implicated in this specific interactions. However, its RING sequence is peculiar as it presents an extended second loop if compared to the class D and E interacting TRIM proteins [4]. We could observe that overexpression of TRIM9 within the cells induces a relocation of UBE2G2 that is recruited in a specific manner in the TRIM9 cytoplasmic bodies. TRIM9 is a neuron specific component of a SNARE complex associated with synaptic vesicle release control [39]. UBE2G2 is one of the two E2 enzymes involved in endoplasmic reticulum-associated degradation (ERAD) [40]. It is tempting to speculate that TRIM9 might cooperate 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, the above activity requires the presence of D and E classes of UBE2 enzymes [41, 44]. We added activity with UBE2N to these data. 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 data clearly show that not all 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 localisation within HeLa cells further suggesting that also *in vivo* TRIM32 may take advantage of the use of UBE2V1 and V2 for its activity. Noteworthy, the heterodimer UBE2N/V1 is specifically involved in NFκB pathway, the same in which TRIM32 also participates through its ability to control Piasy 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 enzymes

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 further complicated 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.

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## FIGURE LEGENDS

**Figure 1.** TRIM proteins directly interact with UBE2 enzymes. A) Schematic representation of the results obtained with the binary two-hybrid system. The TRIM and the UBE2 (E2) clones tested are indicated; the letters below the E2s indicate the direction of the two-hybrid experiments: a, B42-AD and b, LexA-DBD. The asterisk indicates the RING-less TRIM proteins. The interaction strength and reproducibility are indicated by arbitrary scores in the range of 0-1, represented also by the color-scale shown at the bottom. Grey cells indicate no interaction; white cells indicated non-tested pairs. B and C) Two-hybrid panels showing either TRIM18 (B) or TRIM32 (C) domain deleted mutants, shown in the bottom scheme, against the UBE2 enzymes indicated. Blue colonies in X-gal plates (Xgal) and growth on plates lacking leucine (Leu) in the presence of Galactose (Gal) represent positive interaction.

**Figure 2.** E3 ligase activity of TRIM proteins in mammalian cells. *In vivo* ubiquitylation assay of MycGFP(MGFP)-tagged TRIM1, TRIM9, TRIM11, TRIM18,

TRIM27 and TRIM32 full-length (FL) and RING deleted ( $\Delta$ R) forms in HEK293T cells in the presence of HA-ubiquitin (HA-Ub) as indicated. 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) are shown. Asterisks indicate the predominant band(s) observed in the anti-HA immunoblots (see text). Where indicated, cells were treated with the MG132 proteasomal inhibitor.

**Figure 3.** MBP pull-down assay confirms UBE2 and TRIM proteins specific interactions. A) MBP pull-down analysis of eight MycGFP-UBE2 enzymes transiently expressed in HEK293T 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. Immunoblot with anti-Myc antibody of the input lysates is shown in the upper 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 HEK293T cells (N, V1 and V2 as indicated) with MBP-TRIM32 and MBP-TRIM18. MBP was used as control. Legend as in A).

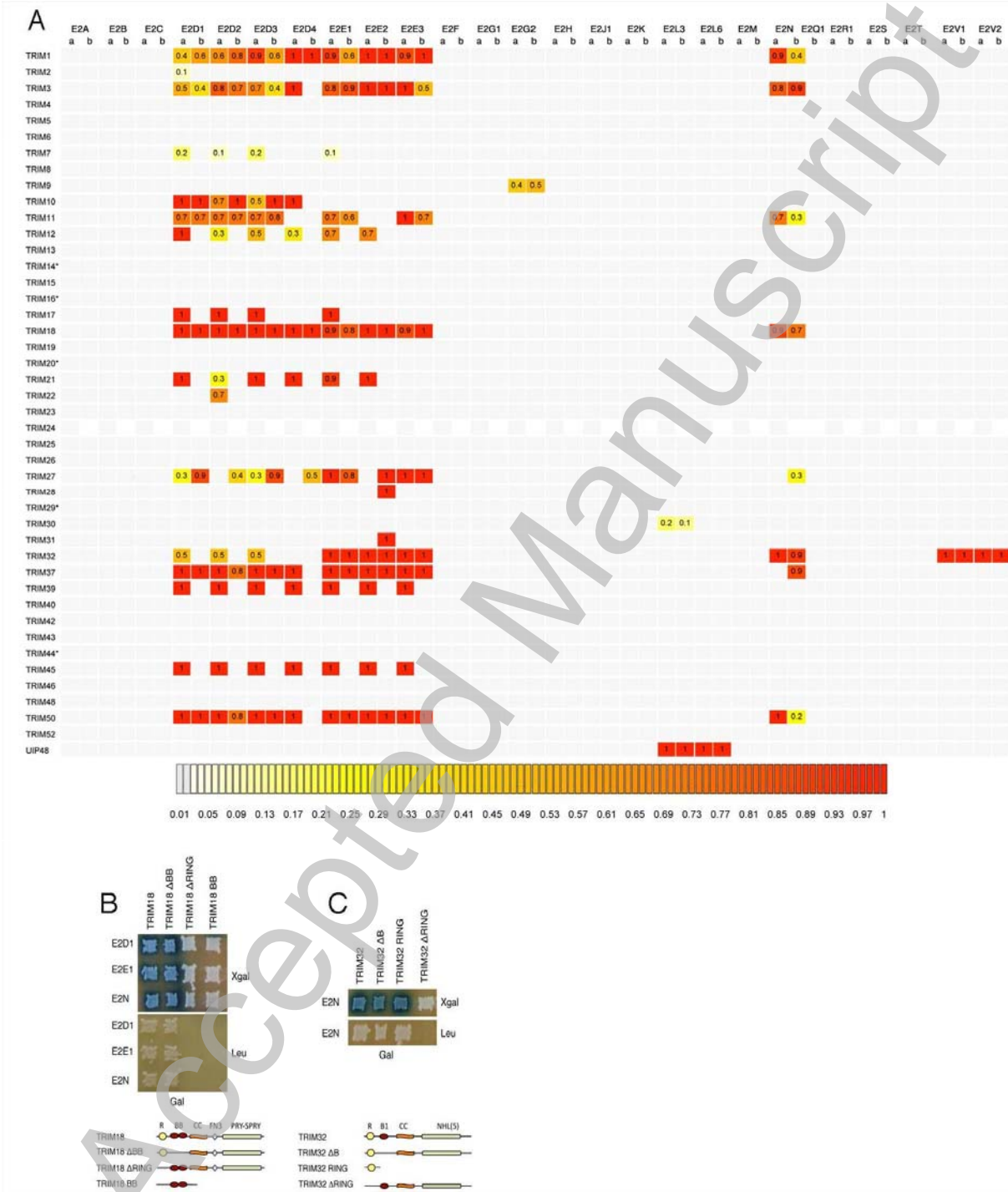
**Figure 4.** TRIM proteins act as ubiquitin E3 ligases *in vitro* using the interacting UBE2 enzymes. A) MBP-TRIM proteins (TRIM1, 9, 11, 18, 27 and 32) were tested for E3 ligase activity in *in vitro* ubiquitylation assay in the presence of the UBE2 enzymes indicated (TRIM+D1, D2, D3, E1, N, G2, J2, L3, L6). As control, the assay was performed without the TRIM protein (lanes with only UBE2 indicated). Immunoblot with anti-Ubiquitin antibody to detect the ubiquitylated species is shown. B) *In vitro* ubiquitylation assay using UBE2N and V1, in the combination indicated, in the presence of MBP-TRIM32. As control, the assay was performed without the TRIM protein, with MBP and with MBP-TRIM18 as indicated. Immunoblots with anti-ubiquitin antibody are shown.

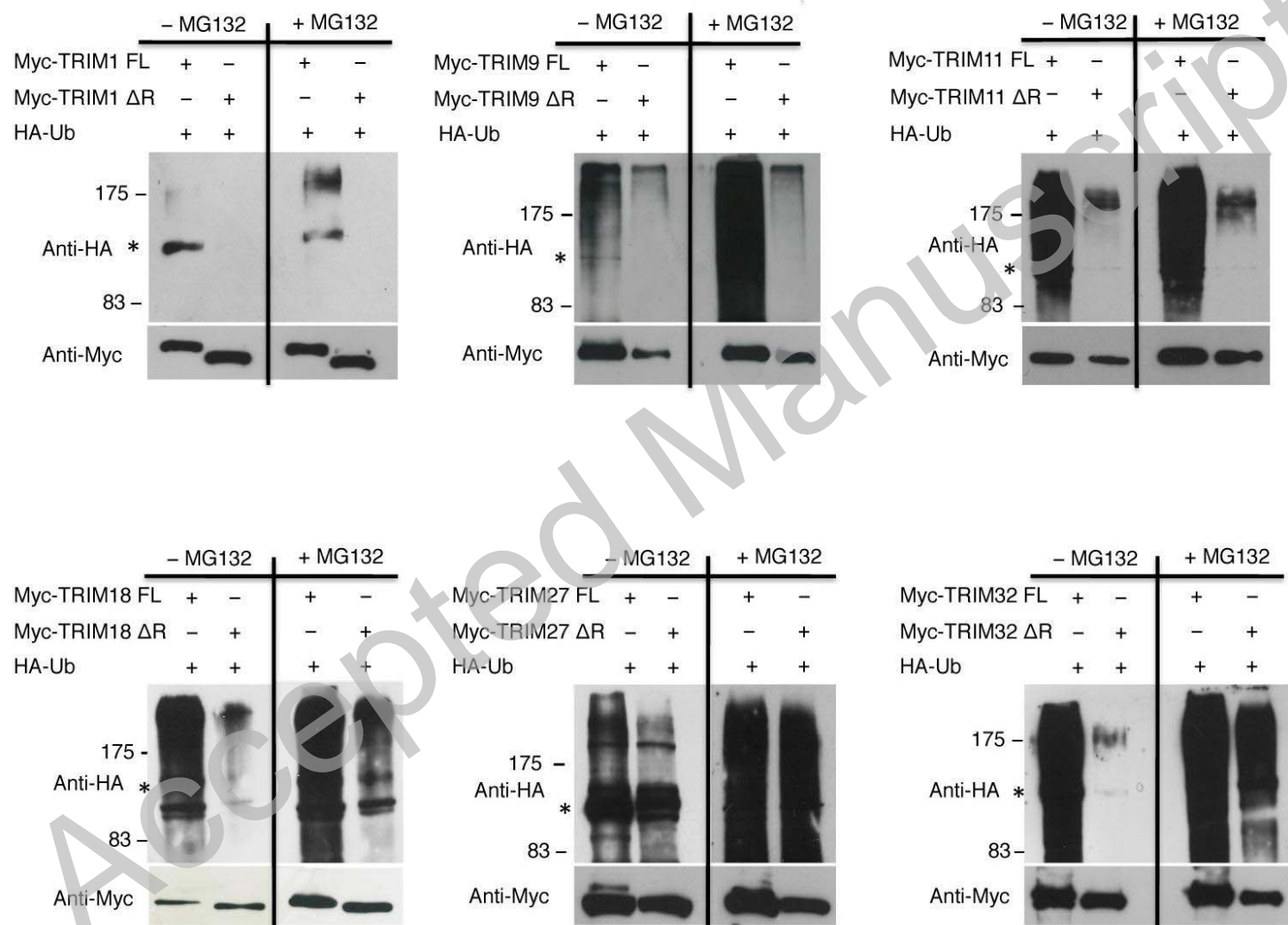
**Figure 5.** TRIM-UBE2 co-localisation in mammalian cells. A-C) TRIM9 co-localises with UBE2G2 in cytoplasmic speckles. A) Immunofluorescence of HeLa cells transfected with either GFP-TRIM9 (upper panel) or GFP-UBE2G2 (lower panel). B) Immunofluorescence of HeLa cells co-transfected with GFP-UBE2G2 (left panel) and HA-TRIM9 (middle panel). The right panel represents the overlay of left 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 panel) and GFP-TRIM9 (middle panel). The right panel represents the overlay of left and middle images. D-J) TRIM32 co-localises with UBE2N, V1, and V2 proteins. D) Immunofluorescence of HeLa cells single transfections with: GFP-TRIM32, HA-UBE2N, GFP-UBE2V1, GFP-UBE2V2. E) Immunofluorescence of HeLa cells co-transfected with GFP-UBE2N (left panels) and HA-TRIM32 (middle panels). The right panels represent the overlay of left 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 panel) and HA-TRIM32 (middle panel). The right panel represents the overlay of left 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. Legend as in G). J) Confocal microscopy images of experiments as in (I).

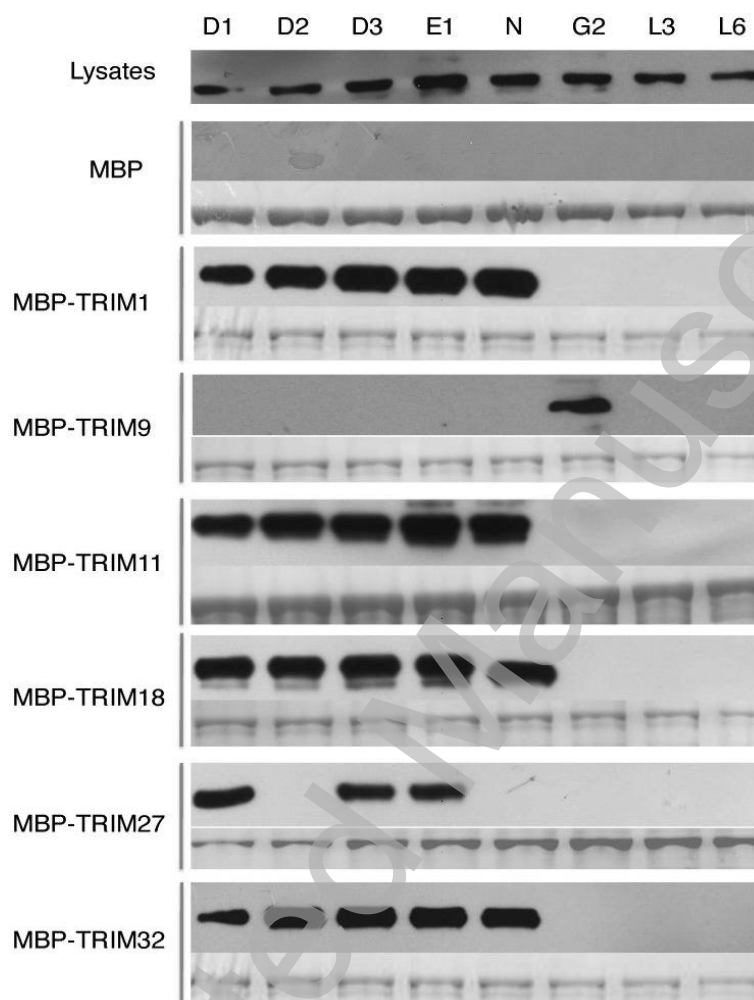
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**A**



**B**

