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Characterisation of human RING finger protein TRIM69, a novel testis E3 ubiquitin ligase and its subcellular localisation

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ABSTRACT

The E3 ubiquitin ligase activity and subcellular localisation of human TRIM69 (hTRIM69) gene were studied. It was found that hTRIM69 mediated ubiquitination in an E2 conjugating enzyme selective fashion in vitro and an intact RING finger domain was indispensible for the process. Further evidences showed that hTRIM69 could mediate ubiquitination in vivo, which could be enhanced by a proteasome inhibitor. hTRIM69 was found to localise in both the cytoplasm and the nucleus in a speckled aggregating pattern, which also required an intact RING finger domain. Collectively, hTRIM69 is a novel E3 ubiquitin ligase identified from human testis and may function to ubiquitinate its particular substrates during spermatogenesis.

1. Introduction

Spermatogenesis is a highly precise temporally regulated process of stem cell spermatogonia to mature spermatozoa in which ubiquitination is involved in almost every stage, including early spermatogonia cell proliferation, spermatocyte development, and the cellular remodelling stage of spermiogenesis differentiation [1].

In previous studies, we identified a series of differentially expressed genes in spermatogenesis through a system combining laser capture microdissection (LCM) and suppressive subtractive hybridisation (SSH) [2–4]. In this study, we isolated a novel gene using this system from the SSH library of human round spermatid-specific cDNA against those of human primary spermatocytes. This novel gene was demonstrated to be the ORF of the human TRIM69 gene (hTRIM69) and assigned GenBank accession No. AY305385. *hTRIM69* maps to chromosome 15q21 and consists of eight exons and seven introns. *hTRIM69* encodes for a member of the TRIM family proteins, which are characterised by a RING finger domain, one or two B-boxes, and an associated coiled-coil region.

There are currently more than 70 known TRIM proteins in humans and mice. On the basis of a variable C-terminus, TRIM proteins can be classified into XI subfamilies [5]. hTRIM69 contains a B30.2 domain and belongs to the C-IV subfamily. TRIM proteins are implicated in various cellular functions, including differentiation, apoptosis, and immunity [6]. The TRIM family represents a class of 'single protein RING finger' E3 ubiquitin ligases [7], which are defined as the largest subfamily of RING finger E3s [8]. Therefore, we inferred TRIM69 might possess an E3 ubiquitin ligase activity.

In our study, hTRIM69 displayed an E3 ubiquitin ligase activity in a RING finger domain-dependent manner. In addition, there are several reports that the subcellular localisations of TRIM family proteins are associated with their functional variations [9,10]. Therefore, we also investigated the subcellular localisation of hTRIM69. In contrast with a RING finger motif mutant or truncation, we found that an intact RING finger motif was also required for hTRIM69 to properly subcellularly localise and to aggregate. In summary, the identification of hTRIM69's E3 ubiquitin ligase activity and its subcellular localisation will contribute to further studies of its function as an E3 ubiquitin ligase during spermatogenesis.

2. Materials and methods

2.1. Plasmids and constructs

cDNA coding sequence of the full-length open reading frame of hTRIM69 was amplified from a human testis cDNA library using RT-PCR with the corresponding primers: (forward primer: GGATCCATGGAGGTATCCACCAAC; reverse primer: CTCGAGTTACTTGTCATCGTCGTCCTTGTAGTCCATCTGTGGATGTAAGATGTG). The PCR products were cloned into the pGEM-T1 vector (Invitrogen, Carlsbad, CA), which was confirmed by sequencing. Wild type hTRIM69

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(hTRIM69WT)was generated by PCR using pGEM-T1-hTRIM69 as a template and then subcloned into a pcDNA6/V5-HisB vector (Invitrogen) at *BamH*I/*Xho*I sites with a N-terminal Flag tag (between *Nde*I and *Hind*III) for expressing Flag-hTRIM69WT. hTRIM69CA (a hTRIM69 point mutant) was generated by PCR using site-directed mutagenesis. In detail, the cDNA of wild-type hTRIM69 was mutated to Ala at Cys-61 and Cys-64 and then subcloned into pcDNA6/V5-Flag-HisB. The following were the relative primers used for the site-directed mutagenesis (forward primer: CAC-AACTTCG CTGAAGCCGCTATCCAAGAC; reverse primer: GTCT TGGATAGCGGCTTCAG CGAAGTTG). hTRIM69ΔR (a deletion of hTRIM69WT from amino acids 1 to 82) was generated by PCR using pGEM-T1-hTRIM69 as a template and then subcloned into pcDNA6/V5-Flag-HisB. The coding regions of hTRIM69WT, hTRIM69CA, and hTRIM69ΔR were subcloned into the pGEX-4T-3 vector at the *BamH*I/*Xho*I sites and into the pEGFP-N1 vector at the *Xho*I/*BamH*I site.

2.2. Antibodies

A rat polyclonal antibody was generated against a polypeptide of the hTRIM69 protein, from amino acids 482 to 495 (PCLNDGGEN-KEPLH). Other antibodies were purchased from companies: anti-Flag and anti-HA (Sigma–Aldrich, St. Louis, MO); anti-ubiquitin, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA); anti-laminA/C (Epitomics) anti-GST (MBL, Woburn, MA).

2.3. Cell culture and transfection

HEK293T and HeLa cells were purchased from the ATCC and cultured under an atmosphere of 5% CO_2 (37 °C) in fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum. The plasmids were transfected into HEK293T cells using the Engreen transfection reagent (Engreen Biosystem Co. Ltd, CN) and into the HeLa cells using Lipofectamine 2000 (Invitrogen).

2.4. In vitro ubiquitination assay

For the assay, one microgram of bacterially produced GST-hTRIM69WT, GST-hTRIM69CA, or GST-hTRIM69ΔR was purified and then incubated in a reaction mixture including: 100 nM human recombinant E1 (ubiquitin-activating enzyme, Sigma–Aldrich), 1 μM each of the different E2 enzymes (ubiquitin conjugating enzyme from Calbiochem and BostonBiochem), 5 μM His-ubiquitin (Sigma–Aldrich), 5 mM ATP, 2.5 mM $MgCl_2$, 1 mM DTT and 50 mM Tris–HCl (pH 7.5), to a final volume of 10 μl for 1 h (37 °C). After terminating the reactions with 10 μl of 2 × SDS sample loading buffer, the reaction products were fractionated by SDS–PAGE (8%) and analysed by immunoblotting with anti-ubiquitin and anti-GST antibodies [11].

2.5. Immunoprecipitation and in vivo ubiquitination assays

Various hTRIM69 recombinant plasmids were transfected into HEK293T cells with or without the HA-Ub expression plasmid. The cells were collected 48 h post-transfection. For the protein stability experiment, at 42 h post-transfection HEK293T cells were treated with the proteasome inhibitor MG132 (20 μM) for 6 h and then harvested for immunoprecipitation. HEK293T cells were lysed with lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 1% SDS), boiled at 100 °C for 10 min and centrifuged at 15.7 × 1000*g* for 15 min at room time. The supernatant (1 mg) was diluted at a ratio 10:1 with dilution buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP40), then added to Flag-M2-agarose (Sigma–Aldrich) and incubated for 1 h at room time. The immunoprecipitates were washed five times with wash buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 0.1% SDS), then subjected to 8% SDS–PAGE and analysed by immunoblotting with anti-HA and anti-Flag antibodies.

2.6. Immunofluorescence analysis

HeLa cells were transfected with Flag-hTRIM69WT, Flag-hTRIM69CA, Flag-hTRIM69ΔR or GFP-hTRIM69WT using the Lipofectamine 2000 reagent. Post-transfection (24 h), cells on coverslips were fixed in 4% formaldehyde for 15 min, permeabilised with 0.5% TritonX-100 in PBS for 10 min, and blocked with 3% BSA in PBS for 30 min (37 °C). The cells were incubated with a mouse anti-Flag primary antibody followed by an Alexa Fluor 594-labelled secondary antibody. Nuclei were counterstained with DAPI (1 μg/ml). Cell immunofluorescence was visualised using confocal microscopy.

2.7. Cell fractionation

HeLa cells were transfected with Flag-hTRIM69WT, Flag-hTRIM69CA, Flag-hTRIM69ΔR or GFP-hTRIM69WT using the Lipofectamine 2000 reagent. Post-transfection (24 h), the cells were collected to separate cytoplasmic and nuclear proteins using a Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) according to the manufacturer's instructions. The extraction was then subjected to SDS–PAGE and analysed by immunoblotting with anti-Flag, anti-tubulin, anti-GAPDH, anti-laminA/C, and a rat anti-hTRIM69 polyantibody.

3. Results

3.1. hTRIM69 catalysed the formation of multiubiquitinylated products

To determine whether hTRIM69 can mediate ubiquitination, purified GST-hTRIM69 produced from *Escherichia coli* was incubated with recombinant E1 enzyme and different recombinant E2s (UbcH6, UbcH1, UbcH2, UbcH3, UbcH5A, UbcH5B, UbcH5C, UbcH7 or UbcH13/UeV1a) in the presence of His-tagged ubiquitin. As shown in Fig. 1A, GST-hTRIM69 catalysed the formation of multiubiquitinylated products only in the presence of UbcH6, UbcH2, UbcH5A UbcH5C, or UbcH13/UeV1a. No products was detected with the other E2s. Because the reaction buffers used in the assays were free of any E3 enzymes other than GST-hTRIM69, these results indicated that hTRIM69 functions as an E3 ubiquitin ligase in cooperation with specific E2s.

To further confirm the role of hTRIM69 in the 'in vitro ubiquitination assay, we determined the minimum requirements for the assay by using one of the selected E2s (UbcH6, UbcH2, UbcH5A, UbcH5C or UbcH13/UeV1a) listed in Fig. 1A. Here, we selected UbcH6 as the E2 enzyme to satisfy the minimum assay requirements. The ubiquitination reaction requires the presence of ATP, E1 enzyme, E2 enzyme, and E3 enzyme. To confirm whether these components are essential for the ubiquitination reaction, hTRIM69 was used as the E3 ligase and incubated in the complete reaction mixture containing ATP, ubiquitin, recombinant E1 enzyme, and UbcH6. Other reactions were performed in incomplete reaction mixtures lacking one of these components. As shown in Fig. 1B, ubiquitinylated products could be detected after incubating hTRIM69 in the complete reaction mixture, whereas no products was detected in the incomplete reaction mixtures. These results further suggest that hTRIM69 possesses an E3 ligase activity and might mediate the formation of multiubiquitinylated products.

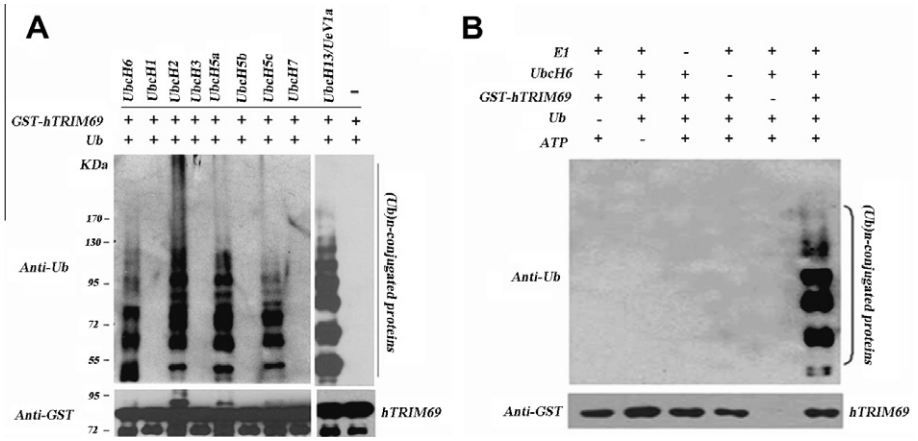

Fig. 1. hTRIM69 mediates E2-dependent ubiquitination in vitro. (A) In vitro ubiquitination assay to screen for E2 collaborating with hTRIM69. GST-hTRIM69WT was incubated with ubiquitin E1 and various E2 enzymes (UbcH6, UbcH1, UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH5c, UbcH7, or UbcH13/Uev1a) or without the E2 enzyme at 37 °C for 1 h, as described in Section 2. The reaction products were resolved by 8% SDS–PAGE and immunoblotted using anti-ubiquitin and anti-GST antibodies. (B) Minimum requirements for the E3 ubiquitin ligase activity of hTRIM69 in vitro. The complete reaction mixture for the 'in vitro ubiquitination assay' was composed of the following components: E1; UbcH6; GST-hTRIM69WT; ubiquitin; and ATP. To confirm the minimal requirements for the E3 ligase activity of hTRIM69, we also performed the assay in incomplete reaction mixtures lacking one of these components. After terminating the reactions, the reaction products were fractionated by SDS–PAGE (8%) and analysed by immunoblotting with anti-ubiquitin and anti-GST antibodies.

3.2. RING finger domain-dependent E3 ubiquitin ligase activity of hTRIM69

hTRIM69 possesses a RING-consensus sequence rich in cysteine residues between amino acid residues 41 and 81. The conserved cysteine (Cys) and histidine (His) residues coordinate with two zinc atoms to maintain the RING domain structure (Fig. 2A). The RING domain plays a critical role in mediating the transfer of ubiquitin to the substrates. To address whether the RING finger domain of hTRIM69 is responsible for its E3 ubiquitin ligase activity, the two adjacent cysteine residues at the conserved zinc-binding position (amino acids 61 and 64) were mutated to alanine residues to generate a hTRIM69 mutant (hTRIM69CA), and the 82 amino acids of the N-terminus of hTRIM69, which contains the RING domain, were deleted to generate a truncation (hTRIM69ΔR), as is illustrated by a schematic representation in Fig. 2A. We then tested whether the mutation or deletion affected the E3 activity of hTRIM69 using the 'in vitro ubiquitination assay'. We selected UbcH13/Uev1a as the E2 enzyme to study the function of the RING domain of TRIM69 on its E3 ligase activity in a cell-free system. GST-tagged hTRIM69WT, hTRIM69CA or hTRIM69ΔR produced from *E. coli* were incubated in the cell-free system described in Section 2. The results indicated that hTRIM69WT catalysed the formation of multiubiquitinylated products. In contrast, hTRIM69CA weakly catalysed the formation of multiubiquitinylated products, and hTRIM69ΔR completely lost its catalysing ability (Fig. 2B). These results indicate that hTRIM69's E3 ligase activity is dependent on its RING domain, suggesting that hTRIM69 is a RING finger E3 enzyme.

3.3. In vivo ubiquitination assay of hTRIM69 in HEK293T cells

To confirm the observed E3 activity of hTRIM69 in vitro, we co-transfected the expression plasmid containing Flag-tagged hTRIM69WT with or without HA-tagged ubiquitin into HEK293T cells. Cell lysates were immunoprecipitated with anti-Flag antibody resin, and the bound proteins were analysed by Western blotting with the anti-HA antibody. As shown in Fig. 3A, the presence of a high molecular weight smear was detected when TRIM69 was co-expressed with HA-ubiquitin in HEK293T cells. In addition, these smear products could be enhanced in the presence of a proteasomal inhibitor (MG132).

To further confirm the role of the RING finger domain in ubiquitination, Flag-tagged hTRIM69WT or hTRIM69CA were co-expressed with or without HA-Ub in HEK293T cells (Fig. 3B). Compared with hTRIM69WT, hTRIM69CA displayed significantly

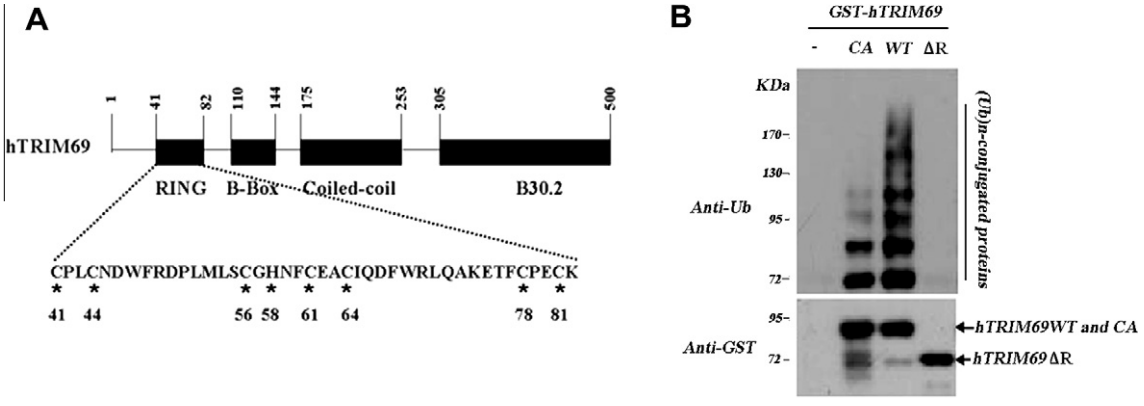

Fig. 2. Role of the RING finger domain in the E3 ubiquitin ligase activity of hTRIM69 in vitro. (A) The domain structure of the entire hTRIM69 molecule. Asterisks indicate conserved Cys and His residues in the RING finger domain. (B) In vitro ubiquitination assay by disruption of the RING finger motif of hTRIM69. Bacterially purified GST-hTRIM69WT, GST-hTRIM69CA, or GST-hTRIM69ΔR was incubated in the reaction mixture, as described in Section 2, with UbcH13/Uev1a as the E2 enzyme. After the reaction, the proteins were analysed by immunoblotting with anti-ubiquitin and anti-GST antibodies.

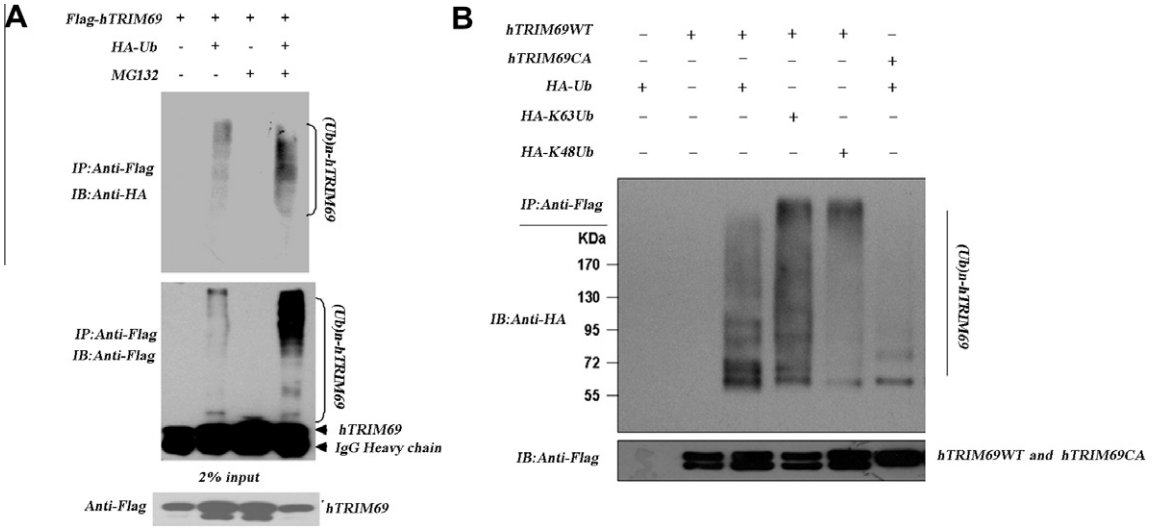

Fig. 3. E3 ubiquitin ligase activity of hTRIM69 in vivo. (A) Flag-tagged hTRIM69WT was co-transfected with a HA-tagged ubiquitin expression vector, or not, into HEK293T cells. Forty-two hours later, the proteasome inhibitor MG132 (20 μM) was added, or not, to the cell cultures, and the cells were further cultured for 6 h and harvested for immunoprecipitation. The precipitates were analysed by immunoblotting with anti-HA and anti-Flag antibodies. The expression of hTRIM69WT in the whole cell lysates was detected using an anti-Flag antibody. (B) HEK293T cells were transfected with plasmids expressing Flag-tagged hTIRM69WT or hTRIM69CA,together with HA-Ub or HA-K63Ub or HA-K48Ub respectively. Cells were harvested for immunoprecipitation 40 h post-transfection, and the precipitates were analysed by immunoblotting using anti-HA and anti-Flag antibodies.

decreased ubiquitination, indicating that the E3 ligase activity of hTRIM69 is also dependent on the RING finger domain in vivo. Ubiquitin contains seven Lys residues, and all seven Lys residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) are likely involved in chain formation in vivo. Currently, Lys48- or Lys63-linked ubiquitin chains are the best characterised [12]. To test whether the ubiquitination mediated by hTRIM69 is associated with these two types of polyubiquitin linkages, we also co-expressed Flag-hTRIM69WT with HA-tagged K63-Ub (containing only the Lys63 residue) or HA-tagged K48-Ub (containing only the Lys48 residue) in HEK293T cells. As shown in Fig. 3B, hTRIM69 could promote K48- and K63-linked polyubiquitination.

3.4. Subcellular localisation of hTRIM69

Previous studies [10,13] found that to accomplish their complex biochemical functional in vivo, TRIM proteins identify and interact with specific cellular compartments in homo or hetero manners. To study this process further, we investigated the subcellular localisation of hTRIM69.

Flag-hTRIM69WT, GFP-hTRIM69WT, Flag-hTRIM69CA, and Flag-hTRIM69ΔR were transfected into HeLa cells respectively, and their subcellular localisations were investigated. Immunofluorescence (IF) staining demonstrated that the two tags of the hTRIM69WT fusion proteins were localised to both the cytoplasm and nucleus (Fig. 4A). The subcellular fractionation assay results confirmed the IF results (Fig. 4B). In addition, the two tagged fusions both formed aggregates (Fig. 4A). However, the ectopic expression of hTRIM69CA and hTRIM69ΔR localised predominantly to the cytoplasm. The IF results indicated that hTRIM69CA could still form aggregates but fewer than hTRIM69WT and that hTRIM69ΔR lost its ability to form aggregates. Furthermore, both cytoplasmic and nuclear proteins were isolated from the HeLa cells ectopically expressing hTRIM69WT, hTRIM69CA or hTRIM69ΔR (Fig. 4B). The amounts of hTRIM69 cytoplasmic and nuclear extracts were similar based on the intensity of western blots signals. In contrast, hTRIM69CA was enriched in the cytoplasm, and hTRIM69ΔR was almost completely localised to the cytoplasm. These results indicate that the RING finger domain is also essential for hTRIM69 localisation and aggregation.

4. Discussion

The mouse counterpart of TRIM69 gene (*mTRIM69)* was originally isolated from a mouse testis cDNA library and is expressed specifically in the spermatid of mouse testis [14]. In terms of its function, there is only one previous report [15]. Human *hTRIM69* gene was isolated by our lab from a human testis subtracted library. Currently, there is no article reporting on its function. Here, we show that hTRIM69 possesses an E3 ubiquitin ligase activity that is dependent on an intact RING finger motif. Disruption by point mutations or deletion of the RING finger motif decreased or abolished the enzyme's catalytic activity.

The E3 activity of hTRIM69 was E2 enzyme-selective. hTRIM69 was clearly active with UbcH6, UbcH2, UbcH5A, UbcH5C, or UbcH13/UeV1a, whereas it displayed no activity with UbcH1, UbcH3, UbcH5B, or UbcH7. This multiple use of E2s is not unique to hTRIM69, and similar enzymatic properties have also been reported for RNF4 and TRIM23 [16,17]. A growing body of evidence suggests the importance of the E2 enzyme as a basis for the type of chain formation [18]. Therefore, on the basis of its ability to selectively use multiple E2s, hTRIM69 might be involved in both of the specific forms of ubiquitin chain modification, i.e., "classical" K48 ubiquitylation, which predominantly leads to substrate degradation, and K63 ubiquitylation, which is associated with endocytosis, cell signalling, and DNA repair [19,20]. The in vivo ubiquitination assay results further support this possibility for hTRIM69, which might be able to catalyse polyubiquitination formation in the presence of only K63-linked ubiquitin (K63Ub) or only K48-linked ubiquitin (K48Ub). In addition, proteasome inhibitor could increase the ubiquitination of hTRIM69, which would indicate that hTRIM69 might target itself for degradation through the UPS. Therefore, self-ubiquitination may be a mechanism to regulate hTRIM69 turnover. However, it is still possible that TRIM69 was ubiquitinated by other cellular ubiquitin ligase.

TRIM proteins exhibit various subcellular localisations and appear to define novel subcellular compartments, which might

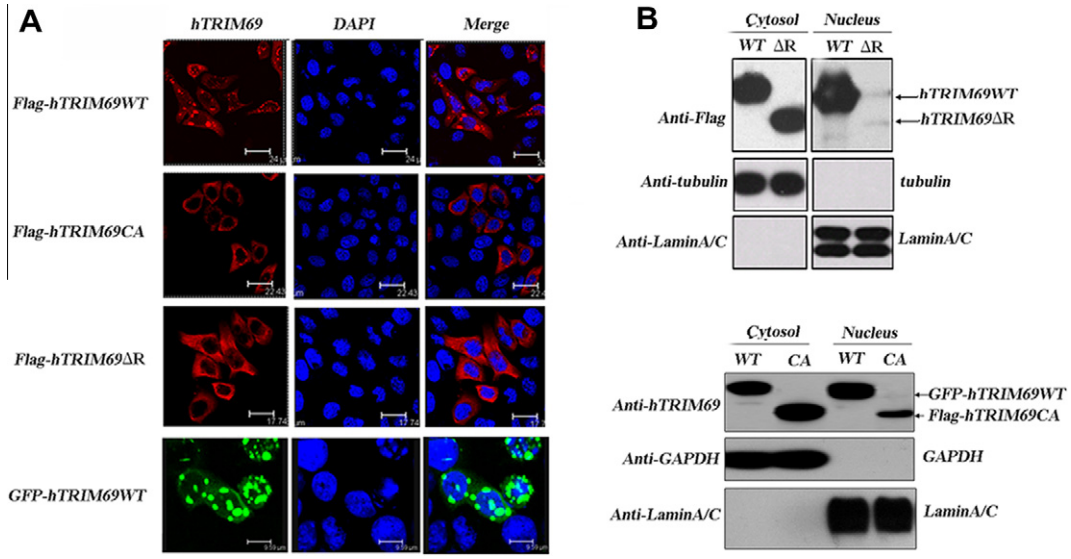

Fig. 4. Subcellular localisation of hTRIM69. (A) HeLa cells were transiently transfected with Flag-tagged expression constructs including hTRIM69WT, hTRIM69CA, hTRIM69ΔR or the GFP-tagged hTRIM69WT expression plasmid. Forty hours post-transfection, the cells were prepared for immunostaining or the cytoplasmic and nuclear fraction separation assay. For immunostaining, the cells were fixed, and hTRIM69WT, its point or deletion mutant was detected using an anti-Flag antibody (red). Nuclei were stained with DAPI (blue). (B) Cytoplasmic and nuclear fractions were prepared and separated by SDS–PAGE and analysed by immunoblotting with anti-Flag and anti-GFP antibodies, respectively. Tubulin or GAPDH was detected as the cytoplasmic marker, and laminA/C was the nuclear marker. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

provide clues for their biological function [10]. Previous investigations have shown that wild type mTRIM69 (mTRIM69WT) overexpression coupled to green fluorescent protein (GFP) in HEK293 cells localised to nuclei with a speckled pattern [15]. In contrast, the GFP tagged hTRIM69 was found both in the cytoplasm and in the nucleus, even if hTRIM69 shared the same amino acid sequence of residues of mTRIM69 (NP_536771) which was hypothesised as a potential nuclear localisation signal (NLS). We also constructed mTRIM69 coupled to a GFP tag and observed its localisation patterns in both HeLa and HEK293 cells, both of which displayed similar localisation patterns to hTRIM69 (data not shown). However, these results contradict with a previous report on mTRIM69 localisation in HEK293 cells. Similar contradictory subcellular localisations have also been observed in another study defining TRIM22 localisation [9]. The explanation for this differential localisation pattern may be the dynamic localisation of mTRIM69 during cell cycle changes, for example, TRIM22 localisation has been shown to change from nuclear at G0/G1 to both nuclear and cytoplasmic during mitosis [21]. To exclude the possible influence of a GFP tag, we also observed the subcellular localisation of Flag-tagged hTRIM69WT in HeLa cells. And the results showed the two different tagged hTRIM69WT fusion proteins displayed a similar subcellular localisation as those from GFP fusion proteins.

In conclusion, although over thirty RING finger proteins have been reported as involved in spermatogenesis in rats, mice, and humans, few have been identified to be E3 ubiquitin ligases [22]. In this study, we cloned the hTRIM69 from a human testis subtracted cDNA library and demonstrated that it possesses an E3 ubiquitin ligase activity. Further studies will focus on the respective roles of the E3 ubiquitin ligase activity of hTRIM69 in spermatogenesis, which may lead to advances in infertility diagnostics and treatment [23].

Acknowledgments

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