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The ability of TRIM3 to induce growth arrest depends on RING-dependent E3 ligase activity

Radhika Raheja[*,†], **Yuhui Liu**[*], **Ellen Hukkelhoven**[*], **Nancy Yeh**[*], and **Andrew Koff**[*,†,1]

[*]Program in Molecular Biology, Memorial Sloan–Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, U.S.A

[†]Graduate Program in Biochemistry, Molecular and Cellular Biology, Weill College of Medicine, Cornell University, New York, NY 10021, U.S.A

Abstract

Mutation of the TRIM (tripartite motif)-NHL family members *brat* and *mei-P26* perturb the differentiation of transit-amplifying progenitor cells resulting in tumour-like phenotypes. The NHL (named after the NCL1, HT2A and LIN41 repeat) domain is essential for their growth suppressive activity, and they can induce cell-cycle exit in a RING-independent manner. TRIM3 is the only *bona fide* tumour suppressor in the mammalian TRIM-NHL subfamily and similar to the other members of this family, its ability to inhibit cell proliferation depends on the NHL domain. However, whether the RING domain was required for TRIM3-dependent cell-cycle exit had not been investigated. In the present study, we establish that the RING domain is required for TRIM3-induced growth suppression. Furthermore, we show that this domain is necessary to promote ubiquitination of p21 in a reconstituted *in vitro* system where UbcH5a is the preferred E2. Thus the ability of TRIM3 to suppress growth is associated with its ability to ubiquitinate proteins.

Keywords

E3 ligase; oligodendroglioma; p21; TRIM3; ubiquitination

INTRODUCTION

The TRIM (tripartite motif) family of proteins is defined by a conserved spatial relationship of a RING, B-box and coiled-coil domain, which can be followed by different functional domains. TRIM proteins have roles in development, patterning, cell division, intracellular trafficking, antiviral response and cancer [1,2]. Whereas many of the members of the TRIM family are ubiquitin ligases, the differentiation-promoting and growth-inhibitory effects of the *Drosophilia* and mammalian TRIM-NHL proteins has been reported to be RING-independent. The NHL (NCL1, HT2A and LIN41 repeat) domain is required for interaction

[1]To whom correspondence should be addressed (a-koff@ski.mskcc.org).

AUTHOR CONTRIBUTION

Radhika Raheja carried out the experiments. Yuhui Liu, Ellen Hukkelhoven and Nancy Yeh provided experimental assistance or reagents throughout the course of the work. Andrew Koff directed the research.

with miRISCs (miRNA-induced silencing complexes), and these complexes can modulate the expression of a number of cell-cycle regulatory genes, including Myc and p21 [3–5]. However, these repeats, which form a scaffold for protein–protein interactions, can also serve as binding sites for substrates of E3 ligases [6,7].

TRIM3 is a mammalian TRIM–NHL family member originally identified as a component of a vesicular trafficking complex in neurons [8], and later suggested to ubiquitinate GKAP (guanylate kinase-associated protein) [9], a neuronal substrate involved in synaptic remodelling. However, TRIM3-knockout mice display no neurological defects [10], and there is no evidence that GKAP and TRIM3 physically interact or that TRIM3 is able to directly ubiquitinate GKAP. On the other hand, the locus encoding TRIM3 is lost in approximately 20 % of human GBMs (glioblastomas) [11], and reducing TRIM3 expression can facilitate tumour development and accelerate the progression of oligodendroglioma in a mouse model [12]. Collectively, these results indicate that TRIM3 is a mammalian tumour suppressor. Thus we wanted to know whether the ability of TRIM3 to induce cell-cycle exit depends on the RING domain. RING-independent growth-suppressive properties would suggest that its tumour suppressive role depended on its interactions with miRISC effector complexes, whereas RING dependence would suggest a role as an E3 ligase.

In the present study, using structure-guided mutational analysis, we determined that the RING was required for the growth arrest activity of TRIM3. Furthermore, we were able to demonstrate that TRIM3 could directly catalyse the transfer of ubiquitin from UbcH5a to p21, a protein necessary for the proliferation of a subset of PDGF (platelet-derived growth factor)-transformed proneural glioma cells. Collectively, the requirement for both the NHL and RING domains of TRIM3 suggests that it functions as an E3 ligase to suppress tumour development.

MATERIALS AND METHODS

Cell culture and mutagenesis

T98G glioma cell lines were purchased from A.T.C.C. (Manassas, VA, U.S.A.) and cultured according to their protocols. Mouse TRIM3 mutants were generated by PCR-based DNA mutagenesis or *in vitro* site-directed mutagenesis as described recently [12].

Immunoblot and BrdU (bromodeoxyuridine) immunofluorescence

Immuoblots and BrdU immunofluorescence assays were carried out as described previously [13,14]. The following antibodies (all from Santa Cruz Biotechnology) were used: anti-p21 antibody (clones M-19, C-19 and F-5), anti-TRIM3 antibody (clone 27), anti-c-Myc antibody (clone 9E10), anti-HA (haemagglutinin) antibody (clone Y-11), anti-tubulin antibody, anti-GST antibody (clone B-14), anti-BSA antibody (clone A23-A/D3) and anti-p27 antibody (clone C-19).

Preparation of *Escherichia coli*-produced recombinant protein

GST- or His-tagged recombinant proteins were produced in *E. coli* using the GST Gene Fusion System (GE Healthcare) and the Probond™ Purification System (Invitrogen)

respectively according to the manufacturers' instructions and extracts were prepared by urea/DTT denaturation and renaturation as described previously [12]. Critical to reducing background ubiquitination, the recombinant His-tagged p21 was clarified by centrifugation at 100 000 ***g*** for 15 min at 4°C and the protein was stored in aliquots of 20 μl at −80°C, which were thawed no more than twice. Proteins prepared in this manner eluted as a well-defined single peak after gel filtration, albeit they were not purified to homogeneity.

In vitro ubiquitination assay

The *in vitro* ubiquitination assay was performed as described previously [15] with the following modifications: ubiquitination reactions in a final volume of 30 μl contained 500 nM recombinant His-tagged p21, 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soya bean trypsin inhibitor, 5 mM ATP, 15 nM ubiquitin-activating enzyme E1 (Boston Biochem), 0.5 μM UbcH5a (Boston Biochem), 10 μM ubiquitin (Boston Biochem) and 78 nM GST-tagged TRIM3 or an equimolar amount of the mutants as described in the Figure legends. These reaction mixtures were incubated for 30 min at 37°C and stopped by adding four loads of SDS sample buffer and immediately boiled for 5 min. The reaction mixture was analysed by SDS/PAGE (12 % gel), transferred to PVDF and had p21 detected by immunoblot and chemoluminescence.

In vitro binding assays

In the *in vitro* binding assays, 500 nM His-tagged p21 was combined with 78 nM GST-tagged TRIM3, or equimolar amounts of the mutants as indicated in the Figure legends, in a final volume of 60 μl containing 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml soya bean trypsin inhibitor. The reaction mixtures were incubated at 37°C for 30 min and incubated with glutathione–agarose beads for an additional 2 h on ice. Immune complexes were washed three times and the proteins eluted by boiling in SDS sample buffer and analysed by SDS/PAGE followed by immunoblotting. TRIM3 proteins were detected using anti-GST antibodies and p21 proteins were detected with anti-p21 antibodies.

RESULTS

The RING domain is necessary for TRIM3-induced growth arrest

The growth-suppressive or differentiation-promoting activity of many of the TRIM-NHL proteins (mei-P26, brat, lin41, nhl2, TRIM32 and TRIM71) is associated with an NHL-dependent and RING-independent interaction with miRISC effector complexes [3–5,16,17]. We have shown previously that the NHL-domain was required for TRIM3-induced growth arrest in glial cells [12]. However, because deletion of the RING, or any mutations that affect the cross-brace structure of the RING, did not express well in those cells [12], we were unable to address whether the growth-suppressive activity of TRIM3 was also RING-dependent. Thus, to investigate the importance of this domain, we generated a collection of six missense mutants (Figure 1A), based on previous studies that mapped E2-binding sites in the RING of c-Cbl, cIAP (cellular inhibitor of apoptosis protein) and BRCA1, E3 ligases that interact with Ubc4/5-like E2s similar to the TRIMs [18–25].

We previously established the NHL-dependent growth-suppressive property of TRIM3 in PDGF-induced proneural glioma cell lines (YH/J12 and T98G) [12]. We transfected T98G cells with plasmids expressing either the wild-type or one of the six TRIM3 missense mutants and measured their effect on BrdU incorporation. A mutant lacking the NHL domain (ΔNHL) was also transfected as a negative control that would not reduce BrdU incorporation [12]. Consistent with our previous results, BrdU incorporation was reduced approximately 3-fold in cells expressing wild-type TRIM3 compared with those cells transfected with an empty vector (Figure 1B). BrdU incorporation was, likewise, reduced in cells expressing either the I50A or L55A mutants. On the other hand, BrdU incorporation was unaffected by the I24A, D27A, V61A or R63A mutants. On the basis of the structure of the BIRC7–UbcH5b–ubiquitin complex, these four mutations were predicted to interfere with E2 binding, whereas the I50A and L55A mutations were not. All of the proteins were expressed at similar levels (Figure 1C). Similar results were obtained in YH/J12 cells (results not shown). Thus the RING domain was necessary for growth arrest.

TRIM3 can ubiquitinate p21

The requirement of the RING domain raised the possibility that TRIM3 might function as an E3 ligase, perhaps recruiting a substrate on its NHL domain and mediating the transfer of ubiquitin from an E2 bound to its RING domain. We hypothesized that a substrate would fulfil three criteria; it must interact with TRIM3, its levels should inversely correlate with TRIM3 and it must be ubiquitinated by TRIM3. It had been demonstrated that p21 binds to TRIM3 in an NHL-dependent manner, and that reducing TRIM3 leads to the accumulation of p21 in both YH/J12 cells and in PDGF-transformed progenitors in mice [12,26].

Although it was relatively easy to establish that p21 ubiquitination was increased when cells were co-transfected with TRIM3 and p21 (results not shown), such an experiment does not establish that TRIM3 directly ubiquitinates p21. Consequently, we set out to establish whether TRIM3 could ubiquitinate p21 using an *in vitro* system completely reconstituted from bacterial proteins. To accomplish this, we supplemented GST–TRIM3/His–p21-binding assays [12] with ATP, E1, E2 and HA-tagged ubiquitin, and measured ubiquitination by p21 immunoblot after the products were resolved by SDS/PAGE and transferred on to a PVDF membrane. After incubation, p21 migrated in two discrete slower migrating bands (30 and 55 kDa) and a high-molecular-mass smear greater than 72 kDa (Figure 2A). Background immunoreactivity detected between 42 and 45 kDa and at 72 kDa was observed in the absence of any individual ubiquitination protein. The nature of these background bands was not clear. Their intensity varied from preparation to preparation of the substrate and the inclusion of the different ubiquitination components. Nevertheless, the 30 kDa, 55 kDa and high-molecular-mass species were indicative of an E1-, E2-, TRIM3- and ubiquitin-dependent reaction. The same pattern of p21 migration was observed when we used a His-tagged TRIM3 instead of GST–TRIM3 (Supplementary Figure S1 at http://www.biochemj.org/bj/458/bj4580537add.htm), suggesting that dimerization through the GST tag was not influencing TRIM3 activity.

The ΔNHL TRIM3 mutant neither induced these modifications nor interacted with p21 (Figure 2B). Additionally, the migration of BSA or the Cdk (cyclin-dependent kinase)

inhibitor p27 was not altered following incubation in these reactions (Figure 2C), indicating that this ligase was not generally promiscuous. Furthermore, the migration of a lysine residue-less mutant of p21 was not affected after incubation in these reactions and lysine residue-less p21 still bound to GST–TRIM3 (Figure 2D). Collectively, these data suggest that the NHL domain might present p21 to a TRIM3–E2 ubiquitination complex.

The 3_{10} helical region facilitates ubiquitination by TRIM3–UbcH5a

Next, we wanted to determine whether these ubiquitinated forms of p21 represented the attachment of polyubiquitin chains. Thus we replaced ubiquitin with lysine residue-less ubiquitin. Lysine residue-less ubiquitin cannot be extended into ubiquitin polymers. Only the 30 kDa species accumulated in these reactions (Figure 3A) indicating that the 55 kDa species and those greater than 72 kDa were due to polymerization of ubiquitin and not the attachment of ubiquitin at multiple sites. Again, the species observed between 42 and 45 kDa were contaminants of the p21 substrate preparation (Figure 2A and Supplementary Figure S1).

We then tested an extensive collection of lysine to arginine mutants in p21 to identify the lysine residue attachment site. A schematic diagram of p21 showing the position of each lysine residue and the nascent secondary structure of the kinase inhibitory domain with the indicated subdomains D1, LH, D2 and the 3_{10} helix is shown (Figure 3B). This Swiss model structure was generated using the p27 kinase inhibitory domain bound to cyclin A–Cdk2 as a template (PDB code 1JSU). The N-terminus of p21 (residues 17–78) can be divided into a similar subdomain structure with the D1 domain assuming an extended conformation when bound to cyclin, which is followed by stabilization of the LH domain and a conformational change in the D2 domain. On the basis of NMR and CD spectroscopy, the LH and 3_{10} subdomains exhibit partial secondary structure even in free p21 [27,28].

To score the effect of each mutation on ubiquitination, we compared the yield of the 30 kDa product in each reaction containing mutant p21, TRIM3, UbcH5a and lysine residue-less ubiquitin to the amount in the reaction containing wild-type p21. The ΔNHL mutant was used as a negative control since it could not catalyse the ubiquitination of p21. Representative autoradiographs are shown with the plotted data as means ± S.E.M. for at least two independent experiments (Figure 3C). Although no single mutation was able to reduce ubiquitination more than 2-fold, the Lys^{91} mutant was generally more affected than the others.

In vitro ubiquitin attachment is generally found in regions rather than at specific amino acids owing to intrinsic protein flexibility [29–33]. For example, SCF^{SKP2} ubiquitinates two lysine residues (Lys^{156} and Lys^{158}) in the C-terminal region [34]. Thus we first examined the ability of TRIM3–UbcH5a to ubiquitinate the K156R/K158R double mutant and a quintuple mutant (K15R/K74R/K91R/K136R/K149R). Ubiquitination was severely reduced by the quintuple mutant compared with the double mutant, which was only reduced 2-fold. Ubiquitination was practically eliminated in a quadruple mutant (K15R/K74R/K91R/K136R) as well.

Double- and triple-mutant analyses refined further this ubiquitination domain. Ubiquitination was reduced approximately 4-fold in the K74R/K91R double mutant and approximately 2-fold in the K15R/K74R, K15R/K136R, K74R/K136R double mutants or the K15R/K74R/K136R triple mutant. Additionally, ubiquitination of a mutant in which all lysine residues except for Lys^{91} were replaced by arginine was also severely reduced. None of these mutations affected p21 binding *in vitro* (results not shown). Collectively, these data suggested that the area around the 3_{10} helix of free p21 might be a preferred site of ubiquitin attachment by the TRIM3–UbcH5a complex or alter the nascent secondary structure; moving the targeted lysine residues away from the E2.

UbcH5a is a preferred E2 for TRIM3-dependent p21 ubiquitination

The combination of E2 and E3 determines the topology and length of ubiquitin chain conjugation to a substrate [35–37]. TRIM3 can interact with a number of E2s in the Ubc4/5-like clade including UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH8, UbcH9 and UbcH13 [35]. UbcH3 can work in partnership with UbcH5a to extend ubiquitin chains [38]. Thus we wanted to know which E2 enzymes could work with TRIM3 to ubiquitinate p21. We obtained a collection of eight E2s (UbcH2, UbcH3, UbcH5a, UbcH5b, UbcH6, UbcH8, UbcH10 and UbcH13) from commercial vendors and first assayed them for TRIM3-dependent ubiquitin polymerization activity by incubating them in the presence of E1 and HA–ubiquitin without the p21 substrate (Figure 4A). As a negative control we included UbcH2 or UbcH10, which are not known to interact with TRIM3 [35], and we included another reaction in which no E2 was added. As expected, few if any polyubiquitin conjugates were formed in reactions containing UbcH2, UbcH10 or no E2. UbcH8 was also inactive suggesting that the enzyme preparation might not be good. UbcH5a, UbcH5b and UbcH6 were strongly active followed by UbcH3 and UbcH13 which had intermediate levels of activity.

To confirm that the activity was dependent on the presence of the E3 ligase, we looked at the ability of UbcH5a, UbcH5b and UbcH6 to catalyse polyubiquitin chain formation in the absence of TRIM3 and p21 (Figure 4B). As a negative control we included UbcH8 or UbcH10, and we had a reaction which did not contain any E2. Surprisingly, UbcH6 had activity, whereas the others did not. We did not pursue the E3-independent UbcH6 autoubiquitination activity further as it had been previously observed by others [39–41].

We then asked which of the E2s were able to ubiquitinate p21 in the presence of TRIM3. Of three with the most activity (UbcH5a, UbcH5b and UbcH6), UbcH5a was the best (Figure 4C). Because UbcH5a can catalyse the formation of multiple types of ubiquitin linkages, we were unable to establish the nature of the linkage *in vitro*. Regardless, the data are consistent with the interpretation that UbcH5a works with TRIM3 to ubiquitinate p21.

The RING is required for ubiquitination of p21

We next wanted to determine whether the RING–E2 interactions were required for p21 ubiquitination. A mutation that disrupts the cross-brace structure of the RING domain (C22A/C25A) in TRIM3 reduced the accumulation of the 30 kDa and 55 kDa species and the high-molecular-mass smear (Figure 5A). On the basis of the interaction of c-Cbl, cIAP

and BRCA1 with their E2s, we made seven additional mutants that should disrupt the ability of E2 enzymes to bind to the TRIM3 RING domain (I24A, L26A, D27A, N48A, Y49A, V61A and R63A). All but N48A and Y49A reduced ubiquitination of p21 (Figure 5B). On the other hand, we also made three additional mutants, C31A, I50A and L55A that were not predicted to interfere with E2 binding. Whereas C31A and I50A did not affect ubiquitination, the monoubiquitinated 30 kDa species accumulated in the reaction with the L55A mutant (Figure 5B). Likewise, the 30 kDa species accumulated in reactions containing the N48A and Y49A mutants, which were expected to eliminate E2 binding based on other studies using different E3–E2 pairs.

To determine whether the accumulation of the 30 kDa product reflected an effect of the mutations on processivity, we repeated the ubiquitination reactions and allowed them to progress for up to 120 min (Figure 5C). Both wild-type and I50A mutant TRIM3 catalysed rapid ubiquitination of p21 into all the higher-molecular-mass forms within 30 min, at which time no further ubiquitination was observed. On the other hand, only the monoubiquitinated 30 kDa product was detected during the first 30 min when N48A, Y49A and L55A TRIM3 mutants were used; however, polyubiquitinated species began to appear with time. None of the mutations affected the binding of TRIM3 to p21 (Figure 5D). Thus mutations at Asn^{48}, Tyr^{49} and Leu^{55} affect the processivity of the ubiquitination reaction.

Modelling of the TRIM3 RING domain/UbcH5a interface on the BIRC7–UbcH5b~ubiquitin structure (Figure 5E) shows that Asn^{48} and Tyr^{49} lie in the vicinity of E2 and ubiquitin. However, L55A faces away from this interface, so how it affected processivity was unclear. Regardless, TRIM3 has a RING-dependent E3 ligase activity and this activity is correlated with its ability to suppress BrdU incorporation, a marker of cell proliferation.

DISCUSSION

TRIM proteins have roles in development, patterning, cell division, intracellular trafficking, antiviral response and cancer (reviewed in [1,2]). A role for the TRIM–NHL proteins in tumorigenesis is consistent with phenotypes associated with the loss of the *Drosophila* orthologues *brat* and *mei-P26* (reviewed in [2,5,42,43]). In *brat* mutant flies secondary neuroblasts undergo excessive proliferation and fail to differentiate into ganglion mother cells in a timely fashion leading to an increase in type II neuroblasts generating a brain-tumour-like phenotype. In *mei-P26* mutant flies, ovarian stem cells excessively proliferate as well. In both of these examples, a RING-independent mechanism regulating an miRISC effector complex affects cell-cycle-specific transcriptional programmes which can accelerate Myc turnover [5]. Mammalian TRIM32 also interacts with miRISCs in neural stem and progenitor cells and promotes ubiquitination of Myc when overexpressed in HEK (human embryonic kidney)-293T and HeLa cells [4]. Mammalian TRIM71 induces proliferation by suppressing the expression of p21 mRNA through NHL domain-dependent interactions with Ago2 in pluripotent embryonic stem cells [3]. However, of the mammalian TRIM–NHL proteins, only TRIM3 is a *bona fide* tumour suppressor supported by mouse modelling and by genome copy number analysis in human PDGF-induced oligodendroglioma [11,12].

We have not been able to identify miRISC proteins in TRIM3 immunoprecipitates analysed by either immunoblotting or MALDI–TOF-MS in our glial cell systems, although known interactions with actinin V in the CART complex were observed (E. Hukkelhoven and A. Koff, unpublished work). We have shown that TRIM3 not only induces growth arrest in transit-amplifying progenitor cells that are dependent on p21 for proliferation, it can also inhibit proliferation in cells in which p21 has a more conventional growth inhibitory role [12]. Thus TRIM3 may negatively affect growth in multiple ways: first, RING-dependent and due to ubiquitination of one or more target proteins, and secondly, RING-independent and associated with the formation and activation of specific miRISCs.

The TRIM3–UbcH5a complex joins a list of E3–E2 pairs that can ubiquitinate p21. SCF^{SKP2} associates with the E2 Cdc34 (cell division cycle 34) [44], and $CRL4^{CDT2}$ associates with the E2 UbcH8 to ubiquitinate p21 [45]. UbcH5a and UbcH5b are almost 90 % identical [46] so it was surprising that we observed differences in the ability of these enzymes to ubiquitinate p21. However, this is not unprecedented. For example, the U-box protein CMPG1 undergoes autoubiquitination with UbcH5b and UbcH5c, but not UbcH5a [47]. UbcH5b and UbcH5c can also polyubiquitinate IκBα (inhibitor of nuclear factor κB α), whereas UbH5a cannot [48]. UbcH5a is much better at promoting autoubiquitination of the RING H2 protein WSSV249 than UbcH5b [49], and UbcH5a is utilized for the autoubiquitination of the HECT (homologous with E6-associated protein C-terminus)-like E3 ligase SopA although UbcH5b is not [50]. It remains to be determined how the dynamics of E2–E3 pairing impinges on substrate ubiquitination.

Two lines of evidence suggest that contaminants in our preparations would not affect our interpretations about the ubiquitination of p21 by TRIM3. First, experimentally, we used large collections of mutant and wild-type TRIM3 and p21, which elute as well-defined peaks during gel filtration and were purified in an identical manner. Nevertheless, it is formally possible that the domain encompassing Lys^{74}/Lys^{91} is not itself ubiquitinated, but rather the mutations in this domain altered the secondary structure of the protein to move the acceptor lysine residues away from the bound E2. Secondly, the effects of the missense mutations in the RING domain are consistent with those predicted from the structure of other RING E3–E2 pairs. Thus we are confident that contaminants in these preparations do not affect our interpretation that TRIM3 is an E3 ligase that can ubiquitinate p21.

In contrast with other studies of p21 ubiquitination which focus on p21 bound to either cyclin–Cdk complexes [SCF^{SKP2} (Skp1/cullin/F-box protein Skp2) and APC/C^{Cdc20} (anaphase-promoting complex/cyclosome and its activator Cdc20)] or PCNA (proliferating-cell nuclear antigen; $CRL4^{CDT2}$) [34,44,51–57], the studies reported in the present paper focus on the ubiquitination of free p21. Such ubiquitination can occur in non-cycling cells that lack cyclin–Cdk complexes. In these cells, TRIM3 would be able to bind to p21 and ubiquitinate lysine residues in the kinase inhibitory domain, which is inaccessible when p21 is bound to cyclin–Cdk complexes [58]. Consequently, we propose that TRIM3 establishes the amount of p21 available for Tyr^{76} phosphorylation [59] such that mitogen-induced expression of cyclin D1 can promote the formation of active cyclin D1–Cdk4–p21 ternary complexes and drive quiescent cells into the cell cycle.

We are aware that our work does not establish p21 as a critical target of TRIM3-dependent ubiquitination *in vivo*. E3 ligases typically have multiple targets, and reducing TRIM3 can induce tumours in cells that lack p21, as well as in those that express p21 [12]. Overexpression of TRIM3 can induce growth arrest in cells that express p21, as well as in cells that do not express p21 (Y. Liu, R. Raheja and A. Koff, unpublished work). Thus, although we have endeavoured to identify a mutant of p21 that can still support cell proliferation and yet be resistant to the effect of TRIM3 ubiquitination, this is a sisyphean task owing to the nuanced manner by which p21 plays a growth-promoting role. p21 must be phosphorylated on Tyr^{76} to support cyclin D1–Cdk4 assembly and activity [59]. Because the structure and not the charge of the 3_{10} helix is so important, aspartate, glutamate and alanine residue substitutions only partially ameliorate the inhibitory effect of the protein when overexpressed [59]. When the levels of p21 exceed the capacity of the cell to carry out this modification, inhibitory non-phosphorylated protein accumulates and the cells undergo cell-cycle arrest. Nevertheless, data in the present study show that TRIM3 has a RING-dependent E3 ligase activity and that this activity can contribute to its ability to cause growth arrest. Consistent with this, missense mutations in the RING domain of TRIM3 have been identified recently in tumours [60,61].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BrdU	bromodeoxyuridine
Cdc	cell division cycle
Cdk	cyclin-dependent kinase
cIAP	cellular inhibitor of apoptosis protein
GBM	glioblastoma
GKAP	guanylate kinase-associated protein
HA	haemagglutinin
HEK	human embryonic kidney

miRISC	miRNA-induced silencing complex
PDGF	platelet-derived growth factor
TRIM	tripartite motif
ΔNHL mutant	mutant lacking the NHL domain

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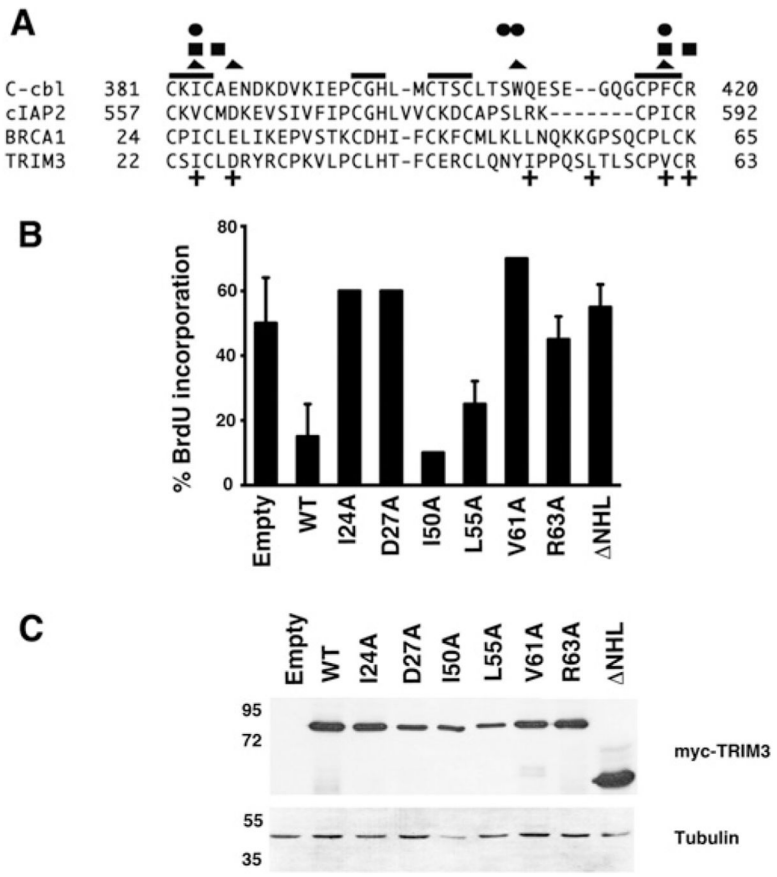

Figure 1. Mutations that disrupt E2 binding to the RING domain of TRIM3 reduce its ability to suppress DNA replication

(**A**) Alignment of the RING domains of c-cbl, cIAP2, BRCA1 and TRIM3. The bars above the alignment indicate the cross-brace structural elements of the RING. The shapes above the alignment indicate the residues required for E2 binding (triangle, cIAP2; circle, c-cbl; square, BRCA1). The TRIM3 mutants generated are indicated as plus symbols below the alignment. (**B**) BrdU incorporation was measured as the percentage of labelled T98G cells following transfection with cDNA vectors expressing Myc-tagged mutants or wild-type protein (WT), or an empty vector as described in the Materials and methods section. The data were compiled from three independent experiments with means ± S.D. shown. (**C**) The amount of the transfected protein was determined by immunoblotting with anti-Myc antibody. Tubulin was the loading control. The molecular mass in kDa is indicated.

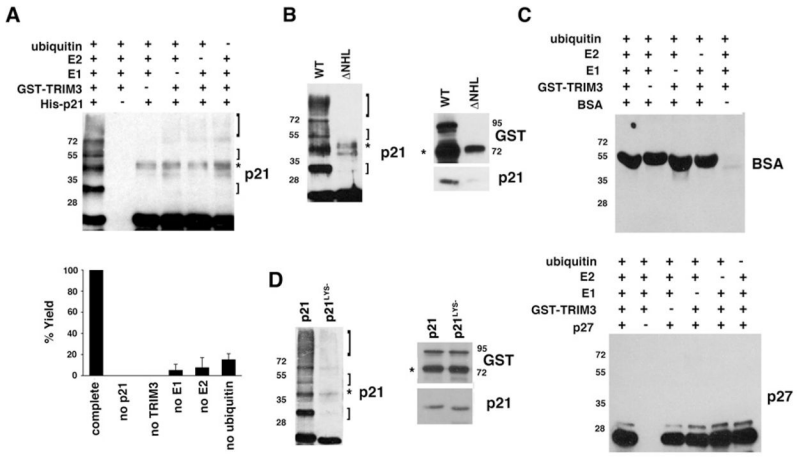

Figure 2. TRIM3 can ubiquitinate p21 *in vitro*

(**A**) Top panel, E1, E2 (UbcH5a), GST–TRIM3, His-tagged p21 and ubiquitin were combined as described in the Materials and methods section and the effect on p21 migration determined by anti-p21 antibody immunoblot. The molecular-mass markers are indicated on the left-hand side in kDa. Bottom panel, data from at least three different independent experiments were compiled and the amount of the modified slower migrating p21 species indicated by the brackets was measured with ImageJ 1.4 (NIH), with data shown as means ± S.D. The asterisk on the right-hand side indicates a contaminant present in the p21 preparations as discussed in the Results. (**B**) The NHL domain is required for ubiquitination. Left panel, GST–TRIM3 (wild-type; WT) or ΔNHL mutants were incubated with His–p21 in the presence of E1, E2, ubiquitin and ATP and the effect on p21 migration was determined by immunoblotting. Right panel, GST-tagged wild-type and ΔNHL mutant TRIM3 were incubated with His–p21 under the same ubiquitination condition and the interaction of TRIM3 and p21 was measured by immunoblot following adsorption to glutathione–agarose beads. The asterisk indicated in the left panel marks a proteolytic fragment of TRIM3 that lacks the NHL domain (E. Hukkelhoven and A. Koff, unpublished work). (**C**) The migration of BSA (top panel) and p27 (bottom panel) are not changed when incubated in the ubiquitination reaction. These panels are arranged as (**A**) except BSA or p27 were used as a substrate and detected by specific antibodies as indicated on the right-hand side. (**D**) Lysine residues on p21 are required for ubiquitination. This is arranged as described for (**B**), but the reactions contained either wild-type (p21) or lysine residue-less ($p21^{LYS-}$) as indicated above each lane. Antibodies used for blotting are shown on the right-hand side.

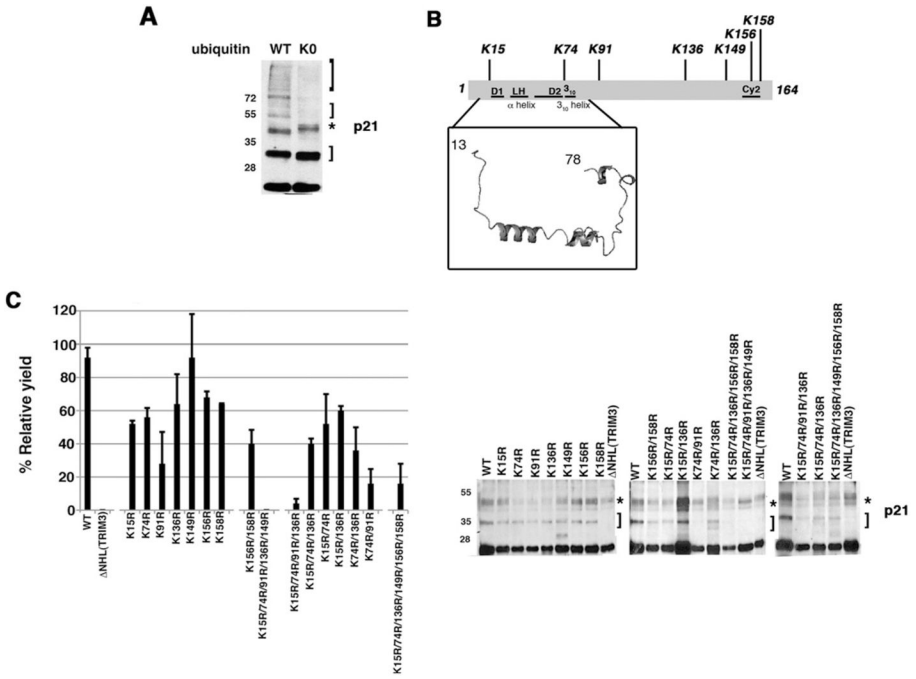

Figure 3. p21 mutational analysis

(**A**) Lysine residue-less ubiquitin (K0) was substituted for wild-type ubiquitin (WT) in a reaction containing ATP, E1, GST–TRIM3 and His-tagged p21. The products were resolved by SDS/PAGE and p21 detected by immunoblotting. Molecular-mass markers are indicated on the left-hand side in kDa. The asterisk represents the non-specific anti-p21 antibody reactive material and the brackets are the regions where ubiquitinated products are observed in reactions containing wild-type protein. (**B**) Structure of the predicted kinase inhibitory domain and positions of lysine residues in p21 [62]. (**C**) *In vitro* ubiquitination reactions were carried out using GST–TRIM3, UbcH5a, E1, K0 ubiquitin and the p21 mutants indicated above each lane in the right-hand panel or below the histogram in the left-hand panel. To plot the yield of ubiquitinated product, we calculated the ratio of 30 kDa monoubiquitinated species to unmodified p21 in each lane and normalized this to the amount in the lanes containing wild-type p21. The asterisk and brackets on the p21 immunoblots on the right-hand side represent the non-specific anti-p21 antibody reactive material and the areas quantified as monoubiquitinated p21 respectively.

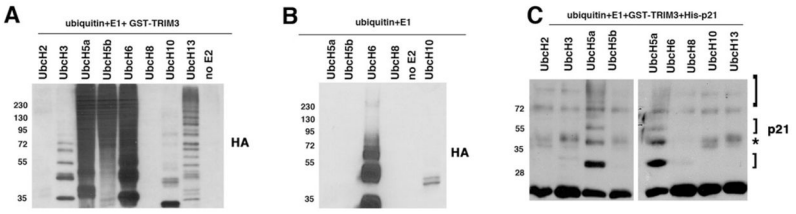

Figure 4. UbcH5a is a preferred E2 in the TRIM3-dependent p21 ubiquitination reaction

(**A**) GST–TRIM3, E1, ATP and HA-tagged ubiquitin were incubated with the E2s indicated above each lane, but no p21, and the extent of ubiquitin polymer formation assessed by immunoblotting with an anti-HA antibody as indicated on the right-hand side. Molecular-mass markers are shown on the left-hand side in kDa. (**B**) E1, ATP and HA-tagged ubiquitin were incubated with the E2s indicated above each lane, but no GST–TRIM3 or p21 were included, and the extent of ubiquitin polymer formation assessed by immunoblotting with an anti-HA antibody as indicated on the right-hand side. Molecular-mass markers are shown on the left-hand in kDa. (**C**) GST–TRIM3, E1, ATP, HA-tagged ubiquitin and His-tagged p21 were incubated with the E2s indicated above each lane and the extent of p21 ubiquitination determined by immunoblotting with anti-p21 antibodies as indicated on the right-hand side. The background contaminant in p21 preparations is indicated with the asterisk and the ubiquitinated products are indicated by the brackets. Molecular-mass markers are shown on the left-hand side in kDa.

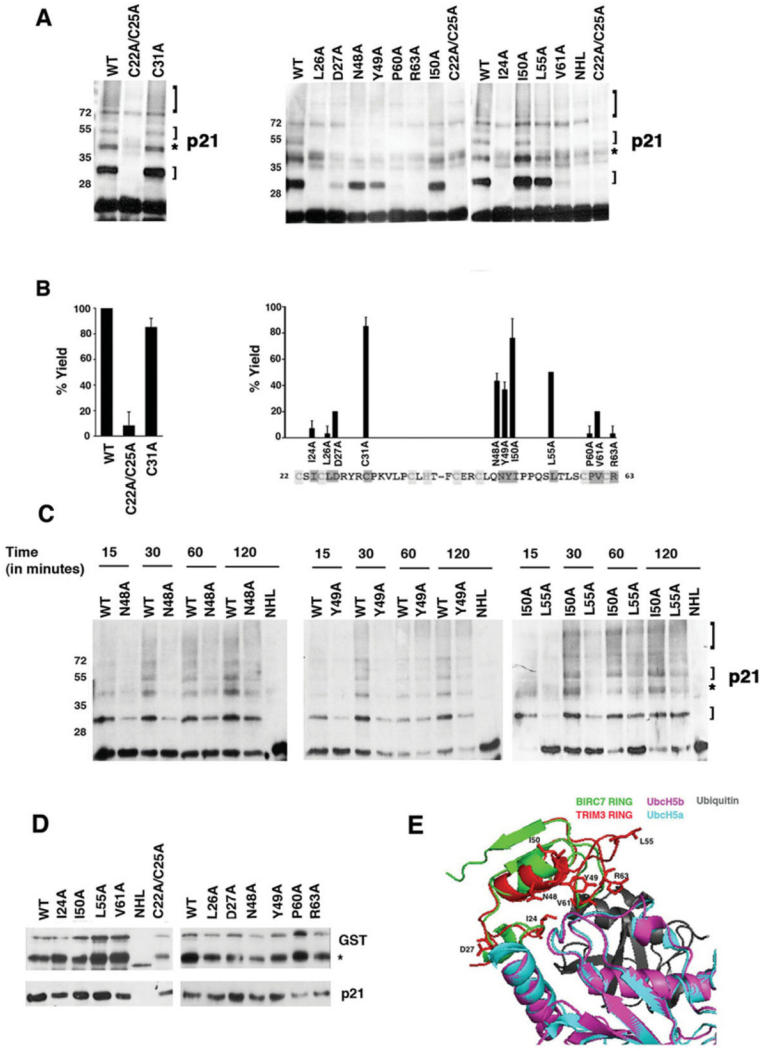

Figure 5. Mutational analysis of TRIM3

(**A** and **B**) E1, ATP, HA-tagged ubiquitin, His-tagged p21 and UbcH5a were incubated with the GST–TRIM3 mutants indicated above each lane and the extent of p21 ubiquitination measured by anti-p21 antibody immunoblot. Molecular-mass markers are shown on the left-hand in kDa. The amount of ubiquitinated p21 accumulating in the bracketed regions was quantified as described, with data in (**B**) shown as means ± S.D. (**C**) E1, ATP, HA-tagged ubiquitin, His-tagged p21 and UbcH5a were incubated with GST–TRIM3 or mutant proteins as indicated above each lane and the extent of p21 ubiquitination measured at different times by p21 immunoblot. (**D**) p21 binding. GST–TRIM3 (wild-type; WT) or the TRIM3 mutants indicated above each lane were incubated with His–p21 and collected on glutathione–agarose before immunoblotting with the antibodies indicated on the right-hand side. The asterisk identifies a TRIM3 proteolytic fragment lacking the NHL domain present in our preparations (E. Hukkelhoven and A. Koff, unpublished work). (**E**) A model of the structure of the TRIM3 RING domain with UbcH5a was generated using Swiss Modeler [63–65] and laid over the structure of the RING domain of BIRC7 (green) and ubiquitin-charged UbcH5b (pink) (PDB code 4AUQ [66]). The RING domain of TRIM3 is coloured red and

the TRIM3 residues mutated to alanine are coloured black. UbcH5a is coloured cyan (PDB code 2C4P).