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TRIM22 E3 ubiquitin ligase activity is required to mediate antiviral activity against encephalomyocarditis virus

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The interferon (IFN) system is a major effector of the innate immunity that allows time for the subsequent establishment of an adaptive immune response against a wide-range of pathogens. Their diverse biological actions are thought to be mediated by the products of specific but usually overlapping sets of cellular genes induced in the target cells. Ubiquitin ligase members of the tripartite motif (TRIM) protein family have emerged as interferon (IFN)-induced proteins involved in both innate and adaptive immunity. In this report, we provide evidence that TRIM22 is a functional E3 ubiquitin ligase that is also ubiquitinated itself. We demonstrate that TRIM22 expression leads to a viral protection of HeLa cells against encephalomyocarditis virus infections. This effect is dependent upon its E3 ubiquitinating activity since no antiviral effect was observed in cells expressing a TRIM22-deletion mutant defective in ubiquitinating activity. Consistent with this, TRIM22 interacts with the viral 3C protease (3C^{PRO}) and mediates its ubiquitination. Altogether, our findings demonstrate that TRIM22 E3 ubiquitin ligase activity represents a new antiviral pathway induced by IFN against picornaviruses.

INTRODUCTION

Picornaviruses include important human pathogens such as rhinovirus, poliovirus and hepatitis A virus, as well as significant insect, plant and agricultural pathogens, such as foot-and-mouth disease virus (Whitton *et al.*, 2005). Encephalomyocarditis virus (EMCV) is the prototype of the cardiovirus subgroup of picornaviruses. Its genome consists of a single-strand of messenger-sense RNA. Viral proteins are expressed from a large open reading frame encoding a giant precursor polyprotein (approx. 255 kDa), which is processed through a series of proteolytic cleavages to produce both capsid and non-structural proteins (Palmenberg, 1990). The majority of the processing reactions are carried out by the 3C protease ($3C^{PRO}$), which acts both inter- and intramolecularly to produce polyprotein precursors as well as the mature $3C^{PRO}$ polypeptide (Palmenberg *et al.*, 1979). Cleavage of the polyprotein normally occurs between glutamine–glycine or glutamine–serine amino acid pairs, which are usually flanked by proline residues (Palmenberg *et al.*, 1984). Besides its commitment to the processing of the viral polyprotein, picornaviral $3C^{PRO}$ can also facilitate virus replication by altering fundamental cellular processes such as transcription and translation of host cell mRNA (Barral *et al.*, 2007; Ehrenfeld, 1982; Kuyumcu-Martinez *et al.*, 2004; Neznanov *et al.*, 2005; Shen *et al.*, 1996; Yalamanchili *et al.*, 1996, 1997). Because the regulated proteolysis mediated by $3C^{PRO}$ is a critical feature in both the processing of picornaviral polyprotein and the inhibition of cellular defences, $3C^{PRO}$ constitutes an important target for host antiviral innate pathways. Consistent with this, the degradation of EMCV $3C^{PRO}$ by the ubiquitin/26S proteasome system has been reported (Losick *et al.*, 2003; Schlax *et al.*, 2007). Although the precise role of this degradation during virus replication is still unknown, a possible role in the prevention of premature cell death or in the host antiviral defence has been proposed (Losick *et al.*, 2003). Ubiquitin-dependent degradation of the $3C^{PRO}$ was reported to be mediated in part by the E3 α /Ubr1, E3 ubiquitin ligase, in conjunction with the isoform of E2 ubiquitin-conjugating enzyme Ubch1 (Glickman & Ciechanover, 2002; Lawson *et al.*, 2001). However, EMCV $3C^{PRO}$ has also been shown as a substrate for a Ubch5a-dependent pathway (Lawson *et al.*, 1999) mediated by an unknown E3 ligase. Altogether, these data strongly suggest that the enhancement of $3C^{PRO}$ degradation by the activation of alternative pathways of ubiquitination, involving different E2 enzymes and E3 ligases, may be part of the host antiviral response induced by interferon (IFN) against picornavirus infection.

Recently, many tripartite motif (TRIM) proteins have emerged as IFN-induced proteins involved in various cellular processes, including cell proliferation and antiviral activities

(Meroni & Diez-Roux, 2005; Nisole *et al.*, 2005). TRIM proteins contain several structurally related domains, including a cluster of highly conserved RING-finger domain, one or two B-box domains and a predicted coiled-coil region (Meroni & Diez-Roux, 2005). The RING-finger domain of several TRIM proteins has been shown to possess an E3 ubiquitin ligase activity that governs a cascade of ubiquitin transfer reactions to specific proteins leading to tight control of the concentration or the subcellular location of cellular target proteins (Gack *et al.*, 2007; Kallijarvi *et al.*, 2005; Kong *et al.*, 2007; Kudryashova *et al.*, 2005). The coiled-coil domain is described as a folding motif important for self-association and oligomerization (Meroni & Diez-Roux, 2005). In addition, some TRIM proteins contain a C-terminal SPRY domain proposed to be involved in protein–protein interactions and RNA binding (Hilton *et al.*, 1998; Ponting *et al.*, 1997). TRIM22 (also known as Staf50) was first identified to be an IFN-induced human protein that represses transcription directed by the long terminal repeat (LTR) promoter region of human immunodeficiency virus type 1 (HIV-1) (Tissot & Mechti, 1995). Recently, TRIM22 has been reported to be a natural antiviral effector of both HIV-1 replication and particle production (Barr *et al.*, 2008; Bouazzaoui *et al.*, 2006). The effect of TRIM22 was abolished by mutation of amino acids Cys15 and Cys18 of its RING-finger domain, suggesting that functional ubiquitin ligase activity is required for TRIM22-mediated antiviral activities. In addition to antiviral properties, TRIM22 has also been shown to be a p53 target gene involved in the control of proliferation of myeloid cells and implicated in haematopoietic and T-cell differentiation (Gongora *et al.*, 2000; Obad *et al.*, 2004, 2007a, b).

In this study, we analysed the involvement of TRIM22 in the innate antiviral immune response against picornaviruses. We reported that TRIM22 is an E3 ubiquitin ligase whose expression leads to noticeable antiviral effects towards EMCV infection in HeLa cells. This effect is dependent on its E3 ligase activity through ubiquitination of the viral 3C^{PRO}. Altogether, our findings demonstrate that TRIM22 E3 ubiquitin ligase activity represents a new antiviral pathway in the mechanism of antiviral defence induced by IFN against picornaviruses.

METHODS

Cell culture, transfection and virus stocks.

Human HeLa and HEK293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transient transfection experiments were performed in six-well plates by using the Lipofectamine Plus Reagent method (Invitrogen). Twenty-four hours after transfection, cells were washed twice in PBS and infected with EMCV in DMEM supplemented with 10% FBS. EMCV stocks were prepared from supernatants of virus-infected L929 cells.

Virus stocks and virus yield assays.

EMCV was prepared from supernatants of virus-infected L929 cells. Infected cells were then frozen and thawed three times. The supernatants were serially diluted, and the virus titres were measured alternatively by a plaque assay on L929 cells as previously described (Blondel *et al.*, 1988) or by the end-point method (Milhaud *et al.*, 1983).

Plasmid constructs.

TRIM22 constructs were derived by PCR amplification using pCMV-SPORT6-TRIM22 (IMAGE consortium; Open Biosystems). The inserts to construct p3XFlag-TRIM22, p3XFlag-TRIM22 Δ RING, p3XFlag-TRIM22Nter and p3XFlag-TRIM22Cter expression vectors were generated by PCR amplification using *PfuUltra* DNA polymerase (Stratagene) with specific primers carrying *HindIII* and *SalI* sites, and subsequently cloned into the *HindIII* and *SalI* sites of p3XFlag-myc-CMV-24 expression vector (Sigma Aldrich). The EMCV 3C^{PRO} expression vector was derived by PCR amplification from pEC9 plasmid encoding the whole EMCV genome and cloned into pcDNA3.1myc-his vector (Invitrogen). The nucleotide sequence of all the constructs was confirmed by DNA sequencing (GenomeExpress). The plasmid pMT123 driving the expression of HA-tagged 8 \times ubiquitin [HA-(Ub)₈] was a generous gift from D. Bohmann (Rochester, New York, USA). The expression vectors for His-ubi and His-ubiK7R were a generous gift from M. T. Burgering (Utrecht, The Netherlands). pEC9 plasmid was a generous gift from A. Palmenberg (Madison, Wisconsin, USA).

Antibodies.

Polyclonal anti-EMCV-3C^{PRO} antibodies were prepared and purified as previously described (Lawson *et al.*, 1994). Mouse monoclonal antibodies directed against EMCV capsid protein VP1 were prepared as previously described (Borrego *et al.*, 2002). Anti-Flag monoclonal and polyclonal antibodies, anti- α -tubulin monoclonal antibody, peroxidase-

conjugated anti-mouse and anti-rabbit IgG (whole molecule) secondary antibodies were purchased from Sigma Aldrich. Anti-HA 12CA5 mouse monoclonal antibody was purchased from Roche Diagnostic and anti-ubiquitin antibody was purchased from Santa Cruz Biotechnology.

Immunoprecipitation and protein analysis.

HeLa or HEK293T cells were resuspended in lysis buffer consisting of PBS buffer containing 1% NP-40, 1 mM DTT, 100 μ M PMSF and protease inhibitor cocktail (1 tablet per 10 ml; Roche Diagnostics). Supernatant (10000 **g**) was prepared and used for immunoprecipitation. The extracts were incubated for 1 h at 4 °C with specific antibodies bound to sheep anti-mouse or anti-rabbit IgG-coupled magnetic beads (Dynabeads; Invitrogen). The beads were washed five times in lysis buffer. The immunoprecipitated proteins were resuspended in 20 μ l loading buffer [10 mM Tris/HCl pH 6.8, 1% SDS, 5 mM EDTA and 50% glycerol (v/v)], incubated 5 min at 95 °C, fractionated by SDS-PAGE and transferred onto PVDF membranes. After a blocking step, the membranes were incubated with the appropriate antibody and then developed using a enhanced chemiluminescent detection system (ECL+Plus; Amersham Pharmacia Biotech). For Ni-NTA (Qiagen) pull-down experiments, the cells were lysed in denaturing buffer (6 M guanidine/HCl, 100 mM Na₂HPO₄/NaH₂PO₄ at pH 8.0, 10 mM Tris/HCl at pH 8.0, 0.2% Triton X-100) and ubiquitinated proteins were precipitated using Ni-NTA agarose.

RESULTS

TRIM22 is a RING-finger E3 ubiquitin ligase

Several RING-finger domain containing proteins are known to act as ubiquitin (E3) ligases (Gack *et al.*, 2007; Kallijarvi *et al.*, 2005; Kong *et al.*, 2007; Kudryashova *et al.*, 2005). This occurs through a three-step process involving ubiquitin activating (E1) and ubiquitin-conjugating (E2) enzymes constitutively expressed in the cells and an ubiquitin E3 ligase that catalyses direct transfer of the activated ubiquitin from E2- to E3-bound substrates thereby providing the specificity for the target proteins. To determine whether the RING-finger domain of TRIM22 can confer to its ubiquitin ligase activity, Flag-TRIM22 fusion protein and HA-tagged ubiquitin polypeptide (HA-ubi) were co-expressed in HeLa cells. Subsequently, whole-cell extracts were prepared and analysed by immunoblotting with anti-HA monoclonal antibodies in order to detect HA-tagged ubiquitin conjugated proteins. As shown in Fig. 1(b), ubiquitin conjugates were produced in cells transfected with HA-ubi alone, reflecting ubiquitination by endogenous ubiquitin ligating enzymes. The level of conjugates was markedly greater in the presence of Flag-TRIM22. The presence of a high-molecular-mass smear of ubiquitinated proteins demonstrates that TRIM22 engenders robust ubiquitination in HeLa cells. The levels of Flag-TRIM22 were monitored by probing the membrane with anti-Flag antibody (Fig. 1b). Extracts from cells expressing Flag-TRIM22 or HA-ubi alone, used as negative controls, contained much smaller quantities of ubiquitin-protein conjugates. To evaluate the implication of the RING-finger domain in the ubiquitin-conjugating activity of TRIM22, we constructed an expression vector for expressing a TRIM22 version deleted of the first 59 aa residues corresponding to the RING-finger domain (Fig. 1a). HA-ubi was co-expressed with Flag-TRIM22 Δ RING and the ubiquitin-conjugated proteins were analysed as previously described. As expected, no ubiquitination activity was detected with TRIM22 Δ RING as compared with the wild-type TRIM22 (Fig. 1b, upper panel), whereas both proteins were expressed at the same level (Fig. 1b, lower panel). These data strongly suggest that TRIM22 is an ubiquitin E3 ligase that catalyses ubiquitin-conjugation of cellular proteins and that the RING-finger domain is required for its activity.

As ubiquitin E3 ligases are frequently ubiquitinated themselves, an event linked to their regulation, we next evaluated whether TRIM22 is ubiquitinated. To this end, we generated two additional expression vectors for both TRIM22 N-terminal region comprising the RING-finger domain and the B-box domain (Flag-TRIM22Nter) and for TRIM22 C-terminal region comprising the coiled-coil and the SPRY domains (Fig. 2a). His-ubiquitin (His-ubi) was co-expressed with Flag-TRIM22, Flag-TRIM22Cter or Flag-TRIM22Nter in HEK293T

cells. Cells were collected (10%) and whole-cell extracts were analysed by Western blotting using anti-Flag antibodies (Fig. 2b). In the presence of His-ubi, additional high-molecular-mass bands were revealed with Flag–TRIM22 and Flag–TRIM22Nter, suggesting that Flag–TRIM22 and the RING-finger domain of TRIM22 are ubiquitinated. Subsequently, the remaining 90% of cells were lysed in a denaturing buffer containing 6 M guanidine/HCl to dissociate ubiquitinated proteins bound to Flag–TRIM22, and ubiquitinated proteins were precipitated using Ni–NTA beads and analysed at first by 8% SDS-PAGE to resolve high-molecular-mass ubiquitin-conjugated TRIM22 products. Western blot detection using anti-Flag antibodies indicated that TRIM22 was polyubiquitinated (Fig. 2c). To resolve low-molecular-mass products, such as ubiquitinated TRIM22Nter, the three last samples shown in Fig. 2(c) were reanalysed by 12% SDS-PAGE. Western blot detection using anti-Flag antibodies revealed that the TRIM22 RING-finger domain (Flag–TRIM22Nter) appeared to be conjugated to one ubiquitin molecule at only one site (Fig. 2d). As an analysis of the TRIM22 amino acid sequence revealed the presence of 37 lysine residues, our finding may reflect the ability of TRIM22 to be polyubiquitinated in one or a few residues or to be monoubiquitinated in many residues. To evaluate these two possibilities, we used an His-ubiquitin construct (His-ubiK7R) that undergoes only monoubiquitination because all seven lysine residues of ubiquitin have been mutated to arginines (van der Horst *et al.*, 2006). Ni–NTA pull-down assays using this modified His-ubi resulted in a pattern of two prominent ubiquitinated forms of Flag–TRIM22 with relative molecular masses corresponding to the molecular mass of Flag–TRIM22 plus approximately 8000 or 16000 Da (Fig. 2e). These data indicated that TRIM22 is essentially ubiquitinated at two major sites and undergoes mono- and polyubiquitination.

TRIM22 promotes the ubiquitination of EMCV 3C protease in HeLa cells

To evaluate the ability of TRIM22 to mediate EMCV 3C^{PRO} ubiquitination in intact cells, we constructed expression vectors for EMCV 3C^{PRO} in pcDNA3.1-mycHis. Flag–TRIM22 and 3C^{PRO}-mycHis were co-expressed in HeLa cells in the presence or absence of His-ubi. After transfection (24 h), a treatment with the proteasome inhibitor MG132 (10 µM) for 14 h was performed in order to ensure 3C^{PRO} accumulation. Cells were subsequently lysed in denaturing buffer containing 6 M guanidine/HCl and ubiquitinated proteins were precipitated using Ni–NTA beads. When 3C^{PRO} was expressed alone, Western blot detection using anti-3C^{PRO} antibodies revealed 3C^{PRO} and additional high-molecular-mass bands (Fig. 3) consistent with 3C^{PRO} ubiquitination by endogenous ubiquitin and ubiquitin ligases, as previously described (Lawson *et al.*, 1994; Schlax *et al.*, 2007). When 3C^{PRO} was co-expressed with His-ubi, the intensity of the bands was enhanced and a slight decrease in their apparent mobility, due to the His-tag present on the transfected His-ubi, was observed. Interestingly, Flag–TRIM22 expression was associated with a significant and noticeable increase in 3C^{PRO} ubiquitination, both in the presence or absence of His-

ubi. These data demonstrate that TRIM22 expression enhances 3C^{PRO} ubiquitination in intact cells.

TRIM22 interacts with 3C^{PRO}

The transient association of E3 ligases with a potential recognition site on their target proteins is a prerequisite for the ubiquitin transfer reaction to occur. To verify whether 3C^{PRO} interacted with TRIM22, whole-cell extracts from Flag-TRIM22, Flag-TRIM22Cter or Flag-TRIM22Nter expressing HeLa cells were incubated with 1 μM purified bacterially expressed 3C^{PRO}. The resulting reaction mixtures were subjected to immunoprecipitations using monoclonal anti-Flag antibodies and the immunoprecipitates were analysed by Western blotting for the presence of 3C^{PRO} using anti-3C^{PRO} polyclonal antibodies. Extracts from HeLa cells expressing the human pre-mRNA cleavage factor IM25 (Flag-IM25), a non-3C^{PRO} substrate protein, was used as a negative control. As shown Fig. 4, 3C^{PRO} was strongly co-precipitated with Flag-TRIM22 and Flag-TRIM22Cter. No co-precipitation was detectable with extracts from Flag-TRIM22Nter or Flag-IM25 expressing HeLa cells. No co-precipitation was also observed with extract from cells transfected with the p3XFlag empty vector (data not shown). These data demonstrate that the C-terminal domain of TRIM22 can tightly associate with 3C^{PRO}. Because ubiquitin-protein ligases must recognize and bind to their substrates, the enhancement of 3C^{PRO} ubiquitination by TRIM22 suggests that TRIM22 may function as an E3 ligase in the 3C^{PRO} ubiquitination process.

TRIM22 confers protection against EMCV infection in transiently transfected HeLa cells

Our findings that TRIM22 can promote 3C^{PRO} ubiquitination suggested that it could be an active player in the antiviral action mediated by IFNs against picornaviruses. The potential antiviral activity of TRIM22 against EMCV was evaluated by transient transfection experiments in HeLa cells. Flag-TRIM22, Flag-TRIM22Cter or Flag-TRIM22Nter were expressed in HeLa cells. Subsequently, the cells were infected with EMCV at an m.o.i. of 1. Various times after infection, viral replication was monitored by Western blot analysis using mouse monoclonal antibodies directed against EMCV capsid protein VP1 (Borrego *et al.*, 2002). The results of a typical experiment, presented in Fig. 5, show that the cells transfected with the wild-type Flag-TRIM22 expressing construct exhibited a significant reduction in virus protein expression as compared with cells transfected with the empty p3XFlag vector. No protective effect was observed in HeLa cells expressing either Flag-TRIM22Cter or Flag-TRIM22Nter, suggesting that TRIM22 antiviral activity was mediated through its ubiquitin ligase activity. Similar results were obtained in three independent experiments.

Constitutive TRIM22 expression confers protection against EMCV infection in HeLa cells

Because in transient transfection experiments only part of the cell population is transfected and expresses the transgene, the antiviral effect observed is underestimated. Thus, to evaluate further TRIM22 antiviral activity, two HeLa cell clones (T7 and T8) constitutively overexpressing Flag–TRIM22 and two clones overexpressing either TRIM22Nter (N5) or TRIM22Cter (C7) were isolated and subjected to EMCV infection at an m.o.i. of 0.1 for 16 h (Fig. 6a). Virus titres were then measured using the end-point method with L929 cells. TRIM22 clearly induces an antiviral state that leads to a 4.6- (T7) or 2.9 (T8)-fold reduction in viral charge. According to the data presented in Fig. 5, the fact that TRIM22Nter and TRIM22Cter deletion mutants did not exhibit any antiviral effects, strongly suggested that TRIM22 antiviral effect is dependent on both the integrity of its E3 ligase activity (TRIM22Nter) and of its ability to interact with 3C^{PRO} (TRIM22Cter).

TRIM22 anti-EMCV effects were also evaluated by monitoring the time-dependent accumulation of 3C^{PRO} and its precursor 3CD during the infection of either control cells or HeLa clone T7. Western blot detection using anti-3C^{PRO} antibodies revealed that the level of detectable 3C^{PRO} and 3CD were strongly reduced by the presence of Flag–TRIM22 (Fig. 6b). 3C^{PRO} and 3CD appeared 6 h post-infection in normal cells and remained barely detectable in Flag–TRIM22 expressing cells.

Altogether, our data obtained either on transiently TRIM22-transfected HeLa cells or in HeLa cells constitutively expressing TRIM22, highlight the TRIM22-mediated antiviral effects against EMCV infection. Our data also suggest that these effects are clearly dependent on TRIM22 E3 ligase integrity, and that they could be in part mediated by TRIM22-dependent ubiquitination of the viral 3C protease.

DISCUSSION

Type I IFN plays a central role in the innate immune response to virus infection, in part by stimulating the synthesis of hundreds of effector proteins (Der *et al.*, 1998; Samuel, 2001). The huge diversity of virus families and the fact that viruses have developed strategies to circumvent the antiviral activities of IFN imply that mammalian cells use various alternative strategies to interfere with viral multiplication. Many E3 ubiquitin ligase members of the TRIM family are inducible by IFNs, providing candidate mediators for the IFN-induced antiviral state (Nisole *et al.*, 2005). Some recent studies have highlighted the role of ubiquitin ligases in both innate and adaptive immunity, as well as in immune evasion (for review see Liu, 2004). In this report, we provide evidence that TRIM22 E3 ubiquitin ligase activity participates in a novel antiviral pathway as part of the mechanism of IFN action against picornaviruses.

We demonstrate a crucial role for the RING-finger domain in TRIM22 ubiquitin E3 ligase activity. Indeed, ubiquitin ligase activity of a TRIM22 mutant lacking the RING-finger domain (TRIM22 Δ RING) was strongly reduced in comparison with the wild-type TRIM22. These data are consistent with other E3 ligases of the TRIM family, where the RING-finger domain has been shown to mediate the interaction with E2 ubiquitin-conjugating enzymes (Meroni & Diez-Roux, 2005). As described for other E3 ubiquitin ligases, TRIM22 is ubiquitinated, an event that may be relevant to its regulation. According to our data, pending submission of our manuscript, TRIM22 has been reported to be a novel RING-finger E3 ubiquitin ligase (Duan *et al.*, 2008). In this study, using GST–TRIM22 purified from GST–TRIM22 transfected COS cells as E3 source, the authors find that TRIM22 underwent self-ubiquitination in combination with the E2 enzyme UbcH5b, in an *in vitro* ubiquitination assay. In addition, because polyubiquitinated forms of TRIM22 seemed stabilized by proteasome inhibitor the authors concluded that TRIM22 targets itself for proteasomal degradation. However, polyubiquitination of proteins normally results in proteasome-mediated protein degradation, whereas monoubiquitination may mediate signalling functions (reviewed by Sigismund *et al.*, 2004). As the described stabilization of TRIM22 appeared only very partial, these data suggested that at least part of TRIM22 ubiquitination could participate to the regulation of its activity. In accordance with this hypothesis, we found that TRIM22 is likely ubiquitinated at two major sites. One site located in the RING-finger region undergoes monoubiquitination. As RING-finger ubiquitin E3 ligase-mediated ubiquitination occurs through a multi-step process involving binding of ubiquitin-conjugating (E2) enzymes to the RING-finger domain of the E3, this monoubiquitination might be involved in TRIM22 regulation by modulating the E2–E3 interaction. The second site, located in the C-terminal region, undergoes polyubiquitination

that might be involved in the control of TRIM22 stability or interaction with substrate proteins. This possibility is supported by the observation that the RING-finger region was ubiquitinated, while having lost the ability to catalyse the ubiquitination of other substrate proteins. This suggests that the TRIM22 C-terminal structurally related coiled-coil and SPRY domains are required for protein substrate recognition. Interestingly, the fact that Flag–TRIM22Cter which has lost the RING-finger-mediated ubiquitin ligase activity was not ubiquitinated suggests that TRIM22 ubiquitination was the result of autoubiquitination and not ubiquitination by other constitutively expressed ubiquitin ligases present in the cell extracts. Altogether, our data clearly demonstrate that TRIM22 is a RING-finger E3 ubiquitin ligase.

We demonstrated that stable and constitutive expression of TRIM22 confers a significant protection against EMCV infection in HeLa cells, providing a new antiviral pathway against picornaviruses. The same experiments were performed in transiently transfected cells, demonstrating that the protective effect was due to TRIM22 expression and was not a characteristic of the selected clones. In host cells, during virus infection the destruction of the short-lived picornaviral 3C^{PRO} is mediated by the ubiquitin/26S proteasome system (Losick *et al.*, 2003; Schlax *et al.*, 2007). Although the precise role of 3C^{PRO} degradation is still unknown, we proposed its involvement in the host antiviral response. Consistent with this hypothesis, in this study we found that TRIM22 specifically interacts with 3C^{PRO} and that 3C^{PRO} ubiquitination can be enhanced by TRIM22 in HeLa cells. We demonstrated that functional TRIM22 E3 ligase is required for TRIM22-mediated antiviral activity against EMCV infection. Indeed, the mutant of TRIM22 lacking the RING-finger domain and the ubiquitin ligase activity did not interfere with virus replication. In addition, the N-terminal region of TRIM22 did not exhibit antiviral effects, suggesting that the coiled-coil and SPRY domains are required for TRIM22–protein substrate recognition, as described for some E3 ligases (Meroni & Diez-Roux, 2005). Accordingly, we found that the C-terminal, but not the N-terminal region of TRIM22, can bind 3C^{PRO}. These data strongly support the idea that TRIM22 antiviral activity is mediated through the ubiquitination of the viral protease. However, we cannot totally exclude that TRIM22 could mediate 3C^{PRO} ubiquitination through the activation of other cellular E3 ligase activities. As TRIM E3 ligases are known to form homo- and heterodimers, TRIM22 may also act by mediating 3C^{PRO} recognition by unknown TRIM E3 ligases. Indeed, 3C^{PRO} has been shown to be ubiquitinated through several different ubiquitinating pathways, not only by Ubr1. Lawson *et al.* (1999) published data that showed that both Ubr1- and a Ubch5a-dependent unknown E3 ligases target EMCV 3C^{PRO}. In addition, the existence of other unidentified E3 ligases targeting 3C^{PRO} ubiquitination can not be excluded. Duan *et al.* (2008) reported that TRIM22 E3 ligase activity was dependent on Ubch5b as an E2 partner. Because Ubch5a and Ubch5b are functionally homologous, we can hypothesize that the unknown E3 ligase may be TRIM22.

Additional experiments are required to clarify this point and to clearly demonstrate whether or not the direct conjugation of ubiquitin to 3C^{PRO} by TRIM22 E3 ligase activity is involved in the TRIM22-mediated antiviral activity against EMCV infection.

Although E3 ubiquitin ligases are involved in non-lysosomal protein degradation, many studies point to the involvement of some ligases in many facets of cell biology unrelated to proteolysis, including transcription and trafficking (Ben-Neriah, 2002; Sigismund *et al.*, 2004; Woelk *et al.*, 2007). In general, polyubiquitination of proteins results in proteasome-mediated protein degradation, whereas monoubiquitination may mediate other regulating functions (Ben-Neriah, 2002). Surprisingly, we found that TRIM22 expression was associated with an increase of 3C^{PRO} ubiquitination in HeLa cells with a pattern corresponding to the addition of one to three ubiquitin molecules, suggesting that TRIM22 mediates the monoubiquitination of the viral protein rather than polyubiquitination. The precise identification of 3C^{PRO}-binding sites on TRIM22 and of the exact 3C^{PRO} amino acid residues involved in ubiquitin conjugation will greatly enhance our understanding of the antiviral effects mediated by TRIM22 against EMCV. On the basis of these observations, it is reasonable to speculate that TRIM22 antiviral activity can be mediated through limited 3C^{PRO} ubiquitination, resulting in altered proteinase function or subcellular localization rather than in proteasome-mediated degradation. Several forms of 3C^{PRO} are synthesized during virus replication because picornaviral polyproteins are cleaved sequentially through a series of intermediates (Hall & Palmenberg, 1996). Thus, it will be very interesting to evaluate whether these different 3C^{PRO} containing polyproteins can serve as substrates for TRIM22-catalysed ubiquitination.

TRIM22 also exhibits antiviral properties against other RNA viruses. TRIM22 has been shown to interfere with HIV-1 infection by either LTR promoter repression (Tissot & Mechti, 1995), inhibition of viral replication (Bouazzaoui *et al.*, 2006) or reduction of particle production (Barr *et al.*, 2008). Interestingly, the C-terminal SPRY domain of TRIM proteins is proposed to be involved in protein–protein interactions and RNA binding (Hilton *et al.*, 1998; Ponting *et al.*, 1997). So, it is tempting to speculate that RNA genomic viruses might be preferential targets for TRIM22 antiviral activity. The SPRY domain of TRIM22 might be required for sensing viral RNAs and promoting ubiquitination of viral proteins. Obviously, further studies are needed to test TRIM22 antiviral activity against other families of RNA viruses and to clarify the exact mechanism by which TRIM22 elicits its antiviral effects. These studies will lead to the identification of a set of new cellular or viral TRIM22 substrates that could be new targets for TRIM22-mediated ubiquitin conjugation.

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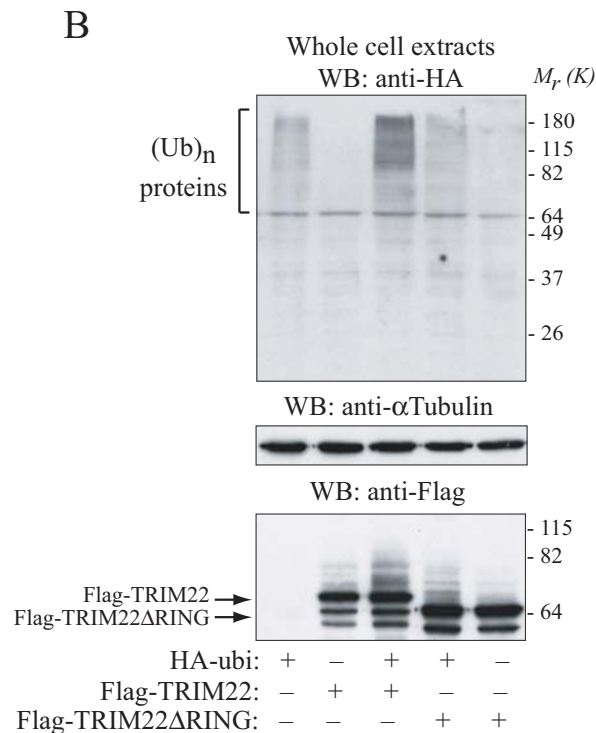
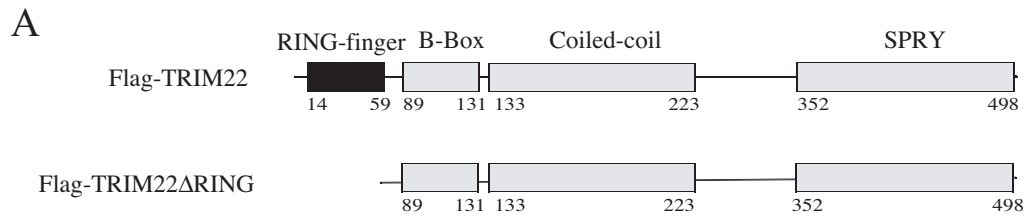


Fig. 1. TRIM22 catalyses the ubiquitination of cellular proteins. (a) Schematic representation of TRIM22 and TRIM22-deletion mutant. The tripartite motifs (RBCC) comprising the RING-finger domain, the B-box domain, the coiled-coil domain and the SPRY domain are represented. (b) Flag-TRIM22 and Flag-TRIM22 Δ RING were co-expressed in HeLa cells in the presence or absence of HA-(Ub)₈. Subsequently, whole-cell extracts (WCE) were prepared and analysed by Western blotting (WB) using anti-HA (upper panel), anti- α -tubulin (middle panel) or anti-Flag (lower panel) antibodies. Ubiquitinated proteins [(Ub)_n-protein] are indicated.

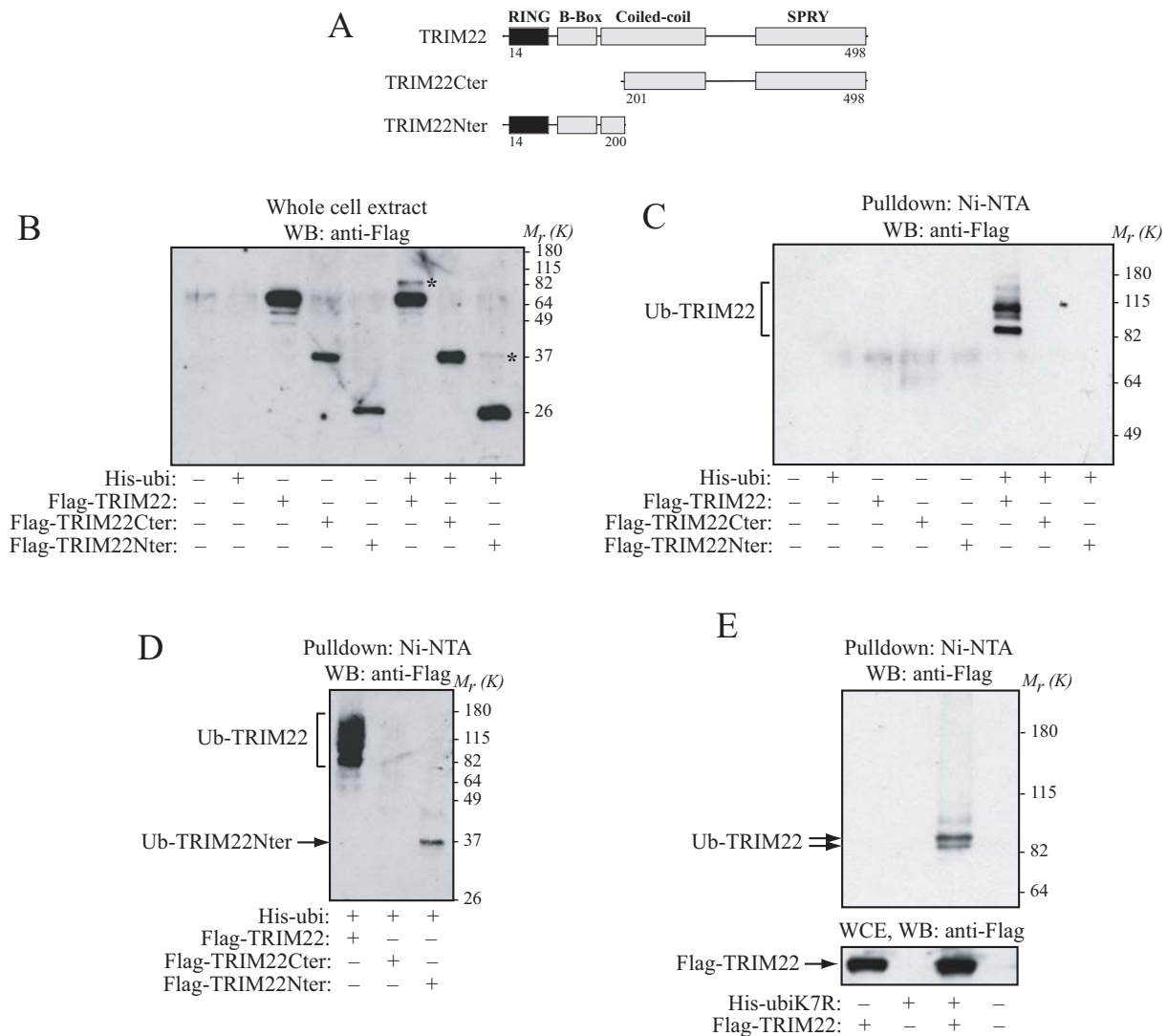


Fig. 2. TRIM22 is self-ubiquitinated. (a) Schematic representation of TRIM22 and TRIM22-deletion mutants. (b) Flag-TRIM22, Flag-TRIM22Cter or Flag-TRIM22Nter were co-expressed with His-ubi in HEK293T cells. Subsequently, whole-cell extracts (WCE) were prepared from a 10% fraction of the cells and were analysed by Western blotting (WB) using anti-Flag antibodies. Asterisks (*) indicate the location of additional ubiquitinated forms of Flag-TRIM22 and Flag-TRIM22Nter. (c) The remaining 90% of the cells were lysed in denaturing conditions and ubiquitinated proteins were precipitated using Ni-NTA agarose. Purified ubiquitinated proteins were analysed by WB by 8% SDS-PAGE for the presence of ubiquitinated forms of Flag-TRIM22, Flag-TRIM22Cter or Flag-TRIM22Nter

(Ub-TRIM22) using anti-Flag antibodies. (d) Purified ubiquitinated proteins corresponding to the three last samples analysed on Fig. 2(c) were reanalysed by 12% SDS-PAGE in order to detect ubiquitinated forms of Flag-TRIM22Nter (Ub-Flag-TRIM22Nter). (e) Flag-TRIM22 was co-expressed in HEK293T cells with a mutant ubiquitin in which all seven lysine residues have been mutated to arginines (His-ubiK7R). Ni-NTA pull-down assays were performed as described in (b) and ubiquitinated protein analysed by WB for the presence of ubiquitinated forms of Flag-TRIM22 (Ub-TRIM22) using anti-Flag antibodies. Flag-TRIM22 expression was verified by WB of WCE using anti-Flag antibodies.

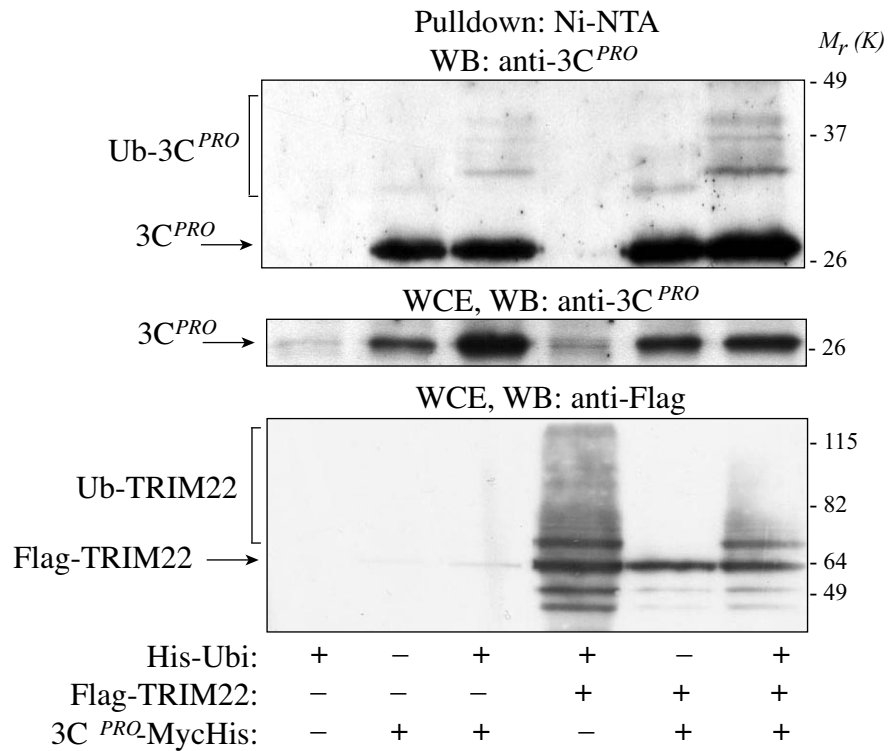


Fig. 3. TRIM22 catalyses the ubiquitination of EMCV 3C^{PRO} in HeLa cells. Flag-TRIM22 and EMCV 3C^{PRO}-His were co-expressed in HeLa cells in the presence or absence of His-ubi. The cells were treated with 10 μM MG132 for 14 h and, subsequently, 90% of the cells were lysed in denaturing conditions and ubiquitinated proteins were precipitated using Ni-NTA agarose. Purified ubiquitinated proteins were analysed by Western blotting (WB) for the presence of 3C^{PRO} and ubiquitinated forms of 3C^{PRO} (Ub-3C^{PRO}) using anti-3C^{PRO} antibodies (upper panel). Whole-cell extracts (WCE) were prepared from the remaining 10% fraction of the cells and analysed by WB for the presence of 3C^{PRO} (middle panel) and Flag-TRIM22 (lower panel). The 3C^{PRO} antibody-reactive bands in lanes 1 and 4 of the anti-3C^{PRO} WB of Fig. 3, are cellular proteins that frequently cross-react with the antibodies and co-migrates with the EMCV 3C^{PRO} (Schlax *et al.*, 2007).

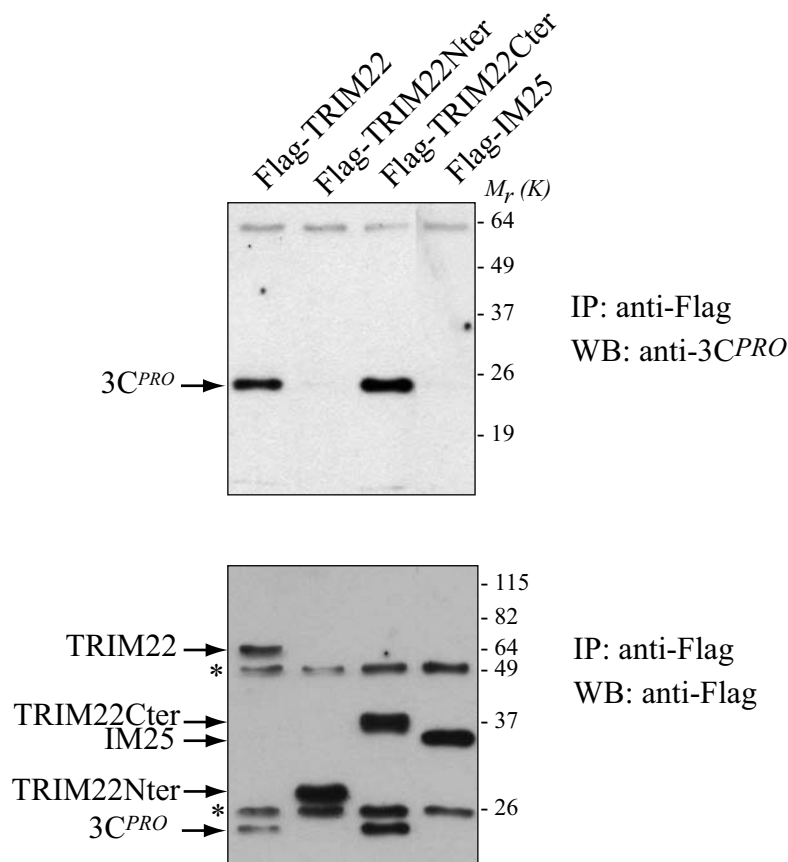


Fig. 4. TRIM22 interacts with EMCV 3C^{PRO}. Whole-cell extracts from HeLa cells expressing either Flag-TRIM22, Flag-TRIM22Cter, Flag-TRIM22Nter or Flag-IM25 were incubated with 1 μ M purified bacterially expressed 3C^{PRO}. The resulting reaction mixtures were subjected to immunoprecipitations (IP) using monoclonal anti-Flag antibodies, and the immunoprecipitates were analysed by Western blotting (WB) for the presence of 3C^{PRO} using anti-3C^{PRO} polyclonal antibodies. Extract from HeLa cells expressing the human pre-mRNA cleavage factor IM25 (Flag-IM25), a non-3C^{PRO} substrate protein was used as a negative control. The blot was reprobed with anti-Flag antibodies to verify Flag constructs expression. Asterisks (*) indicate immunoglobulin chains.

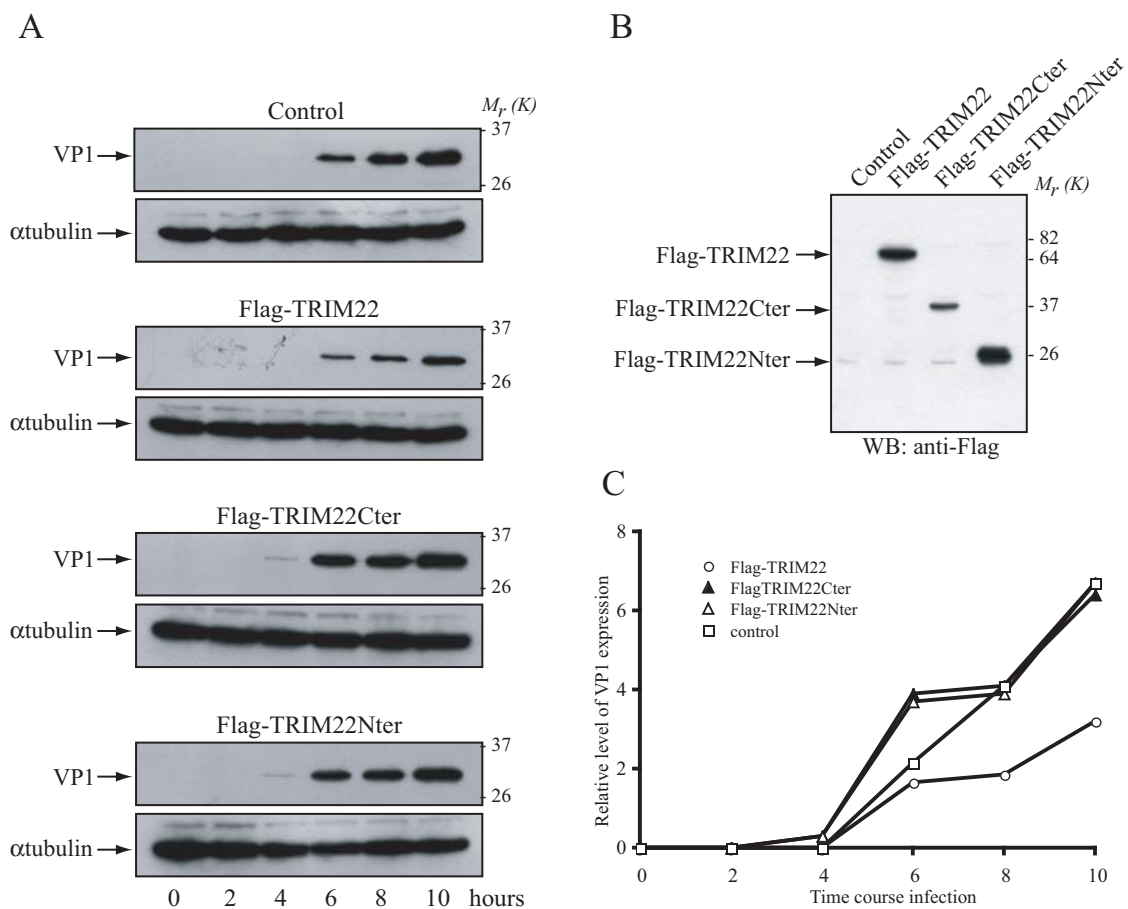
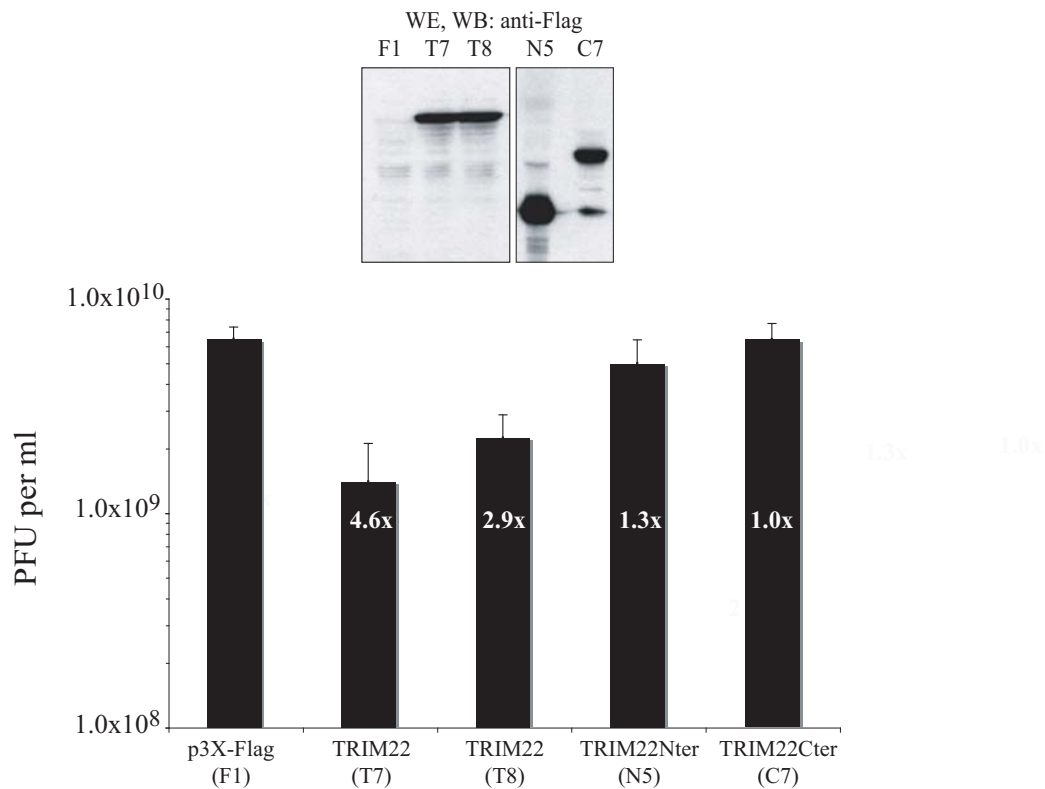


Fig. 5. TRIM22 confers resistance to EMCV infection in transiently transfected HeLa cells. (a) p3XFlag, Flag-TRIM22, p3XFlag-CterTRIM22 or p3XFlag-NterTRIM22 were expressed in HeLa cells. Cells transfected with the p3XFlag empty vector were used as a negative control. The cells were then infected with EMCV at an m.o.i. of 1. Whole-cell extracts were prepared at the indicated times and analysed by Western blotting (WB) for the presence of the EMCV capsid protein VP1, using anti-VP1 and anti- α -tubulin antibodies. (b) Whole-cell extracts described in (a) were analysed by WB for the presence of Flag-TRIM22, Flag-TRIM22Cter or p3XFlag-TRIM22Nter using anti-Flag antibodies. (c) The VP1 expression levels presented in (a) were quantified by scanning the bands from immunoblot autoradiograms using the NIH ImageJ 1.38x tool, with the α -tubulin bands as an internal calibration control. Each WB membrane was treated simultaneously with the same antibody incubation time and subsequent washes. Exposure with ECL was also done in parallel, and the autoradiograms were developed with the same exposure time. The variations of the relative levels of VP1 expression during the time course of EMCV infection are presented.

A



B

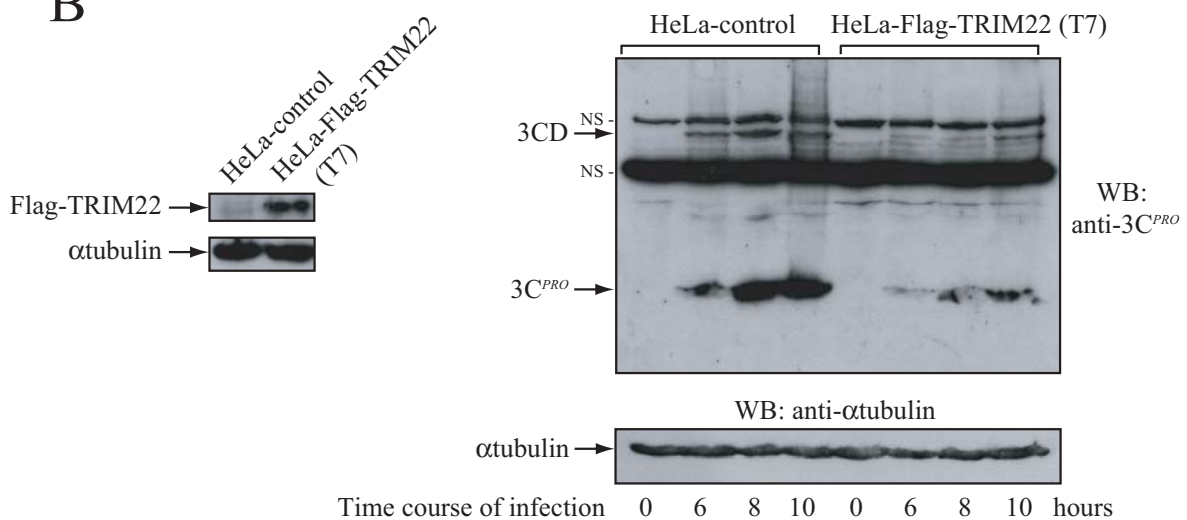


Fig. 6. Constitutive expression of TRIM22 confers resistance to EMCV infections. (a) HeLa cells constitutively expressing p3XFlag (F1), Flag-TRIM22 (clones T7 and T8), Flag-TRIM22Nter (N5) or Flag-TRIM22Cter (C7) were infected with EMCV at an m.o.i. of 0.1 for 16 h. Virus titre was determined by the end-point method on L929 cells. Inset, the level of TRIM22 expression of each clone was analysed by Western blotting (WB) using whole-cell extracts (WCE) probed with anti-Flag antibodies. Folds of virus titre reduction with respect to F1 titre are indicated on the histogram. (b) Left panel, WCE from HeLa cells

(HeLa-control) or Flag–TRIM22 constitutively expressing HeLa cells (HeLa-Flag–TRIM22, clone T7) were analysed by WB for the presence of Flag–TRIM22 using anti-Flag and anti- α -tubulin antibodies. Right panel, the cells were then infected with EMCV at an m.o.i. of 1. WCE were prepared at the indicated time and analysed by WB for the presence of EMCV $3C^{PRO}$ using anti- $3C^{PRO}$ and anti- α -tubulin antibodies. $3C^{PRO}$ and the precursor 3CD are indicated. NS, Non-specific bands revealed by anti- $3C^{PRO}$ antibodies.