

# The p53 target gene TRIM22 directly or indirectly interacts with the translation initiation factor eIF4E and inhibits the binding of eIF4E to eIF4G

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**Background information.** The interferon (IFN)-inducible protein TRIM22 (Staf50) is a member of the tripartite motif protein family and has been suggested a role in the regulation of viral replication as well as of protein ubiquitylation. In addition, we have previously shown that TRIM22 is a direct target gene for the tumour suppressor p53. Consistently, over-expression of TRIM22 inhibits the clonogenic growth of monoblastic U937 cells, suggesting anti-proliferative or cell death-inducing effects.

**Results.** Here, we demonstrate that TRIM22 directly or indirectly interacts with the eukaryotic translation initiation factor (eIF)4E, and inhibits the binding of eIF4E to eIF4G, thus disturbing the assembly of the eIF4F complex, which is necessary for cap-dependent translation. Furthermore, TRIM22 exerts a repressive effect on luciferase reporter protein levels and to some extent on radiolabelled methionine incorporation. Even though all nuclear mRNAs are capped, some are more dependent on eIF4F than others for translation. The translation of one of these mRNAs, IRF-7C, was indeed found to be repressed in the presence of TRIM22.

**Conclusions.** Our data suggest TRIM22 to repress protein translation preferably of some specific mRNAs. Taken together, we show that TRIM22 represses translation by inhibiting the binding of eIF4E to eIF4G, suggesting a mechanism for regulation of protein translation, which may be of importance in response to p53 and/or IFN signalling.



Supporting Information available online

## Introduction

We have previously identified the interferon (IFN)-inducible protein TRIM22 (Staf50) as a target gene for the tumour suppressor p53 (Obad et al., 2004). In the absence of IFN or p53 stimulation, TRIM22 is constitutively expressed in lymphoid tissues such as

peripheral lymph nodes, thymus and spleen, as well as in peripheral blood mononuclear leukocytes and in the ovary and lung (Tissot and Mechti, 1995). The specific molecular function of TRIM22 has not yet been elucidated, but TRIM22 has been shown to be involved in repression of viral replication (Barr et al., 2008; Bouazzaoui et al., 2006; Eldin et al., 2009; Gao et al., 2009; Kajaste-Rudnitski et al., 2011; Singh et al., 2011). Furthermore, we have previously shown that over-expression of TRIM22 inhibits the clonogenic growth of monoblastic U937 cells,

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**Abbreviations used:** eGFP, enhanced green fluorescent protein; IFN, interferon; PML, promyelocytic leukemia.

and also that TRIM22 is downregulated during T lymphocyte activation, indicating anti-proliferative or apoptotic effects (Gongora et al., 2000; Obad et al., 2004, 2007).

TRIM22 belongs to the TRIM protein family, named based on a characteristic tripartite motif (TRIM), including a RING finger, one or two B-boxes and a coiled-coil domain. RING domains are often associated with ubiquitin E3-ligase activity, responsible for ubiquitylation of target proteins, marking them for destruction in the proteasome or altered activity (Joazeiro and Weissman, 2000). Many TRIM family members have been shown to exhibit RING domain-dependent E3-ligase activity, including TRIM22 (Duan et al., 2008; Eldin et al., 2009; Meroni and Diez-Roux, 2005).

The TRIM protein family consists of more than 70 proteins, involved in diverse biological processes such as apoptosis, cell proliferation and viral defence (Meroni and Diez-Roux, 2005; Sardiello et al., 2008). Several TRIM protein family members are also associated with cancer. Promyelocytic leukemia (TRIM19) and Ret finger protein (TRIM27) form oncogenic fusion proteins with retinoic acid receptor  $\alpha$  and Ret proto-oncogene, respectively, whilst estrogen-responsive finger protein (TRIM25) and TRIM32 enhance proliferation and survival of breast tumour growth and squamous cancer cells, respectively (Cao et al., 1998, 1996; Horn et al., 2004; Urano et al., 2002).

PML is the most well studied TRIM protein. PML has been shown to inhibit protein translation, and to induce cell cycle arrest, cell death and viral restriction (Salomoni et al., 2008). The translational regulatory activity of PML is mediated through its interaction with the eukaryotic translation initiation factor (eIF)4E. When binding to eIF4E, PML induces a conformational change, resulting in a 100-fold reduction in the cap-binding affinity of eIF4E (von der Haar et al., 2004). This gives PML the ability to repress eIF4E-mediated mRNA transport from the nucleus to the cytoplasm as well as to repress translation initiation in the cytoplasm (Culjkovic et al., 2006; Kentsis et al., 2001). eIF4E-binding proteins generally interact with eIF4E through the YXXXL $\Phi$  motif (where X is any residue and  $\Phi$  is leucine, methionine or phenylalanine) (Sonenberg and Gingras, 1998). However, PML does not contain this motif

but instead binds to eIF4E through its RING domain (Cohen et al., 2001).

Regulation of protein translation plays a critical role in many fundamental biological processes, such as cell growth, development and viral replication but also in response to cell stress (Holcik and Sonenberg, 2005). During transcription, most cellular mRNAs are capped with an m<sup>7</sup>GpppN-cap at the 5' terminus and a poly(A)tail at the 3' terminus, after which the modified mRNA is transported to the cytoplasm where translation occurs. Capped translation is dependent on an array of eIFs, for example eIF4E, eIF4G, eIF4A etc. (Van Der Kelen et al., 2009).

eIF4E is a central effector molecule in response to cell stress, regulating both the transport of certain mRNAs from the nucleus into the cytoplasm, as well as the initiation of translation by binding to the mRNA m<sup>7</sup>G-cap. Binding between eIF4G and the eIF4E–mRNA complex results in a stronger association with the cap. Together with eIF4A, which unwinds secondary structures of the mRNA, eIF4E and eIF4G constitute the translation initiation factor complex eIF4F. The regulatory protein 4E-BP1 inhibits translation by binding to eIF4E, interfering with its interaction with eIF4G (Topisirovic et al., 2011; Van Der Kelen et al., 2009).

Although all capped mRNAs require eIF4F, certain mRNAs are more dependent on eIF4F than others for translation to occur. These mRNAs are often described as 'weak' mRNAs, and have a more complex secondary structure. These 'weak' mRNAs are not characterised by low stability or abundance, but are rather inherently weakly translated. As a general rule, complex hairpin structures upstream of the start codon repress translation, and often these 'weak' mRNAs have oncogenic potential. In contrast, transcripts of housekeeping genes such as GAPDH mRNA are efficiently translated even in the presence of low eIF4F levels. Consistently, an increase in either amount or activity of eIF4E leads to increased translation of a subset of mRNAs, rather than elevated rates of global translation. However, the mRNA target repertoire of eIF4E may differ between different tissue types (Hsieh and Ruggero, 2010; Livingstone et al., 2010; Richter and Sonenberg, 2005).

Interestingly, PML is in many aspects remarkably similar to TRIM22. Both are TRIM proteins induced by p53 and IFNs, containing a p53-response element

in intron 1 in their genetic sequence (Carthagena et al., 2009; de Stanchina et al., 2004; Obad et al., 2004). Furthermore, they are both involved in viral defence. However, in an evolutionary perspective, they are quite distantly related with barely detectable homology (Sardiello et al., 2008). Given the connection of both p53 and IFNs to regulation of cell growth and proliferation, and to the response to cell stress, we wondered whether TRIM22 also might be able to bind to eIF4E and repress translation in a manner similar to PML. Indeed, our data show that TRIM22 directly or indirectly interacts with eIF4E and represses protein translation. However, in contrast to PML, which interrupts the binding of eIF4E to the mRNA m<sup>7</sup>G-cap structure, we show that TRIM22 inhibits the binding of eIF4E to eIF4G. Consistently, we demonstrate that TRIM22 represses IRF-7, a 'weak' mRNA highly dependent on eIF4F for translation. Moreover, also dissimilar from PML, TRIM22 inhibits translation in a RING domain independent manner. Taken together, our data increase our understanding of the function of TRIM22 and suggest a mechanism by which TRIM22 influences protein translation, which may be of significance in response to p53 and/or IFN signalling.

## Results

### TRIM22 interacts with eIF4E independently of its RING domain

Translation initiation factors are necessary for cap-dependent translation to occur. For example, the translation initiation factor eIF4E must bind to the mRNA m<sup>7</sup>G-cap for translation to be initiated. PML (TRIM19) has been shown to bind to eIF4E through its RING domain, repressing cap-dependent protein translation (Kentsis et al., 2001). To study whether TRIM22 also interacts with eIF4E, TRIM22 was co-transfected together with eIF4E into 293T/17 cells after which sequential immunoprecipitation and Western blotting were performed. A TRIM22 construct with deleted RING domain (TRIM22-delRING) was utilised to determine if the RING domain is necessary also for the ability of TRIM22 to interact with eIF4E. As demonstrated in Figure 1, specific bands were obtained in reciprocal experiments, revealing that both full-length TRIM22 and, interestingly also TRIM22-delRING, were pulled down together with eIF4E. Hence, TRIM22 inter-

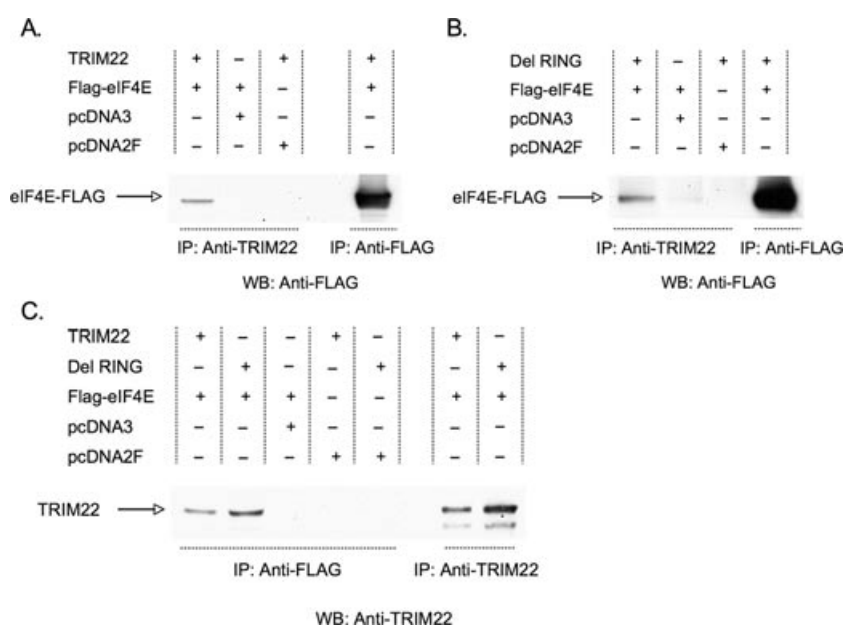
acts directly or indirectly with eIF4E and moreover, unlike PML, the interaction is independent of the RING domain of TRIM22. However, the possibility that RNA might mediate the interaction between TRIM22 and eIF4E cannot be excluded, since RNase was not included in the experiments.

### TRIM22 inhibits the binding of eIF4E to eIF4G

Since PML binds to eIF4E and represses the ability of eIF4E to bind to the mRNA m<sup>7</sup>G-cap (von der Haar et al., 2004), and since TRIM22 also interacts with eIF4E, our next question was whether TRIM22 also represses the cap-binding ability of eIF4E. To that end, TRIM22 or TRIM22-delRING were transfected into 293T/17 cells, after which the cells were lysed. To isolate cap-binding fractions of eIF4E in the presence and absence of TRIM22, equal protein amounts were precipitated with the mRNA cap-analogue 7-methyl-GTP sepharose 4B. However, neither full-length TRIM22 nor TRIM22-delRING showed any effect on the amount of precipitated eIF4E (Figure 2A), indicating that TRIM22 does not affect the binding of eIF4E to the mRNA m<sup>7</sup>G-cap. Interestingly, both TRIM22 as well as TRIM22-delRING were precipitated with the m<sup>7</sup>G-cap analogue, confirming the interaction of TRIM22 with the cap-binding fraction of eIF4E (Figure 2A). However, TRIM22 was not unspecifically precipitated by sepharose, as shown in Figure 1C, talking against an unspecific interaction of TRIM22 with sepharose 4B. In order to study whether TRIM22 affects the interaction of eIF4E with other binding partners, levels of the scaffold protein eIF4G and the inhibitory protein 4E-BP1 were analysed. Since eIF4E precipitates with the cap-analogue 7-methyl-GTP sepharose 4B, also the binding partners of eIF4E, for example eIF4G and 4E-BP1, are precipitated. Interestingly, a significantly lesser amount of eIF4G was 7-methyl-GTP precipitated in cells transfected with TRIM22 or TRIM22-delRING (Figure 2A), suggesting TRIM22 to interfere with the binding of eIF4E to eIF4G, a step vital for translation to occur. This step is regulated by several proteins, for example 4E-BP1, binding the same region of eIF4E as does eIF4G (Asnaghi et al., 2004). However, the amount of precipitated 4E-BP1 was not diminished in the TRIM22-transfected cells, suggesting that TRIM22 does not affect the binding of 4E-BP1 to eIF4E (Figure 2A).

**Figure 1 | TRIM22 binds to the translation initiation factor eIF4E independently of its RING domain**

(A) 293T/17 cells were co-transfected either with FLAG-eIF4E and TRIM22 (lanes 1 and 4), FLAG-eIF4E and empty pcDNA3 (lane 2) or TRIM22 and empty pcDNA2F (lane 3). After 48 h, cells were harvested, lysed in lysis buffer and subjected to immunoprecipitation with TRIM22 (Abnova) or FLAG antibody as indicated. The cell lysates were subjected to Western blotting with FLAG antibody (lane 1–4). (B) 293T/17 cells were co-transfected either with FLAG-eIF4E and TRIM22-delRING (lanes 1 and 4), FLAG-eIF4E and empty pcDNA3 (lane 2) or TRIM22-delRING and empty pcDNA2F (lane 3). After 48 h, cells were harvested, lysed in lysis buffer and subjected to immunoprecipitation with TRIM22 (Abnova) or FLAG antibody as indicated. The cell lysates were subjected to Western blotting with FLAG antibody (lanes 1–4). (C) 293T/17 cells were co-transfected either with FLAG-eIF4E and TRIM22 (lanes 1 and 6), FLAG-eIF4E and TRIM22-delRING (lane 2 and 7), FLAG-eIF4E and empty pcDNA3 (lane 3), TRIM22 and empty pcDNA2F (lane 4) or TRIM22-delRING and empty pcDNA2F (lane 5). After 48 h, cells were harvested, lysed in lysis buffer and subjected to immunoprecipitation with FLAG or TRIM22 (Atlas) antibody as indicated. The cell lysates were subjected to Western blotting with FLAG antibody. In (C), IP and Western blotting were performed using the One-hour complete IP-Western kit, to avoid heavy and light IP chains.



The tumour suppressor p53 has been shown to repress translation by degradation of eIF4G (Constantinou and Clemens, 2007). To exclude the possibility that TRIM22 induces degradation of eIF4G, TRIM22 or TRIM22-delRING was transfected into 293T/17 cells and the levels of eIF4G were analysed by Western blot. As shown in Figure 2B, an effect on the amount of eIF4G in cells expressing TRIM22 or TRIM22-delRING was not demonstrated, suggesting the TRIM22-related effects on levels of cap-precipitated eIF4G not to be mediated by increased degradation of eIF4G. Altogether, these results suggest that TRIM22 directly or indirectly interacts with eIF4E, without affecting its interaction with the mRNA m<sup>7</sup>G-cap structure. Furthermore, our data suggest that TRIM22 inhibits the binding of eIF4E

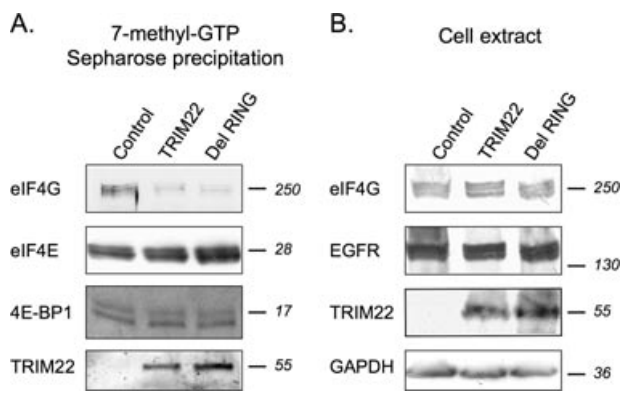
to eIF4G, and that this occurs independently of the RING domain of TRIM22.

**TRIM22 represses protein translation from a luciferase reporter construct**

As TRIM22 directly or indirectly interacts with eIF4E and inhibits the binding of eIF4E to eIF4G, thus affecting initiators of protein translation, we next examined the effect of TRIM22 on protein translation. Therefore, a luciferase reporter system was used as an assay of protein translation by estimation of luminescence in combination with measurement of luciferase mRNA levels after transfection of luciferase reporter constructs. For that purpose, TRIM22 was co-transfected into 293T/17 cells together with pGL3/Waf1/Luc or with pGL3/Bax/Luc

**Figure 2 | TRIM22 inhibits the binding of eIF4E to eIF4G, without affecting the levels of eIF4G**

(A) 293T/17 cells were transfected with TRIM22, TRIM22-delRING or control plasmid (pcDNA3). After 48 h, cells were lysed and total protein amount was measured. Equal protein amounts were precipitated with 7-methyl-GTP Sepharose 4B, each sample was divided into two parts and resolved on 10–20% SDS-PAGE (eIF4E, 4E-BP1 and TRIM22) or 6% SDS-PAGE (eIF4G). (B) 293T/17 cells were transfected with TRIM22, TRIM22-delRING or control plasmid (pcDNA3). After 48 h, samples were harvested and resolved on either 10–20% (TRIM22 and GAPDH) or 6% (eIF4G and EGF receptor) SDS-PAGE gels and subjected to Western blotting. The EGF receptor and GAPDH were used to estimate equal input. Molecular weight markers (kDa) to the right.



expressing firefly luciferase after a p21 or bax promoter sequence. After 48 h, luciferase activity was measured by luminescence and luciferase mRNA levels were determined by real-time PCR. Indeed, luciferase activity from both constructs was down-regulated in the presence of TRIM22 (Figure 3A), although luciferase mRNA levels were not affected (Figure 3B), indicating that TRIM22 represses expression of luciferase on the post-transcriptional level. Protein levels were also visualised by Western blot using an anti-luciferase antibody, confirming the down-regulation of luciferase protein in the presence of TRIM22 (Figure 3C). The effect was not specific to either p21 or bax promoters since several promoters were assessed with the same result (data not shown). Moreover, the TRIM22-mediated effect on luciferase activity was dependent on the amount of transfected TRIM22 cDNA, confirming a dose–response effect of TRIM22 on protein translation (Figures 3D–3F).

Hence, our data indicate that TRIM22 represses protein expression by a post-transcriptional mechanism.

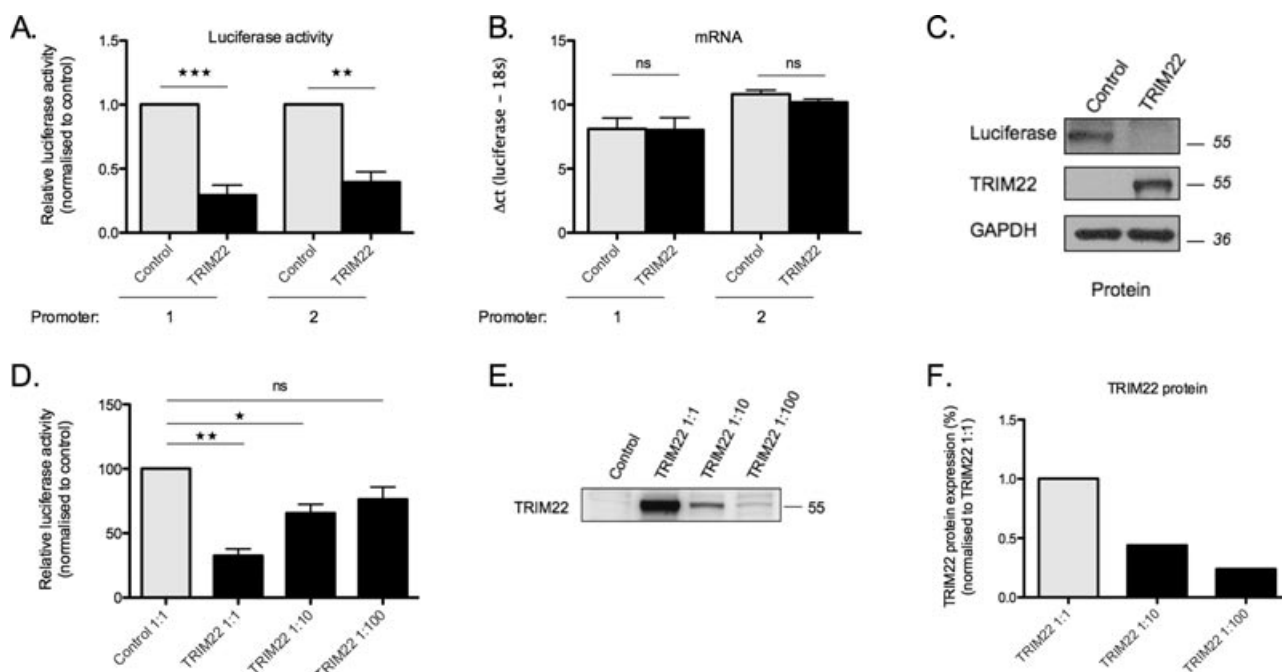
### TRIM22-mediated repression of protein expression is independent of its RING domain

To determine whether the repressive effects on luciferase protein levels were dependent on the RING or SPRY domain of TRIM22 (a schematic representation of the domains of TRIM22 is visualised in Figure 4A), we used the RING-deletion mutant, as well as a SPRY-deletion mutant of TRIM22 in the luciferase translation assay. Since the RING domain is required for the ubiquitin E3-ligase activity of TRIM22 (Duan et al., 2008; Eldin et al., 2009), deletion of the RING domain abolishes the ubiquitin E3-ligase activity of TRIM22. As expected, full-length TRIM22 repressed luciferase activity. Also TRIM22 lacking the RING domain repressed the reporter activity, suggesting that the RING domain is dispensable for the repressive effect on protein expression (Figure 4B). This also suggests luciferase not to be degraded by the E3-ligase activity of TRIM22. However, the TRIM22-delSPRY exhibited a significantly weaker repressive effect, demonstrating specificity for the translational repression of full-length TRIM22 and TRIM22-delRING. These data are consistent with our previous results showing the RING domain to be dispensable for both eIF4E binding and disruption of the binding of eIF4E to eIF4G (Figures 1 and 2). The deletion of the RING domain from TRIM22 results in a protein of approximately 7 kDa less molecular weight. Still, TRIM22-delRING was visualised with approximately equal size as full-length TRIM22 on Western blot (Figure 4C). Therefore, cDNA sequencing of both constructs was performed, showing the expected sequence. Moreover, *in vitro* translated TRIM22-delRING migrated faster than *in vitro* translated full-length TRIM22 with expected differences in molecular weight, even though *in vivo* translated full-length TRIM22 and TRIM22-delRING were visualised with equal size on the same protein gel (Supplementary Figure S1). We cannot explain why proteins of expected different molecular weight after expression *in vivo* migrate at a similar rate on the gel as observed in Figure 4C and Supplementary Figure S1.

To further exclude the possibility that TRIM22 represses luciferase through degradation, we investigated the protein stability of luciferase in the

**Figure 3** TRIM22 represses luciferase protein levels but does not affect luciferase mRNA levels

(A) 293T/17 cells were co-transfected with pGL3/Waf1/Luc (promoter 1) or pGL3/Bax/Luc (promoter 2) together with TRIM22 or a control plasmid (empty pcDNA3). After 48 h, luciferase activity was measured. Error bars represent SEM,  $n = 6$  (1) or  $n = 5$  (2), each performed in duplicate. (B) 293T/17 cells were co-transfected with pGL3/Waf1/Luc (promoter 1) or pGL3/Bax/Luc (promoter 2) together with TRIM22 or a control plasmid (empty pcDNA3). After 48 h, RNA was extracted and subjected to real-time RT-PCR to determine luciferase expression. Error bars represent SEM,  $n = 6$  (1) or  $n = 4$  (2), each performed in triplicate. (C) 293T/17 cells were transfected with pGL3/Waf1/Luc together with TRIM22 or a control plasmid (empty pcDNA3). After 48 h, samples were harvested and subjected to Western blotting. (D) 293T/17 cells were co-transfected with pGL3/Waf1/Luc together with decreasing amounts of TRIM22 or a control plasmid (empty pcDNA3). After 48 h, luciferase activity was measured. Error bars represent SEM,  $n = 4$ , each performed in duplicate. The value of each dilution was normalised against the control. (E) The cell lysates from (D) were subjected to Western blotting. (F) TRIM22 protein levels, as determined by densitometry, from (E). \*\*\* $P < 0.0005$ , \*\* $P < 0.005$  and \* $P < 0.05$  according to paired  $t$ -test. Molecular weight markers (kDa) to the right.



presence of TRIM22 and TRIM22-delRING. To this end, cycloheximide was added to 293T/17 cells co-transfected with TRIM22/TRIM22-delRING together with pGL3/Waf1/Luc. Thereafter luciferase activity was measured by luminescence. Cycloheximide completely interrupts protein translation, resulting in diminishing levels of proteins at a rate depending on how fast the proteins are degraded. As expected, neither the presence of TRIM22 nor TRIM22-delRING did accelerate the degradation of luciferase (Supplementary Figure S2). However, as previously shown (Figures 3 and 4), both TRIM22 and TRIM22-delRING did repress basal luciferase activity at time 0 h (data not shown). In conclusion, our data support that TRIM22 does not enhance the

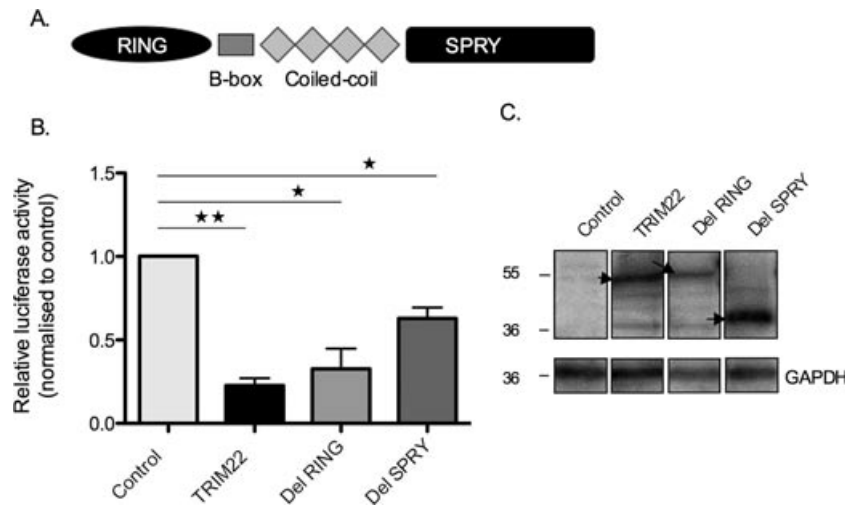
degradation of luciferase protein. This is consistent with our finding that the effect of TRIM22 on protein levels is independent of its RING domain, and hence also of its E3-ligase activity.

#### TRIM22 moderately represses total protein synthesis

As we had observed that TRIM22 reduced luciferase protein translation, we asked whether this effect is specific for luciferase protein or whether it extends to other proteins as well. Therefore, a protein synthesis assay was performed. Since PML is implicated in translational repression, enhanced green fluorescent protein (eGFP)-tagged PML was used as a positive control (Cohen et al., 2001).

**Figure 4 | TRIM22 and TRIM22-delRING repress luciferase activity, whereas TRIM22-delSPRY represses luciferase activity to a moderate extent**

(A) Schematic representation of the domains of TRIM22. (B) 293T/17 cells were co-transfected with pGL3/Waf1/Luc together with TRIM22, TRIM22-delRING, TRIM22-delSPRY or a control plasmid (empty pcDNA3). After 48 h, luciferase activity was measured. Error bars represent SEM,  $n = 3$ , each performed in duplicate. (C) The cell lysates from (B) were subjected to Western blotting as described in 'Materials and Methods' normal type. Error bars represent SEM,  $n = 3$ , each performed in duplicate. \*\*\* $P < 0.0005$ , \*\* $P < 0.005$  and \* $P < 0.05$  according to paired  $t$ -test. Molecular weight marker (kDa) to the left.



To that end, 293T/17 cells were transfected with eGFP-tagged TRIM22, PML or eGFP alone. After 24 and 72 h, the cells were pulsed with [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine for 2 h. In order to avoid results being skewed by possible cell death, the fraction of dead cells was estimated by the cell death markers Annexin V and 4',6-diamidino-2-phenylindole (DAPI). No TRIM22-related effects on cell viability could be observed, and the amount of cell death in either cell population was never higher than 10% (data not shown). To analyse the effects of TRIM22 on total protein translation, equal amounts of gated viable eGFP, TRIM22-eGFP or PML-eGFP cells were sorted by flow cytometry, and trichloroacetic acid (TCA) precipitation of proteins was performed. The precipitated radioactive labelling, reflecting the amount of newly synthesised proteins, was then measured in a scintillator. PML repressed radiolabelled methionine incorporation to a modest, but however not statistically significant, degree (Figure 5). This is compatible with the documented role of PML in translation, since PML mainly represses translation of certain mRNAs (Culjkovic et al., 2006). In the presence of TRIM22, a repression of methionine incorporation was observed, revealing that TRIM22 does

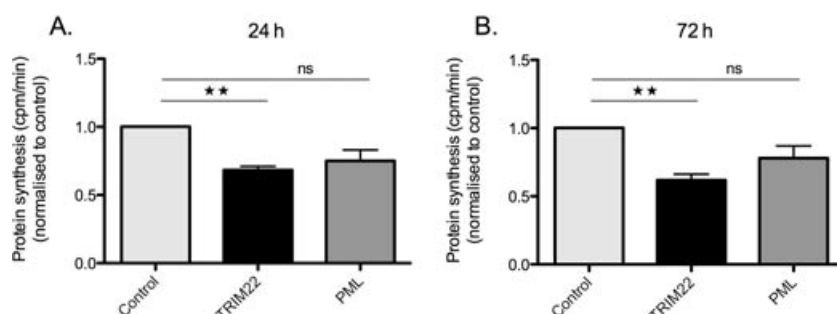
repress total protein synthesis to a statistically significant extent. In order to study whether TRIM22 represses translation initiation, also polysome fractionation experiments were performed. Compatible with our previous results, expression of TRIM22 induced a slight increase in the accumulation of 80S ribosomes (Supplementary Figure S3). Although not entirely conclusive, these results are consistent with the moderate effects of TRIM22 on total protein translation, and support that TRIM22 may inhibit translation initiation.

#### TRIM22 represses translation of IRF-7C

The modest effect of TRIM22 on total translation in the  $^{35}$ S-labelling experiment and in particular in the case of polysome fractionation experiments suggests that all translation might not be equally affected by TRIM22. Indeed, disturbance of the binding between eIF4E and eIF4G is known to particularly affect certain mRNAs with a more complex secondary structure. IRF-7 is recognised to be one of these 'weak' mRNAs with a complex secondary structure and has been shown to be strongly regulated on the translational level (Colina et al., 2008; Erickson and Gale, 2008; Sonenberg, 2008). Therefore, we

**Figure 5 | TRIM22 represses total protein synthesis**

293T/17 cells were transfected with eGFP (control), eGFP-TRIM22 or eGFP-PML and incubated for 24 or 72 h followed by incubation with 60  $\mu$ Ci/ml of [ $^{35}$ S]methionine/[ $^{35}$ S]cysteine for 2 h. 50,000 eGFP expressing, and DAPI and Annexin V negative, cells were sorted in triplicates using a FACS Aria, whereupon total protein was precipitated with TCA and radioactivity of labelled protein was measured. The value of TRIM22 and PML was normalised to the control. Error bars represent SEM,  $n = 3$ . \*\* $P < 0.005$  according to paired  $t$ -test.



transfected 293T/17 cells with eGFP-tagged TRIM22 or eGFP alone, sorted for eGFP-expressing cells and performed Western blot with an IRF-7 antibody. Jurkat cells treated with IFN $\alpha$  for 24 h were used as positive controls for the IRF-7C bands. 293T/17 cells only express the small IRF-7 isoform C (Zhao et al., 2010), which indeed was repressed by TRIM22 (Figures 6A and 6B). To determine whether this repression is regulated on the translational or transcriptional level, we also analysed the amount of IRF-7 mRNA. No effect on IRF-7 mRNA levels could, however, be observed (Figure 6C), demonstrating that TRIM22 inhibits translation rather than transcription of IRF-7 mRNA. However, as demonstrated in Figures 2B, 3C, 4B, 6A and 6D, TRIM22 did not have any effect on the protein levels of the housekeeping gene GAPDH or histone H3. Because of a non-complex secondary structure of its mRNA, GAPDH is known to be readily translated even in the presence of low eIF4F levels, (Hsieh and Ruggero, 2010; Livingstone et al., 2010; Richter and Sonenberg, 2005). Probably also histone H3 is easily translated due to its basic function in the cells. To exclude the possibility that TRIM22 degrades IRF-7C, we also analysed cells transfected with TRIM22-delRING (Figures 6D and 6E). In this experiment, we co-transfected TRIM22 or TRIM22-delRING together with eGFP, where after eGFP expressing, cells were sorted on a fluorescence-activated cell sorting (FACS) Aria and subjected to Western blotting. Indeed, also TRIM22-delRING transfected cells ex-

hibited a reduced amount of IRF-7C protein. Since the RING domain is necessary for the ubiquitin E3-ligase activity of TRIM22, this suggests IRF-7C not to be degraded in the presence of TRIM22. In conclusion, these data further strengthens the idea of TRIM22 as a regulator of translation of certain mRNAs with a higher dependence on eIF4F. It might seem contradictory that both TRIM22 and IRF-7C are induced by IFN $\alpha$ , since according to our conclusion TRIM22 is expected to counteract the translation of IRF-7C. Therefore, we investigated the levels of IRF-7C and TRIM22 protein in Jurkat cells treated with 10, 100 and 1,000 U/ml IFN $\alpha$  (Supplementary Figure S4). As expected, we demonstrate a dose-dependent increase of TRIM22 upon treatment with 10–1000 U/ml IFN $\alpha$ . Interestingly, however, while IRF-7C also increases upon treatment with 10–100 U/ml IFN $\alpha$ , IRF-7C levels clearly decrease with 1,000 U/ml IFN $\alpha$ , a concentration resulting in the highest levels of TRIM22 (Supplementary Figure S4), consistent with our conclusion that high levels of TRIM22 inhibit translation of IRF-7C.

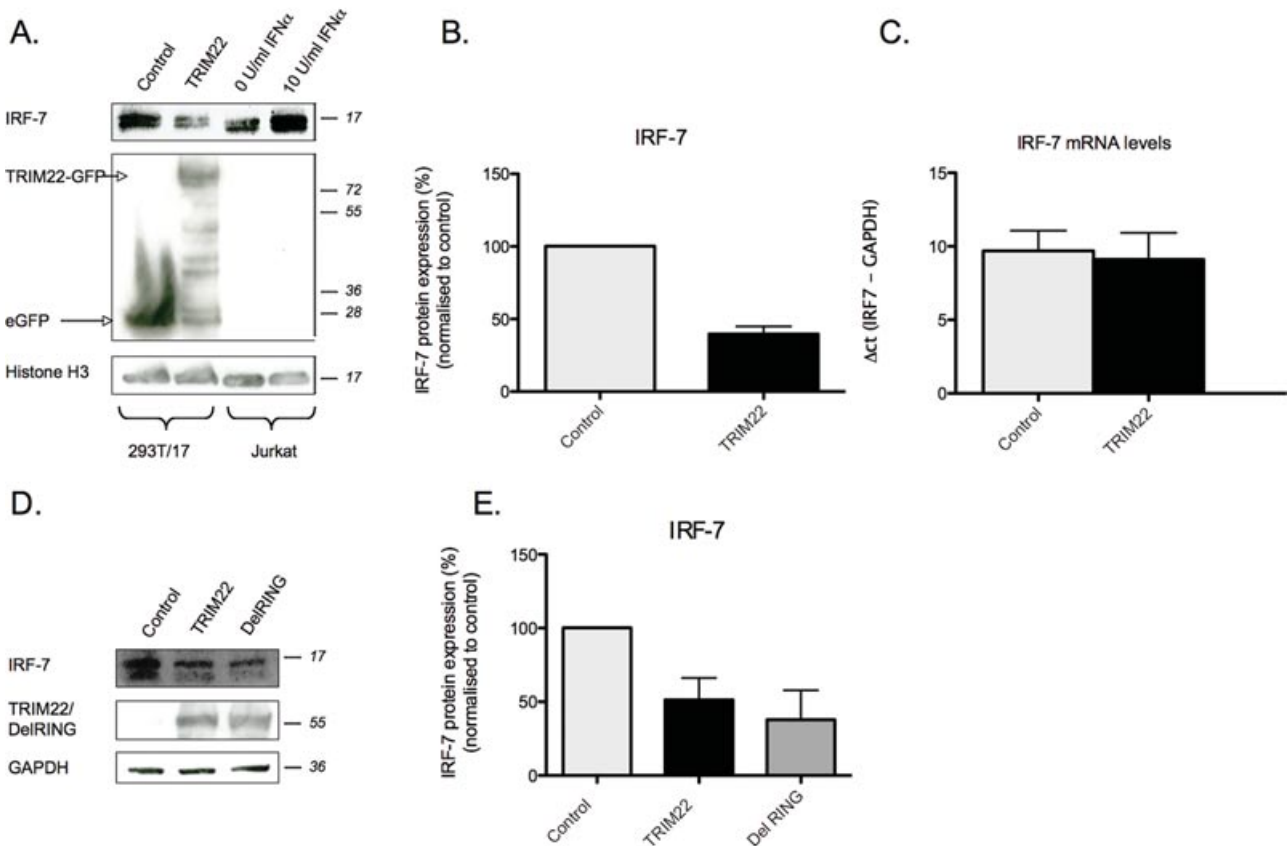
**Discussion**

Regulation of protein translation is central to proliferating cancer cells as well as for viral replication. In this context, both p53 and IFNs are important orchestrators, and several translation regulatory molecular mechanisms downstream of p53 and IFN signalling have been identified. For example, p53 can inhibit protein translation by cleavage of the scaffold protein



**Figure 6 | TRIM22 represses translation of IRF-7C protein**

(A) 293T/17 cells were transfected with eGFP or eGFP-TRIM22. After 48 h, equal amounts of cells were sorted and subjected to Western blotting with anti-GFP, as described in experimental procedures. As control, un-transfected Jurkat cells were incubated with and without 10 U/ml IFN $\alpha$  during 24 h. Histone H3 is used as an equal loading control. One out of three representative experiments is shown. Molecular weight markers (kDa) to the right. (B) Mean IRF-7C protein levels, as determined by densitometry, from three separate experiments, normalised to the control. Error bars represent SEM. (C) 293T/17 cells were transfected with eGFP or eGFP-TRIM22. After 48 h, equal amounts of cells were sorted on a FACS Aria, RNA was extracted and subjected to real-time RT-PCR to determine IRF-7 mRNA expression. Error bars represent SEM,  $n = 3$ , each performed in triplicate. (D) 293T/17 cells were co-transfected with TRIM22 and eGFP, TRIM22-delRING and eGFP or eGFP alone. After 48 h, equal amounts of eGFP-positive cells were sorted and subjected to Western blotting as described in experimental procedures. GAPDH is used as an equal loading control. One out of three representative experiments is shown. Molecular weight markers (kDa) to the right. (E) Mean IRF-7C protein levels, as determined by densitometry, from three separate experiments, normalised to the control. Error bars represent SEM.



eIF4G or dephosphorylation of 4E-BP1, and IFNs can disturb protein synthesis through induction of PKR, inhibiting eIF2 $\alpha$  (Constantinou and Clemens, 2007; De Benedetti and Baglioni, 1984; Der and Lau, 1995). Similar to eIF4E, eIF2 $\alpha$  is extensively regulated in response to cell stress, and is a part of the 43S pre-initiation complex. Here, we suggest a mechanism for translational regulation, possibly me-

diated by p53 and/or by IFNs. We show here that the IFN-inducible p53 target gene TRIM22 represses translation of the reporter protein luciferase as well as it modestly represses total radiolabelled methionine incorporation. In addition, we demonstrate that the direct or indirect interaction of TRIM22 to the translation initiation factor eIF4E inhibits the binding of eIF4E to the scaffold protein eIF4G. This may result

in inhibition of the formation of the eIF4F complex, which is essential for cap-dependent translation to occur. Although an IRES-containing mRNA has not been utilised as a control for the luciferase experiments (Figure 3), this points towards a role of TRIM22 as a regulator of cap-dependent translation. Indeed, we demonstrate TRIM22 to repress translation of IRF-7C, an mRNA well known to be highly dependent upon eIF4F for its translation by means of a complex secondary structure (Colina et al., 2008; Erickson and Gale, 2008; Sonenberg, 2008). Consequently, our data suggest TRIM22 to repress translation of a subset of mRNAs, which are more dependent on eIF4F levels, and perhaps also have a more complex secondary structure. Hence, radiolabelled methionine incorporation experiments where TRIM22 indeed did repress translation to a statistically significant degree probably reflects repressed translation of mRNAs more dependent on eIF4F for their translation. It is well documented that dephosphorylated 4E-BP1 binds to eIF4E, inhibiting its interaction with eIF4G (Van Der Kelen et al., 2009). Our data show that TRIM22 inhibits the binding of eIF4E to eIF4G, without affecting the amount of 4E-BP1 binding to eIF4E. This is in line with previous data by Moerke et al. (2007), who utilised a synthetic small inhibitor of the eIF4E–eIF4G interaction, without affecting the levels of 4E-BP1 binding to eIF4E. However, our data rely on over-expressed proteins and the effect of TRIM22 at physiological levels remains to be determined.

The IFN-inducible p53 target gene PML (TRIM19) is similar to TRIM22 in several ways. PML has also been shown to inhibit cap-dependent protein translation through direct interaction with eIF4E. However, PML binds eIF4E through its RING domain, and induces a conformational change of eIF4E, leading to a reduction of the cap-binding ability of eIF4E. Our data show that, unlike PML, the RING domain of TRIM22 is dispensable for eIF4E interaction, and that TRIM22 does not interfere with eIF4E binding to the mRNA m<sup>7</sup>G-cap. These differences, however, are not very surprising since TRIM22 and PML, in spite of their many similarities, are evolutionarily quite distantly related (Sardiello et al., 2008).

By means of its RING domain, TRIM22 has also been shown to function as an ubiquitin E3-ligase, inducing proteasomal protein degradation through tar-

geting selected proteins with ubiquitin (Duan et al., 2008; Eldin et al., 2009). It could be argued that the downregulation of global protein levels in the presence of TRIM22 in our assays could be due to TRIM22-mediated proteasomal degradation. However, we have shown a specific inhibitory mechanism of TRIM22 with crucial components of the translational machinery, talking against effects merely related to protein degradation. Moreover, the RING domain of TRIM22 is dispensable for both its eIF4E interaction capability as well as for its repressive effects on luciferase and IRF-7C protein levels, inconsistent with an effect dependent on the ubiquitin E3-ligase activity of TRIM22 (Duan et al., 2008; Eldin et al., 2009). In addition, cycloheximide experiments suggest the half-life of luciferase protein not to be affected by TRIM22, again supporting that the observed effects are independent of increased protein degradation.

eIF4E binding to the mRNA m<sup>7</sup>G-cap structure is necessary both for initiation of translation as well as for export of selected mRNAs from the nucleus to the cytoplasm. Since PML primarily is localised to the nucleus, PMLs main role in translational regulation has been suggested to be inhibition of mRNA transport (Flenghi et al., 1995; Lai and Borden, 2000). In contrast, TRIM22, present in both nucleus and cytoplasm (Pettersson et al., 2010), interferes with the binding of the eIF4E m<sup>7</sup>G-cap mRNA complex to eIF4G, suggesting that the primary role of TRIM22 in regulation of protein synthesis is inhibition of translation initiation in the cytoplasm.

Our data point at possible important functions of TRIM22 in the process of malignant transformation. The inhibition of the formation of the eIF4F complex is central for suppression of tumour progression, and both eIF4E and eIF4G are upregulated in many cancers, as well as increased formation of the eIF4F complex (Silvera et al., 2010). Here, TRIM22, induced by p53 or by IFNs, may function as an inhibitory factor.

In regulation of viral protein synthesis, inhibition of the eIF4F complex is more multi-faceted. In some cases, certain viruses may hijack the intrinsic translational machinery of the cell. For example, herpesvirus, translated from capped and polyadenylated mRNA, have evolved mechanisms to maintain an efficient cap-dependent translation dependent on the eIF4F complex (Smith et al., 2008). Also, in a recent study,

a synthetic inhibitor of eIF4E and eIF4G binding was shown to inhibit coronavirus replication (Cencic et al., 2011). Therefore, TRIM22 could function as an inhibitor of viral replication, by interfering with eIF4E and eIF4G.

In other cases, viral infection turns off intrinsic protein synthesis in favour of synthesis of viral proteins. For example, poliovirus infection results in the cleavage of eIF4G and nuclear re-localisation of eIF4E, resulting in the shut-off of host cell protein synthesis (Sukarieh et al., 2010). In this case, TRIM22 could hypothetically stimulate viral replication. Hence, although our data may indicate a possible molecular mechanism for the demonstrated anti-viral features of TRIM22, still much remains to be understood regarding the specific role of TRIM22 in viral protein synthesis.

In summary, our data suggest that TRIM22 may influence the assembly of the eIF4F translation initiation factor complex, and demonstrate functional consequences of this regulation. Hence, we show that TRIM22 directly or indirectly interacts with eIF4E and disrupts the binding of eIF4E to eIF4G. Consistently, over-expression of TRIM22 suppresses protein synthesis of certain mRNAs. Future research may reveal which consequences these observations may have for malignant transformation and viral replication.

## Material and methods

### Cell lines and culture conditions

The human embryonic kidney cell line 293T/17 (American Type Culture Collection [ATCC]) was maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% foetal bovine serum (FBS). The human T lymphocyte cell line Jurkat (ATCC) was cultured in Roswell Park Memorial Institute medium 1640 (Gibco) supplemented with 10% FBS.

### Cloning of TRIM22 and deletion mutants

The TRIM22 (CMV-SPORT 6) expression plasmid was kindly provided by Dr Nadir Mechti (University of Montpellier, France). Open reading frame TRIM22 was cloned from the CMV-SPORT 6 plasmid to the pcDNA3 plasmid using the following primers; 5'-GAC ATT AAG CTT GCC ACC ATG GATT TC TCA GTA AAG GTA GAC-3' (forward), 5'-CTG TAA CTC GAG TCA GGA GCT CGG TGG GCA CAC AGT CA-3' (reverse). TRIM22-delRING was constructed using the TRIM22 (CMV-SPORT 6) expression plasmid as a template, with the following primers; (aa 64–498) 5'-GAC ATT AAG CTT GCC ACC ATG CAG CCT GGG AAC CTC CGA CCT AA'3 (forward), 5'-CTG TAA CTC GAG TCA GGA GCT CGG TGG GCA CAC AGT CA-3' (reverse). TRIM22-delSPRY was constructed using the following primers (aa 1–351) 5'-GAC

ATT AAG CTT GCC ACC ATG GATT TC TCA GTA AAG GTA GAC-3' (forward), 5'-CTG TAA CTC GAG TCA AGA GAA ATA TTG GCA GCC G-3' (reverse). TRIM22-delCoiled-coil was performed with the following primers; (aa 1–137 and 249–498) 5'-TAT TCA AAG CTT GATT TC TCA GTA AAG GTA GAC-3' (forward), 5'-TCA ATC ACA TCC TGG ACC ACC TCG TTT ATG CGG AAT GT-3' (reverse) and 5'-CGC ATA AAC GAG GTG GTC CAG GAT GTG ATT-3' (forward), 5'-CTG TAA CTC GAG TCA GGA GCT CGG TGG GCA CAC AGT CA-3' (reverse). These two PCR products were ligated. The PCR products were cleaved with XhoI and HindIII and ligated into an empty pcDNA3 plasmid. cDNA sequencing and protein *in vitro* translation controlled the integrity of the TRIM22 and TRIM22-delRING constructs.

Open reading frame TRIM22 was also cloned into the pEGFP-N1 N-terminal protein fusion vector (Clontech), generating the fusion protein TRIM22-eGFP.

### Plasmids and reporter vectors

pcDNA2F/FLAG-eIF4E (murine) was kindly provided by professor Katherine Borden (University of Montreal, Canada). The luciferase reporter vectors pGL3/Waf1/Luc, and pGL3/Bax/Luc, were kindly provided by Professor Moshe Oren (Weizmann Institute of Science, Israel).

### Transient transfections

293T/17 cells were transfected either with Polyfect Transfection Reagent kit (Qiagen), Attractene Transfection Reagent kit (Qiagen) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To investigate the dose-response effect in Figure 3D, cells were transfected with 1:10 and 1:100 the amount of TRIM22 vector instructed by the manufacturer. The amount of total cDNA was balanced by addition of empty pcDNA3 plasmid.

### Immunoprecipitation and immunoblotting

Cells were lysed on ice in lysis buffer (containing 250 mM NaCl, 20 mM Na-phosphate, pH 7.0, 30 mM sodium pyrophosphate, pH 7.0, 5 mM EDTA, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF and 0.1% NP-40 supplemented with a protease inhibitor cocktail [Complete, Roche Diagnostics]). The crude lysate was centrifuged at 16,100 × *g* at 4°C for 20 min. For immunoprecipitation, equal amounts of the cell lysates were incubated with protein-A sepharose™ CL-4B (GE Healthcare Bio-Sciences AB) and protein-G PLUS-Agarose (sc-2002, Santa Cruz Biotechnology Inc.), and 1 μg of either mouse anti-TRIM22 (Abnova Corporation) or mouse anti-FLAG M2 (Sigma-Aldrich) at 4°C overnight where after samples were subjected to SDS-PAGE. To avoid the heavy and light chains from the IP antibody, when visualising TRIM22, the One-hour IP-Western kit (GenScript) was used.

### 7-methyl-GTP Sepharose assay

Cells were lysed on ice in lysis buffer and the crude lysate was centrifuged (see above). The protein concentration was measured with Pierce 660 nm protein assay (Thermo Scientific) in a NanoDrop ND-1000 ultraviolet-visible spectrophotometer (NanoDrop Technologies). Equal protein amounts were incubated with 50 μl 7-methyl-GTP Sepharose 4B (GE Healthcare) at 4°C

## TRIM22 inhibits translation initiation

overnight, where after the samples were centrifuged and washed with lysis buffer three times. Each sample was divided into two and resolved on 10–20% or 6% SDS-PAGE gels depending on protein size.

### Western blot

Samples were subjected to SDS-PAGE on precast Tris–glycine gels (Invitrogen and Lonza) and transferred to Hybond™-ECL™ nitrocellulose membranes (Amersham Biosciences). Immunoreactive bands were visualised by EZ-ECL Western blot detection kit (Biological Industries) or SuperSignal® West Dura Extended Substrate (Thermo Scientific). Quantification was performed using the software Image Lab or Quantity One (BioRad).

### Antibodies

The following antibodies were used: rabbit anti-TRIM22 (Atlas Antibodies AB), mouse anti-TRIM22 (Abnova Corporation), mouse anti-FLAG M2 (Sigma–Aldrich), rabbit anti-eIF4G, rabbit anti-eIF4E, rabbit anti-4E-BP1, rabbit anti-EGF receptor (all from Cell Signalling), goat polyclonal anti-luciferase pAb (Promega Corporation), mouse monoclonal antibody GAPDH (6C5): sc-32233, mouse monoclonal antibody IRF-7 (F-1): sc-74471 (both from Santa Cruz Biotechnology Inc.), mouse anti-GFP (Roche) and rabbit polyclonal antibody Histone H3 (ab1719) (Abcam).

### Luciferase assay

The Dual Luciferase Reporter Assay System (Promega) was used according to the manufacturer's instructions. The luminescence was measured for 10 s with a GloMax™ 20/20 luminometer (Promega). The Renilla luciferase plasmid was used as a control in all experiments but normalisation was not performed since the Renilla luciferase activity was affected in the same manner as the other plasmids.

### RNA isolation and real-time reverse transcription PCR

Total RNA was extracted with the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems), using 1  $\mu$ g of total RNA. Real-time PCR was performed using TaqMan probe-based chemistry (Applied Biosystems). The probes for TRIM22 (Hs01001178\_m1), IRF-7 (Hs01014809\_g1), the endogenous control 18S (Hs99999901\_s1) and the endogenous control GAPDH (Hs99999905\_m1) were purchased as assay-on-demand gene expression products (Applied Biosystems). The probe for luciferase was purchased as Custom Taqman Gene Expression Assay (Applied Biosystems). The primers used for the Luciferase probe was GAGGAGCCTTCAGGATTACAAGATT (forward) and GCTTTTGGCGAAGAAGGAGAATAG (reverse). The amplification reactions were performed in triplicates in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. The endogenous genes 18S or GAPDH were used as internal controls.

### Fluorescence-activated cell sorting

Cell sorting was performed on a FACS Aria (BD Bioscience Immunocytometry systems). For sorting of viable cells only, the cells were incubated with Annexin V-APC (BD) accord-

ing to the manufacturer's instructions. Just prior sorting, DAPI (3  $\mu$ M, Molecular Probes) was added to the cells. eGFP-positive, Annexin-V-negative and DAPI-negative cells were FACS sorted and subjected to TCA precipitation.

### TCA precipitation

Cells labelled with [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (Amersham) were lysed on ice in radioimmune precipitation assay buffer (containing 0.15 M NaCl, 30 mM HEPES, pH 7.3, 1% Triton-X [v/v], 1% sodium deoxycholate [w/v] and 0.1% SDS [w/v] supplemented with protease inhibitor cocktail). Proteins were precipitated with TCA, washed twice with acetone and resuspended in 0.1 M Tris–HCl, pH 8. The proteins were added to liquid scintillation fluid (Beckman Coulter) and counted in a scintillation counter (Wallac Guardian 1414 Liquid Scintillation Counter, PerkinElmer).

### Author contribution

J.P. designed the concept of the research, planned and carried out the experiments and wrote the article; M.A. contributed to the concept of the research; C.S. contributed by carrying out experiments; T.O. contributed with FACS expertise; U.G. and K.D. designed the concept of the research and supervised the writing of the article.

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### Conflict of interest statement

The authors have declared no conflict of interest.

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