Induction of cell cycle arrest by the dual activity of TRIM8 on p53 and DNp63

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The p53 oncosuppressor protein plays a crucial role in the protection of genome integrity and inactivation of its pathway appears to be a common, if not universal, feature of human cancer[1,2]. The contribution of its relative p63 to onset and tumour progression is less established[3,4]. However, the TAp63 isoforms possess a p53-like anti-oncogenic activity, while N-terminal truncated ΔNp63 are characterized by pro-oncogenic potential: the relative balance between the TA and ΔN isoforms has been shown to be critical in tumour development and/or progression[5,6]. Here we describe a previously unknown function for the human TRIM8 gene, a member of Tripartite Motif protein (TRIM) family, as a new key node necessary to enhance p53 oncosuppressor activity and, at the same time, to down-modulate oncogenic ΔNp63α activity. The TRIM protein family is defined by the presence of a RING domain, one or two B-boxes and a Coiled-Coil region[7]. TRIM8 is located at 10q24.3, a region frequently associated to rearrangements and deletions in different cancer cells, particularly gliomas[8,9]. It has been reported that TRIM8 decreases the protein stability of SOCS-1 (suppressor of cytokine signalling-1)[10] and PIAS3 (protein inhibitor of activated STAT3)[11] suggesting a possible E3-ligase activity for the RING domain of TRIM8. We found that TRIM8 overexpression induces MDM2 degradation, which results in increased p53 protein levels and activity, leading to cell cycle arrest and reduction of cellular proliferation; at the same time, the oncogenic ΔNp63α isoform is degraded through the proteasome. Interestingly, TRIM8 silencing prevents p53 activation and ΔNp63α degradation after UV exposure and Nutlin-3 treatment. Finally, we found that TRIM8 expression is strongly down regulated in patients affected by glioblastomas, a cancer in which p53 mutations are rarely encountered. These results reveal a previously unknown regulatory pathway controlling p53 and p63 activities and suggest TRIM8 as a novel therapeutic target to simultaneously enhance p53 oncosuppressor and impair ΔNp63α oncogenic activities.

p53 and p63 are central hubs in controlling cell proliferation and an array of post-translational modifications and protein-protein interactions modulates their stability and activities in order to avoid malignant transformation[12]. Approximately 50% of human tumours have inactivating mutations in the p53 gene, but this percentage rises up to 90% if we consider the inactivation of proteins involved in p53 regulatory pathway. Tight control of p53 cellular levels is primarily achieved through its ubiquitin-mediated proteasomal degradation[12]. Although MDM2 has been demonstrated to be the most important regulator of p53 turnover[14], there are growing evidences that p53 stability is more complex than originally anticipated and other important modulators control p53 functionality[15-17]. The challenge is the discovery of additional p53-inactivating pathways that might account for escape from p53 control in malignancy with wild type p53. Here, we report the identification of TRIM8, belonging to TRIM protein family, as a good candidate for this role, since a number of observations from our and other laboratories suggest that TRIM8 might be involved in a tumour suppression mechanism[18]. We investigated this involvement by analysing the effect of TRIM8 over-expression or knockdown on cellular proliferation rate in different TRIM8 expressing cell lines (Fig.1a). The levels of exogenously expressed TRIM8 protein and the abrogation of TRIM8 endogenous expression were confirmed by western blotting and qRT-PCR (Supplementary Fig.1). We observed suppression of cell proliferation in p53wt cell lines (HCT116, MCF-7, U2OS) but not in p53-/- backgrounds (HCT116 p53-/-, H1299, Saos-2) (Fig.1a). Conversely, silencing of TRIM8 by specific short hairpin RNA (shRNA) resulted in increased cell proliferation rate only in p53wt cells (Fig.1a), suggesting that the suppression of cell proliferation observed upon TRIM8 overexpression was p53-dependent. Then we sought whether the suppression of cell proliferation was due to apoptosis or cell cycle arrest. Immunochemical determination of cytoplasmic histone-associated-DNA fragments showed that p53 induced apoptosis in HCT116 cells in a dose dependent manner, but when p53 was co-transfected with TRIM8 the number of apoptotic cells significantly decreased (Fig.1b). Flowcytometric analysis showed that HCT116 cells overexpressing p53 yielded a 26% increase in G1 phase compared to control cells (Fig.1c). The effect resulted amplified in cells overexpressing both p53 and TRIM8 (40% increase in G1 phase with respect to control cells) (Fig.1c). Together these results demonstrated that the suppression of cell proliferation upon TRIM8 overexpression is due to a p53-dependent cell cycle arrest. In fact, as shown in Fig. 1b and 1c, TRIM8 overexpression induced stabilization of endogenous as well as transfected p53, enhancing its half-life from ~30 to ~120 min (Fig.1d and Supplementary Fig.2). Consistently, TRIM8 ablation induced a significant decrease of p53 stability (Fig.1d, and Supplementary Fig.2). Interestingly, the increased p53 levels were paralleled by an increase in the levels of Ser-15 and Ser-20

phosphorylated p53, a marker of the activation status of p53 for cell cycle arrest activity, but not of Ser-46, linked to p53 apoptotic activity (Fig.1d, and Supplementary Fig.2)[19-21]. Remarkably, we found that TRIM8 overexpression decreased MDM2 protein levels and TRIM8 ablation increased MDM2 levels, resulting in p53 destabilization (Fig.1d).

The p53 phosphorylation on Ser-15 and Ser-20 and the p53-mediated cell cycle arrest suggested that the expression of the genes involved in cell cycle arrest and in the related pathways (e.g. p21, GADD45) but not the one involved in apoptosis (e.g. BAX, PUMA) should increase upon TRIM8 overexpression and p53 activation. To assess this hypothesis, we performed qRT-PCR to analyze the expression of different p53 transcriptional targets in HCT116 p53 wt and in HCT116 p53-null cell lines (Fig. 1e) upon TRIM8 overexpression or silencing. As shown in Fig1e, upon TRIM8 overexpression, p21 and GADD45 mRNA levels increased in HCT116-p53wt but not in HCT116-p53(-/-) cell lines, while the expression levels of BAX and PUMA genes were not affected in both cell lines. On the contrary, we found that TRIM8 silencing in HCT116 p53wt cells led to decreased p21 and GADD45 expression. Similar results were obtained in different cell lines (supplementary Fig.3). These data were supported by reporter assay experiments (Fig.1f), which demonstrated that p53 significantly increased its ability to transactivate the p21 and GADD45 p53REs when co-expressed with TRIM8. Conversely, activation of the BAX promoter by p53 was decreased by TRIM8 co-expression.

Altogether these results indicate that TRIM8 activates p53 toward a specific cell cycle arrest program.

In order to identify the domain of TRIM8 responsible for the observed effects on p53, we transfected HCT116 p53-wt cells with different TRIM8 deletion mutants. Interestingly, only the cells overexpressing TRIM8 without the RING domain (TRIM8-ΔRING) did not show p53 stabilization and escaped the p53-mediated suppression of cell proliferation, as showed by Western blot and MTT assays (Fig.2a). Also reporter assay experiments (Fig.2b) demonstrated that TRIM8-ΔRING mutant was unable to mediate the activation of p21 and GADD45 p53REs.

Next we addressed whether TRIM8 interacts with p53. Co-immunoprecipitation experiments demonstrated that TRIM8 directly interacts *in vivo* with p53 (Fig.2c). The regions involved in this physical interaction are aminoacids 291-317 in p53 and the coiled-coil domain and the two B-boxes in TRIM8, as revealed by the use of p53 and TRIM8 deletion mutants (Fig.2d).

As it is known that p53 controls the expression of many proteins involved in the regulation of its own stability and activity, we verified whether TRIM8 is a direct target gene of p53 family members. To this purpose, we queried the p53FamTag database[22] and we identified four p53 Responsive Elements (REs) in the intron 1 of the TRIM8 gene; p53RE1-TRIM8 is the closest to

TSS (Transcription Start Site) (Fig.3a and supplementary Fig.4). The *in vivo* binding of p53 and p63 to these p53REs was verified by Chromatin ImmunoPrecipitation (ChIP) analysis and luciferase assays, which indicated that these p53REs are functionally active for both p53 and p63, although with different efficiencies (Fig.3a, 3b and supplementary Fig.4). The activation was dependent on functional p53 and p63 since the transactivation defective mutants, p53H175, TAp63αR279Q and ΔNp63αR279Q were unable to activate the reporter constructs (Fig.3a and Supplementary Fig.4). Moreover, p53RE1mut-TRIM8, carrying a deletion of one decamer out of three (Fig.3a), was weakly responsive to both wt p53 and p63. Subsequently, the dependence of TRIM8 expression by p53 and p63 was further corroborated in 293-Trex cells stably transfected with p53 and p63 under the control of a tetracycline inducible promoter. p53 and p63 overexpression resulted in increased TRIM8 mRNA levels (Fig.3c), while TRIM8 expression decreased upon p53 or p63 silencing in MCF-7 cells (Fig.3d).

Because there is a fine and very complex interplay between the TA and ΔN isoforms of the p53 family members, we investigated the effect of TRIM8 overexpression on the oncogenic ΔNp63α, the main p63 isoform expressed in cell cultures. We found that, in MCF-7 and U2OS cell lines, TRIM8 silencing resulted in increase of endogenous or transfected ΔNp63α protein levels with a parallel increase in cell proliferation (Fig.1a and Fig.3e). On the other hand, TRIM8 overexpression, induced a dose dependent degradation of endogenous and transfected ΔNp63α, but not ΔNp63γ (Fig. 3e, f). TRIM8 overexpression resulted in ΔNp63 degradation also in SAOS-2 cells, demonstrating that the mechanism is p53-independent (data not shown). Consistently, ΔNp63α transactivation potential on specific p63 target genes, e.g. ADA and CCND3[21], decreased upon TRIM8 overexpression (Supplementary Fig.5). The observation that ΔNp63γ was unaffected by TRIM8 overexpression suggested that the C-terminal region of ΔNp63α is required for TRIM8 mediated degradation. Remarkably, the mutant ΔNp63α K637R, in which the sumoylation site was mutated[23], was still sensitive to TRIM8 overexpression. Conversely, the mutant ΔNp63αK494R/K505R was partially unaffected by TRIM8 overexpression, while the mutant ΔNp63αK193R/K194R/K494R/K505R was completely resistant to TRIM8-mediated degradation. Altogether these results suggest that K494R and K505R are involved in ΔNp63α degradation upon TRIM8 overexpression. Treatment of U2OS and MCF-7 cells with the proteasome inhibitor MG132 reversed TRIM8-mediated ΔNp63α degradation, providing evidences that p63 degradation was proteasome-dependent (Fig. 3g and Supplementary Fig5).

Next, we found that endogenous TRIM8 expression was modulated by p53 in response to U.V. and Nutlin-3 treatment, a small pharmacologic MDM2-antagonist molecule[24]. In HCT116 and in MCF7 cells a p53-dependent increase of TRIM8 expression was observed after U.V. irradiation and

Nutlin-3 treatment, which it was not observed in p53 null background (HCT116 p53-/- and H1299 cell lines) (Fig.3h, lower panel-TRIM8 and Supplementary Fig.6). Interestingly p53 activates TRIM8 transcription only when the stress was modulated to cause cell cycle arrest (20 J/m^2 U.V. and 10 μM Nutlin-3) and not apoptosis (80 J/m^2 U.V. and 20 μM Nutlin-3). Most importantly in HCT116 p53wt cells, TRIM8 silencing prevented ΔNp63α degradation, p53 stabilization, p53 phosphorylation on Ser-15 and Ser-20, but not on Ser-46, and p53-dependent activation of p21, GADD45 but not BAX gene expression (Fig.3h).

Altogether these results demonstrate that TRIM8 levels are relevant to the p53-mediated cellular responses to stresses.

Finally, the relative expression of TRIM8 in a cohort of 45 patients affected by gliomas was analysed by qRT-PCR, since TRIM8 is located within the 10q24.3 brain cancer LOH region and in gliomas p53 gene is rarely mutated. Remarkably, we found a strong reduction of *TRIM8* expression in these patients and TRIM8 levels inversely correlated ($P<0.01$) with the histological tumour grade and the major clinical-pathological parameters (Fig.4). Therefore, we suggest that in these tumours, TRIM8 down-regulation causes the attenuation of p53 oncosuppressor activity and the association of decreased TRIM8 expression with poor prognosis further argues for its clinical relevance.

Collectively, our data reveal a new p53 tumour suppressor circuit and suggest TRIM8 as an interesting therapeutic target for restoring p53 function in tumours with wild type p53.

Methods Summary

Cell lines and primary glioma cells were cultured as described previously[26]. Tumour samples were obtained during surgery from patients of the Neurosurgery Department, Casa Sollievo della Sofferenza Hospital (San Giovanni Rotondo, Italy). Informed consent was obtained for all patients before the surgery as approved by the ethics board. All tumors were histologically classified according to the WHO classification of tumors. Samples were collected immediately after surgical resection and processed.

Procedures for cell treatments and transfections, cell proliferation assays by MTT reduction, immunoblotting, flow cytometric analysis, chromatin immunoprecipitation (ChIP), RNA extraction and Real Time PCR were also performed as described previously[23].

Co-immunoprecipitation experiments were performed as described previously[26].

Specific siRNA for p53 and p63 and controls were as described previously[23]. TRIM8- specific shRNAs were from Origene.

For luciferase assays, all constructs used in this study were sequence verified and the procedures were also described**.** Transient reporter assays were performed as previously described[23] and transfection efficacy was determined by renilla activity.

For immunoblotting, chromatin immunoprecipitation and co-immunoprecipitation, a list of the antibodies and reagents used is reported in Supplementary Information.

Real-time PCR for p21 (also known as CDKN1A), BAX, PUMA (also known as BBC3), and GADD45 was performed using TaqMan Gene Expression Assays (Applied Biosystems).

For immunochemical determination of cytoplasmic histone-associated-DNA fragments, the levels of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates was determined 48h after the transfections by using the Cell Death Detection ELISA^{PLUS} (Roche™) according to the manufacturer's instructions.

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Supplementary Information is linked to online version of the paper at www.nature.com/nature

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Author contributions M.F.C., L.M., T.L. and F.G. performed experimental work. L.G. planned and supervised the p63 experiments. A.M., G.P., E.S. performed data analysis. A.T. and G.M. planned and supervised the project, performed data analysis and wrote the manuscript.

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Figure legends

Figure 1. Trim8 induces p53 activation and p53-dependent cell cycle arrest. (a) Cell proliferation was measured by MTT reduction in the indicated cell lines 72h after transfection with pcDNA3 control vector, pcDNA3-FLAG-TRIM8, pRS control vector or specific TRIM8-shRNA. **(b)** In HCT116-p53wt transfected for 72h with the indicated control or recombinant vectors, apoptosis was measured by immunochemical determination of cytoplasmic histone-associated-DNA fragments. **(c)** Flow cytometric analysis of HCT116-p53wt cells transfected with the indicated control and recombinant vectors. **(d)** MCF-7 were transfected as indicated and immunoblotted as shown. p53 half-life was measured by treating MCF-7 cells with cycloheximide (CHX) for the indicated time. **(e)** qRT-PCR of the indicated p53 target genes in HCT116-p53wt and in HCT116-p53-/- transfected for 72h with pcDNA3 control vector, pcDNA3-FLAG-TRIM8, pRS control vector or specific TRIM8-shRNA. **(f)** Luciferase assay. p53-null H1299 were co-transfected with pcDNA3 control vector, pcDNA3-p53, pcDNA3-p53 plus pcDNA3-TRIM8, or pcDNA3-TRIM8 with pGL3-basic-luciferase reporter constructs containing p21, BAX, and GADD45 p53REs. The levels of exogenously expressed proteins in H1299 cell line were controlled by WB. Cells were lysed and luciferase expression was determined as described.

Figure 2. TRIM8 interacts with p53 and the TRIM8-RING domain is essential to induce p53-dependent cell cycle arrest. (a) Cell proliferation was measured by MTT reduction in the HCT116-p53wt cell line transfected for 72h with pcDNA3 control vector or with TRIM8-deletion mutants, lacking the two B-boxes (TRIM8-ΔBB), the coiled coil domain (TRIM8-ΔCC), the RING domain (TRIM8-ΔRING), or the RFP domain (TRIM8-ΔRFP). **(b)** Luciferase assay. p53-null H1299 were co-transfected with pcDNA3 control vector, pcDNA3-p53, pcDNA3-p53 and pcDNA3-TRIM8, or pcDNA3-TRIM8 deletion mutants, with pGL3-basic-luciferase reporter constructs containing p21 or GADD45 promoters. The levels of exogenously expressed proteins in H1299 cells were controlled by WB. Cells were lysed and luciferase expression was determined as described. **(c)** Co-immunoprecipitation of TRIM8 and p53 in HEK293 cells. HEK293 cells were co-trasfected with plasmids expressing EGFP or EGFP-p53 together with FLAG-TRIM8 for 48 h. Cells were then exposed to MG132 (20 μM) for an additional 4 h before harvest. The cell lysates were either separated by SDS-PAGE followed by immunoblotting, or immunoprecipitated by FLAG or EGFP antibody, followed by immunoblotting with EGFP or FLAG antibody. **(d)** Co-immunoprecipitation of p53 deletion mutants and TRIM8 (left panels). The various p53 constructs used in co-immunoprecipitation experiments are shown in the upper left panel. HEK293 cells were transfected with FLAG-TRIM8 and EGFP-p53 or with the indicated p53 deletion mutants. The

lysates were subjected to immunoprecipitation with FLAG antibody, and corresponding p53-tagged proteins, were analyzed by immunoblotting using EGFP or HA antibodies. Co-immunoprecipitation of TRIM8 deletion mutants and EGFP-p53 (right panel). TRIM8 deletion mutants used in co-immunoprecipitation studies are shown in the upper right panel. R, RING-finger motif; B1, B2, B boxes; C-C, coiled-coil domain; RFP, C-terminal domain. HEK293 cells were transfected with EGFP or EGFP-p53, together with plasmids expressing FLAG-TRIM8 deletion mutants for 48 h and the lysates were immunoprecipitated with a FLAG antibody. The bound proteins were detected by EGFP antibody.

Figure 3. TRIM8 is a p53 and p63 target gene and its silencing prevents p53 activation and ΔNp63α degradation after UV exposure and Nutlin-3 treatment. (a) Schematic map of the human TRIM8 genomic region containing the four putative p53 REs (TRIM8-p53RE1-4). In vivo recruitment of p53, p53H175R mutant, TAp63α and ΔNp63α to TRIM8-p53RE1 in living cells present in TRIM8 gene by Chromatin-imuunoprecipitation assay. **(b)** Luciferase assay. Effect of p53, p53H175R mutant, TAp63α, ΔNp63α and their R279Q mutants on the transcriptional activity of TRIM8-p53RE1 and of TRIM8-p53RE1mut. p53-null H1299 cells were co-transfected with plasmids expressing p53, TAp63α, ΔNp63α wt or their mutated forms (p53H175R, TAp63α-R279Q or ΔNp63α-R279Q) and pGL3-Basic or pGL3-Basic-TRIM8-p53RE1 luciferase reporter. The luciferase activities were measured 48 h later. The data represent the mean (+/-) the standard deviation) of three separate experiments. **(c)** TRIM8 qRT-PCR in 293-Trex cell line stably transfected with control vector, p53, -TAp63α, -ΔNp63α. **(d)** TRIM8 qRT-PCR in MCF-7 cell line transfected with non specific scramble RNA or specific p53 or p63-siRNA**.** (e) MCF-7 were transfected as indicated and immunoblotted as shown. (f) U2OS cells were transiently co-transfected with ΔNp63α, ΔNp63γ or the related mutants ΔNp63αK637R, ΔNp63αK494/505R and ΔNp63αK193/194/494/505R (20 ng) and increasing amount of TRIM8 expression plasmid (10, 20, 40 ng). WB analysis was performed with the indicated antibodies. (g) U2OS cells were transiently co-transfected with ΔNp63α construct (20 ng) and increasing amount of TRIM8 expression plasmid (10-20-30 ng), in presence or absence of the proteasome inhibitor MG132. WB analysis was performed on cells extracts with the indicated antibodies.

(**h**) HCT116-p53wt cells were transfected with pRS control vector or specific TRIM8-shRNA and exposed to UV or Nutlin-3 treatment (24h) as indicated. The proteins were immunoblotted as indicated (upper panel). qRT-PCR of TRIM8, p21, bax and GADD45 transcripts (lower panel).

Figure 4. TRIM8 expression level in glioma cell lines established from human glioma surgery specimens at different grade was determined by qPCR. Grade I-III (14) and IV (31) samples were compared with respect to human normal brain (quanti??).