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ORIGINAL PAPER

TRAP1 controls cell cycle G2–M transition through the regulation of CDK1 and MAD2 expression/ubiquitination

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Abstract

Regulation of tumour cell proliferation by molecular chaperones is still a complex issue. Here, the role of the HSP90 molecular chaperone TRAP1 in cell cycle regulation was investigated in a wide range of human breast, colorectal, and lung carcinoma cell lines, and tumour specimens. TRAP1 modulates the expression and/or the ubiquitination of key cell cycle regulators through a dual mechanism: (i) transcriptional regulation of *CDK1*, *CYCLIN B1*, and *MAD2*, as suggested by gene expression profiling of TRAP1-silenced breast carcinoma cells; and (ii) post-transcriptional quality control of CDK1 and MAD2, being the ubiquitination of these two proteins enhanced upon TRAP1 down-regulation. Mechanistically, TRAP1 quality control on CDK1 is crucial for its regulation of mitotic entry, since TRAP1 interacts with CDK1 and prevents CDK1 ubiquitination in cooperation with the proteasome regulatory particle TBP7, this representing the limiting factor in TRAP1 regulation of the G2–M transition. Indeed, TRAP1 silencing results in enhanced CDK1 ubiquitination, lack of nuclear translocation of CDK1/cyclin B1 complex, and increased MAD2 degradation, whereas CDK1 forced up-regulation partially rescues low cyclin B1 and MAD2 levels and G2–M transit in a TRAP1-poor background. Consistently, the CDK1 inhibitor RO-3306 is less active in a TRAP1-high background. Finally, a significant correlation was observed between TRAP1 and Ki67, CDK1 and/or MAD2 expression in breast, colorectal, and lung human tumour specimens. This study represents the first evidence that TRAP1 is relevant in the control of the complex machinery that governs cell cycle progression and mitotic entry and provides a strong rationale to regard TRAP1 as a biomarker to select tumours with deregulated cell cycle progression and thus likely poorly responsive to novel cell cycle inhibitors.
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Introduction

Deregulation of cell cycle progression is a general feature of human cancer cells due to aberrant activity of cyclin-dependent kinases (CDKs), CDK inhibitors (CDKis), and cyclins [1]. Indeed, the deregulated activity of cell cycle regulators contributes to uncontrolled proliferation of cancer cells, thus providing attractive pharmacological targets [1,2]. These issues are extremely relevant in the perspective of the recent development of the dual CDK4/6 inhibitor palbociclib and other CDKis, with established activity in specific human malignancies [2,3].

TRAP1 (TNFα receptor-associated protein 1) is a member of the HSP90 protein family responsible for co-translational quality control of specific client proteins [4,5]. TRAP1 is expressed at a low level in normal non-proliferating cells [6] and is aberrantly up-regulated in several human malignancies (i.e. colorectal, breast, lung, and prostate carcinomas) [6–10], and in lung carcinoma cells, TRAP1 silencing results in arrest/delay of cell proliferation [9]. Indeed, TRAP1 was originally identified as a stress protein interacting with RB1 and responsible for the refolding of denatured RB1 [11], even though the functional consequence of this regulation is still unclear. Our group recently reported that the down-regulation/inhibition of TRAP1 results in the attenuation of ERK phosphorylation and cell cycle progression in colorectal [12], breast [12], and thyroid [13] carcinoma cells, and this

correlates with a wide reprogramming of genes regulating the cell cycle machinery and that central step in this process is TRAP1 quality control on BRAF [12]. Recent reports highlighted TRAP1 involvement in several other functions of tumour cells [14,15] and, among others, adaptive responses and protection from apoptosis induced by environmental stress conditions such as oxidative [6,16] and ER stress [4,17,18], drug resistance [7,8,19,20], stemness [15,21], and glycolytic/oxidative balance [22–25]. Based on these premises, the molecular mechanism responsible for TRAP1 regulation of the cell cycle was further investigated. Here, we report that the TRAP1 network regulates the expression and the ubiquitination of key cell cycle regulators and that this mechanism is responsible for mitotic entry and transit.

Materials and methods

Tumour specimens

Sixty CRCs and corresponding normal, non-infiltrated peritumoural mucosa were collected consecutively between 2014 and 2015 at the General Surgery Unit of the University of Foggia; 46 paraffin-embedded LC specimens were consecutively collected in 2015 at the Pathology Units of the University of Foggia. Fifty-seven BCs, collected consecutively in 2015, were obtained from the Tissue Biobank of the IRCCS-CROB of Rionero in Vulture. Surgical specimens were collected after removal of tumours and immediately frozen in liquid nitrogen. Tumours were classified according to the WHO classification for CRC, LC, and BC, respectively [26–28]. Patients' characteristics are reported in the supplementary material, Table S3. All patients gave their informed written consent to use biological specimens for investigational procedures, according to the IRCCS-CROB Ethics Committee approval for the Tissue Biobank.

Immunoblot analysis

Preparation of cell lysates, protein immunoprecipitation, and immunoblot analysis were carried out as previously reported [12,20]. Nuclear and cytosolic fractions were purified by a Qproteome Mitochondria Isolation kit (Qiagen, Hilden, Germany). Where indicated, protein levels were quantified by densitometric analysis using Quantity One 4.5 software (Bio-Rad Laboratories GmbH, München, Germany). Primary antibodies are reported in the supplementary material, Supplementary materials and methods.

Cell cycle analysis

Cells were incubated in a culture medium supplemented with 20 μM 5-bromo-2'-deoxyuridine (BrdU) for 20 min and harvested. Subsequently to incubation in 3 N HCl solution for 30 min at room temperature, cell pellets were incubated in the presence of anti-BrdU FITC (Becton Dickinson, Franklin Lakes, USA) for 1 h in the dark and with 6 μg/ml propidium iodate for 20 min, and finally evaluated using the FACsCalibur™ (Becton Dickinson).

Tissue microarray-based immunohistochemistry

A tissue microarray (TMA) (Galileo TMA CK 3500 Tissue Microarrayer, ISE TMA Software; Integrate Systems Engineering, Brugherio (MI), Italy) was constructed as previously reported [29]. H&E staining of a 4-μm TMA section was used to verify all samples. Immunohistochemical analysis was performed using a Ventana Benchmark® XT autostainer and the standard linked streptavidin–biotin horseradish peroxidase technique (LSAB-HRP) [29].

Detailed methods for cell cultures, chemicals, plasmid generation and transfection procedures, RNA extraction and reverse transcription–quantitative PCR (RT-qPCR) analysis, antibodies for immunoblotting, fluorescence microscopy, flow cytometry and immunohistochemistry analyses, confocal microscopy and proximity ligation assay, microarray expression analysis, and statistical analyses are provided in the supplementary material, Supplementary materials and methods.

Results

TRAP1 silencing induces wide reprogramming of genes involved in cell cycle progression

Previous studies suggest that TRAP1 regulates cell cycle progression by modulating RAF/ERK signalling and reprogramming the expression of key genes responsible for cell cycle regulation [9,12]. In addition, several putative TRAP1-interacting proteins were previously reported by our group [20] and, among others, CDK1 and MAD2, two master regulators of mitotic entry and transit [30,31]. Both findings prompted us to focus on TRAP1 regulation of the G2–M transition. To this end, a whole-genome gene expression analysis was performed in MCF7 cells upon TRAP1 knockdown (supplementary material, Figure S1A) and wide reprogramming of gene expression was observed with 787 up-regulated and 871 down-regulated genes (supplementary material, Table S1). Microarray data have been submitted to Array Express under accession number E-MTAB-3584. Interestingly, the Ingenuity Pathway Analysis (IPA) identified 'Cell Cycle' and 'Cell Growth and Proliferation' as the most significant biofunctions modulated in our data set (Figure 1A), with about 200 up/down-regulated genes involved in cell cycle control and proliferation (supplementary material, Figure S1B). Consistently, Gene Ontology (supplementary material, Figure S2A) and DAVID (supplementary material, Table S2) analysis identified 'cell proliferation' and 'cell growth' among several GO categories and IPA identified 'Mitotic Roles of Polo-like kinases', a signalling pathway coordinating mitotic entry [32], as the

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second most significant pathway regulated by TRAP1 (Figure 1B) and the MAPK pathway as the second top upstream regulator (supplementary material, Figure S2B). The latter evidence is consistent with our previous observation that cell cycle regulation by TRAP1 occurs as an event downstream to its quality control on BRAF and the modulation of ERK phosphorylation [12,19]. It is noteworthy that specific regulators of the G2–M checkpoint were down-regulated in TRAP1-silenced cells: (i) CDK1 and cyclin B1 (*CCNB1*), two master regulators of the G2–M transition [30]; and (ii) MAD2 (*MAD2L1*), an essential spindle checkpoint protein which regulates the progression through the prometaphase-to-anaphase transition downstream of CDK1/cyclin B1 complex formation [31] (supplementary material, Figure S1B). The down-regulation of these genes was confirmed in TRAP1-silenced MCF7 cells by RT-qPCR (supplementary material, Figure S3A). Furthermore, TRAP1 silencing by siRNA (Figure 1C–G) or shRNA (supplementary material, Figure S3B) in multiple *in vitro* systems, i.e. BC ER-positive MCF7 (Figure 1C and supplementary material, Figure S3B) and HER2-positive SKBR3 (Figure 1D), LC A459 (Figure 1E), CRC HCT116 (Figure 1F), and HT29 cells (Figure 1G), always resulted in the down-regulation of CDK1, cyclin B1, and MAD2, and in the parallel reduction of Thr-161 CDK1 phosphorylation, a molecular event responsible for the nuclear translocation of the CDK1/cyclin B1 complex [33]. Consistently, the transfection of TRAP1 cDNA resulted in higher protein levels of CDK1, pCDK1, cyclin B1, and MAD2 in BC MCF7, SKBR3, and CRC HCT116 cells (supplementary material, Figure S3C–E). Furthermore, TRAP1 silencing was confirmed to induce attenuation of the S phase and arrest in the G2–M phase in BC MCF7 (supplementary material, Figure S3F, G) and LC A459 cells (supplementary material, Figure S3H) [12]. In addition, arrest in the G1–S phase was also observed in TRAP1-silenced cells (supplementary material, Figure S3F–H), this representing an issue that will be the subject of further characterization. Taken together, these data suggest that TRAP1 modulates specific genes responsible for entry in mitosis across different tumour cell models.

TRAP1 controls entry into mitosis through regulation of CDK1, cyclin B1, and MAD2

In order to study the role of TRAP1 in the G2–M transition, we addressed the issue of whether TRAP1 silencing arrests the cell cycle in the G2 phase or during mitosis. Thus, TRAP1-silenced MCF7, HCT116, and A459 cells were cultured in the presence of colcemid, an agent that arrests the cell cycle in metaphase [34], and were evaluated for the Ser-10 phosphorylation of Histone H3, a specific marker of cell transit through mitosis [35]. Interestingly, while the phosphorylation of Histone H3 was enriched in control siRNA cells synchronized in metaphase, TRAP1-silenced cells, cultured in the same experimental conditions, showed significantly lower levels of Histone H3 phosphorylation (Figure 2A and supplementary material, Figure S4A, B). In parallel experiments, flow cytometric evaluation of phospho-Histone H3 was used to distinguish the fraction of cells in mitosis from cells in the G2 phase [36]. Interestingly, colcemid synchronization favoured significant accumulation of cells in mitosis in control siRNA cells, as indicated by the increase of cells with positive phospho-Histone H3 staining (Figure 2B and supplementary material, Figure S4C, D). By contrast, TRAP1-silenced cells, cultured in the presence of colcemid, showed lower levels of cells with phospho-Histone H3 positive staining (Figure 2B and supplementary material, Figure S4C, D), suggesting that TRAP1 down-regulation arrests cells in the G2 phase before mitotic entry. Consistently with a block of the cell cycle that prevents entry in mitosis, a significantly lower number of metaphases were observed in TRAP1-silenced MCF7 cells cultured in the presence of colcemid (Figure 2C).

These data suggest that TRAP1 regulates both the G2–M transition and the expression of CDK1 and cyclin B1, two master regulators of the G2–M transit [30]; we therefore questioned whether the kinetics of arrest of the cell cycle upon TRAP1 silencing is dependent on CDK1 down-regulation/inhibition. Thus, MCF7 cells were synchronized using hydroxyurea, an agent that blocks the G1–S transition [34], and cell cycle progression was monitored upon hydroxyurea release and further incubation in the presence of the CDK1 inhibitor RO-3306 [37] or the dual HSP90/TRAP1 inhibitor HSP990 [12] (Figure 2D). Indeed, control MCF7 cells exhibited rapid entry into the S phase upon hydroxyurea release, with the majority of the cell population in the S phase between 4 and 8 h after hydroxyurea removal and entry in the G2 phase after 10 h (Figure 2D). Conversely, MCF7 cells treated with RO-3306 or HSP990 showed a delay in G2-phase entry, with the majority of the cells still in the S phase 10 h after hydroxyurea release (Figure 2D; two-way ANOVA test, $p < 0.0001$). Interestingly, this delay in G2-phase entry correlated with reduced expression and phosphorylation of CDK1 at 10 h after hydroxyurea release in cells exposed to HSP990 or RO-3306 (Figure 2E). These data suggest that the kinetics of arrest of cells upon TRAP1 inhibition is consistent with down-regulation/inhibition of CDK1.

To further address the role of TRAP1 regulation of the mitotic transit, TRAP1-silenced MCF7 cells were cultured in the presence of colcemid and analysed at different time points after colcemid release (supplementary material, Figure S5A). Interestingly, MCF7 cells transfected with control siRNA exhibited a rapid exit from mitosis, with the majority of the cell population in the G1 phase 8 h after colcemid release (supplementary material, Figure S5A, upper panel). By contrast, TRAP1-silenced cells showed a delay in the G2–M transition, with the majority of the cell population still in the G2–M phase 8 and 10 h after colcemid release (supplementary material, Figure S5A, lower panel; two-way ANOVA test, $p < 0.001$). Consistently, TRAP1-silenced

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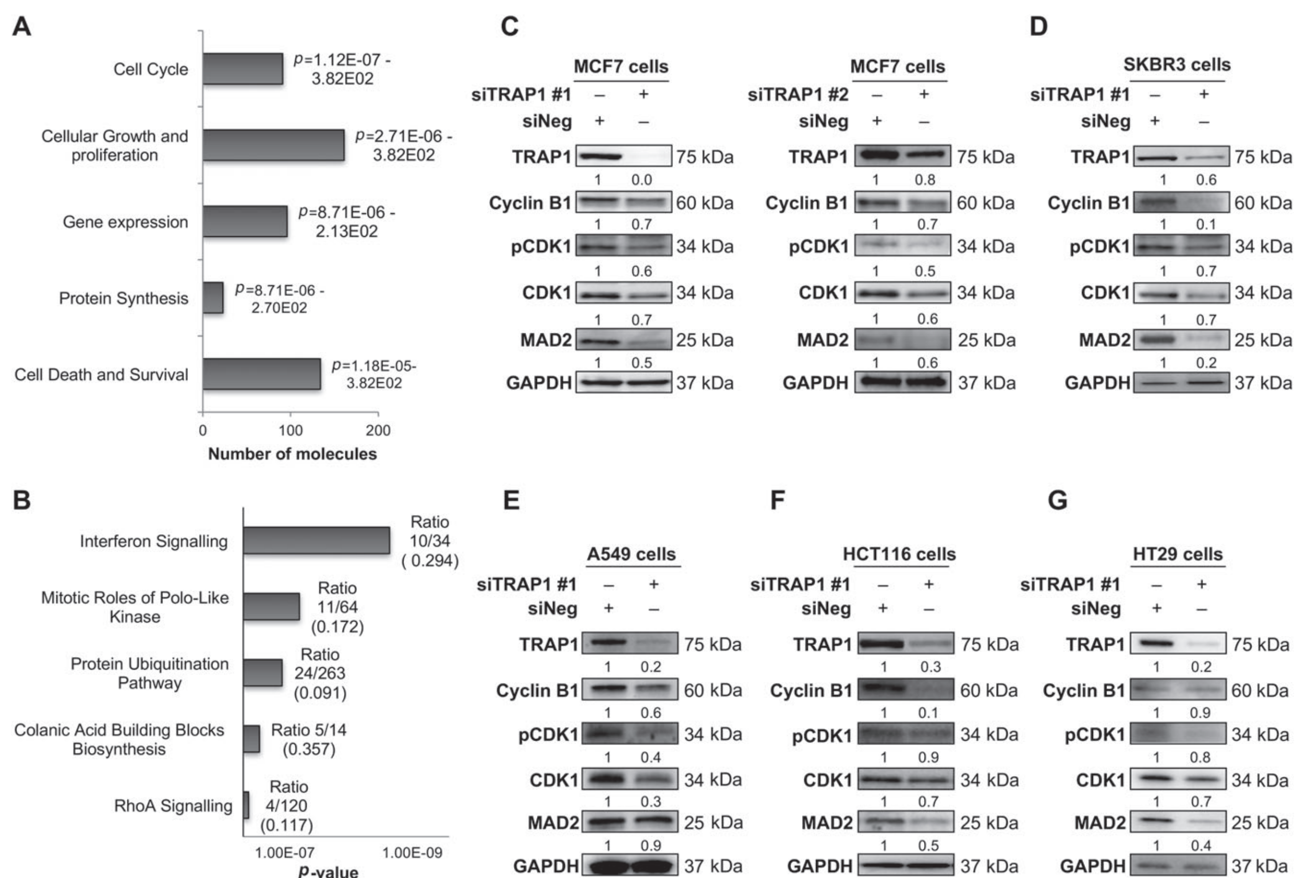

Figure 1. TRAP1 silencing results in cell cycle inhibition and reprogramming of gene expression. (A, B) The top predicted biofunctions (A) and top predicted signalling pathways (B) associated with genes modulated in TRAP1-silenced MCF7 cells. IPA software. Bar graphs represent *P* values for each biofunction/signalling pathway. (C–G) TRAP1, CDK1, pCDK1, cyclin B1, MAD2, and p21 immunoblot analysis in breast MCF7 (C) and SKBR3 (D), lung A549 (E), colon HCT116 (F), and HT29 (G) carcinoma cells transfected with control or TRAP1 siRNAs.

cells showed lower levels of phospho-Histone H3 either in the presence of colcemid or after colcemid removal (supplementary material, Figure S5B), thus confirming that TRAP1 silencing prevents entry in mitosis, as well as lower levels of CDK1 and cyclin B1 compared with control cells upon colcemid block and at early time points after colcemid release (supplementary material, Figure S5B). Of note, TRAP1-silenced cells cultured after colcemid release showed a significant down-regulation of MAD2 (supplementary material, Figure S5B). These data support the concept that TRAP1 silencing prevents entry into mitosis, with accumulation of cells in the G2 phase, and this correlates with lack of CDK1, cyclin B1 and MAD2 expression.

TRAP1 silencing results in impaired formation of the CDK1/cyclin B1 complex in nuclei

The CDK1/cyclin B1 complex translocates to the nucleus during G2–M transition, this representing a critical event for mitotic entry [30] and induction of MAD2 expression [31]. Thus, subcellular fractions were obtained from TRAP1-silenced MCF7 cells cultured in standard medium for 9 h after hydroxyurea release (Figure 3A, upper panel). Interestingly, immunoblot analysis showed lower levels of CDK1 and cyclin B1 in nuclei derived from TRAP1-silenced cells (Figure 3A, lower panel). In parallel experiments, the intracellular distribution of CDK1 was studied by confocal microscopy in TRAP1-silenced MCF7 cells cultured for 9 h after hydroxyurea removal (Figure 3B). Interestingly, while control cells showed positive CDK1 staining, with a nuclear localization more evident after hydroxyurea release (Figure 3B, left panel), TRAP1-silenced cells exhibited a lack of CDK1 staining with absent nuclear localization (Figure 3B, right panel). MAD2 immunostaining was evaluated in MCF7 cells synchronized using colcemid (Figure 3B). It is noteworthy that control MCF7 cells synchronized in metaphase showed fragmented nuclei and positive MAD2 staining, consistent with the phase-specific expression of this protein [31] (Figure 3B, left panel). Conversely, fragmented nuclei and MAD2 expression were absent in TRAP1-silenced MCF7 cells (Figure 3B, right panel). These data suggest that the assembly and the nuclear translocation of the CDK1/cyclin B1 complex are impaired in TRAP1-silenced cells and this correlates with a delay in mitotic entry and lack of expression of MAD2.

CDK1 and MAD2 are TRAP1-interacting proteins

The interaction between TRAP1 and CDK1 and MAD2 was evaluated by co-immunoprecipitation experiments

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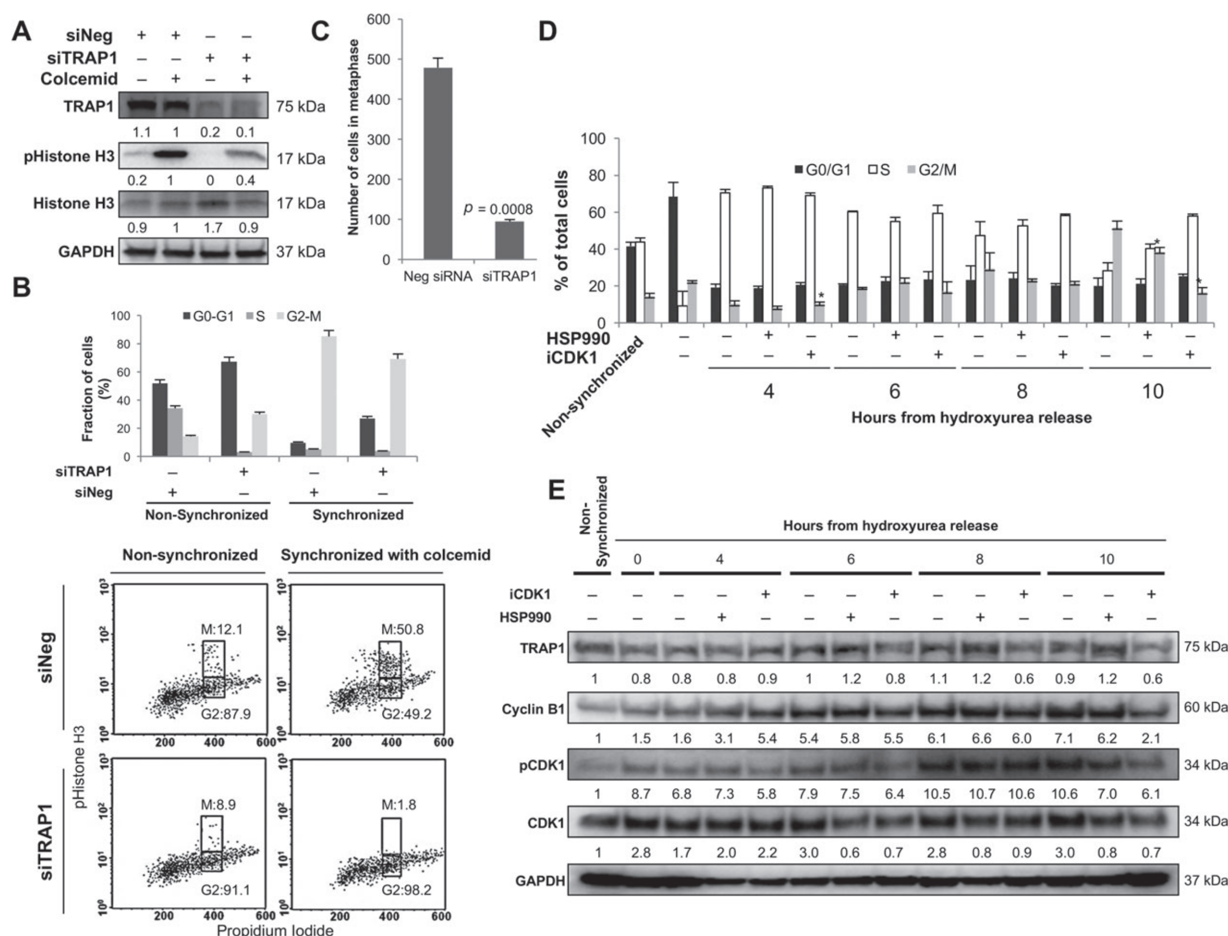

Figure 2. TRAP1 silencing arrests the cell cycle in the G2 phase modulating the expression of CDK1, cyclin B1, and MAD2. (A–C) TRAP1, Histone H3, and pHistone H3 immunoblot analysis (A); flow cytometric analysis of pHistone H3 expression (B); and number of metaphases (C) in MCF7 cells transfected with control or TRAP1 siRNAs and cultured in the presence and absence of 134.5 nM colcemid for 15 h. (B) Inset: cell cycle distribution of MCF7 cells cultured as indicated above. (D) Cell cycle distribution of MCF7 cells transfected with control or TRAP1 siRNAs and cultured for 15 h in the presence of 1 mM hydroxyurea and further incubated, after hydroxyurea release, in the presence and absence of 200 nM HSP990 or 10 μM RO-3306. *P* values indicate the statistical significance with respect to cells transfected with control siRNA exposed to the same experimental conditions. $^*p < 0.0001$. (E) TRAP1, CDK1, pCDK1, and cyclin B1 immunoblot analysis in MCF7 cells cultured as described in D.

and the *in situ* proximity ligation assay in MCF7 cells cultured under normal conditions or synchronized using colcemid. Interestingly, TRAP1 co-immunoprecipitated with both CDK1 and MAD2, as well as with the CDK1-interacting protein cyclin B1 [30] (Figure 3C). Intriguingly, the fraction of CDK1, cyclin B1, and MAD2 interacting with TRAP1 was enriched in cell synchronized using colcemid (Figure 3C). Furthermore, TRAP1 and CDK1 or MAD2 immunostaining showed a close proximity between these proteins in cells synchronized using colcemid, and notably these interactions occurred outside fragmented nuclei (Figure 3D). This evidence suggests that TRAP1 forms a complex with CDK1 or MAD2 and that this interaction is maximal during the G2–M transition.

CDK1 and MAD2 are regulated by TRAP1 networks at the post-transcriptional level

TRAP1 is responsible for the quality control of a network of client proteins, whose expression is higher in a TRAP1-rich background and their ubiquitination increased upon TRAP1 silencing [4,5,12]. This regulation is mediated by TRAP1 interaction with the proteasome regulatory particle TBP7 [38], whose silencing results in enhanced ubiquitination of TRAP1 client proteins [4]. Of note, RT-qPCR analysis showed no major changes in the mRNA levels of *CDK1*, cyclin B1 (*CCNB1*) or MAD2 (*MAD2L1*) in TPB7-silenced cells (Figure 4A), whereas immunoblot analysis showed a protein expression profile similar to TRAP1-silenced cells with down-regulation of the three proteins (Figure 4B). Hence, in addition to the previously described transcriptional regulation, these data suggest that the TRAP1/TBP7 network regulates CDK1 and MAD2 at the post-transcriptional level. Thus, the ubiquitination levels of CDK1 and MAD2 were evaluated in TRAP1- or TBP7-silenced MCF7 cells by ubiquitin immunoblot analysis of their immunoprecipitates. Interestingly, either TRAP1 or TBP7 silencing resulted in increased ubiquitination of CDK1 and MAD2 (Figure 4C), this confirming that the

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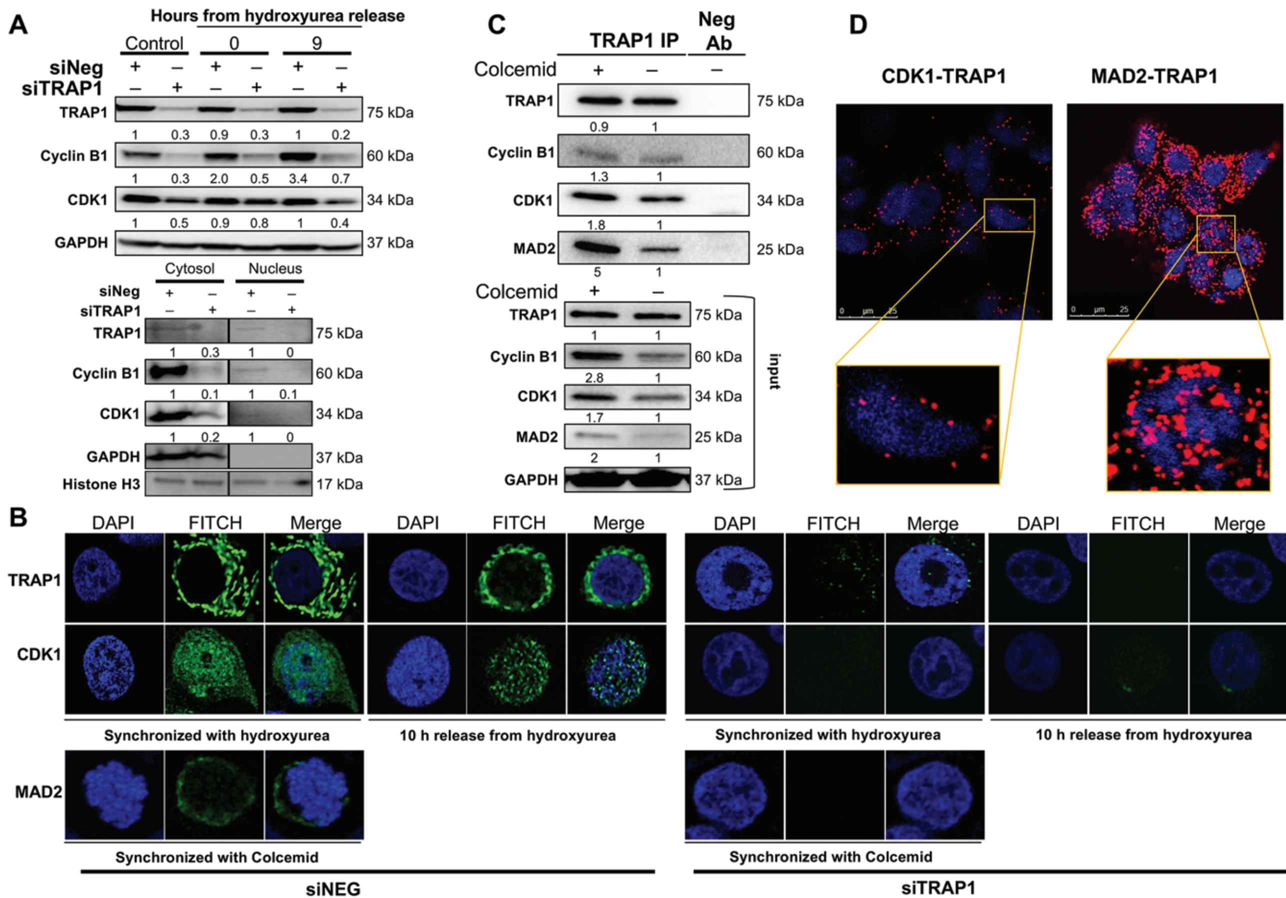

Figure 3. TRAP1 silencing results in impaired formation of the CDK1/cyclin B1 complex. (A) Upper panel: TRAP1, CDK1, and cyclin B1 immunoblot analysis in MCF7 cells transfected with control or TRAP1 siRNAs and cultured for 15 h in the presence of 1 mM hydroxyurea or 9 h after hydroxyurea release. Lower panel: TRAP1, CDK1, and cyclin B1 immunoblot analysis in cytosolic and nuclear fractions obtained from MCF7 cells transfected with control or TRAP1 siRNAs, synchronized with hydroxyurea, and cultured for 9 h after hydroxyurea release. (B) Representative confocal microscopy images showing TRAP1 and CDK1 subcellular distribution in MCF7 cells transfected with control or TRAP1 siRNAs and cultured in the presence of 1 mM hydroxyurea or 9 h after hydroxyurea release, and MAD2 subcellular distribution in MCF7 cells transfected with control or TRAP1 siRNAs and cultured with 134.5 nM colcemid for 15 h. (C) TRAP1 IPs were obtained from total cell lysates of MCF7 cells cultured in standard conditions or in the presence of 134.5 nM colcemid for 15 h. Inputs: TRAP1, CDK1, cyclin B1, and MAD2 immunoblot analysis in MCF7 cells cultured as previously described. (D) Representative fluorescence images (upper panels) and close-up (lower panels) showing proximity ligation assay signals (red) detected in MCF7 cells incubated with 134.5 nM colcemid for 15 h and stained with TRAP1 and CDK1 (left panels) or MAD2 (right panels) antibodies. Nuclei are DAPI-labelled (blue). Scale bar = 20 μm.

two proteins are regulated at the post-transcriptional level by the TRAP1/TBP7 network. Since cyclin B1 stability/ubiquitination is tightly regulated upon formation of a complex with CDK1 and phosphorylation by Cdc25 [39], the cyclin B1 ubiquitination level was also evaluated and consistently with the reduction of CDK1 expression, its ubiquitination was enhanced in conditions of TRAP1 or TBP7 silencing (Figure 4C). Finally, TBP7 silencing (Figure 4D), as well as the transfection of a TBP7 deletion mutant, lacking the TRAP1-binding domain, with dominant negative activity over endogenous TBP7 [4] (supplementary material, Figure S6A, B), induced a significant reduction of the proportion of cells in the S phase, with accumulation of cells in the G0–G1 and G2–M phases – a cell cycle distribution observed under TRAP1-silencing conditions, thus confirming the relevance of this post-transcriptional control for TRAP1 regulation of the cell cycle. These data suggest that TRAP1 post-transcriptional quality control on CDK1 and MAD2 contributes to its regulation of the G2–M transition and that this mechanism is complementary to the transcriptional regulation.

CDK1 regulation is critical for TRAP1-dependent control of the G2–M transition

To evaluate the functional relevance of CDK1 regulation for TRAP1 control of the G2–M transition, in further experiments, cell cycle progression through mitosis was monitored upon transfection of CDK1 in TRAP1-interfered MCF7 cells synchronized using colcemid and after colcemid release (Figure 5A, B). Interestingly, CDK1 up-regulation partially rescued the low levels of cyclin B1, MAD2, and phospho-Histone H3 in conditions of TRAP1 knockdown (Figure 5A). Furthermore, while TRAP1-interfered cells showed a delay of cell cycle progression through mitosis, the majority of the cells still being in the G2 phase 6 h after colcemid release (56.9% versus 51.6%; Figure 5B), CDK1-transfected cells showed a more

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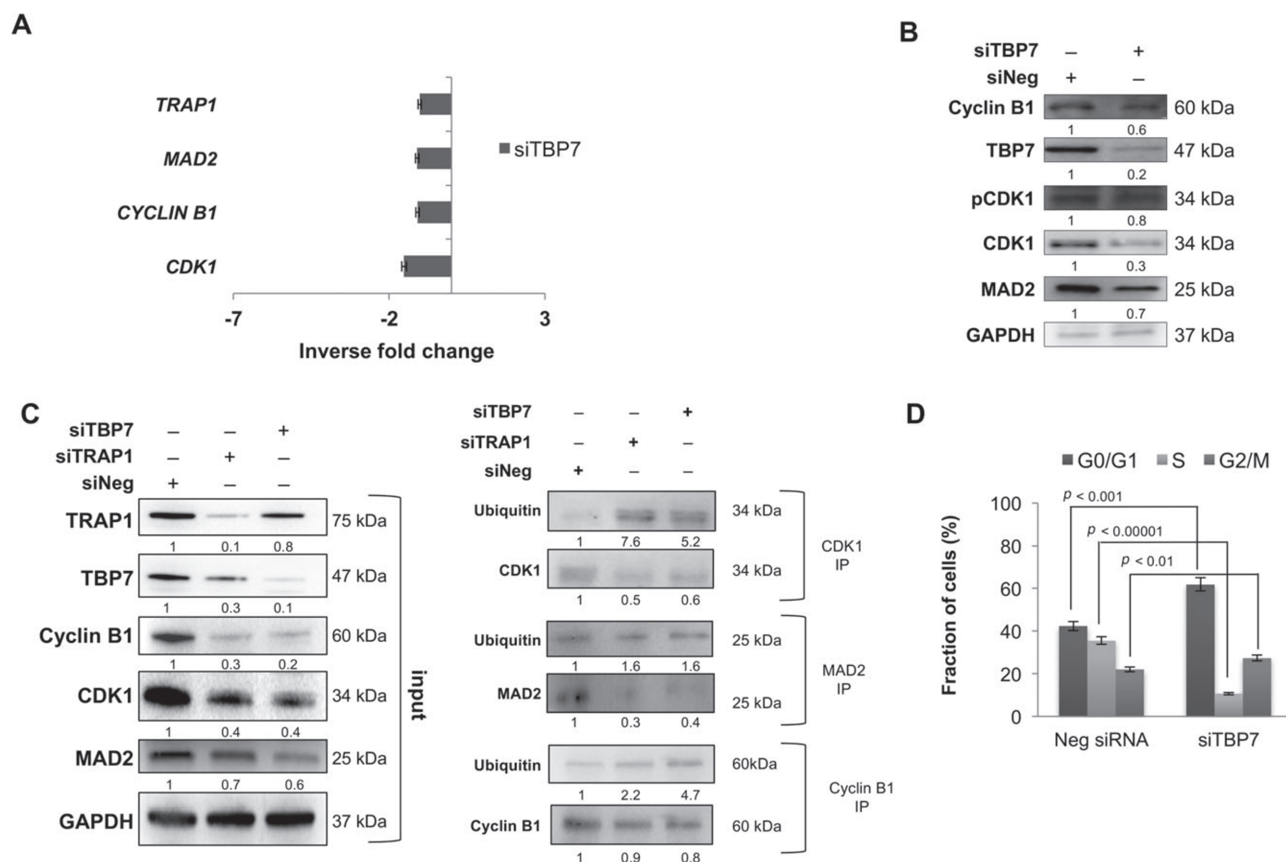

Figure 4. The TRAP1 network is responsible for post-translational quality control of CDK1 and MAD2. (A, B) *CDK1*, *CYCLIN B1*, and *MAD2* real-time RT-PCR (A) and immunoblot (B) analysis in MCF7 cells transfected with control or TBP7 siRNAs. (C) CDK1, MAD2, and cyclin B1 IPs were obtained from total cell lysates of MCF7 transfected with control, TRAP1 or TBP7 siRNAs and incubated with 10 μM MG132 for 2 h before cell lysis. IPs were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Densitometric band intensities represent ratios between ubiquitinated bands and total proteins in IPs. Inputs: TRAP1, TBP7, CDK1, cyclin B1, and MAD2 immunoblot analysis in MCF7 cultured as previously described. (D) Cell cycle distribution of MCF7 cells transfected with control or TBP7 siRNAs.

rapid transit through mitosis in a TRAP1-poor background (68.1% versus 47.9%; Figure 5B). A parallel higher increase of the G0–G1 fraction was observed in conditions of CDK1 up-regulation and TRAP1 interference (29.4% versus 42.2%) compared with pMock control TRAP1-interfered cells (38.7% versus 37.4%; two-way ANOVA test, $p < 0.01$). Consistently, CDK1 up-regulation rescued the G2-phase cell cycle arrest in a TRAP1-poor background in LC A549 and CRC cells, as demonstrated by the increase of phospho-Histone H3-positive cells in TRAP1-silenced CDK1-transfected cell lines (supplementary material, Figure S7). To confirm this observation, mitotic counts were evaluated upon transfection of CDK1 in TRAP1-interfered MCF7 cells synchronized using colcemid and after colcemid release (Figure 5C). Indeed, mitotic counts were similar in TRAP1-silenced cells upon colcemid synchronization (Figure 5C, left panel) and after colcemid release (Figure 5C, right panel), confirming the arrest of the cell cycle in the G2 phase. By contrast, the up-regulation of CDK1 in a TRAP1-poor background favoured a rapid transit through mitosis, with a 50% reduction of cells in metaphase after colcemid removal (Figure 5C, right panel). Taken together, this evidence suggests that TRAP1 regulation of CDK1 is the limiting factor in its control of G2–M transit. Consistently, TRAP1 up-regulation significantly impaired the capacity of the CDK1 inhibitor RO-3306 to block the cell cycle in the G2 phase in MCF7 (Figure 5D), HCT116 (supplementary material, Figure S8A), and A549 cells (supplementary material, Figure S8B).

TRAP1 regulation of CDK1, cyclin B1, and MAD2 is widely conserved in human colorectal, lung, and breast carcinomas

In order to address the relevance of TRAP1 regulation of the cell cycle in human malignancies, lung and breast carcinomas were analysed for TRAP1 expression and this was correlated with the proliferation index Ki67. Patients' characteristics are reported in the supplementary material, Table S3. Interestingly, the Spearman rank test showed a significant correlation between the TRAP1 and Ki67 levels in two series of 46 LCs ($\rho = 0.7$, $p < 0.0001$; Figure 6) and 57 BCs ($\rho = 0.28$, $p = 0.04$; supplementary material, Figure S9A). Consistently, Cohen's kappa coefficient analysis showed a moderate association between TRAP1 and Ki67 in BCs (supplementary material, Table S4). Thus, TRAP1-high lung, colon, and breast carcinomas were

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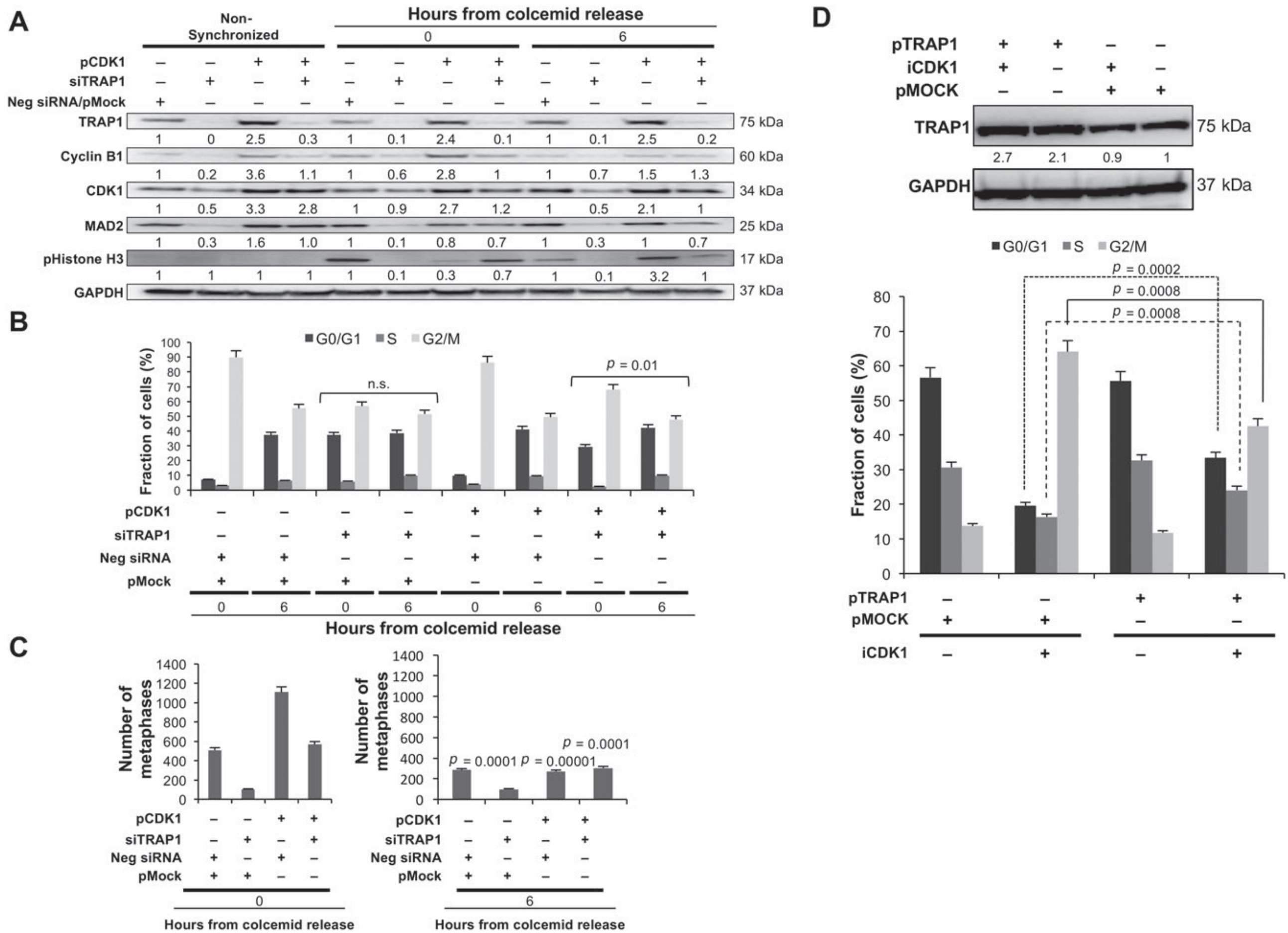

Figure 5. CDK1 regulation is critical for TRAP1-dependent control of the G2–M transition. (A) TRAP1, CDK1, cyclin B1, MAD2, and pHistone H3 immunoblot analysis in TRAP1-silenced MCF7 cells, transfected with pMock or CDK1 cDNA and cultured for 15 h in the presence of 134.5 nM colcemid or 6 h after colcemid release. (B) Cell cycle distribution of MCF7 cells cultured as described in A. *P* values indicate the statistical significance between CDK1- and Mock-transfected TRAP1-silenced cells upon synchronization with colcemid and after colcemid release. (C) Number of metaphases in TRAP1-silenced MCF7 cells transfected with pMock or CDK1 cDNA, and cultured for 2 h in the presence of 134.5 nM colcemid (left panel) or 6 h after colcemid release (right panel). *P* values indicate the statistical significance with respect to cells synchronized with colcemid and exposed to the same experimental conditions. (D) Cell cycle distribution of MCF7 cells transfected with pMock or TRAP1 cDNA and treated with 10 μM RO-3306. Inset: TRAP1 immunoblot analysis in MCF7 cells treated as previously described.

further evaluated for the expression of CDK1 and/or MAD2 in comparison with TRAP1-low tumours by TMA (LCs) and immunoblot (BCs and CRCs) analysis, respectively. It is noteworthy that a significant correlation was observed between TRAP1 and CDK1 expression levels in human LCs ($\rho = 0.66$, $p < 0.0001$; Figure 6), as well as between TRAP1 and CDK1 ($\rho = 0.46$, $p = 0.01$) or MAD2 protein levels ($\rho = 0.64$, $p = 0.0002$) in BCs (supplementary material, Figure S9A). Cohen's kappa coefficient analysis showed a moderate/large association between TRAP1 and CDK1 and/or MAD2 in BCs (supplementary material, Table S4). Finally, a striking statistically significant co-expression and a large association were observed between TRAP1 and CDK1 ($\rho = 0.62$, $p = 9.84\text{E-}08$) or MAD2 ($\rho = 0.50$, $p = 6.57\text{E-}05$) in CRCs, CDK1 and MAD2 being constantly up-regulated in TRAP1-positive CRCs (supplementary material, Figure S9B, Table S4). These data strongly support the concept that TRAP1 regulation of the cell cycle through CDK1 and MAD2 is widely conserved in human malignancies.

Discussion

This study provides the first evidence that TRAP1 is relevant in the control of key cell cycle regulators and that TRAP1/TBP7 quality control of CDK1 and MAD2 contributes mechanistically to the regulation of mitotic entry and transit. Indeed, the involvement of TRAP1 in regulation of the cell cycle has been hypothesized by early studies, since TRAP1 was proposed as the molecular chaperone of RB1 [11], a nuclear protein which prevents cell cycle progression towards the S phase [40]. Since RB1 stabilization requires TRAP1 translocation into the nucleus and occurs during heat shock and mitosis [11] or hypoxia [41], it has been proposed that TRAP1 regulation of RB represents the molecular basis for its capacity to block S-phase entry under stress conditions [42]. This mechanism may be relevant in human tumours with reduced TRAP1 expression, where TRAP1 down-regulation could provide a proliferative advantage to tumour cells, due

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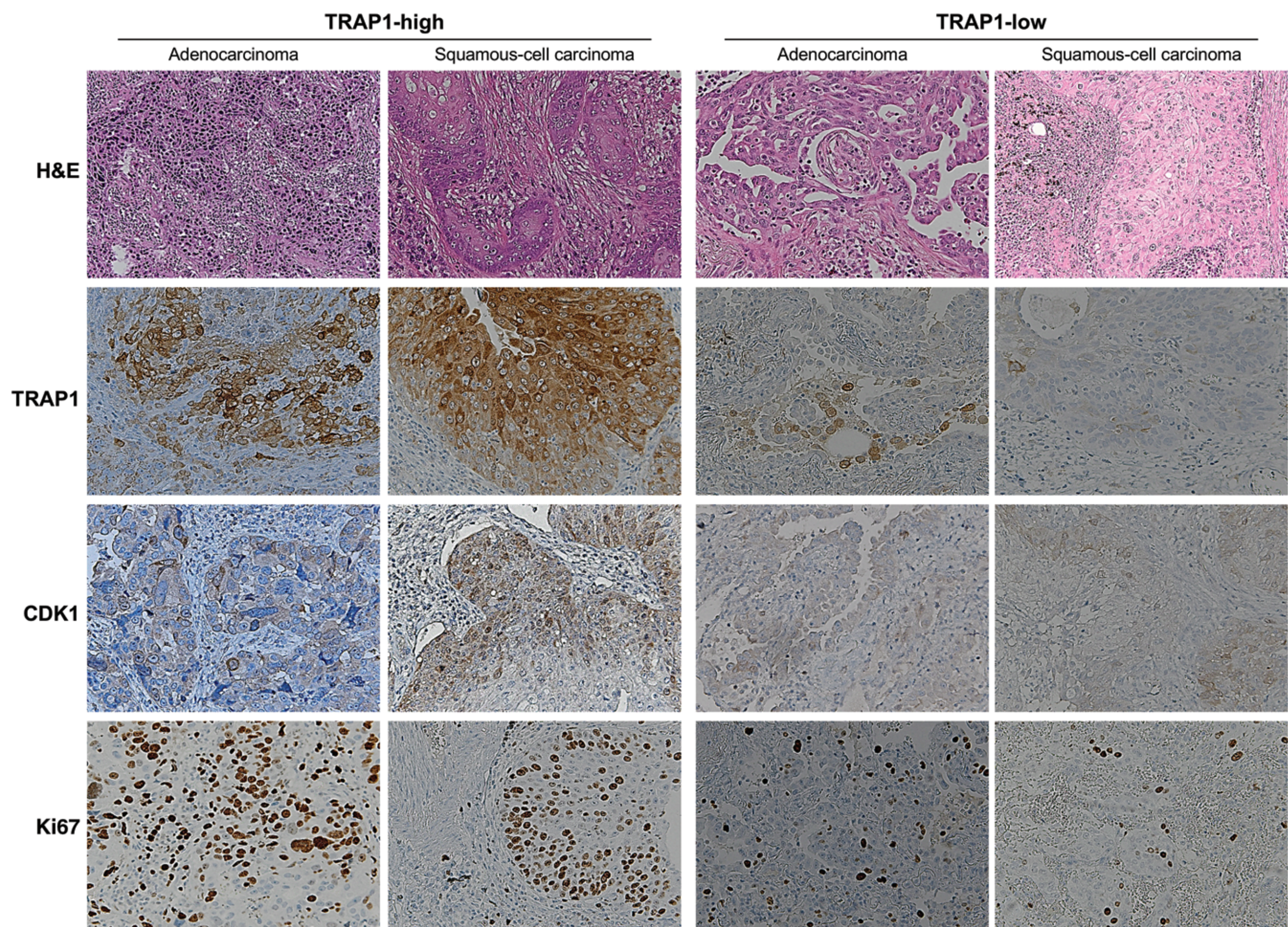

Figure 6. TRAP1 expression correlates with Ki67 proliferation index and CDK1 levels in human lung carcinomas. TRAP1, Ki67, and CDK1 tissue microarray immunohistochemistry in four cases of LCs.

to the lack of RB stabilization [42]. Recent studies suggested that TRAP1 is up-regulated in several human malignancies [6,8–10,16] and high TRAP1 expression correlates with increased cell proliferation in LC [9]. In such a context, we reported that TRAP1 regulation of the cell cycle occurs as a downstream event to its control on RAF/ERK signalling in BC and CRC [12]. Since no changes in RB1 stability were observed in TRAP1-silenced cells cultured in standard conditions (data not shown), the molecular mechanism responsible for TRAP1 modulation of the cell cycle was further addressed by whole-genome gene expression profiling of TRAP1-silenced MCF7 cells. Indeed, TRAP1 indirectly regulates the transcription of several genes involved in cell cycle progression and, more specifically, in the G2–M transition: CDK1, cyclin B1, and MAD2, cell cycle regulators responsible for mitotic entry and transit [30]. Complementary to this transcriptional regulation, post-transcriptional quality control on CDK1 and MAD2 was observed, as an additional mechanism responsible for TRAP1 control of the G2–M transition. In such a perspective, this study provides, to our knowledge, the first mechanistic demonstration that TRAP1 governs the intricate machinery responsible for mitotic entry, and this occurs through a dual, complementary control on CDK1 and MAD2 expression, both at the transcriptional and at the post-transcriptional level. Indeed, TRAP1 interacts with both CDK1 and MAD2 and its silencing results in (i) reduced mRNA and protein levels of both genes, (ii) an increase of their ubiquitination, and (iii) lack of formation of the CDK1/cyclin B1 complex. Experiments based on TBP7 silencing allowed us to dissect the complexity of this mechanism, since mRNAs encoding for CDK1 and MAD2 were unchanged but their protein levels were reduced in parallel with enhanced ubiquitination and inhibition of the G2–M transition, as observed under TRAP1 interference conditions. Thus, these observations suggest that the quality control played by the TRAP1/TBP7 network on CDK1 and MAD2 is a mechanism that contributes to TRAP1 control on mitotic entry. What is puzzling is the understanding of the role of cyclin B1 in TRAP1 regulation of the G2–M transition, as both the mRNA and the protein levels are down-regulated in TRAP1-silenced cells. However, the interaction between TRAP1 and cyclin B1 is not predicted by mass spectroscopy analysis of TRAP1 co-immunoprecipitates from osteosarcoma cells [20], but cyclin B1 was detectable in TRAP1 immunoprecipitates together with CDK1 and MAD2 in MCF7 cells. Indeed, cyclin B1 stability depends on the formation of a complex with CDK1 and a phosphorylation event by Cdc25 [39]. Thus, our data do not establish

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whether cyclin B1 is a TRAP1-interacting protein or its co-immunoprecipitation with TRAP1 is due to its interaction with CDK1 and whether the increased cyclin B1 ubiquitination levels observed under TRAP1/ TBP7 silencing conditions are dependent by loss of TRAP1 quality control or reduced levels of CDK1, this representing a limitation of our study. However, experiments with CDK1 re-expression in a TRAP1-low background showed that CDK1 represents the limiting factor in TRAP1 control of the G2–M transition and that its up-regulation partially rescues the low levels of cyclin B1 and MAD2. Thus, our data allow us to conclude that TRAP1 regulation on mitotic entry relies on CDK1 quality control and, secondarily, on the assembly and nuclear translocation of the CDK1/cyclin B1 complex and MAD2 transcription/degradation (supplementary material, Figure S10).

Previous reports suggest that CDK1 stability is controlled by HSP90 chaperones [43] and their inhibition results in CDK1 ubiquitination and G2–M cell cycle arrest [44,45]. More recently, it has been suggested that CDK1 plays a central role in coupling mitotic entry with gene transcription, mitochondrial bioenergetics, and protection from apoptosis [46–49]. Lack of CDK1 expression results in impaired oxidative phosphorylation [46,47], suggesting that CDK1 is critical in stimulating mitochondrial bioenergetics under the condition of high energy demand as the mitotic transit [46]. Since TRAP1 is involved in remodelling of cancer cell metabolism [18,22,23], our data support the hypothesis that TRAP1 regulation of CDK1 represents a central mechanism in the multifaceted roles of TRAP1 in human malignancies [14].

What is clinically relevant is the observation that TRAP1 regulation of the cell cycle is conserved in BCs, LCs, and CRCs. Indeed, our data reveal a significant correlation between high TRAP1 expression and high proliferation index in BCs and LCs, and between TRAP1 expression and CDK1 and/or MAD2 levels in BCs, CRCs, and LCs. The concordance between *in vitro* and *in vivo* data strongly support the hypothesis that TRAP1 control of the cell cycle relies on common mechanisms conserved across several human malignancies, all characterized by TRAP1 up-regulation [7–9]. Furthermore, these data are relevant in the perspective of recent studies showing promising anti-tumour activities of CDK inhibitors [2,3] and suggest that TRAP1 deserves to be evaluated as a biomarker to select human malignancies with deregulated cell cycle control. In this regard, the observation that TRAP1 up-regulation partially impairs the activity of CDK1 pharmacological agents suggests that human malignancies with high TRAP1 expression are likely to be more resistant to anti-cancer agents targeting the cell cycle regulatory machinery.

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Author contributions statement

The authors contributed in the following ways: study concept and design: LS, FE, and ML; acquisition of data: LS, FM, VC, GP, VLB, EL, AP, DSM, CM, FN, GL, and MRA; analysis and interpretation of data: LS, VS, DSM, PB, and ML; drafting of the manuscript: LS and ML; critical revision of the manuscript: LS, FE, and ML; acquisition of funding: FM, FE, ML; study supervision: ML.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. TRAP1 silencing results in reprogramming of the genes involved in cell cycle regulation

Figure S2. TRAP1 silencing results in reprogramming of the cell functions in breast carcinoma cells

Figure S3. TRAP1 silencing/up-regulation results in modulation of the cell cycle regulators in breast, lung, and colorectal carcinoma cell lines

Figure S4. TRAP1 silencing arrests the cell cycle in the G2 phase in lung and colorectal carcinoma cells

Figure S5. TRAP1 controls entry into mitosis through the regulation of CDK1, cyclin B1, and MAD2

Figure S6. Cell cycle distribution and TBP7 immunoblot analysis of MCF7 cells transfected with TBP7 cDNA or the ΔTBP7 deletion mutant

Figure S7. CDK1 regulation is critical for TRAP1-dependent control of the G2–M transition in colorectal and lung carcinoma cells

Figure S8. TRAP1 up-regulation partially impairs the activity of the CDK1 inhibitor RO-3306

Figure S9. TRAP1 expression correlates with CDK1 or MAD2 levels in human breast and colorectal carcinomas

Figure S10. TRAP1 exerts dual controls on cell cycle progression

Table S1. Genes differently expressed in MCF7 TRAP1-silenced cells

Table S2. The top five predicted biofunctions associated with genes modulated in TRAP1-silenced MCF7 cells as assessed by the DAVID platform

Table S3. Demographic and clinicopathological characteristics of the patients

Table S4. Cohen's kappa coefficient association analysis of TRAP1 and Ki67, CDK1 or MAD2 expression levels in the cohorts of 57 breast and 60 colorectal cancers

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