Proteomic Identification of Chaperonin-containing Tail-less Complex Polypeptide-1 Gamma Subunit as a p53-responsive Protein in Colon Cancer Cells

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Abstract. *We recently reported that functional loss of p53 altered the responses of human HCT116 colon cancer cells to apoptosis triggers. To examine the molecular basis underlying the differential responses to drug treatment in the cancer cells, we performed a proteomic analysis in order to compare the protein expressions between human colon cancer cells with and those without p53 ($p53^{+/+}$ and $p53^{-/-}$) respectively. We identified two isoelectric variants of the chaperonin-containing tail-less complex polypeptide-1 gamma subunit (TCP-1γ) from the cells, and confirmed that the two isoelectric variants were phosphorylated at tyrosine residue(s). The $p53^{-/-}$ cells had higher protein concentration of the more acidic variant of TCP-1γ compared to their $p53^{+/+}$ counterparts. Moreover, TCP-1γ was found to be co-localized with centrosome. Our results implicate a novel cell signaling loop in HCt116 cells involving p53 and TCP1-γ phosphorylation, which may be related to regulation and action of centrosomes.*

p53 was discovered in 1979 (1) and has been studied intensively since then due to its important roles in cell physiology and pathophysiology. One of the most recognized biological activities of p53 is its involvement in the control of cancer development, which is of great clinical significance. Approximately half of all human carcinomas, including colon cancer, are found to have defect(s) in p53. Novel therapeutic methods targeting p53 or its signaling network are being developed for the management of cancer (2, 3).

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At the cellular level, p53 is a pro-apoptotic protein that promotes cell death. Under normal physiological conditions, p53 activity is kept at a low level to maintain normal tissue homeostasis, which is achieved mainly through its binding to the negative regulators. Mouse double minute 2 for example, binds to p53 to inhibit p53 transcriptional functions and targets p53 for proteasomal degradation (4); enhancing MDM2 activity can lead to cell death, while inhibiting MDM2 promotes carcinogenesis. This interaction forms a feedback regulation loop, MDM2 itself being a transcriptional target of p53. An increase in p53 activity leads to an increase in expression of MDM2, which in turn inhibits p53 function. In addition to MDM2, posttranslational modification (*i.e.* phosphorylation) (5) and subcellular localization (6) of p53 can also modify its biological activities.

p53, as a transcription factor, exerts diverse biological functions through regulating the expression of various genes (7, 8). At least 129 human or viral genes have p53-responsive elements, showing the broad effect of p53. The most common molecular mechanism of p53 trans-regulatory activity is probably transactivation: p53 interacts with and recruits translation elements to promoter-enhancer regions to initiate transcription (9). Transrepression is another established model of p53 transregulation, however, the molecular mechanisms are less clear. The biological activities of genes under p53 transregulation are often linked to apoptosis, senescence or cell-cycle arrest.

Apoptosis can be subjectively categorized as being p53-dependent or p53-independent. p53-dependent apoptosis typically occurs by the intrinsic mitochondrial pathway. Physical, chemical and biological stress can activate p53, which in turn transactivates various apoptotic genes such as BCL-2 for cell death. This is an important mechanism to ensure that damaged cells are removed properly (10). However, other signal transduction routes of p53-dependent apoptosis have also been shown. In our recent studies using the human colon cancer HCT116 cell line as an experimental model, we noted that knockout of p53 gene ($p53^{-/-}$) makes cells less responsive to chemical stimuli (11, 12), which is

in keeping with findings of many other reports. We thus performed a proteomic analysis in order to compare the protein expressions of $p53^{-/-}$ HCT116 cells and their p53 wild-type counterparts.

Materials and Methods

Chemicals, antibodies, cell culture and sample preparation. Chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Pte. Ltd, Singapore). Those for proteomic analysis were provided by GE Healthcare Biosciences (GE Healthcare Biosciences, Singapore). Antibodies against γ-tubulin (# sc-7396) and chaperonin-containing tail-less complex polypeptide-1 γ (TCP-1 γ; # sc-13878) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Phospho-Tyrosine (# 9411) and Phospho-Threonine (# 9386) were from Cell Signaling Technology (Beverly, MA, USA). Two HCT116 human colon cancer cell lines were used in the study, kindly provided by Dr. B. Vogelstein (School of Medicine, the John Hopkins University, Baltimore, MD, USA): cells with wild-type p53 ($p53^{+/+}$) and cells with knockout of p53 ($p53^{-/-}$). The cells were maintained in the modified McCoy's 5A medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37˚C. The modified McCoy's 5A, penicillin-streptomycin, and L-glutamine were purchased from GIBCO (Invitrogen Singapore PTE, Singapore). Fetal bovine serum was a product of Hyclone (Logan, UT, USA). Cell cultures at approximately 70% confluence were used for all the experimental treatments.

For proteomic analysis, samples were prepared following a procedure previously described (8, 10). For sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), cells were lysed in RIPA buffer which contained 1% NP-40, 0.5% DOC, 0.1% SDS in phosphate buffer saline (PBS), with phenylmethanesulfonyl fluoride (50 mM), leupeptin (1 mg/ml), sodium vanadate (200 mM), and aprotinin (2.1 mg/ml). Supernatants (10000 ×*g*, 5 min) were collected and stored at –20˚C until use. Protein quantification for western blot analysis was performed by Bradford assay using Coomassie reagent from Pierce Biotechnology (Rockford, IL, USA).

Two dimensional gel electrophoresis (2-DE). 2-DE analyses (11, 13) were performed using IPG polyacrylamide strips with a linear pH gradient from 4 to 7 as the first-dimensional gels. IPG strips were rehydrated in a reswelling buffer that contained 8 M urea, 2% CHAPS, 0.5% pharmalyte, 0.2% dithiothreitol (DTT) for isoelectric focusing using an IPGphor machine (500 V for 1 h, 1000 V for 1 h, 3500 V for 1 h, gradient 8000 V for 1 h and 24 000 Vhour). Total cellular protein samples were applied using the cup loading method. Focused IPG strips were frozen at –20˚C until use. Before the second-dimensional run, IPG strips were equilibrated twice (each for 15 min) with SDS in 6 M urea, 30% glycerol, 2% SDS, 50 mM Tri pH 8.8; the first and second equilibrations were performed with 1% DTT and 4% iodoacetamide, respectively. Equilibrated strips were placed on top of 12% SDS-PAGE (Bio-Rad PROTEAN II xi cell, Bio-Rad, Singapore) and sealed with 0.5% agarose, containing a trace amount of bromophenol blue in running buffer (24 mM Tris, 200 mM glycine, 0.1% SDS) for the second-dimensional separation (12 mA for 2 h then 24 mA till the bromophenol blue reached the bottom of the electrophoresis cell). External molecular weight calibration was performed by running molecular weight standard ladders (Bio-Rad, CA, USA) in parallel with the first-dimensional gels during second-dimensional separation. Values of p*I* were calibrated by plotting the proteins of interest against the pH scale of IPG 4-7. Peptides separated on 2-D gels were visualized by the silver staining method.

Gel images were captured using an ImageScanner (GE Health Sciences, Singapore) and analyzed using the software package ImageMaster 2D Platinum (version 4.9; GE Health Sciences, Singapore). Spots of interest were excised with a scalpel for in-gel tryptic digestion, which involved destaining (30 mM potassium ferricyanide and 50 mM sodium thiosulfate till colorless), washing (twice with 25 mM ammonium bicarbonate), air drying, and overnight digestion at 37˚C in 25 mM ammonium bicarbonate containing 10 ng/ml of trypsin.

Peptide mass fingerprinting (PMP). PMF involved mass determination of tryptic fragments using MS in combination with mass database matching for peptide identifications. MS was performed using an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Framingham, MA, USA). Tryptic peptide mixtures (0.5 ml) were spotted on a 192-well target plate and crystallized with 0.5 ml of CHCA matrix solution (5 mg/mL). MS data were automatically acquired with a trypsin autodigest exclusion list and the ten most intense ions were selected for MS/MS. The collision gas was nitrogen air and the energy was 1 kV. Interpretation was carried out using the GPS Explorer software and database searching was done using the MASCOT program (Matrix Science, London, UK). Combined MS and MS/MS searches were conducted with the following settings: NCBI database, all entries, peptide tolerance at 200 ppm, MS/MS tolerance at 0.5 Da, carbamidomethylation of cysteine (fixed modification), and methionine oxidation (variable modifications).

SDS-PAGE and western blot analysis. Western blot analysis was performed as described previously (11, 13). Briefly, cell lysates were subjected to 15% PAGE then transferred onto PVDF membranes. After blocking for 1h with 5% dry milk in TBST solution (50 mM Tris/ HCl pH 7.4, 150 mM NaCl, 0.1% Tween-20), membranes were washed three times using TBST, exposed to primary antibody overnight at 4˚C with gentle shaking, washed with TBST three times, exposed to secondary antibody for 1 h, washed three times again in TBST, and eventually subjected to chemiluminescence detection using the SuperSignal Substrate Western Blotting Kit (Pierce Biotechnology, Rockford, IL, USA). Primary antibodies were purchased from Cell Signaling.

Immunofluorescent staining and confocal microscopy. Apoptosis was evidenced and quantified using *In Situ* Cell Death Detection Kit terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay from Roche Diagnostics (Penzberg, Germany), followed by confocal microscopy. Briefly, HCT116 cells were grown in Labtek Chamber Slides (Rochester, NY, USA), were washed once with PBS and were fixed in 100% methanol at –20˚C for 10 min. The fixed cells were blocked in blocking buffer (1% bovine serum albumin, 0.05% Tween-20 in 1× PBS) for 1 h, followed by three washes with 1× PBS. The cells were then incubated with the TUNEL staining solution together with antibodies of interest overnight at 4˚C, then washed three times with 1× PBS before being mounted with UltraCruz Mounting Medium (Santa Cruz Technology, CA, USA) containing DAPI. The cells were promptly viewed under confocal microscopy. The cells were regularly checked to ensure that they remained adherent to the slides during the staining procedure.

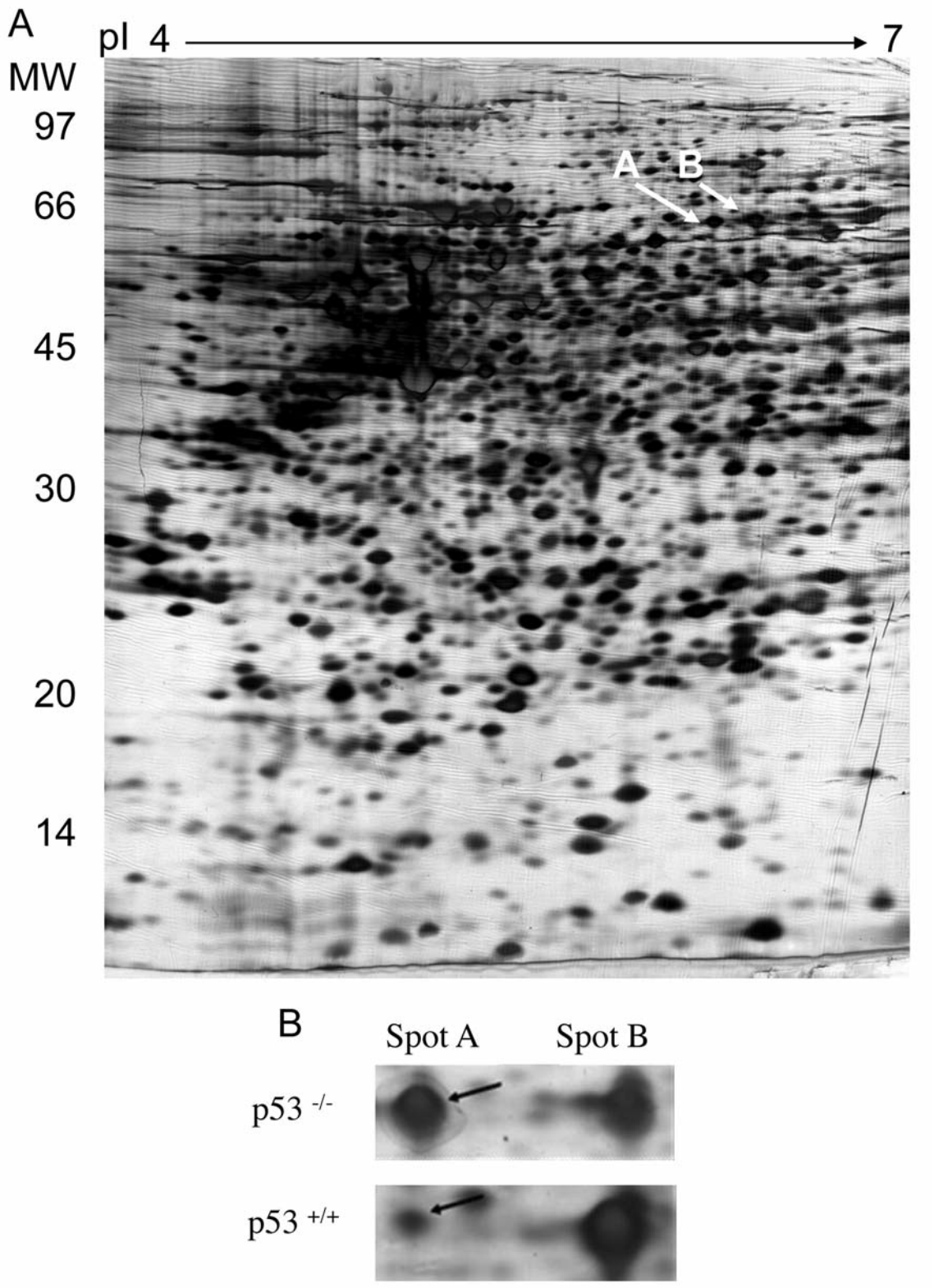

Figure 1. *Identification of a peptide differentially expressed between HCT116 p53$^{+/+}$ and p53$^{-/-}$ cells using two dimensional gel electrophoresis. A: Overall view of a representative 2-D gel image of a lysate from HCT116 p53$^{-/-}$ cells. B: Regional view of peptide spot A separated on the 2-D gels from lysates of p53$^{+/+}$ and p53$^{-/-}$ cells respectively. The optical density of peptide spot A was higher in p53$^{-/-}$ cells than that in the p53$^{+/+}$ cells. Spot B was next to spot A, with a similar molecular weight, but being slightly towards the basic side on the pI scale.*

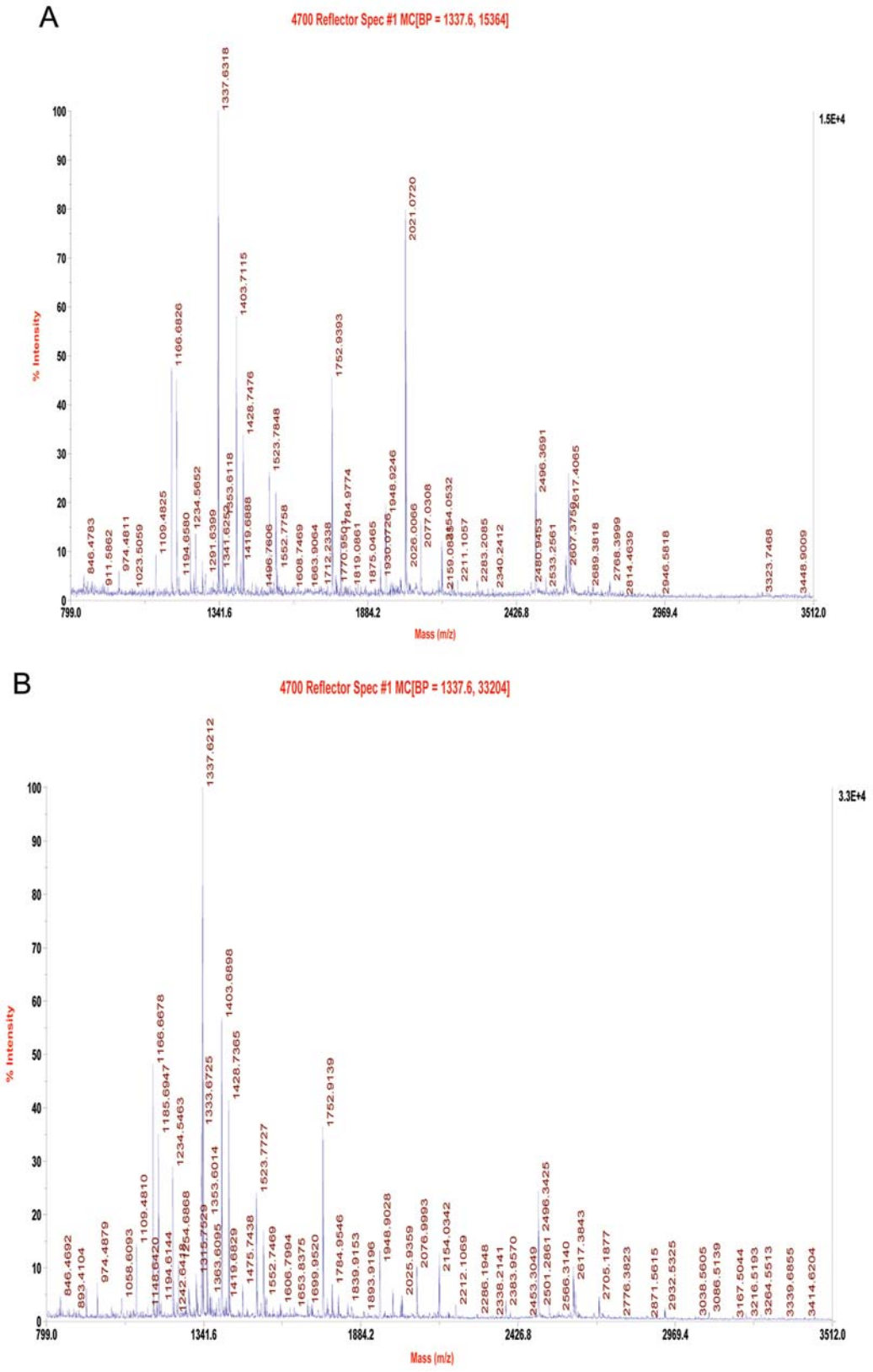

Figure 2. *Molecular characterization of peptide spots of interest (spots A and B, Figure 1) as TCP-1γ using peptide mass fingerprinting. Mass spectra for spots A (A) and B (B) respectively.*

Results

2-DE analysis reveals a peptide spot up-regulated in p53$^{-/-}$ cells. To screen for p53-regulated peptides, we examined and compared the protein compositions of p53$^{+/+}$ and p53$^{-/-}$ cells by 2-DE (Figure 1). We found that a peptide spot (spot A, Figure 1) from p53$^{-/-}$ cells was increased in its optical density by greater than three-fold, compared to that from p53$^{+/+}$ cells. The molecular weight of the peptide spot was estimated to be slightly less than 66 kDa in size through external calibration and its p*I* value was between 6 and 7 (Figure 1).

Molecular characterization identifies the peptides of interest as TCP-1γ. Via PMF (Figure 2A), we identified the peptide spot of interest as TCP-1γ. The MASCOT score was 743 (p<0.5) and the coverage was 50%. The theoretical molecular weight and pI value of TCP-1γ are 60 kDa and 6.1 respectively. The calibrated molecular weight and pI value on our gel (Figure 1) are close to the theoretical calculations, supporting the characterization of spot A as TCP-1γ.

Functional loss of p53 is associated with up-regulation of the more acidic phospho-variant of TCP-1γ. We next performed western blot analysis using a specific antibody to examine whether the protein concentration of TCP-1γ was up-regulated in the p53$^{-/-}$ cells. However, the results showed that that the p53$^{-/-}$ and p53$^{+/+}$ cells had comparable levels of TCP-1γ (Figure 3A). Thus, the increase in the protein concentration of TCP-1γ in gel spot A (Figure 1) might be due to protein modification such as phosphorylation. Indeed, further PMF characterization revealed that a more basic neighboring spot of similar molecular weight (spot B, Figure 1) was also TCP-1γ (Figure 2B). The MASCOT score and coverage were 726 and 36% respectively. The PMF characterization of the spots A and B as TCP-1γ isoelectric variants was confirmed by 2-D gel and western blot analysis using an antibody specific to TCP-1γ (Fig. 3B). The western blot analysis also supported the view from gel image analysis that the p53$^{-/-}$ cells had higher protein concentration of the acidic variant of TCP-1γ compared to p53$^{+/+}$ cells (Figure 1).

Moreover, spots A and B, the variants of TCP-1γ, were also found to be reactive to an antibody specific to phospho-tyrosine on 2D gel-western blot analysis (Figure 3C). They were not detected by an antibody against phospho-threonine (data not shown). The results indicate that the colon cancer cells had at least two phosphorylated variants of TCP-1γ. Functional loss of p53 might promote phosphorylation of TCP-1γ.

TCP-1γ is localized in centrosomes. Phosphorylation of proteins is known to act as a signal for subcellular localization. In light that the p53$^{-/-}$ cells have a higher level of centrosome amplification (14), we performed fluorescent microscopy to examine the subcellular distribution of TCP-1γ in both p53$^{-/-}$ and p53$^{+/+}$ cells, in particular in relevance to its relationship with centrosomes. As shown in Figure 4A, TCP-1γ was distributed comparatively evenly in the cytoplasm in the cancer cells in interphase. However, it was found more in the perinuclear region in other mitotic phases. More interestingly,

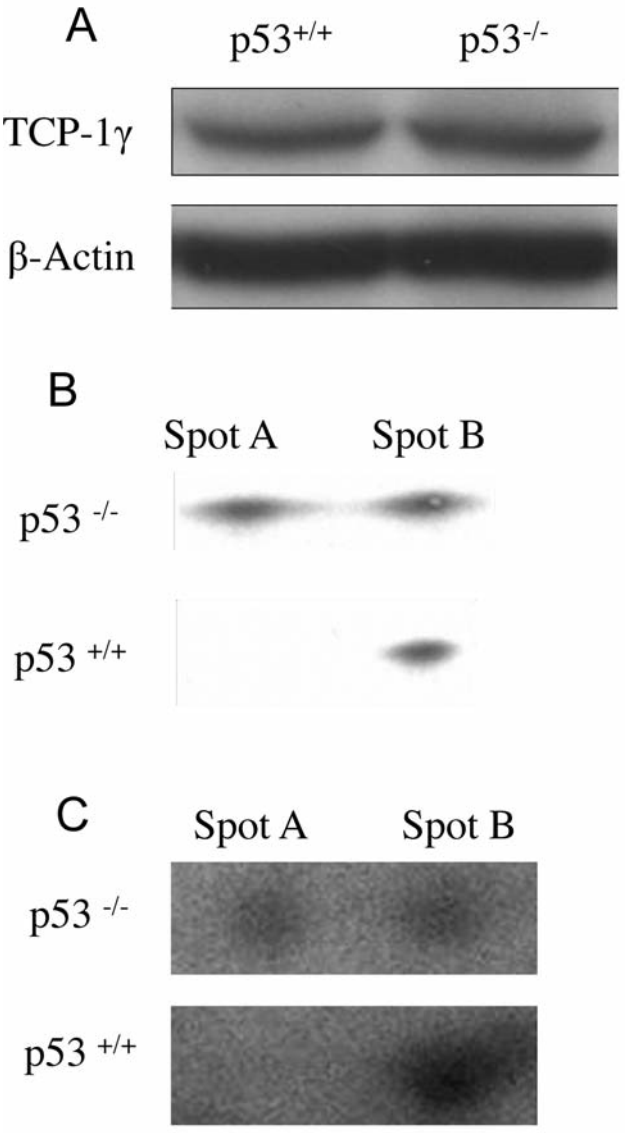

Figure 3. *Peptide spots A and B are two tyrosine-phosphorylated isoelectric variants of chaperonin-containing tail-less complex polypeptide-1γ (TCP-1γ). A: The protein concentration of TCP-1γ is comparable in p53$^{+/+}$ and p53$^{-/-}$ HCT116 cells, as assessed using western blot analysis. B: Detection of the two TCP-1γ variants using 2-DE in combination with western blot analysis. This confirms the peptide mass fingerprinting identification of the two TCP-1γ variants and the up-regulation of the more acidic variant in p53$^{-/-}$ cells. C: The two TCP-1γ variants are phosphorylated on tyrosine as detected using 2-DE and western blot analyses.*

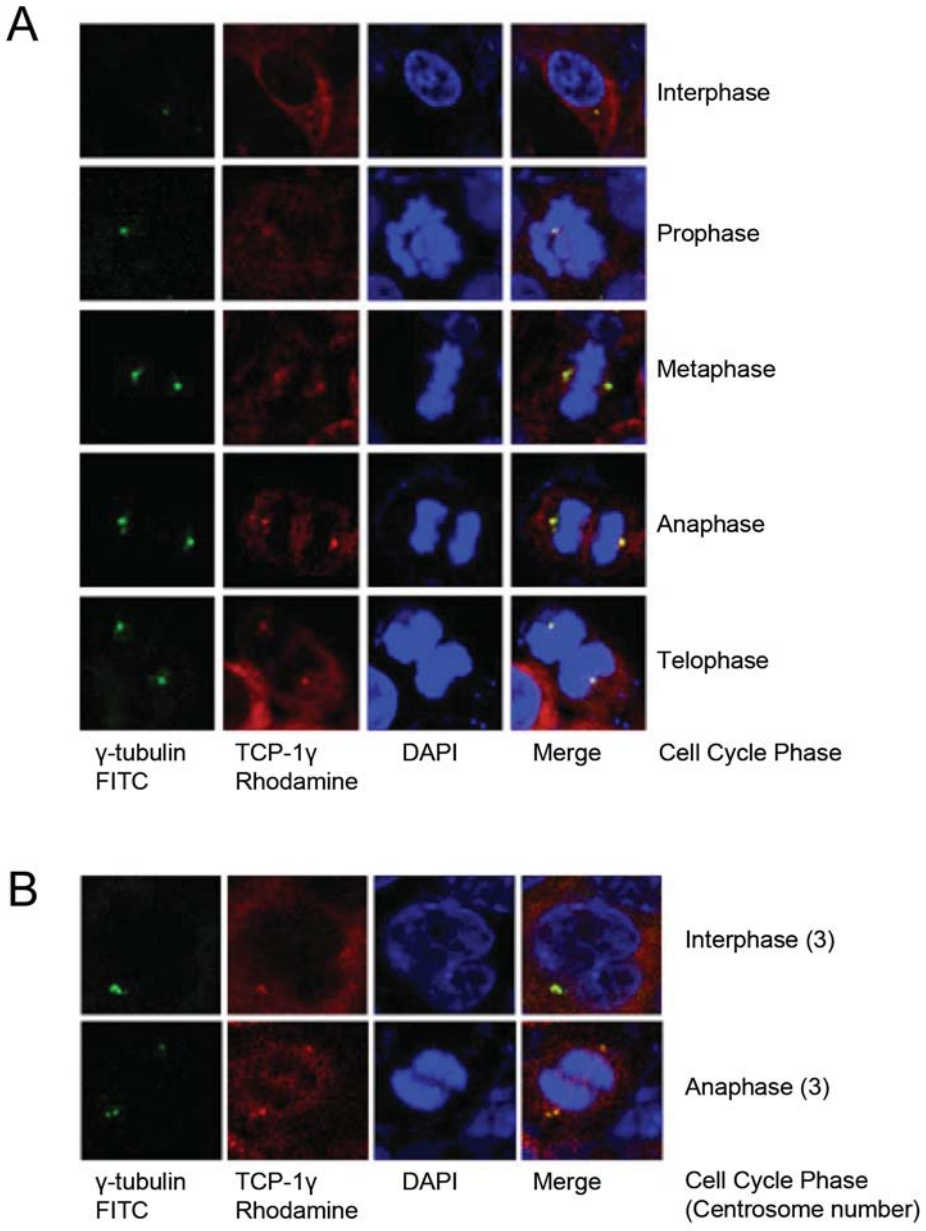

Figure 4. *Chaperonin-containing tail-less complex-1γ is localized to the centrosomes throughout different phases of the cell cycle in HCT116 colon cancer cells. A: Cells in different stages of the cell cycle. B: Cells with centrosome amplification.*

it co-localized with the centrosome, irrespective of cell cycle phase and the presence or absence of centrosome amplification (Figure 4B). The status of p53 did not affect the intracellular distribution of TCP-1γ (data not shown).

Discussion

The reversible phosphorylation of proteins is a critical post-translational modification that plays a major role in many vital cellular processes. The presence or absence of phosphate groups on amino acid side chains, such as serine, threonine and tyrosine, is used to modulate protein activity and propagate signals within cellular pathways and networks (15). In the present study, we have characterized two isoelectric variants of TCP-1γ with tyrosine phosphorylation. The protein level of the more acidic variant is up-regulated in $p53^{-/-}$ cells compared to the one in the $p53^{+/+}$ counterpart. This is compatible with a view that functional loss of p53 is associated with phosphorylation or hyperphosphorylation of TCP-1γ. TCP-1γ is a protein rich in potential phosphorylation

sites and putative motifs for various kinases. Further molecular characterizations such as radioactive sequencing may not only confirm the p53-regulated phosphorylation of TCP-1γ but will also rend possible to reveal the nature of phosphorylation (tyrosine, serine, or combination), as well as phosphorylation site(s).

TCP-1γ is a subunit in a large molecular chaperone known as chaperonin-containing tail-less complex polypeptide-1, which maintains cellular protein folding homeostasis by assisting the biogenesis of many proteins. These barrel-shaped molecules undergo large conformational changes to encapsulate and release bound substrate proteins during their ATP-dependent folding cycle (16). Although initially proposed to fold only actin and tubulin, numerous non-cytoskeletal substrate proteins of TCP-1γ have been identified, including cyclin E, CDC20 and the Von Hippel-Lindau tumor suppressor, as well as some proteins with tryptophan-aspartic acid repeats. However, there is no direct evidence that TCP-1γ alone or as part of TCP-1 complex plays a role in p53-regulated biological functions such as apoptosis, senescence and/or cell cycle regulation.

Because phosphorylation of proteins is known to play a signaling role in subcellular localization, we proceeded in examining the subcellular location of TCP-1γ. Interestingly, we found that TCP-1γ not only lies in the cytosol, but was also co-localized with centrosomes, irrespective of the status of p53 and the presence of centrosome amplification. This is in keeping with a previous report that TCP-1γ is detected in centrosome-enriched preparations (17). Our data, together with observations from others (18), indicate that the TCP-1 complex is in the centrosome or closely associated with it. However, we were unable to differentiate the centrosomal location of TCP-1γ variants.

The centrosome has been a focus of studies in the field of cancer research, primarily because of its speculated role in the origins of genetic instability and carcinogenesis. Centrosome amplification, defined as the presence of three or more centrosomes in each cell, is a phenomenon frequently observed in a large number of human tumors but not in normal cells (19). Studies have shown that centrosome amplification is exaggerated in cells with loss or mutations of *p53*. p53 regulation of p21/WAF1 and subsequent downstream effects on CDK2-cyclin E has been shown to mediate, but only to a certain extent, the process of centrosome duplication (20). Notably, protein phosphorylation is a common event in centrosome amplification. Specifically, loss of p53 has been associated with hyperphosphorylation of centrosome-associated proteins (21). In our studies, we have noted the evidence for concomitant increase in centrosome amplification (14) and TCP-1γ phosphorylation (Figures 1-4) in HCT116 $p53^{-/-}$ cells. More studies are required to examine the relationship amongst p53, TCP-1γ phosphorylation, centrosomal location of TCP-1γ and centrosome amplification.

In conclusion, our proteomic analysis revealed that *p53* loss in HCT116 colon cancer cells is associated with up-regulation of a phosphorylated TCP1-γ variant. The functional implications of this cell signaling loop, in particular in relevance to the centrosome and its associated activities, remain to be investigated.

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