

Supplemental Information

Interaction of p53 with the CCT Complex Promotes Protein Folding and Wild-Type p53 Activity

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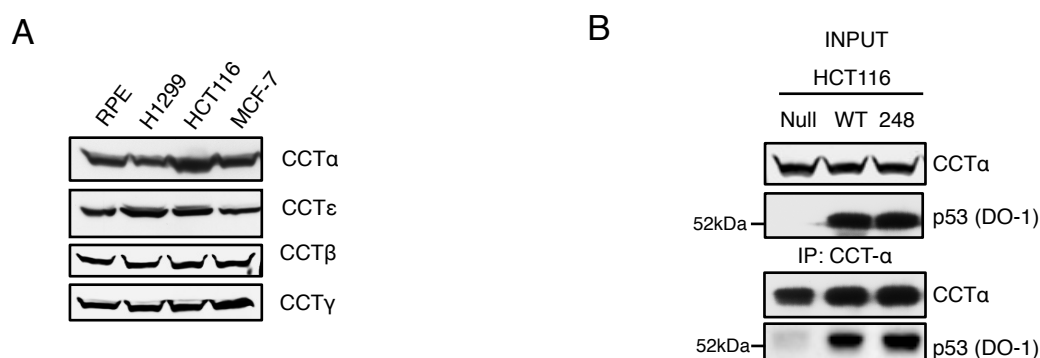


Figure S1 (related to Figure 1). Interaction of CCT α with Wild-Type and Mutant p53

(A) Protein expression levels of different endogenous CCT subunits (α , β , γ and ϵ) were analysed in RPE, H1299, HCT116 and MCF-7 cells by western blot. (B) Immunoprecipitation of p53 with CCT α in HCT116 without p53 (null) or HCT116 expressing endogenous wild-type p53 and mutant p53 (248W). Top panel shows total CCT α and p53 input, bottom panel immunoprecipitation of CCT α and co-precipitation of p53.

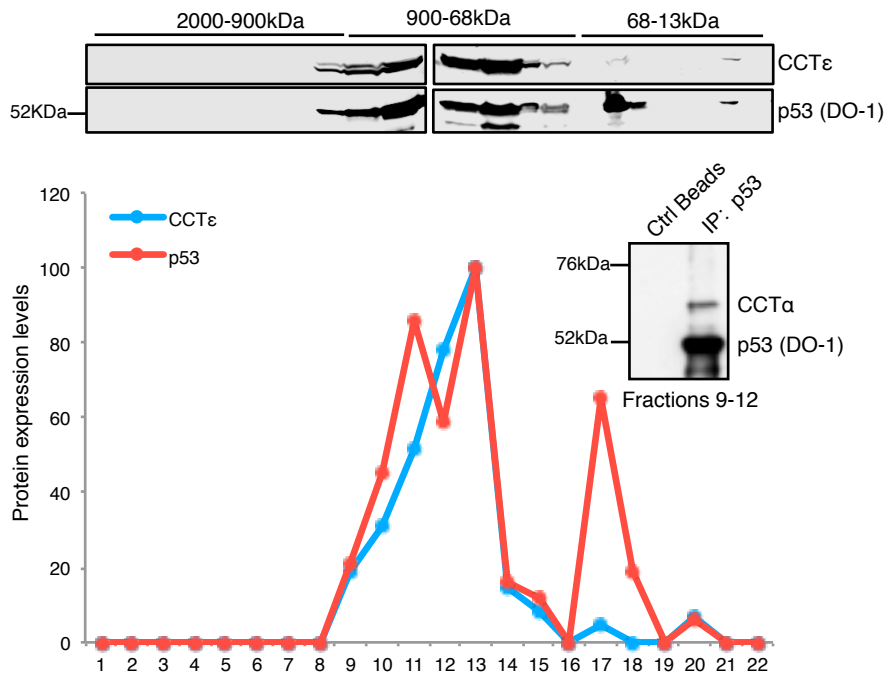


Figure S2 (related to Figure 2). p53 Interacts with the Whole CCT Complex

Lysate from H1299 cells transiently transfected with wild-type p53 was separated by superose 6 gel chromatography, and p53 and CCTε subunit expression assessed in fractions 1-22 by western blotting. p53 was immunoprecipitated from fractions 9-12 with DO-1 antibody, then analyzed for CCTα and p53 expression by western blotting.

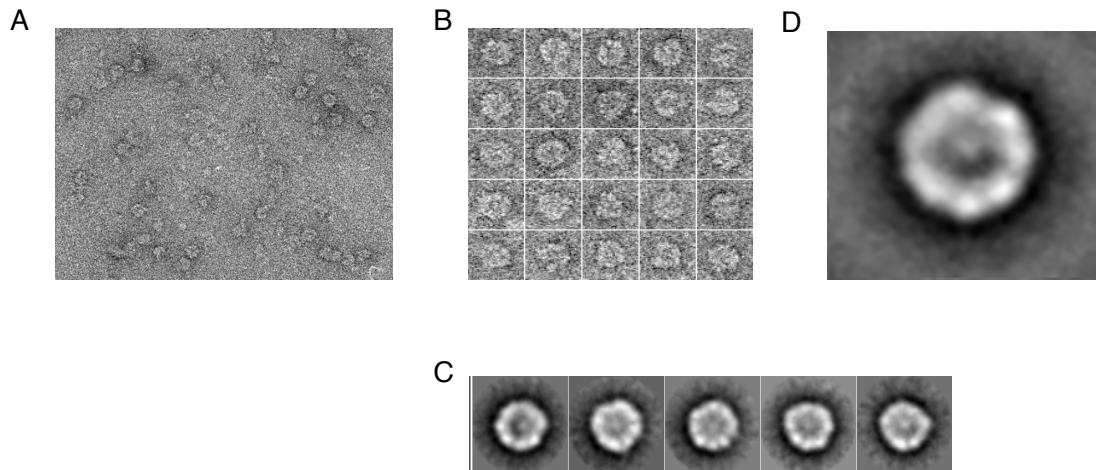


Figure S3 (related to Figure 3). Electron Microscopy and Image Processing of CCT:p53

(A) Representative areas of CCT:p53 and the immunocomplex formed by CCT:p53:anti-CCT ϵ particles. (B) Gallery of selected particles. (C). 2-D maximum likelihood classification of the particles (five classes are represented for CCT:p53 complexes. (D) Final average images of the CCT:p53 complexes.

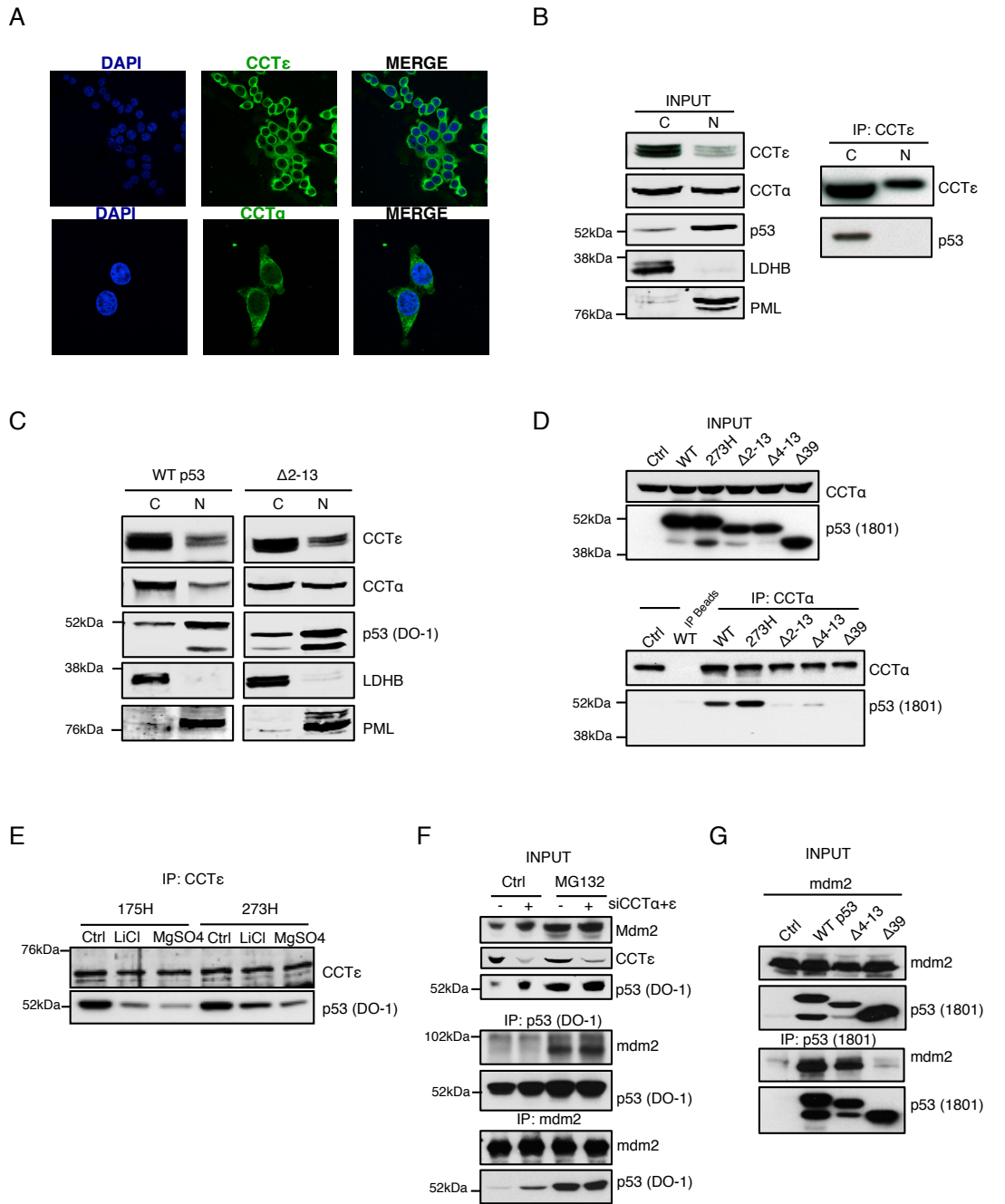


Figure S4 (related to Figure 4). Subcellular Localization and Interaction of p53 and CCT

(A) Localization of CCT α and CCT ϵ was determined by immunofluorescence in wild-type p53 expressing HCT116 cells. (B) Subcellular fractionation was performed in wild-type p53 expressing HCT116 cells. Input and immunoprecipitated cytosolic and nuclear proteins were analysed by western blot with specific antibodies to p53, CCT α , CCT ϵ ,

PML (nuclear control) and LDHB (cytosolic control). (C) p53 null HCT116 cells were transfected with wild-type and $\Delta 2-13$ p53 expression constructs and subcellular localization of proteins determined as in (B). (D) CCT α binding to p53 N-terminal deletion constructs ($\Delta 2-13$, $\Delta 4-13$ and $\Delta 39$) and 273H was assessed in H1299 cells. The second lane shows lysates from wild-type p53 transfected cells, immunoprecipitated only with protein sepharose beads as a control and the rest of the lanes were immunoprecipitated with crosslinked CCT α antibody. CCT α and p53 proteins were visualized by western blot with 1801 and CCT α antibodies. (E) Mutant p53 proteins (175H and 273H) from transiently transfected H1299 cells were co-precipitated with CCT ϵ , then washed with LiCl (0.2M) or Mg₂SO₄ (0.15M) and analysed by western blotting for p53 or CCT ϵ expression. (F) HCT116 cells were transfected with control siRNA and siRNA targeting CCT $\alpha+\epsilon$. After 48 hours cells were treated with 10 μ M of the proteasome inhibitor MG132 for 6 hours. Lysates were subjected to immunoprecipitation of p53 or Mdm2 and total protein levels and immunoprecipitates were detected by western blot with CCT ϵ , Mdm2 and p53 specific antibodies. (G) Mdm2 was co-transfected with wild-type p53 and deletion constructs $\Delta 4-13$ and $\Delta 39$ p53 in H1299 cells. After 24 hours cells were harvested and p53 was immunoprecipitated with 1801 antibody. Protein binding of p53 to Mdm2 and total expression levels were visualised by western blot with antibodies for p53 and Mdm2.

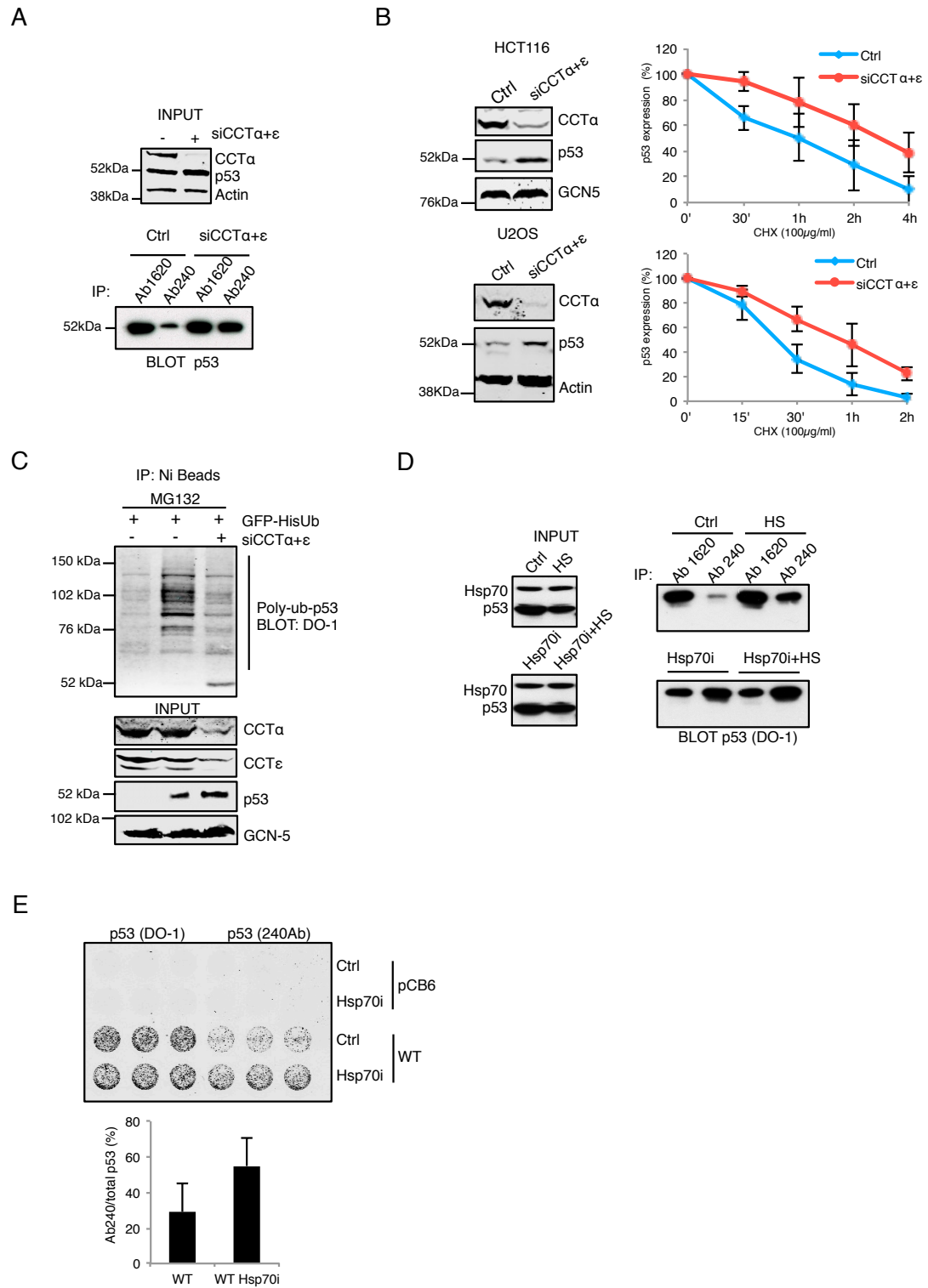


Figure S5 (related to Figure 5). Effect of CCT Depletion on p53 Turnover and Hsp70 Inhibition on p53 Conformation

(A) A2780 cells were transfected with siRNA targeting CCT α and ϵ subunits. Top panel shows input levels of CCT α and p53, bottom panel shows p53 precipitated with the

conformational antibodies Ab1620 or Ab240, detected by western blot using p53 1C12 antibody. (B) The effect of depletion of CCT α and ϵ subunits in HCT116 (top) and U2OS (bottom) cells on p53 stability was determined by western blot with DO-1 antibody (left panels). Cells were treated with cycloheximide (CHX) for 0-4 hours as indicated. Protein levels were analyzed by western blot and p53 expression was quantified and normalized to actin or GCN-5 expression (right panels). Error bars correspond to the mean \pm SEM from three independent experiments. (C) HCT116 null (first lane) or HCT116 cells expressing endogenous wild-type p53 (lanes 2 and 3) were transfected with control siRNA or siRNA targeting CCT α and ϵ subunits in combination with GFP-HisUb. Ubiquitinated proteins were purified with nickel coupled magnetic beads in denaturing conditions. Endogenous ubiquitinated (upper panel) and total (lower panel) p53 protein levels were analyzed by western blot. (D) H1299 transfected with wild-type p53 were treated for 16 hours with an inhibitor of Hsp70/Hsc70 (Hsp70i) followed by heat shock (HS) at 39°C for 4 hours. Lysates were immunoprecipitated with the conformational antibodies and p53 was detected by western blot with DO-1 antibody (E) Cells were seeded into a 96 well plate, transfected with pCB6 (Ctrl) and wild-type p53 (WT), and treated with Hsp70i as in (D). After the treatment, cells were fixed and stained with DO-1 or Ab240, followed by incubation with a green fluorescent secondary antibody. Fluorescent signal corresponding to total p53 and unfolded p53 were quantified by the Li-Cor scanning system. The graph represents the quantification of Ab240 reactive signal as a percentage of total p53 signal normalized for the background signal detected in cells expressing empty plasmid, and shows the mean \pm SEM of three replicates from three independent experiments.

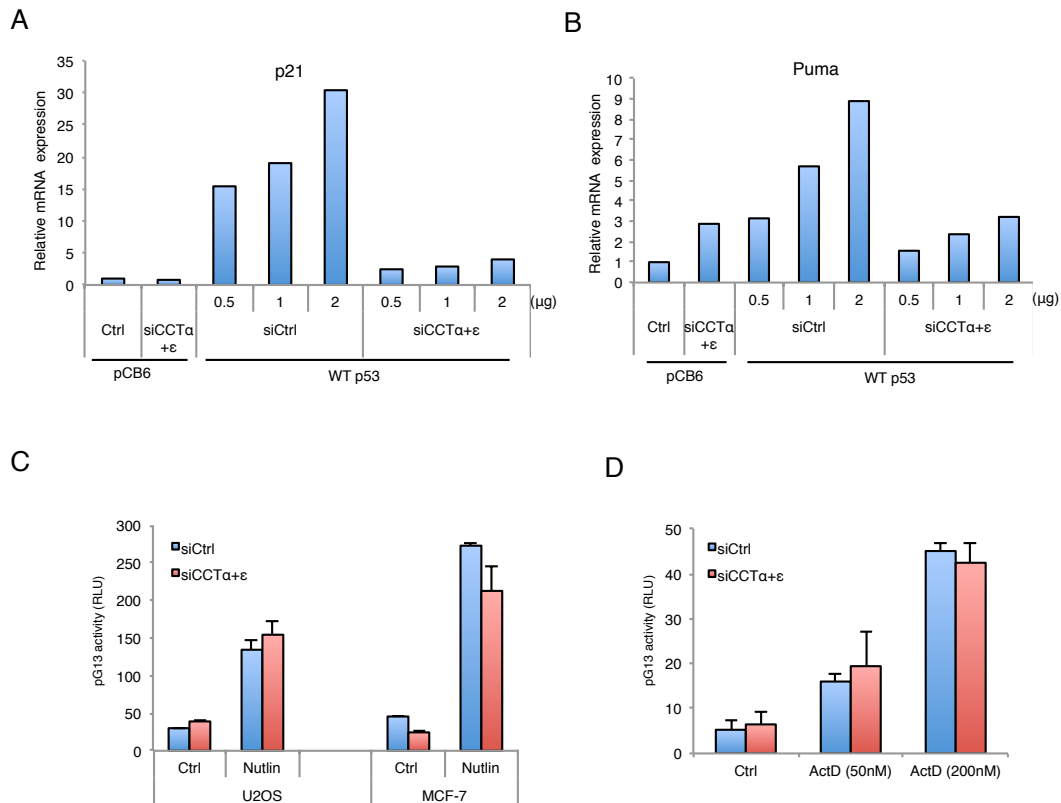


Figure S6 (related to Figure 6). CCT Depletion Decreases p53 Transcriptional Activity

(A and B) H1299 cells were depleted of CCT α and ϵ by siRNA, followed by transfection with empty vector pCB6 (Ctrl) or wild-type p53. After 48 hours, cells were harvested and mRNA expression of p53-target genes p21 (A) and Puma (B) was detected by qRT-PCR. Results were normalized to RPLPO expression. (C) U2OS and MCF-7 cells were depleted of CCT α and ϵ using siRNA, then transfected with pG13 Firefly luciferase and TK Renilla luciferase followed by treatment with Nutlin, as indicated. Firefly luciferase activity was normalised for Renilla luciferase expression and expressed as relative luciferase units (RLU). The graph shows the mean +/-SEM of three replicates from three independent experiments. (D) U2OS cells were transfected as in (C), treated for 24 hours with concentrations of actinomycin D (ActD) that were too low to inhibit transcription in general, but sufficient to activate p53, followed by measurement of luciferase activity. The graph shows the mean +/-SEM of three replicates from three independent experiments.

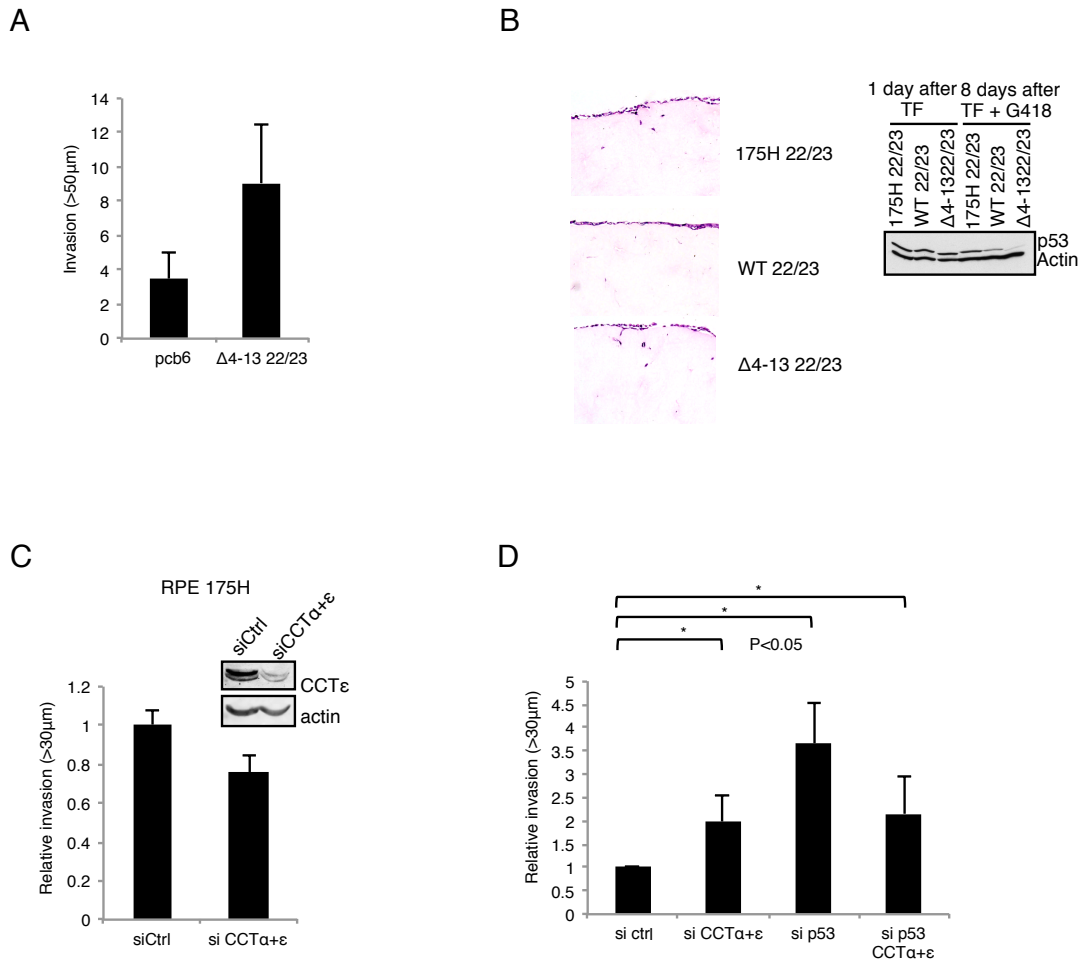


Figure S7 (related to Figure 7). Loss of CCT Binding Enhances p53's Ability to Promote Invasion

(A) Invasion into Matrigel was assessed in H1299 cells transfected with Δ4-13 22/23 compared to cells expressing pCB6 (Ctrl) after 48 hours. Graphs show the mean +/-SEM from three independent experiments. (B) Invasion of H1299 cells transfected with the constructs as indicated in organotypic assays was determined after 9 days in the presence of G418 selection. Expression levels of p53 from parallel cell cultures from the same transfection were tested after 1 day following transfection or after 8 days following transfection and selection with G418. (C) Invasion into Matrigel over 30µm was determined in RPE cells expressing mutant p53 175H after depletion of CCTα and ε.

Protein expression levels were detected by western blot with CCT ϵ and Actin antibody. The graph shows the mean \pm SEM from three independent experiments. (D) Invasion of parental (wild-type p53 expressing) RPE cells in which p53 and/or CCT $\alpha+\epsilon$ were depleted using siRNA targeting p53 and CCT $\alpha+\epsilon$ was tested as in (C). The graph shows the mean \pm SEM from four independent experiments and * indicates $p < 0.05$.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture and reagents

H1299, HCT116, A2780, U2OS and MCF-7 cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% Glutamine at 37°C and 5% CO₂. Infinity™ Telomerase-immortalised RPE cells were obtained from Clontech and cultured in DMEM/F12 HAM supplemented with 10% FBS, 1.6% Sodium bicarbonate and 1% Glutamine at 37°C and 5% CO₂. Stable H1299 cells expressing pCB6 and 273H were described previously (Muller et al., 2009). H1299 cells expressing Δ39 were generated by selecting transfected cells 24 hours after transfection in 500ng/ml G418 for 10 days. Stable doxycycline-inducible monoclonal Tet-on H1299 cells expressing wild-type p53 or 273H p53 were generated as described previously (Nakano et al., 2000).

Cells were treated with 5μM of the Mdm2 inhibitor Nutlin-3a (Cayman Chemical), with 10μM of the proteasome inhibitor MG132 (Sigma) and 200g/μl of cycloheximide (Sigma). 1mM and 0.2mM of nucleotides ATP (Sigma), ADP (Sigma) respectively and Proteinase K as indicated (Sigma) were used for the *in vitro* reactions. To inhibit the chaperone activity of Hsp70/Hsc70, 40μM of the inhibitor VER-155008 (Tocris Bioscience) which is able to bind to the ATPase site of both Hsc/p70 described previously (Massey et al., 2010).

Plasmids, sequences and transfections

Expression plasmids for p53 wild-type, p53-273H, p53-175H, p53-175 22/23, p53-22/23, p53Δ43, p53Δ327, p53Δ347, p53Δ370, p53ΔI, p53ΔII, p53ΔIII, p53ΔIV, p53ΔV, p53Δ28-40, p53Δ41-49, p53Δ39 have been described previously (Lin et al., 1995; Marston et al., 1994; Muller et al., 2009). Plasmid pCHDM1A-wild-type Mdm2 has been described previously (Chen et al., 1995). pTRE-tight expression vectors were obtained

from Clontech and p53 was cloned into this vector using the BamH1 and EcoR1 restriction sites. pG13-luciferase construct, containing generic p53 response elements (El-Deiry et al., 1993) was provided by Dr Bert Vogelstein and the pGFP-HisUb construct was provided by Dr Ron Hay. $\Delta 2-13$, $\Delta 19-28$, $\Delta 2-13,22/23$, $\Delta 4-13$ and $\Delta 4-13 22/23$ mutations in p53 were generated by site-directed mutagenesis using pCB6 vector and the following oligos: **$\Delta 2-13$** , *fw* GCC TTC CGG GTC ACT GCC ATG CTG AGT CAG GAA ACA TTT TCA G *rev* C TGA AAA TGT TTC CTG ACT CAG CAT GGC AGT GAC CCG GAA GGC; **$\Delta 4-13$** *fw* GCC AGA CTG CCT TCC GGG TCA CTG CCA TGG AGG AGC TGA GTC AGG AAA CAT TTT CAG ACC TAT GG *rev* CCA TAG GTC TGA AAA TGT TTC CTG ACT CAG CTC CTC CAT GGC AGT GAC CCG GAA GGC AGT CTG GC; **$\Delta 19-28$** , *fw* CCC CCT CTG AGT CAG GAA ACA AAC AAC GTT CTG TCC CCC TTG C *rev* G CAA GGG GGA CAG AAC GTT GTT TGT TTC CTG ACT CAG AGG GGG; **$\Delta 2-13,22/23$** , *fw* GTC AGG AAA CAT TTT CAG ACC AAT CGA AAC TAC TTC CTG AAA AC *rev* GT TTT CAG GAA GTA GTT TCG ATT GGT CTG AAA ATG TTT CCT GAC **$\Delta 4-13$** *fw* AGA TCA CTA GAA GCT ATG GAG GAG CT *rev* CCG GAT TGC CAA GCT GTC TGA GTC AG

Depletion of p53 and CCT subunits α and ϵ expression was achieved by transient transfection with Hiperfect for 48 hours (Invitrogen). 20nM of specific siRNA targeting p53 was used *fw* GAC UCC AGU GGU AAU CUA C(TT) and 10nM of a pool of different small interfering RNA duplexes (SMARTpool SantaCruz) were used for CCT subunits: **CCT α** , *fw* GGA UGA UAU UGG UGA UGU A(TT), *fw* GAA GCA GUG CGU UAU AUC A(TT), *fw* GAA GUC AAA UGG AGA GUA U(TT) **CCT ϵ** , *fw* CUG CUC GUG UUG CUA UUG A(TT) , *fw* GAA GCA ACA GCA UGU CAU A(TT), *fw* CCA CUU CUG UGA UUA AGU A(TT).

Transfection of p53 constructs was performed using GeneJuice (Novagen) according to the manufacturers' instructions and the expression levels were assessed 24 hours later. Alignment of p53 sequences of different species was performed using BOXSHADE 3.21.

Mass spectrometry

For the mass spectrometry analysis, three 15cm plates of H1299 cells were used per condition. Following transfection with empty vector, Flag-wild type p53 and Flag-175H p53 plasmids for 24 hours, cells were treated with 10 μ M of proteasome inhibitor MG132 for 4 hours and harvested in lysis buffer (20mM HEPES pH7.4, 150mM NaCl, 2mM EDTA and 0.5% NP-40). Lysates were precipitated with Protein G beads coupled to Flag M2 Ab (Sigma) for 2 hours 4°C. Beads were washed in lysis buffer twice and eluted in the same buffer with the addition of 200 μ g/ml of Flag-peptide for 45 minutes 4°C. Samples were run on a 4-12% gradient gel, fixed and stained with Brilliant Blue G-Colloidal solution according to manufacturer's protocol (Sigma). The gel lanes were fractionated into 10 slices. Proteins in each slice were reduced (10mM DTT, 50mM ammonium bicarbonate (AB) for 20 minutes at 56°C), alkylated (55mM iodoacetamide, 50mM AB for 1 hour at room temperature) and digested (modified porcine trypsin (Promega) for 90 minutes at 37°C). Digested material was extracted from gel pieces with 0.1% trifluoroacetic acid, 5% MeCN (Sigma), and concentrated to 20 μ l volume. The digest was subject to separation on an Ultimate 3000 nanoLC (Dionex, Sunnyvale, CA). Acquired tandem mass spectra were exported from Analyst using the script Mascot.dll 1.6b23 (matrix Science, London, UK). Protein identification was obtained using Mascot 2.3.02 software (Matrix Science). Scaffold (version Scaffold_3.1.2, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm, resulting in a peptide false discovery rate (FDR) of 1.0% (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 4 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).

Immunoprecipitation, western blot and protein stability analysis

For immunoblotting, cells were collected by centrifugation, washed with cold PBS and lysed in NP-40 lysis buffer (50mM Tris-HCl pH=7, 150mM NaCl, 1mM EDTA and 1% NP-40 supplemented with a complete protease inhibitor cocktail). 50µg total proteins were separated in SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk and 0.2% tween20 in TBS and western blot analysis was carried out according to the method described previously (Kubbutat et al., 1999). The following monoclonal and polyclonal antibodies were used to detect all human proteins: anti-p53 DO-1 (Vojtesek et al., 1992), anti-p53 1801 (Banks et al., 1986), anti-p53 CM-1 (Novocastra); anti-p53 clone 1C12 (Cell Signaling), polyclonal anti-p53 FL-393 (Santa Cruz), anti-p21 (SantaCruz), polyclonal anti-Puma (Sigma), monoclonal anti-Mdm2 SMP-14 (SantaCruz), monoclonal anti-Mdm2 Ab1 and Ab2 (Calbiochem), anti-Actin (Miliopore), anti-GCN-5 (SantaCruz), anti-CCT α (Serotec), anti-CCT β (Cell Signaling), anti-CCT γ (SantaCruz), mouse anti-CCT ϵ (Serotec), goat anti-CCT ϵ clone C-15 (SantaCruz) and anti-Hsp70 (BD bioscience), polyclonal anti-Hsp70 (ENZO), polyclonal anti-PML (Santa Cruz) and anti-LDHB clone 2H6 (Sigma). Secondary antibodies were incubated for 1 hour at RT in 5% milk followed 5 washes of 5 minutes with tween-TBS buffer. Secondary HRP-coupled antibodies anti-rabbit, mouse and goat (Amersham) and secondary HRP-coupled anti mouse IgG light chain (BD Pharmigen) antibody were used when proteins were visualized with ECL reagents (MiliPORE). IRDye secondary antibodies 640CW and 800CW (Li-Cor) were used when protein were visualized by the ODYSSEY® CLx infrared system (Li-Cor). Intensity values were quantified with Image J64 software for the bands visualized with ECL and with Image Studio software 2.0.

Immunoprecipitation to determine the conformation of p53 protein was performed by using monoclonal antibodies DO-1, 1801, Ab240 (Calbiochem) and Ab1620 (Calbiochem). CCT-p53 complexes were precipitated using goat anti-CCT ϵ (SantaCruz)

and crosslinked mouse anti-CCT α (Enzo). Cell lysates containing 1.5-2 mg of total protein were incubated overnight with the appropriate antibody following addition of 20 μ l of Protein G conjugated with magnetic beads (Dynabeads) or 40 μ l of agarose Protein G equilibrated in lysis buffer (1:1). Beads were washed by vortexing in lysis buffer three times, resuspended in 4X or 1X concentrated Laemmli buffer and boiled at 95°C 5 minutes. To analyze the contribution of hydrophobic bonds in CCT-p53 binding, precipitates were washed 3 times with the following chaotropic salts at different concentrations: 0.25M LiCl and 0.15M Mg₂SO₄ (Sigma).

To measure protein stability, cells were treated for the indicated times with 50 μ g/ml of cycloheximide (Sigma). Cells were harvested and processed for western blot as described above. Actin was used as a loading control, and bands were quantified using Li-Cor software by normalizing to the band intensity of the sample without cycloheximide treatment. Results are expressed as a percentage of intensity relative to the untreated sample

***In vitro* translation and biochemical methods**

In vitro translation of p53 was performed in TNT[®] SP6 couple reticulocyte lysate system (Promega) using the constructs PGEM-wild-type p53, Δ 2-13 and Δ 4-13 as a template. 5 μ l of each reaction was used to validate the expression of p53 and CCT subunits. To test the binding of endogenous CCT ϵ and *in vitro* translated p53, 20 μ l of reticulocyte lysate were used.

CCT conformational changes and p53 folding were carried out as described (Frydman and Hartl, 1996; Meyer et al., 2003). *In vitro* translated p53 was incubated for 90 minutes at 30°C in a reaction buffer (50mM Tris HCl pH 7.5, 150 mM NaCl, 5mM MgCl₂, 0.1 mM PMSF, 0.5% NP-40 and 10% Glycerol) with 200ng of CCT purified protein. The

CCT-p53 complexes were incubated for 30 minutes at 30°C with 1mM of ATP alone or in presence of a γ -phosphate analogue AIFx (5mM $\text{Al}(\text{NO}_3)_3$ + 30mM NaF) (Sigma) which replaces Pi released in the ATPase reaction and traps CCT complex in a close conformation. Proteolytic digestion of the complex was performed with addition of 0.5 $\mu\text{g}/\text{ml}$ of proteinase K (Sigma) for 10 minutes at 25°C. Reaction was stopped by addition of 10mM of PMSF.

***In vitro* CCT/p53 binding assays**

CCT was purified from bovine testis as described (Cuellar et al., 2008). The CCT-p53 complexes were formed by denaturing human p53 protein in 6M guanidine hydrochloride and subsequent 100-fold dilution in buffer (50 mM Tris pH 7.4, 500 mM NaCl, 5 mM MgCl_2 , 2 mM DTT) containing 2 mM purified CCT (1:10 chaperonin:p53 molar ratio) for 30 minutes at 30°C. Aliquots were then subjected to native electrophoresis using 4% (w/v) acrylamide gels run at 90-100 V for 4-5 hours. The chaperonin band, clearly separated in the gel from the unbound p53 protein, was stained with Bio-Safe Coomassie (BIO-RAD) and subsequently excised, dehydrated at 95°C for 5 minutes and rehydrated with denaturing loading electrophoresis buffer. Samples were boiled for 5 minutes and loaded onto an 8% (w/v) SDS-PAGE. After the electrophoresis was performed, the gels were blotted electrophoretically for 50 minutes to a 0.2mm nitrocellulose membrane (BIO-RAD) using a Trans-Blot SD Transfer Cell (BIO-RAD). The nitrocellulose membrane was subsequently blocked by washing the membrane with WB buffer (3% skimmed milk, 0.05% Tween-20 in PBS buffer) for one hour at room temperature. The membrane was then incubated with a 1:1000 dilution of mouse monoclonal anti-p53 antibody followed by a 1:5000 dilution of a sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare). Immunoblots were developed with the ECL Plus chemiluminescence reagent (GE Healthcare) and visualized with a Storm 860 PhosphorImager (Amersham Biosciences).

Electron microscopy and image processing

Samples (either CCT or CCT:p53) were applied onto carbon-coated copper grids previously glow-discharged and stained with 2% uranyl acetate. Micrographs were taken under minimal dose conditions on Kodak S0-163 film, in a JEOL JEM 1200EXII microscope with a tungsten filament operated at 100 kV and at 60000 magnification. Micrographs were digitalized in a Zeiss SCAI scanner with a sampling window corresponding to 7Å/pixel for all specimens. Individual particles were manually selected using XMIPP software package (Sorzano et al., 2004). Image classification was performed using a free-pattern maximum-likelihood multi-reference protocol (Scheres et al., 2005), which gave rise to homogeneous populations, which were then averaged to generate a final two-dimensional average image.

Gel chromatography

Analytical gel filtrations of lysates were performed using Superose 6 10/300 GL (GE Healthcare) with a following running buffer: 50mM Tris-HCl pH 7.0, 250mM NaCl and 1mM DTT. Experiments were conducted with a flow rate of 0.5ml/minute and an injection volume of 500µl. The molecular masses of separated fractions were deduced from calibration curve of Superose 6 10/300 GL column. Fractions of 1ml were collected and precipitated by incubation at 4°C for 1 hour with 10% trichloroacetic acid (TCA). Precipitates were rinsed twice with pure acetone, dried for 2 minutes at 90°C, resuspended in Laemmli buffer and boiled for 5 minutes.

Ubiquitination in cells

HCT116 cells expressing endogenous wild-type p53 were transfected with siRNA against CCT α and ϵ subunits for 48 hours. pGFP-HisUb plasmid was transfected for 24 hours before harvest and expression of the GFP construct was verified by fluorescence

microscopy. Cells were treated with 10 μ M of MG132 for 4 hours, harvested in UBA buffer (6M Guanidinium HCl, 300mM NaCl, 50mM phosphate pH 8.0, 100 μ g/ml N-ethylmaleimide (NEM)) and sonicated twice for 10 seconds at 40% amplitude. Cell lysates were incubated overnight with His-tag matrix Dynabeads (Invitrogen), then washed once with UBA buffer, UBB buffer (1:1 UBA:UBC) and once with UBC (300mM NaCl, 50mM phosphate pH 8.0, 100 μ g/ml NEM). Beads were resuspended in Laemmli buffer and ubiquitin protein precipitates separated by SDS-PAGE and visualized by western blot with the p53 antibody DO-1.

mRNA gene expression analysis

RNA was prepared using an extraction column according to the manufacturer's instructions (Qiagen). Complementary DNA was generated using first-strand cDNA kit (Invitrogen) according manufacturing instructions on 2 μ g DNase-treated total RNA. Quantitative RT PCR (qRT-PCR) analysis was performed with 2 μ l of a 100 \times dilution of the cDNA, 2 μ M of each primer and carried out with the DyNAmo SYBR Green two-step qRT-PCR kit (Thermo Scientific). Accumulation of fluorescent products was monitored by real-time PCR using a Chromo4 reader (Bio-Rad), melting curves determined and was analysed with the Opticon Monitor3 software.

qRT-PCR cycling parameters were 15 minutes at 95 $^{\circ}$ C; 40 cycles of 20 seconds at 94 $^{\circ}$ C, 30 seconds at 60 $^{\circ}$ C, and 30 seconds at 72 $^{\circ}$ C; and 10 minutes at 72 $^{\circ}$ C. The relative quantification of gene expression was performed using the $\Delta\Delta C_T$ method, with normalization of the target gene to the ribosomal control gene RPLP0. Results are presented as a fold induction between indicated conditions. The following primer sequences to detect p21 and Puma genes were used **p21**: *fw* CTG GAG ACT CTC AGG GTC GAA A *rev* GAT TAG GGC TTC CTC TTG GAG AA, **Puma**: *fw* GAC GAC CTC AAC GCA CAG TA *rev* CAC CTA ATT GGG CTC CAT CT.

Luciferase assays

H1299 cells were grown to 70% confluence in 24 well plates and transfected with 400ng pG13 luciferase construct and 50ng Renilla luciferase construct in combination with 0.2-1 μ g of the different p53 constructs. After 24 hours cells were washed with PBS and harvested in Promega lysis buffer according to manufacturer's protocol using Promega substrates. Firefly luciferase measurements were corrected for Renilla luciferase values and expressed as relative light units (RLU). Expression of p53 deletions were analysed by western blot using 10% of the total lysates.

Immunofluorescence

For immunofluorescence, H1299 and HCT116 cells were seeded on 25 mm² coverslips at 70% confluence. Cells were washed 4 times with PBS, fixed in 4% PFA for 10 minutes, and permeabilised with 0.01% Triton X-100 in PBS for 10 minutes. Cells were washed in PBS 4 times before blocking for 30 minutes in 1% FCS in PBS, followed by incubation overnight at 4°C in 1% BSA-PBS with CCT α (1:100 Serotec) and CCT ϵ (1:50 SantaCruz) antibodies. After 3 washes with PBS, cells were incubated for 1 hour with the secondary antibody alexa 488-conjugate to a dilution of 1:500. Cells were rinsed 5 times and incubated in a DAPI solution (1:2000) for 10 minutes in the last wash. Cells were mounted on microscope slides with mounting fluid (Vectashield) and visualized using an inverted confocal fluorescence microscope (Fluoview FV1000, Olympus).

In-Cell western™ assay

The conformation of p53 in cells was determined in an In-cell western assay according to the manufacturers' instructions (Li-Cor). 5000 cells were seeded in 96 CellBIND surface plates (Corning) and transfected for 24 hours with the p53 constructs as indicated. Cells were fixed in 4% para-formaldehyde (Sigma) in PBS for 10 minutes at

4°C and blocked for 1 hour at RT with blocking buffer (PBS pH=7, 0.4% TRITON X-100(Sigma) and 1% BSA (Sigma). Primary antibodies (p53 polyclonal (Santa Cruz) and 1620 (Calbiochem)) were incubated for 2 hours at RT followed by 3 washes with blocking buffer. IREDye secondary antibodies 800CW or 640CW (Li-Cor) were incubated for 45 minutes at RT and cells were scanned in ODDYSEY CLx infrared system and the intensity of the fluorescence in cells was quantified with Image Studio Software 2.0 (Li-Cor).

Invasion and migration assays

Matrigel assays were performed as previously described (Caswell et al., 2008). Matrigel (BD Biosciences) was supplemented with 25µg/ml of fibronectin (FN, Sigma) and polymerized at 37°C for 45 minutes in transwell inserts (Corning). The plates were inverted and 2.5×10^4 H1299 cells or 3.5×10^4 RPE cells in 100µl were seeded on the base. The inserts were placed in serum free medium and topped with medium containing 10% FCS and a mixture of chemo attractants: 25ng/ml EGF and 10ng/ml HGF (Sigma). The remaining cells were re-plated and used to check protein expression by western blot analysis. Migrating cells were stained after 3 days with Calcein (Invitrogen) and visualized using an inverted confocal microscope (Leica2) in serial sections at 10µm intervals. Quantification of invading cells was determined using ImageJ software, in which intensity was measured using the area calculator plugin software. The percentage invasion was determined by measuring the total intensity of all slides beyond 30-50µm compared to the total intensity of all slides.

Invasion in organotypic assays was performed as previously described (Edward et al., 2005). In brief, rat-tail collagen was isolated and allowed to contract in the presence of telomerase-immortalized fibroblasts to generate organotypic plugs. Next, 5×10^4 H1299 cells stably expressing p53 constructs or H1299 cells transfected with the indicated p53 constructs were seeded on top of these plugs for 16 hours. Plugs and cells were then

placed on a grid with 10% FCS containing medium at the bottom to allow cells to invade into the plugs in an air/liquid interface. Nine days after transfection cells were harvested in 4% PFA, embedded in paraffin and stained with haematoxylin and eosin. Representative images of invading cells were taken with the Olympus BX51 FL microscope.

Cell migration was tracked by wound scratch assays in which H1299 cells were monitored by time-lapse imaging. 250,000 cells were plated in a 6 well dish 48 hours before a wound scratch was made using a pipette tip. Cells were thoroughly washed after scratching and maintained at 5% CO₂ at 37°C in medium with 1% FCS and then monitored using an inverted microscope (Nikon TE2000). Images were taken with the 20X objective were obtained every 10 minutes for the duration of 16 hours. Movies were generated with the software ImageJ and in total 108 cells from three independent experiments were tracked. Migration analysis of individual cells was performed by quantification of the directionality using the plugins manual tracking and chemotaxis tool in the software program ImageJ.

Statistical analysis

Statistically significant differences were determined using a Student t-test

SUPPLEMENTAL REFERENCES

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