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The adapter protein CD2AP binds to p53 protein in the cytoplasm and can discriminate its polymorphic variants P72R

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Proline-rich motifs are widely distributed in eukaryotic proteomes and are usually involved in the assembly of functional complexes through interaction with specific binding modules. The tumour - suppressor p53 protein presents a proline-rich region that is crucial for regulating apoptosis by connecting the p53 with a complex protein network. In humans, a common polymorphism determines the identity of residue 72, either proline or arginine, and affects the features of the motifs present in the polyproline domain. The two isoforms have different biochemical properties and markedly influence cancer onset and progression. In this article, we analyse the binding of the p53 proline-rich region with a pool of selected polyproline binding domains (i.e. SH3 and WW), and we present the first demonstration that the purified SH3 domains of the CD2AP/Cin85 protein family are able to directly bind the p53 protein, and to discriminate between the two polymorphic variants P72R.

Keywords: CD2AP/p53 polymorphism/P72R/polyproline/SH3 domains.

Abbreviations: DBD, DNA-binding domain; GST, glutathione-*S*-transferase; POD, peroxidase; PBS, phosphate-buffered saline.

The p53 protein forms a homotetramer in most vertebrate species and also in some unicellular organisms, although not in yeast (*1*). Since its discovery in 1979, countless papers have been published on the role of this crucial protein in both physiological and pathological conditions. Despite this vast amount of knowledge, many questions remain unanswered.

It was recently reported that the ancestral function of this conserved gene was not related to tumour development (*1*). However, it later evolved as a genome guardian, showing evident tumour-suppressing activity. More than 35,000 mutations have been described in different cancers, many of which are somatic missense mutations with oncogenic functions (*2*, *3*). A common genetic polymorphism occurs at codon 72 of human p53 with two alleles encoding either a proline (P) or an arginine (R). Over the past decade, the two resulting variants have been extensively reported to influence cancer susceptibility, as well as other pathologies. Although the P allele is considered the ancient allele (the R variant has not been described in other primates (*4*)), the genotype frequencies in Europe are 60% R/R, 30% R/P and 10% P/P, suggesting that the R variant confers some advantages in this geographical background. By contrast, in African areas, the frequency of the P allele is higher (*5*, *6*). According to these observations, it has been hypothesized that UV radiation exposure or low average winter temperatures could influence the frequencies of the two variants (*7*, *8*).

A large number of genetic studies and meta-analyses have suggested an association of the polymorphism at codon 72 with the onset and/or the progression of tumours. In pioneering works, homozygous arginine was indicated as a risk factor in the HPV-associated tumourigenesis, because of the enhanced susceptibility of the protein to E6-mediated degradation (*9*, *10*). Other epidemiological studies have reported significantly lower or higher frequencies of R/R in non-HPV patients, depending on the subtype of cancer and on the ethnicity of the population analysed (*11–16*). Moreover, the polymorphism has been reported to profoundly influence survival some years after cancer diagnosis, longevity and recurrent failure of *in vitro* fertilization (*5*, *17*). These epidemiological studies were supported by the findings that the polymorphism at codon 72 makes the two resulting p53 isoforms biologically different in terms of apoptotic potential (*18*, *19*) under both normoxia and hypoxia (*20*, *21*), and differently able to induce cell senescence (*22*). More recently, it has been reported that p53 can also interact with proteins involved in protection and replication of mitochondrial DNA (*23*, *24*). All these observations suggest that the substitution of a single amino acid in position 72 induces substantial changes in the biochemical properties of the protein. The region surrounding codon 72 is rich in proline residues, and it has been suggested to be an important signalling motif

involved in the control of proliferation in a transcription-independent manner (*25–27*). The region was shown to be required for suppressing tumour cell growth, and to promoting apoptosis in response to treatment with different chemotherapeutic agents (*25*, *27*). Notably, many species ranging from birds to mammals show a proline-rich stretch in the p53 protein; however, the number of PXXP motifs seems to increase during evolution and only in primates does the region contain five motifs, as in humans. These types of motifs have been shown in literature to adopt a distinct secondary structure called a poly-L-proline helix of type II, forming a unique recognition motif for protein interactions (*28*, *29*). They are frequently involved in signalling events by mediating the interaction with binding domains such as SH3 and WW domains (*29–32*). The P72R polymorphism maps in the second PXXP repetition, and it has been described only in the human p53 sequence. Previous studies have demonstrated that the biological differences between the two p53 isoforms cannot be ascribed to changes in the secondary conformation introduced by the amino-acidic substitution at codon 72 (*33*). We considered the possibility that the observed biological differences are due to differential capabilities of these amino acid motifs to bind p53 interactors. So far, few p53 interacting proteins have been shown to discriminate between the two polymorphic variants, and their binding specificity may indeed explain important aspects of the differential behaviour of the two isoforms (*19*, *34–36*). Actually, the interaction was mapped in the proline region only for the SH3 domains of ASPP2 and iASPP, confirming that SH3 modules are involved in the regulation of the p53 network (*19*). According to (*19*), both proteins recognize two regions of the p53: the DNA-binding domain (DBD) domain (*37*) and the proline-rich region, but with different affinity, which gives rise to an interesting regulatory mechanism. In fact, iASPP, which inhibits the apoptotic function of p53 (*38*), binds preferentially to the proline-rich region, with higher affinity for the P72 isoform, whereas ASPP2 that stimulates apoptosis (*39*) preferentially binds to the DBD. As a result, p53R escapes iASPP inhibition and is a better apoptosis inducer.

In this study, we have tested a collection of polyproline binding domains against the two isoforms of the p53 protein, to identify domains that could recognize the two polymorphic variants with different intensity. Exploiting protein domain arrays and a SPOT synthesis approach, we found evidence that the SH3 domains of the adaptor proteins CD2AP/Cin 85 are able to bind to this region. We showed that both the adaptors Cin85 and CD2AP bind to p53 *in vitro* and markedly prefer the R72 variant. Moreover, we mapped this interaction in the proline region of the p53. Finally, we showed that endogenous CD2AP form complexes with transfected p53 in H1299 cell lines.

Materials and Methods

Antibodies

Anti-GST was from GE-Healthcare, polyclonal and monoclonal anti-p53 were from Santa Cruz Biotechnology; Peroxidase-conjugated (POD) anti-rabbit and anti-mouse were from Jackson Immunoresearch, anti-goat was from SIGMA; anti-rabbit-Cy5 conjugated was from Jackson Immunoresearch and anti-mouse AlexaFluor555 was from Molecular Probes. Anti-Cin85 and anti-CD2AP were from Santa Cruz Biotechnology. Anti-Flag was from SIGMA.

Plasmids

The collection of domains used in the protein array experiment was previously collected in our lab (*40*). The two p53 isoforms were amplified by PCR using a cDNA obtained from RT-PCR of total RNA from a heterozygous subject. The primers used were: 5_-CAC CATggAggAgCCgCAgTCAgAT-3_(For) and 5_-TCAgTCTg AgTCAggCCCTTC-3_ (Rev). The PCR products were cloned using pET151 directional TOPO expression kit (Invitrogen) as elsewhere described to obtain the expression plasmids (*33*). CD2AP SH3 domains A, B and C were amplified from an H1299 cDNA library produced in our laboratory, and cloned in pGex2TK. Flag-Cin85 PCDNA and Cool1 beta Pix-SH3 were a generous gift from Prof. I. Dikic.

Cell cultures and transfection

Human embryonic kidney HEK293 cells and human lung carcinoma H1299 p53−/− cells were purchased from ATCC. Cells were maintained in a humidified atmosphere at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (Sigma) and 0.1% penicillin/streptomycin (Invitrogen). Cells (50–70% confluence) were transfected with Lipofectamine 2000 (Invitrogen) or with JetPEI (Polyplus) according to the manufacturer's protocol, and analysed 24 or 48 h post-transfection.

Construction of protein arrays and binding assay

A collection of 46 proline binding domains cloned in pGex vectors (*40*) were expressed in *Escherichia coli* BL21 Rosetta as glutathione-*S*-transferase (GST) fusion products. The proteins were purified by standard procedures with Glutathione Sepharose 4B (GE Healthcare) and eluted from the column with 10 mM glutathione. Their purity was assessed by electrophoresis. Proteins were dialyzed against phosphate-buffered saline (PBS) to remove glutathione and their concentration was estimated with Biorad assay. All the samples were brought to a final concentration of 1 μg/μl and arrayed in quadruplicate using a Microcaster Arrayer (Schleicher e Schuell). Each domain was arrayed twice on the same spot to increase the local concentration of the protein. They were spotted onto aldehyde-displaying glass slides to generate a rectangular array. In the first set of experiments, we arrayed 30 GST-fused domains to generate 2 grids of 4 lines and 32 columns each. Domains were spotted four times in two different lines to assess reproducibility of the binding (data not shown). In the second set of experiments, 46 domains were spotted in 2 grids of 6 lines and 32 columns and each domain was spotted four times (Fig. 1B). Slides were air-dried in NaCl saturated atmosphere and one sample was tested with anti-GST to confirm domains binding.

The assay was performed incubating the slides in blocking solution (BSA 2% in PBS) for 30 min 4°C, then with the p53 protein. The slides were incubated with recombinant p53 expressed in *E. coli* BL21 Rosetta as histidine—tag fusion products and purified by standard procedure. After 2 h at 4°C arrays were washed three times in PBS and incubated with polyclonal anti-p53 (Santa Cruz) diluted 1:1,000 in BSA1% in PBS for 1 h 4°C. Alternatively they were incubated with monoclonal anti-p53 diluted 1:2,000 in the same solution. After three washing in PBS, the slides were incubated with anti-rabbit Cy5-conjugated or alternatively with anti-mouse Alexa-Fluor555 conjugated, in BSA 1% PBS. Signals were detected using a Fluorescence Microarray Scanner (ScanArray Gx Plus from PerkinElmer). For each spot, the software calculates the mean value of all pixel and subtracts the local background. These values were used in subsequent analyses, in which the mean of four spots of the same domain and the standard deviation were calculated (Supplementary Table S1).

Pull-down assay with recombinant p53

E. coli BL21 Rosetta expressing recombinant p53 P isoform or p53 R isoform with histidine tags were lysed by sonication. After centrifugation, extracts were quantified with Biorad assay and incubated for

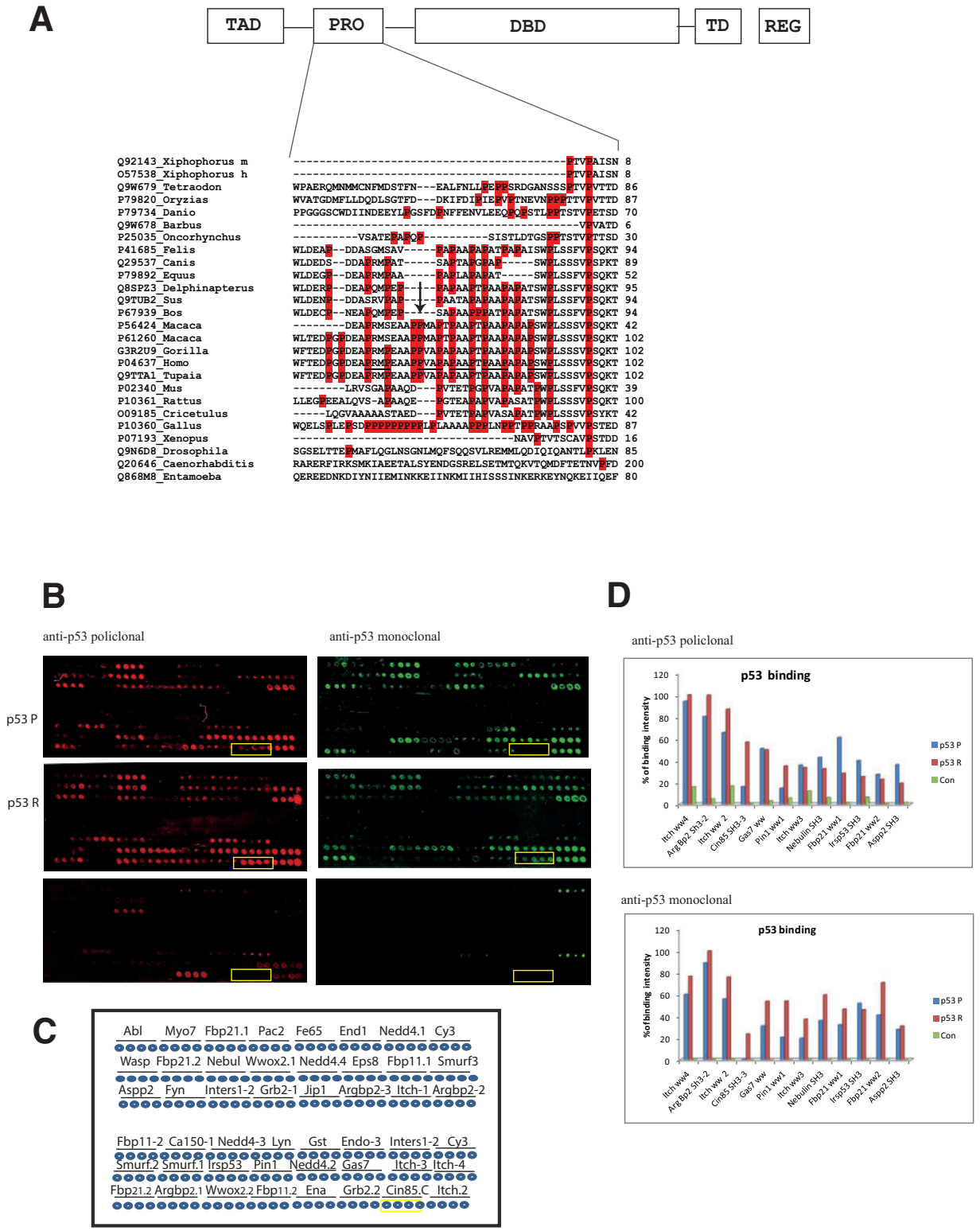

Fig. 1 The p 53 proline-rich binds to SH3 domains. (**A**) The primary structure of p53 proteins from several species were aligned by the Clustal-Omega program. The region corresponding to the proline-rich (aa 60–93) is shown: prolines are highlighted, and the five PXXP motifs of the human sequence are underlined. Position 72 is indicated with an arrow. (**B**) Forty-six WW and SH3 domains were spotted on glass slides and incubated with the p53 protein, P or R isoform, respectively, or with no protein, as control (Con). The binding was revealed with rabbit anti-p53 (on the left) or mouse monoclonal anti-p53 (on the right) followed by anti-rabbit-Cy5 (on the left) or anti-mouse-alexa555 (on the right). A box surrounds the position of the Cin85 SH3-3 domains. (**C**) The box illustrates the position of the domains in the arrays. Each domain is named with the name of the protein and, if it contains more domains, a number is added to identify the position of the domain in the protein. (**D**) The graphics show the mean values of the binding intensities for the preferred binding domains. Data obtained with polyclonal and monoclonal anti-p53 antibody are shown in the upper and lower graphic, respectively. Binding intensities for all domains, mean values and standard deviation are reported in Supplementary Table S1.

2 h at 4°C with 50 µg of the selected GST-fused domain bound to glutathione sepharose beads. The resins were washed four times with 1 ml of PBS buffer 0.05% Tween, and the bound proteins were recovered by boiling in SDS-dye (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 1% Bromophenol Blue, 10% glycerol), analysed by electrophoresis on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. The membranes were blocked for 2 h RT or overnight at 4°C in PBS containing 5% skimmed-milk powder (blocking solution), then incubated for 2 h at 4°C with polyclonal rabbit anti-p53 1:1,000 in blocking solution. After four washes in PBS 0.05% Tween, membranes were incubated with anti-rabbit POD conjugated for 45 min RT in blocking solution, and washed again four times in PBS 0.05% Tween. Bound p53 was revealed by chemiluminescence.

Pull-down assay with p53 expressed in mammalian cells

Transiently transfected Hek293 or H1299 p53−/− cells were washed with PBS and collected by scraping in buffer containing 50 mM Tris pH7.4, 150 mM NaCl, 0.1% NP40, supplemented with protease inhibitors. After centrifugation, cell extracts were quantified with Biorad assay, and 500 µg of total extract was used in pull down experiments with GST-fused domains bound to sepharose beads. After 2 h 4°C the beads were washed repeatedly in 500 µl of the same buffer and the bound proteins were recovered by boiling in SDS-dye. They were analysed by electrophoresis, blotted onto nitrocellulose membranes, and p53 was revealed as described in the previous paragraph.

Immunoprecipitation assay

Transiently transfected H1299 p53−/− cells were washed with PBS and collected by scraping in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% NP40, supplemented with protease inhibitors. The extracts were clarified by centrifugation, and protein concentration was determined by Bradford assay (Bio-Rad). Co-immunoprecipitations were performed on 500 micrograms/1milligram of protein extract, using the primary antibody for 4 h at 4°C, followed by A/G sepharose beads for 1 h at 4°C. For negative controls, sepharose beads with no antibodies were added. After three washes in the lysis buffer, samples were resolved on SDS-PAGE and elettroblotted on nitrocellulose membranes. Membranes were blocked in 5% skimmed milk, and then p53 was revealed with monoclonal anti-p53 followed by anti-mouse peroxidase conjugated. CD2AP and Cin85 were revealed with polyclonal specific antibodies followed by anti-rabbit POD conjugated.

Immunofluorescence

H1299 cells were fixed with 4% of PFA for 5 min and blocked with PBS 1% Serum 10% TritonX-100, 1% for 1 h at RT. The cells were incubated with mouse anti-p53 (1:100 Santa Cruz), and rabbit anti-CD2AP (1:50 Santa Cruz) for 1 h at RT. After three washes, they were incubated with anti-mouse or anti-rabbit Alexa-Fluor-conjugated (Invitrogen) diluted 1:250, 30 min at RT. The samples were washed three times and incubated with Hoescht (1 mg/ml, 5 min at RT), washed and mounted. Fluorescence images were acquired using Automated Microscope DMI 6000B Leica using 40× objective. Confocal microscopy analysis was performed with Confocal Laser Scanning Microscope FV1000 Olympus using 60× objective. Images were analysed with FV1000 Olympus Software. Pearson coefficients were obtained by generating manual region of interests covering the entire cell or selected regions of the cytoplasm.

Peptide arrays and binding assay

The peptide arrays were synthesized on cellulose-(3-amino-2-hydroxy-propyl)-ether. membranes (Intavis, Koeln, Germany). The peptides, 13-amino acid long, were synthesized according to standard spot synthesis protocols, using an automatic spot synthesizer (Intavis). Before use, the membranes were wet in ethanol and washed repeatedly in PBS.

The binding assay was performed incubating the membranes with 5 µg/ml of the GST-fused domain in blocking buffer overnight at 4°C. After washing three times for 10 min with PBS, the anti-GST antibody was added 1/1,000 in blocking buffer for 2 h at room temperature. After three washes with PBS (10 min each), POD-conjugated anti-goat mAb was added 1/5,000 in blocking buffer and incubated for 1 h at 4°C, followed by washing three times with PBS. Positive signals were revealed using a chemiluminescence substrate and the Las-3000 instrument (Fujifilm).

Results

Protein domain arrays reveal new p53 interactors

To identify polyproline binding domains that are able to discriminate between the p53 P and R isoforms, we performed protein array binding assays. The proline-rich region of the p53 protein in birds and mammals contains several PxxP motifs. This region is absent in invertebrates, suggesting that it has gained new functions during the course of evolution (Fig. 1A). The polymorphism at codon 72 changes the second proline motif from PPVAP to PRVAP. We investigated whether any of the proline-rich binding domains involved in signal transduction could recognize this region and discriminate between the two motifs. SH3 and WW domains are well-known binders of proline-rich-motifs and are abundant in human cells. We chose a collection of 46 of these modules, spanning a large spectrum of recognition specificities (*40*), produced them as GST fusions and arrayed them on activated glass slides, to generate domain chips. As previously described, while full length proteins are sometimes badly folded when expressed in heterologous systems and bound on glass slides, protein domains fused to GST usually retain their binding specificity (*41*). Domains were purified from bacterial cultures and all the samples were brought to a final concentration of 1 µg/µl and spotted onto glass slides. GST alone was used as a negative control. A set of six identical arrays was generated to perform each experiment. The arrays were incubated with histidine-tagged p53, P72 or R72 isoforms, and the binding was revealed with anti-p53 antibody, followed by a fluorescent secondary antibody. Two arrays were incubated only with the antibodies to serve as a negative control. To highlight false interactions, two different anti-p53 antibodies were used to reveal the binding (Fig. 1B and C). Domains that yielded higher reactions with both detection methods are reported in Fig. 1D. Interestingly, several WW and SH3 domains were shown to bind to both p53 isoforms; among them, the WW domains of Itch, Fbp21, Pin 1 and Gas7, and the SH3 domains of Argbp2 and Aspp2, which is a well-known p53 binding domain (*37*). However, very few domains could consistently discriminate between the two polymorphic variants, and, among them, the SH3-C domain of Cin85 clearly preferred the p53R variant in all the experiments performed.

Identification of the binding region for SH3 domains on the p53 sequence

To better characterize the positive interactions, we have utilized a different approach, which permits us to map the residues in the polyproline motif of p53 that bind the previously selected domains, if they recognize this region. We synthesized, on membrane support, an array of 14 peptides, 13-amino acids long, reproducing the p53 polyproline region surrounding codon 72. The membranes were probed with the GST-tagged domains that produced the strongest

interactions in the previous experiment (Fig. 1D), and the binding was revealed with anti-GST and secondary antibody conjugated to POD. No signal was detected with the WW domains of Itch, Fbp21, Pin1 or with Fbp11 and Wwox2, suggesting that no interaction occurred between these WW domains and the polyproline region under investigation, when T81 is unphosphorylated, in agreement with previous observations (*42*) (data not shown). On the contrary, the carbossiterminal SH3-C domain of Cin85 showed a strong binding with three of the p53 peptides, all containing the 67-PEAAPR-72 sequence (Fig. 2). Cin85 is a broadly expressed multi-domain protein, which contains three SH3 domains, dubbed A, B and C (*43*). Two homologues have been described in human and mouse cells: Cin85 and CD2-Associated Protein CD2AP (Fig. 3A). It has been reported that the consensus binding motif for the SH3 domains of Cin85 is PXXXPR (*44*). Two motifs matching this consensus are present in the p53 proline-rich: 60-PDEAPRM-66, which is present on both the isoforms, and 67-PEAAPR-72, which only occurs in the R variant. Our data clearly show that only the second motif was recognized by the SH3-C domain of Cin85.

We also tested the N-terminal SH3 domain of Cin85 that has the same binding specificity (*44*, *45*): as expected, we achieved the same result. In both cases, the binding was abolished if the first proline of the motif was deleted (Fig. 2). To analyse the behaviour of the other member of the CMS/Cin85 family (Fig. 3A), we cloned the three SH3 domains of CD2AP from a cDNA library of H1299 cells, and expressed them as GST fusion. The SH3-B domain of CD2AP showed a strong interaction with the same arginine-containing peptide of p53, as Cin85 (Fig. 2).

Furthermore, the SH3 domain of Aspp2 and the second SH3 domain of ArgBP2 recognized the p53 polymorphic region. In agreement with previous observations (*19*, *40*), the SH3 of Aspp2 was able to recognize the polyproline peptides, both with P or R, with a preference for the R isoforms. The SH3-2 domain of ArgBP2 gave only a low signal with the peptide 69-AAP**P**VAPAPAAPTPA-83, while no signal was detected with the SH3-3 domain of the same protein. GST alone was used as a negative control. The binding of Aspp2 with p53 has been already investigated (*19*); therefore, we focused on the SH3 domains of the CMS/Cin85 family.

Interaction of the CMS/Cin85 family members with the p53 isoforms

To further investigate the p53 binding to the family of Cin85/CD2AP, we performed a pull-down assay using recombinant p53 expressed in *E. coli* with a histidine tag, and GST-Cin85 SH3-C domain bound to glutathione sepharose beads. The binding was revealed with anti-p53, followed by incubation with a secondary antibody conjugated with POD. In agreement with the array and SPOT synthesis data, Cin85 SH3-C domain bound to the p53 protein with a strong preference for the R isoform. As shown in Fig. 4A, the first domain of Cin85 (SH3-A) presented the same behaviour. Notably, the SH3 domain of Cool-1 beta Pix, which recognized peptides containing PXXXPR motifs (*46*, *47*), did not bind to the p53R isoform, confirming that the interaction is specific. Bacterially expressed p53 presented several degradation bands, thus we next tested whether the SH3 domains of Cin85 could discriminate between p53 P and R isoforms when they were expressed in a more physiological system. To achieve this, we transfected human HEK 293 cells with p53P and p53R allelic variants and performed a pull-down assay with Cin85 SH3-A and Cin85 SH3-C. Both domains preferentially bound to the p53 R isoform (Fig. 4B). Differential behaviour of the two isoforms has been reported to be cell-type specific (*34*), thus, the same experiment was repeated in H1299 p53−/− cells, where the apoptotic potential of the R isoform has been investigated (*34*). As shown in Fig. 4B, the results were identical.

The SH3-A and SH3-B of CD2AP were also able to bind bacterially expressed p53R in a pull-down assay, where the SH3-B gave a markedly stronger reaction (Fig. 3B). Moreover, all three SH3 domains of CD2AP probed in the H1299 cell extract showed a preference for the R isoform. Once again, the SH3-B is the strongest p53 interactor (Fig. 3C).

CD2AP proteins form in vivo complexes with cytoplasmic p53

To establish if p53 forms a complex *in vivo* with the CMS/Cin85 proteins, we performed co-immunoprecipitation and co-localization analyses. We transiently expressed p53R and p53P in H1299 cells and analysed the co-immunoprecipitates with endogenous Cin85 and CD2AP. As shown in Fig. 5A, no specific association is revealed between endogenous Cin85 and p53. On the contrary, endogenous CD2AP co-immunoprecipitates with both the p53 isoforms, but it shows a marked preference for the p53R isoform (Fig. 5A). Furthermore, although overexpressed, Cin85 does not recognize p53 (Fig. 5C). Thus, we conclude that in the H1299 environment, in unstressed and unstimulated conditions, a fraction of p53 is in complex with CD2AP but not with Cin85. In addition, the p53 R isoform is a better ligand for CD2AP.

To assess the p53 co-localization with CD2AP, we performed immunofluorescence staining of the endogenous CD2AP and transfected p53P or p53R. In agreement with previous reports, CD2AP showed a diffused distribution with a slight accumulation in the cytosolic perinuclear regions (*48*). Localization of over-expressed p53 is mainly nuclear in H1299 cells, but, in some cells, it was also localized in the cytosol. As shown in Fig. 6, the cytosolic form of p53 co-localized with CD2AP. Cytosolic localization of the p53 protein was not unexpected; it has been reported that the protein underwent nuclear-cytoplasmic shuttling in unstressed cells, whereas it is mainly nuclear in immortalized cells (*49–54*). The co-localization was confirmed by confocal microscopy. In Fig. 6B, p53 and CD2AP co-localized in merge panels. The localization was analysed with Olimpo FV1000 software in the selected cytosolic areas (inset circles).

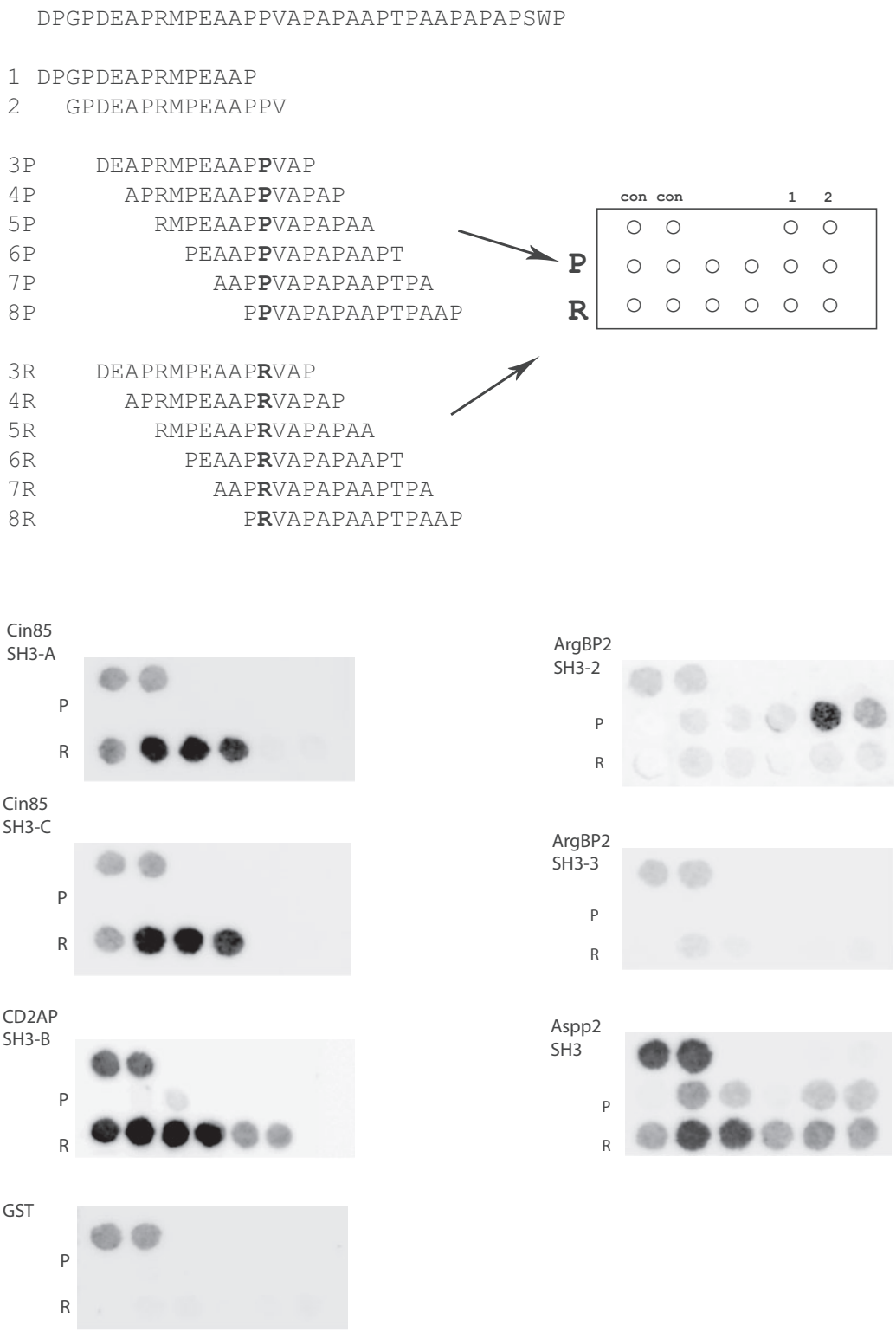

Fig. 2 Cin85 and CD2AP SH3 domains bind to the proline-rich of the p53 R isoform. Arrays of 14 peptides, 13-amino acid long, matching the sequence of p53 polyproline region and surrounding the codon 72 were synthesized and incubated with SH3 domains as indicated. Peptides indicated with *con* are control peptides that bind to GST alone. Peptides 3P to 8R contain, in the position in bold, the 72nd residue, either a proline (P lane) or an arginine (R lane). The binding was revealed with anti-GST followed by a secondary antibody bound to POD. All experiments were repeated at least twice.

As shown, both isoforms are co-localized with CD2AP, with a little increase for the p53 R form (Pearson coefficients 0.81 and 0.84 for P and R, respectively).

Discussion

The p53 protein is a crucial node for the regulation of cell physiology. Although it mainly functions as

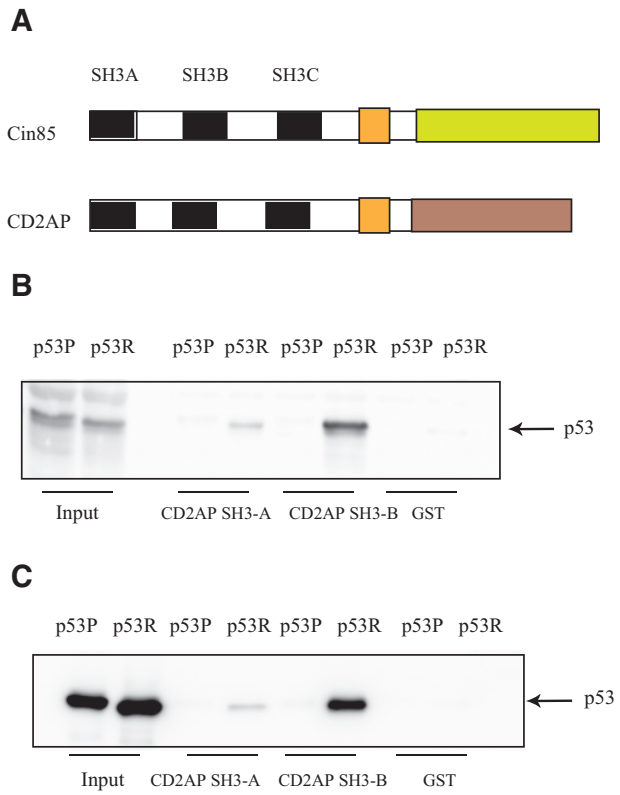

Fig. 3 CD2AP SH3 domains bind to the p53 Arg isoform in pull-down assay. (**A**) Modular organization of Cin85 and CD2AP proteins. (**B**) Resin-bound GST-SH3 domains were incubated with Histidine-tag-p53-expressing bacteria extracts. The blot was revealed with anti-p53. GST conjugated to the resin was used as negative control. (**C**) Resin-bound SH3 domains were incubated with H1299 extracts, transfected with p53 P or R as indicated. GST was used as negative control. Pull down experiments were repeated three times.

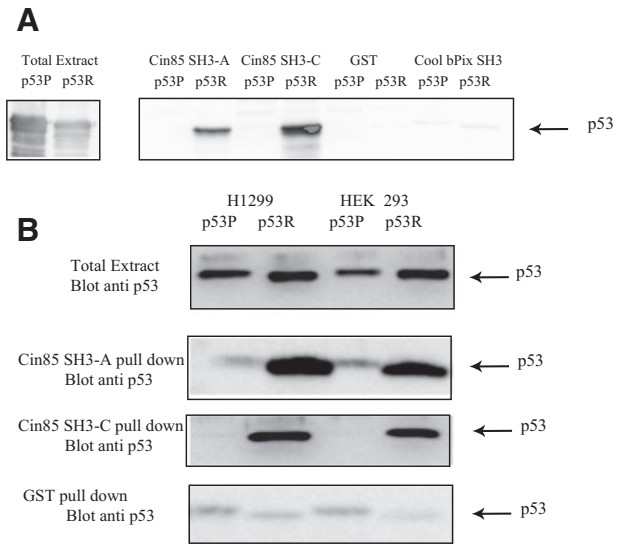

Fig. 4 P53-R binds to Cin85 SH3-A and SH3-C domains, but not to Cool-1 beta Pix SH3 in pull down assay. (**A**) Resin-bound GST-SH3 domains were incubated with Histidine-tag-p53-expressing bacteria extracts. The blot was revealed with anti-p53. Resin-bound GST was used as negative control. (**B**) Resin-bound SH3 domains were incubated with H1299 or HEK293 extracts, transfected with p53 P or R as indicated. The binding was revealed with anti-p53 and resin-bound GST was used as negative control. Pull down experiments were repeated three times.

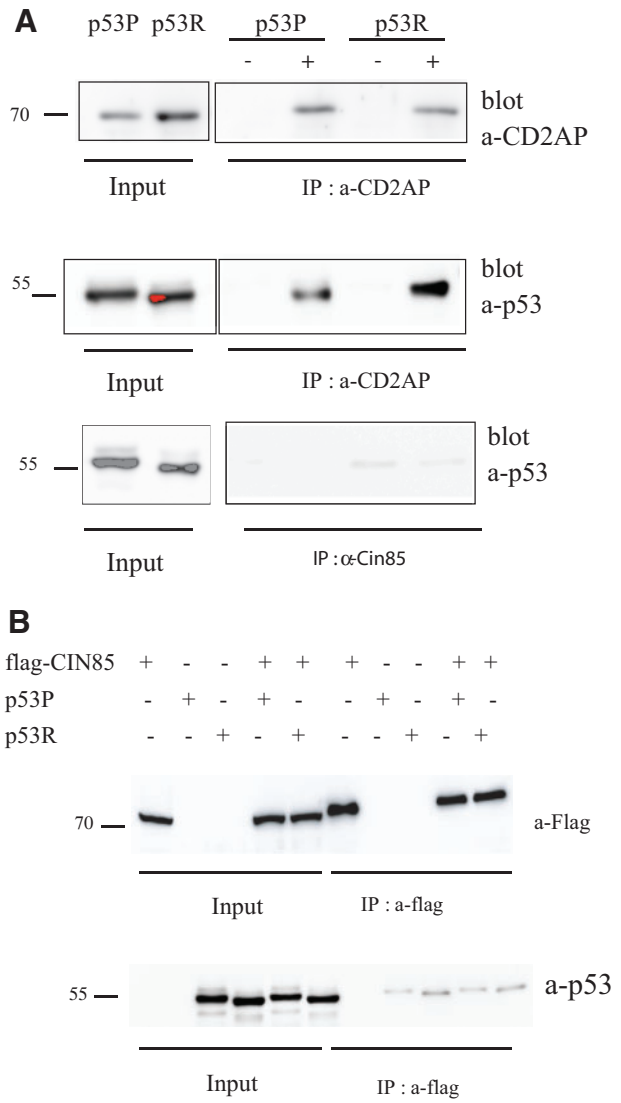

Fig. 5 CD2AP forms a complex with p53. (**A**) Immunoprecipitation of p53-CD2AP complex: H1299 cells were transfected with p53 P or R isoforms. CD2AP or Cin85 were immunoprecipitated with anti-CD2AP or anti-Cin85, respectively, followed by A/G sepharose beads (+), and the blot was revealed with anti-p53 or anti-CD2AP, as indicated. For the controls (−) the extracts were incubated with A/G sepharose beads alone. The experiment shown is representative of three independent experiments. (**B**) H1299 cells were cotransfected with p53P or R and Cin85-Flag. Cin85 was immunoprecipitated with anti-Flag and the blot was revealed with anti-p53 or anti-Cin85 as indicated. No coimmunoprecipitation was observed with Cin85.

transcriptional activator, distinct roles for this protein have been proposed since 1994 (*55–57*). In particular, the proline-rich region of the human p53 has been shown to be dispensable for modulating transcription, while playing an essential role in other functions of the protein (*25*, *27*). Clearly, the P72R polymorphism markedly influences the protein's performance in normal and tumour cells; as such, it has been investigated since it interferes with the ability of p53 to interact with other proteins. Actually, the apoptosis inhibitor iAspp was shown to discriminate between the two polymorphic variants, and to prefer the

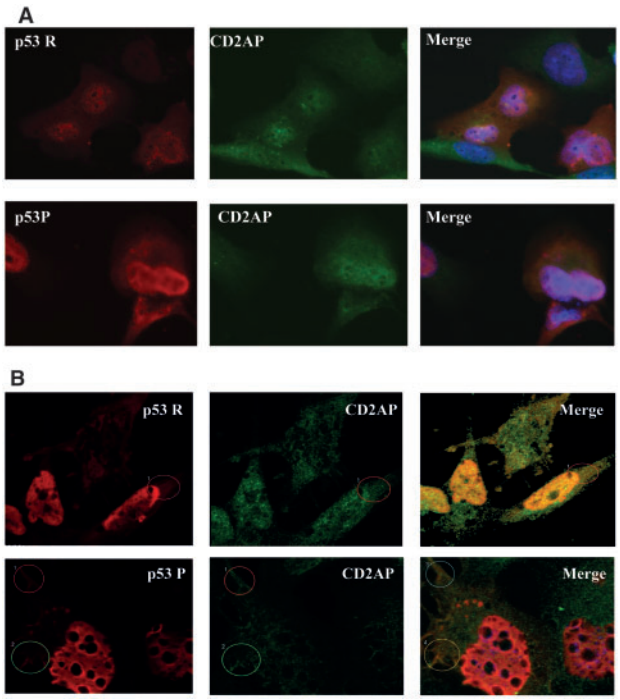

Fig. 6 Colocalization of cytoplasmic p53 with CD2AP. H1299 cells were grown on coverslip and transiently transfected with p53 P or R respectively. Twenty four hours after transfection cells were fixed and the p53 protein was stained with monoclonal anti-p53 followed by anti-mouse, while endogenous CD2AP was stained with anti-CD2AP followed by anti-rabbit. (**A**) The immunofluorescence was visualized with automated fluorescence microscopy. (**B**) Co-localization of p53 with CD2AP was confirmed by confocal microscopy. The staining in the first two columns corresponds to transfected p53, P or R as indicated, and to endogenous CD2AP. The staining in merge panels indicates the presence of both proteins. The inset boxes highlight the areas analysed with Olympus AV1000 software. Pearson coefficients were obtained by generating manual ROIs. (Inset circles). Pearson:0,81 and 0,84 respectively for P and R.

P72p53, whereas the R72 isoform can exert its higher apoptotic potential (*19*). Other interesting molecular models have been proposed to explain the different behaviour of the two isoforms (*34–36*); however, further investigations are needed to address the extent to which the diverse and complex characteristics of the polymorphic variants are integrated in contributing to the final phenotypes. Contradictory results have been obtained with mouse models: some reports concluded that the characteristics of the polymorphic variants are species-specific and not reproducible in mouse models (*58*), while other studies have demonstrated that mouse cells reproduce human results (*36*, *59*). Notably, most studies pointed out that the effect of the polymorphism correlated to cell type, which may be the result of the presence in different tissues of different protein interactors and different expression levels of ubiquitous interactors.

Having excluded the possibility that the difference in biological activity of the two isoforms may be because of the modifications in the secondary structure induced by the P72R polymorphism (*33*), it is plausible that such a difference may be accounted for a diverse subcellular localization of the two isoforms that in turn can be due to a preferential interaction with other factors. In particular, it has been reported that the R isoform is retained at cytoplasmic level more than the P isoform (*34*).

In agreement with this hypothesis, in this article, we identified CD2AP, which is a ubiquitously expressed adaptor protein with three SH3 domains (*43*), as a novel binding protein of cytosolic p53. All three SH3 domains of CD2AP were able to bind to p53 and the second SH3 is the strongest binder, specifically with the p53R isoform. CD2AP has multiple, tissue-specific functions. In T-cells, it has been shown to interact with the cell adhesion molecule CD2 to assist CD2 in clustering and cytoskeletal polarization during antigen recognition, whereas CD2AP-deficient mice showed impaired T-cell function, but died of renal failure (*60*, *61*). More recently, it has been reported that CD2AP is involved in actin remodelling and, similarly to Cin85, in the endocytic degradative pathway of receptors (*62–64*). By virtue of its ability to connect different partner proteins, CD2AP may anchor p53, and in particular the R isoform, in the cytosol. In fact, in non-immortalized and unstressed cells, the p53 is exported to the cytosol where it is rapidly polyubiquitinated and degraded (*53*, *54*, *65*). However, it has been shown (*57*) that a part of the cytosolic pool of p53 undergoes monoubiquitination instead of degradation, and, upon genotoxic stress, is imported into mitochondria where it exerts its pro-apoptotic function.

Mechanisms that regulate the balance between stable, monoubiquitinated and polyubiquitinated p53 are numerous and not completely defined (*57*). Binding to CD2AP may partially protect the p53 from polyubiquitination and degradation. A similar model had been proposed in the past for several cytoplasmic proteins to explain cytosolic localization of the p53, as reviewed in (*52*). Particularly in neuroblastoma cells, the PARC protein has been shown to sequester the p53 in the cytoplasm, inhibiting it (*66*). According to our data, CD2AP preferential interaction with the p53R isoform may allow sequestration of a higher fraction of p53R out of the nucleus, in accordance with the observation that p53R preferentially migrates to mitochondria in response to apoptotic stimuli (*34*). Interestingly, the SH3 domains of CD2AP were shown to bind to monoubiquitin moieties, which could reinforce the binding to ubiquitinated p53 (*67*).

CD2AP directly interacts with both actin and membrane proteins, and it has been proposed as a link between receptors or other membrane proteins (such as nephrin) and the actin cytoskeleton (*64*, *68*). According to our observations, it may also link receptors to the p53 protein. In fact, several reports reveal cross-talking between the cytoplasmic p53 protein and different growth factor receptors, as well as cytokines and Fas receptors (*35*, *55*, *69–71*). In particular, in response to EGF treatment, a cytosolic fraction of p53 was shown to form a complex with clathrin at the plasma membrane in normal and cancer human cells, and to influence EGFR endocytosis (*69*, *71*). Moreover, mutant forms of p53 promote plasma membrane localization

of EGFR and integrins (*72*). We did not observe interaction with Cin85 full-length protein, *in vivo*, even upon induction with EGFR. However, we cannot exclude the possibility that the two proteins might bind in different physiological environments.

CD2AP was shown to regulate the formation and maintenance of cell–cell interaction via Rac 1 (*73*) and to directly bind F-actin to modulate cytoskeleton rearrangements (*74*). Notably, p53 also cooperates with Rac1 in the control of cell proliferation and migration, in normal and invasive cells (*75*, *76*). In p53−/− lymphoma cells, Rac1 deletion was able to abrogate hyperproliferation and to induce apoptosis (*76*). Clearly, the complicated connections between p53 and CD2AP signalling pathways require further study to address the physiological significance of their binding, and to clarify the real capability of the P72R polymorphism to influence the cytosolic functions of the p53 protein.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

References

1. Lu, W.J., Amatruda, J.F., and Abrams, J.M. (2009) p53 ancestry: gazing through an evolutionary lens. *Nat. Rev. Cancer* **9**, 758–762
2. Soussi, T. and Wiman, K.G. (2007) Shaping genetic alterations in human cancer: the p53 mutation paradigm. *Cancer Cell* **12**, 303–312
3. Leroy, B., Fournier, J.L., Ishioka, C., Monti, P., Inga, A., Fronza, G., and Soussi, T. (2013) The TP53 website: an integrative resource centre for the TP53 mutation database and TP53 mutant analysis. *Nucleic Acids Res.* **41**, D962–D969
4. Puente, X.S., Velasco, G., Gutierrez-Fernandez, A., Bertranpetit, J., King, M.C., and Lopez-Otin, C. (2006) Comparative analysis of cancer genes in the human and chimpanzee genomes. *BMC Genom.* **7**, 15
5. Bojesen, S.E. and Nordestgaard, B.G. (2008) The common germline Arg72Pro polymorphism of p53 and increased longevity in humans. *Cell Cycle* **7**, 158–163
6. Sjalander, A., Birgander, R., Kivela, A., and Beckman, G. (1995) p53 polymorphisms and haplotypes in different ethnic groups. *Hum. Hered.* **45**, 144–149
7. Beckman, G., Birgander, R., Sjalander, A., Saha, N., Holmberg, P.A., Kivela, A., and Beckman, L. (1994) Is p53 polymorphism maintained by natural selection? *Hum. Hered.* **44**, 266–270
8. Shi, H., Tan, S.J., Zhong, H., Hu, W., Levine, A., Xiao, C.J., Peng, Y., Qi, X.B., Shou, W.H., Ma, R.L., Li, Y., Su, B., and Lu, X. (2009) Winter temperature and UV are tightly linked to genetic changes in the p53 tumor suppressor pathway in Eastern Asia. *Am. J. Hum. Genet.* **84**, 534–541
9. Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I.M., Matlashewski, G., and Banks, L. (1998) Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* **393**, 229–234
10. Zehbe, I., Voglino, G., Wilander, E., Genta, F., and Tommasino, M. (1999) Codon 72 polymorphism of p53 and its association with cervical cancer. *Lancet* **354**, 218–219
11. Zhou, X., Gu, Y., and Zhang, S.L. (2012) Association between p53 codon 72 polymorphism and cervical cancer risk among Asians: a HuGE review and meta-analysis. *Asian Pac. J. Cancer Prev. APJCP* **13**, 4909–4914
12. Xu, T., Xu, Z.C., Zou, Q., Yu, B., and Huang, X.E. (2012) P53 Arg72Pro polymorphism and bladder cancer risk—meta-analysis evidence for a link in Asians but not Caucasians. *Asian Pac. J. Cancer Prev.: APJCP* **13**, 2349–2354
13. Yang, Z., Nie, S., Zhu, H., Wu, X., Jia, S., Luo, Y., and Tang, W. (2013) Association of p53 Arg72Pro polymorphism with bladder cancer: a meta-analysis. *Gene* **512**, 408–413
14. Walter, S.D., Riddell, C.A., Rabachini, T., Villa, L.L., and Franco, E.L. (2013) Accuracy of p53 codon 72 polymorphism status determined by multiple laboratory methods: a latent class model analysis. *PloS ONE* **8**, e56430
15. Papadakis, E.D., Soulitzis, N., and Spandidos, D.A. (2002) Association of p53 codon 72 polymorphism with advanced lung cancer: the Arg allele is preferentially retained in tumours arising in Arg/Pro germline heterozygotes. *Br. J. Cancer* **87**, 1013–1018
16. Weng, Y., Lu, L., Yuan, G., Guo, J., Zhang, Z., Xie, X., Chen, G., and Zhang, J. (2012) p53 codon 72 polymorphism and hematological cancer risk: an update meta-analysis. *PloS ONE* **7**, e45820
17. Kang, H.J., Feng, Z., Sun, Y., Atwal, G., Murphy, M.E., Rebbeck, T.R., Rosenwaks, Z., Levine, A.J., and Hu, W. (2009) Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 9761–9766
18. Bonafe, M., Salvioli, S., Barbi, C., Trapassi, C., Tocco, F., Storci, G., Invidia, L., Vannini, I., Rossi, M., Marzi, E., Mishto, M., Capri, M., Olivieri, F., Antonicelli, R., Memo, M., Uberti, D., Nacmias, B., Sorbi, S., Monti, D., and Franceschi, C. (2004) The different apoptotic potential of the p53 codon 72 alleles increases with age and modulates in vivo ischaemia-induced cell death. *Cell Death Differ.* **11**, 962–973
19. Bergamaschi, D., Samuels, Y., Sullivan, A., Zvelebil, M., Breyssens, H., Bisso, A., Del Sal, G., Syed, N., Smith, P., Gasco, M., Crook, T., and Lu, X. (2006) iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. *Nat. Genet.* **38**, 1133–1141
20. Sansone, P., Storci, G., Pandolfi, S., Montanaro, L., Chieco, P., and Bonafe, M. (2007) The p53 codon 72

proline allele is endowed with enhanced cell-death inducing potential in cancer cells exposed to hypoxia. *Br. J. Cancer* **96**, 1302–1308

21. Vannini, I., Zoli, W., Tesei, A., Rosetti, M., Sansone, P., Storci, G., Passardi, A., Massa, I., Ricci, M., Gusolfino, D., Fabbri, F., Ulivi, P., Brigliadori, G., Amadori, D., and Bonafe, M. (2008) Role of p53 codon 72 arginine allele in cell survival in vitro and in the clinical outcome of patients with advanced breast cancer. *Tumour Biol.* **29**, 145–151
22. Salvioli, S., Bonafe, M., Barbi, C., Storci, G., Trapassi, C., Tocco, F., Gravina, S., Rossi, M., Tiberi, L., Mondello, C., Monti, D., and Franceschi, C. (2005) p53 codon 72 alleles influence the response to anticancer drugs in cells from aged people by regulating the cell cycle inhibitor p21WAF1. *Cell Cycle* **4**, 1264–1271
23. Altilia, S., Santoro, A., Malagoli, D., Lanzarini, C., Ballesteros Alvarez, J.A., Galazzo, G., Porter, D.C., Crocco, P., Rose, G., Passarino, G., Roninson, I.B., Franceschi, C., and Salvioli, S. (2012) TP53 codon 72 polymorphism affects accumulation of mtDNA damage in human cells. *Aging* **4**, 28–39
24. de Souza-Pinto, N.C., Harris, C.C., and Bohr, V.A. (2004) p53 functions in the incorporation step in DNA base excision repair in mouse liver mitochondria. *Oncogene* **23**, 6559–6568
25. Walker, K.K. and Levine, A.J. (1996) Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15335–15340
26. Ruaro, E.M., Collavin, L., Del Sal, G., Haffner, R., Oren, M., Levine, A.J., and Schneider, C. (1997) A proline-rich motif in p53 is required for transactivation-independent growth arrest as induced by Gas1. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 4675–4680
27. Baptiste, N., Friedlander, P., Chen, X., and Prives, C. (2002) The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. *Oncogene* **21**, 9–21
28. Rath, A., Davidson, A.R., and Deber, C.M. (2005) The structure of ''unstructured'' regions in peptides and proteins: role of the polyproline II helix in protein folding and recognition. *Biopolymers* **80**, 179–185
29. Ball, L.J., Kuhne, R., Schneider-Mergener, J., and Oschkinat, H. (2005) Recognition of proline-rich motifs by protein-protein-interaction domains. *Angew Chem. Int. Ed. Engl.* **44**, 2852–2869
30. Cesareni, G., Panni, S., Nardelli, G., and Castagnoli, L. (2002) Can we infer peptide recognition specificity mediated by SH3 domains? *FEBS Lett* **513**, 38–44
31. Landgraf, C., Panni, S., Montecchi-Palazzi, L., Castagnoli, L., Schneider-Mergener, J., Volkmer-Engert, R., and Cesareni, G. (2004) Protein interaction networks by proteome peptide scanning. *PLoS Biol.* **2**, E14
32. Tonikian, R., Xin, X., Toret, C.P., Gfeller, D., Landgraf, C., Panni, S., Paoluzi, S., Castagnoli, L., Currell, B., Seshagiri, S., Yu, H., Winsor, B., Vidal, M., Gerstein, M.B., Bader, G.D., Volkmer, R., Cesareni, G., Drubin, D.G., Kim, P.M., Sidhu, S.S., and Boone, C. (2009) Bayesian modeling of the yeast SH3 domain interactome predicts spatiotemporal dynamics of endocytosis proteins. *PLoS Biol.* **7**, e1000218
33. Naldi, M., Pistolozzi, M., Bertucci, C., De Simone, A., Altilia, S., Pierini, M., Franceschi, C., Salvioli, S., and Andrisano, V. (2010) Structural characterization of p53 isoforms due to the polymorphism at codon 72 by mass spectrometry and circular dichroism. *J. Pharm. Biomed. Anal.* **53**, 200–206
34. Dumont, P., Leu, J.I., Della Pietra, A.C.3rd, George, D.L., and Murphy, M. (2003) The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. *Nat. Genet.* **33**, 357–365
35. Azzam, G., Wang, X., Bell, D., and Murphy, M.E. (2013) CSF1 is a novel p53 target gene whose protein product functions in a feed-forward manner to suppress apoptosis and enhance p53-mediated growth arrest. *PloS ONE* **8**, e74297
36. Frank, A.K., Leu, J.I., Zhou, Y., Devarajan, K., Nedelko, T., Klein-Szanto, A., Hollstein, M., and Murphy, M.E. (2011) The codon 72 polymorphism of p53 regulates interaction with NF-{kappa}B and transactivation of genes involved in immunity and inflammation. *Mol. Cell. Biol.* **31**, 1201–1213
37. Gorina, S. and Pavletich, N.P. (1996) Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. *Science* **274**, 1001–1005
38. Bergamaschi, D., Samuels, Y., O'Neil, N.J., Trigiante, G., Crook, T., Hsieh, J.K., O'Connor, D.J., Zhong, S., Campargue, I., Tomlinson, M.L., Kuwabara, P.E., and Lu, X. (2003) iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nat. Genet.* **33**, 162–167
39. Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001) ASPP proteins specifically stimulate the apoptotic function of p53. *Mol. Cell* **8**, 781–794
40. Carducci, M., Perfetto, L., Briganti, L., Paoluzi, S., Costa, S., Zerweck, J., Schutkowski, M., Castagnoli, L., and Cesareni, G. (2012) The protein interaction network mediated by human SH3 domains. *Biotechnol. Adv.* **30**, 4–15
41. Espejo, A., Cote, J., Bednarek, A., Richard, S., and Bedford, M.T. (2002) A protein-domain microarray identifies novel protein–protein interactions. *Biochem. J.* **367**, 697–702
42. Mantovani, F., Tocco, F., Girardini, J., Smith, P., Gasco, M., Lu, X., Crook, T., and Del Sal, G. (2007) The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. *Nat. Struct. Mol. Biol.* **14**, 912–920
43. Dikic, I. (2002) CIN85/CMS family of adaptor molecules. *FEBS Lett.* **529**, 110–115
44. Kowanetz, K., Szymkiewicz, I., Haglund, K., Kowanetz, M., Husnjak, K., Taylor, J.D., Soubeyran, P., Engstrom, U., Ladbury, J.E., and Dikic, I. (2003) Identification of a novel proline-arginine motif involved in CIN85-dependent clustering of Cbl and down-regulation of epidermal growth factor receptors. *J. Biol. Chem.* **278**, 39735–39746
45. Moncalian, G., Cardenes, N., Deribe, Y.L., Spinola-Amilibia, M., Dikic, I., and Bravo, J. (2006) Atypical polyproline recognition by the CMS N-terminal Src homology 3 domain. *J. Biol. Chem.* **281**, 38845–38853
46. Hoelz, A., Janz, J.M., Lawrie, S.D., Corwin, B., Lee, A., and Sakmar, T.P. (2006) Crystal structure of the SH3 domain of betaPIX in complex with a high affinity peptide from PAK2. *J. Mol. Biol.* **358**, 509–522
47. Schmidt, M.H., Husnjak, K., Szymkiewicz, I., Haglund, K., and Dikic, I. (2006) Cbl escapes Cdc42-mediated inhibition by downregulation of the adaptor molecule betaPix. *Oncogene* **25**, 3071–3078

48. Havrylov, S., Ichioka, F., Powell, K., Borthwick, E.B., Baranska, J., Maki, M., and Buchman, V.L. (2008) Adaptor protein Ruk/CIN85 is associated with a subset of COPI-coated membranes of the Golgi complex. *Traffic* **9**, 798–812
49. Shaulsky, G., Ben-Ze'ev, A., and Rotter, V. (1990) Subcellular distribution of the p53 protein during the cell cycle of Balb/c 3T3 cells. *Oncogene* **5**, 1707–1711
50. Middeler, G., Zerf, K., Jenovai, S., Thulig, A., Tschodrich-Rotter, M., Kubitscheck, U., and Peters, R. (1997) The tumor suppressor p53 is subject to both nuclear import and export, and both are fast, energy-dependent and lectin-inhibited. *Oncogene* **14**, 1407–1417
51. Wang, L., Wang, M., Wang, S., Qi, T., Guo, L., Li, J., Qi, W., Ampah, K.K., Ba, X., and Zeng, X. (2013) Actin polymerization negatively regulates p53 function by impairing its nuclear import in response to DNA damage. *PloS ONE* **8**, e60179
52. O'Brate, A. and Giannakakou, P. (2003) The importance of p53 location: nuclear or cytoplasmic zip code? *Drug Resist. Updat.* **6**, 313–322
53. Rotter, V., Abutbul, H., and Ben-Ze'ev, A. (1983) P53 transformation-related protein accumulates in the nucleus of transformed fibroblasts in association with the chromatin and is found in the cytoplasm of non-transformed fibroblasts. *EMBO J.* **2**, 1041–1047
54. Ferecatu, I., Bergeaud, M., Rodriguez-Enfedaque, A., Le Floch, N., Oliver, L., Rincheval, V., Renaud, F., Vallette, F.M., Mignotte, B., and Vayssiere, J.L. (2009) Mitochondrial localization of the low level p53 protein in proliferative cells. *Biochem. Biophys. Res. Commun.* **387**, 772–777
55. Bennett, M., Macdonald, K., Chan, S.W., Luzio, J.P., Simari, R., and Weissberg, P. (1998) Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* **282**, 290–293
56. Caelles, C., Helmberg, A., and Karin, M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature* **370**, 220–223
57. Marchenko, N.D., Wolff, S., Erster, S., Becker, K., and Moll, U.M. (2007) Monoubiquitylation promotes mitochondrial p53 translocation. *EMBO J.* **26**, 923–934
58. Phang, B.H. and Sabapathy, K. (2007) The codon 72 polymorphism-specific effects of human p53 are absent in mouse cells: implications on generation of mouse models. *Oncogene* **26**, 2964–2974
59. Leu, J.I., Murphy, M.E., and George, D.L. (2013) The p53 codon 72 polymorphism modifies the cellular response to inflammatory challenge in the liver. *J. Liver* **2**, 117
60. Dustin, M.L., Olszowy, M.W., Holdorf, A.D., Li, J., Bromley, S., Desai, N., Widder, P., Rosenberger, F., van der Merwe, P.A., Allen, P.M., and Shaw, A.S. (1998) A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. *Cell* **94**, 667–677
61. Shih, N.Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M.L., Kanagawa, O., Miner, J.H., and Shaw, A.S. (1999) Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* **286**, 312–315
62. Kirsch, K.H., Georgescu, M.M., Shishido, T., Langdon, W.Y., Birge, R.B., and Hanafusa, H. (2001) The adapter type protein CMS/CD2AP binds to the proto-oncogenic protein c-Cbl through a tyrosine phosphorylation-regulated Src homology 3 domain interaction. *J. Biol. Chem.* **276**, 4957–4963
63. Lynch, D.K., Winata, S.C., Lyons, R.J., Hughes, W.E., Lehrbach, G.M., Wasinger, V., Corthals, G., Cordwell, S., and Daly, R.J. (2003) A Cortactin-CD2-associated protein (CD2AP) complex provides a novel link between epidermal growth factor receptor endocytosis and the actin cytoskeleton. *J. Biol. Chem.* **278**, 21805–21813
64. Lehtonen, S., Zhao, F., and Lehtonen, E. (2002) CD2-associated protein directly interacts with the actin cytoskeleton. *Am. J. Physiol. Renal Physiol.* **283**, F734–F743
65. Ferecatu, I., Rincheval, V., Mignotte, B., and Vayssiere, J.L. (2009) Tickets for p53 journey among organelles. *Front Biosci. (Landmark Ed.)* **14**, 4214–4228
66. Nikolaev, A.Y., Li, M., Puskas, N., Qin, J., and Gu, W. (2003) Parc: a cytoplasmic anchor for p53. *Cell* **112**, 29–40
67. Ortega Roldan, J.L., Casares, S., Ringkjobing Jensen, M., Cardenes, N., Bravo, J., Blackledge, M., Azuaga, A.I., and van Nuland, N.A. (2013) Distinct ubiquitin binding modes exhibited by SH3 domains: molecular determinants and functional implications. *PloS ONE* **8**, e73018
68. Shih, N.Y., Li, J., Cotran, R., Mundel, P., Miner, J.H., and Shaw, A.S. (2001) CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am. J. Pathol.* **159**, 2303–2308
69. Endo, Y., Sugiyama, A., Li, S.A., Ohmori, K., Ohata, H., Yoshida, Y., Shibuya, M., Takei, K., Enari, M., and Taya, Y. (2008) Regulation of clathrin-mediated endocytosis by p53. *Genes Cells* **13**, 375–386
70. Bouleau, S., Parvu-Ferecatu, I., Rodriguez-Enfedaque, A., Rincheval, V., Grimal, H., Mignotte, B., Vayssiere, J.L., and Renaud, F. (2007) Fibroblast growth factor 1 inhibits p53-dependent apoptosis in PC12 cells. *Apoptosis* **12**, 1377–1387
71. Comel, A., Sorrentino, G., Capaci, V., and Del Sal, G. (2014) The cytoplasmic side of p53's oncosuppressive activities. *FEBS Lett.* **588**, 2600–2609
72. Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., Cromer, A., Brugge, J.S., Sansom, O.J., Norman, J.C., and Vousden, K.H. (2009) Mutant p53 drives invasion by promoting integrin recycling. *Cell* **139**, 1327–1341
73. van Duijn, T.J., Anthony, E.C., Hensbergen, P.J., Deelder, A.M., and Hordijk, P.L. (2010) Rac1 recruits the adapter protein CMS/CD2AP to cell–cell contacts. *J. Biol. Chem.* **285**, 20137–20146
74. Gaidos, G., Soni, S., Oswald, D.J., Toselli, P.A., and Kirsch, K.H. (2007) Structure and function analysis of the CMS/CIN85 protein family identifies actin-bundling properties and heterotypic-complex formation. *J. Cell Sci.* **120**, 2366–2377
75. Guo, F. and Zheng, Y. (2004) Rho family GTPases cooperate with p53 deletion to promote primary mouse embryonic fibroblast cell invasion. *Oncogene* **23**, 5577–5585
76. Bosco, E.E., Ni, W., Wang, L., Guo, F., Johnson, J.F., and Zheng, Y. (2010) Rac1 targeting suppresses p53 deficiency-mediated lymphomagenesis. *Blood* **115**, 3320–3328