account for all the cytotoxic effects of cisplatin. Modulators of this p53-independent cell-death pathway, which involves mismatch repair, c-Abl and p73, may be able to increase the response of cancer cells to cisplatin. □

Methods

Cell culture and transfection. Human colon-carcinoma lines HCT116-2(1) and HCT116-3(6) (ref. 8), osteosarcoma line Saos-2, Abl-deficient 3T3 cells, or primary MEFs were cultured in standard medium plus fetal bovine serum. Retroviral-mediated gene transfer was used to reconstitute c-Abl expression in Abl-deficient 3T3 cells[17] and polyclonal populations of reconstituted cells were used. *Abl*$^{-/-}$, *p53*$^{-/-}$ and *MLH1*$^{-/-}$ MEFs were prepared by mating the appropriate heterozygous mutant mice[12–14]. MEFs were prepared between day 11–13 of pregnancy and genotyped by PCR of embryo DNA[12–14]. Transient transfections were performed either with Superfect reagent (Gibco-BRL) or by using calcium phosphate precipitation[18].

Immunoblotting. Endogenous p73 protein was detected by immunoblotting with a monoclonal anti-p73 antibody (ER15), as described[19]. The following antibodies were used: anti-HA (12CA5; Boehringer Mannheim), anti-Abl (8E9; Pharmingen), anti-p53 PAb421 (Oncogene Science), anti-p53 C-19 (Santa Cruz Biotech), anti-MLH1 (Ab-2; Oncogene Science), anti-GFP (Clontech), anti-actin (Oncogene Science). To prepare total proteins, cells were extracted with lysis buffer (50 mM Tris, pH 8, 120 mM NaCl and 0.5% NP-40) and the insoluble pellet discarded after centrifugation. The protein concentration in the soluble fractions was determined by using a BioRad dye-binding assay.

c-Abl tyrosine kinase assay. c-Abl tyrosine kinase activity was measured in an immune-complex kinase assay as described[20]. A fusion protein (CTD–CRK), containing a modified C-terminal-repeat domain of RNA polymerase II (CTD) and the Abl phosphorylation site in the adapter protein CRK (J.G. and J.Y.J.W., unpublished), was used as a specific substrate to assay c-Abl kinase activity.

Measurement of p73 half-life. Decay of p73 protein in cycloheximide (Fig. 2b): cycloheximide (20 μg ml^{-1}) was added to cells 24 h after cisplatin (25 μM) addition and the cellular levels of p73 or actin at different time points were determined by immunoblotting; the amount of p73 relative to actin was normalized to that at the zero-time point. ^{35}S-pulse chase (Fig. 2d): Saos-2 cells were transfected with 3 μg each of pCDNA-HAp73-α with or without 3 μg pMSCV-c-Abl, or with a kinase-defective c-Abl (KD) containing a point mutation in the lysine residue critical for ATP binding. At 24 h post-transfection, cells were labelled with 250 μCi ml^{-1} of Translabel (containing ^{35}S-Met and ^{35}S-Cys; Amersham) for 60 min. Unlabelled methione and cysteine (1 mg ml^{-1}) were added and the cells collected at the indicated times; the ^{35}S-labelled p73-α in the anti-HA immunoprecipitate from each time point was quantified by Phosphoimager and normalized to that of the zero time point.

Cell-death assays. The killing of HCT116 cells by cisplatin (25 μM) was assayed by counting live cells, based on their trypan-blue exclusion. The percentage of live cells was determined by comparing live cells in a cisplatin-treated culture with those in an untreated culture collected at the same time (Fig. 1a). The killing of transfected Abl-deficient 3T3 cells (Fig. 3) was determined by chromatin condensation (Fig. 3a, b) or sub-G1 DNA content (Fig. 3c). In the chromatin-condensation assay, Abl-deficient 3T3 were co-transfected with 0.5 μg pEGFP (Clontech) and 3 μg each of pCDNA3-HA (vector), pCDNA-HAp73-α, pCDNA-HAp73-β or pCDNA3–p53 plasmid, either in the presence or absence of 3 μg MSCV–c-Abl or pMSCV–c-Abl-KD expression vector. At 96 h after transfection, cells were stained with Hoechst 33278 dye. The percentage of viable cells (diffuse Hoechst staining of green cells) after each transfection was determined and normalized to that of the pCDNA3-HA vector-transfected culture. In a fluorescence-activated cell-sorting (FACS) assay, c-Abl-deficient 3T3 cells were co-transfected with 4 μg of the indicated expression plasmids and 1.5 μg of a plasmid encoding a membrane-localized GFP[21]. At 48 h post-transfection, cells were permeabilized in PBS plus 0.1% Triton X-100 and stained with propidium iodide[22]. The DNA content of GFP-positive cells was determined with a FACScan and Cellfit program. The sensitivity of c-Abl-deficient and c-Abl-reconstituted 3T3 cells to cisplatin (Fig. 5c) was determined by Hoechst staining. The sensitivity of MEFs to cisplatin (Fig. 5a, b) was determined by counting the total number of cells using crystal violet stain. MEFs were seeded at 4×10^4 per well into 24-well plates and treated with cisplatin one day later. At the indicated time after cisplatin addition, cells were fixed and stained with 0.1% crystal violet. After extensive washing with PBS, the stain was extracted with 10% acetic acid and the dye concentration was determined from its absorbance at 600 nm. The optical density units of cisplatin-treated wells (in triplicate) at each time point were presented as a percentage of the units recovered from untreated wells (also in triplicate) collected at the same time point, with standard deviations (Fig. 5a, b).

Received 21 April; accepted 6 May 1999.

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Acknowledgements. We thank I. Hunton for the preparation of p53- and c-Abl-deficient MEFs, R. Kucherlapati and R. Kolodner for the MLH1-deficient MEFs, and R. Kolodner for critically reading the manuscript. This work was supported by grants from AIRC and CNR-PF Biotechnologie (to M.L.) and from the NIH (to J.Y.J.W.).

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Interaction of c-Abl and p73α and their collaboration to induce apoptosis

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c-Abl, a non-receptor tyrosine kinase, is activated by agents that damage DNA. This activation results in either arrest of the cell cycle in phase G1 or apoptotic cell death, both of which are dependent on the kinase activity of c-Abl[1]. p73, a member of the p53 family of tumour-suppressor proteins[2,3], can also induce apoptosis[3]. Here we show that the apoptotic activity of p73α requires the presence of functional, kinase-competent c-Abl. Furthermore, p73 and c-Abl can associate with each other, and

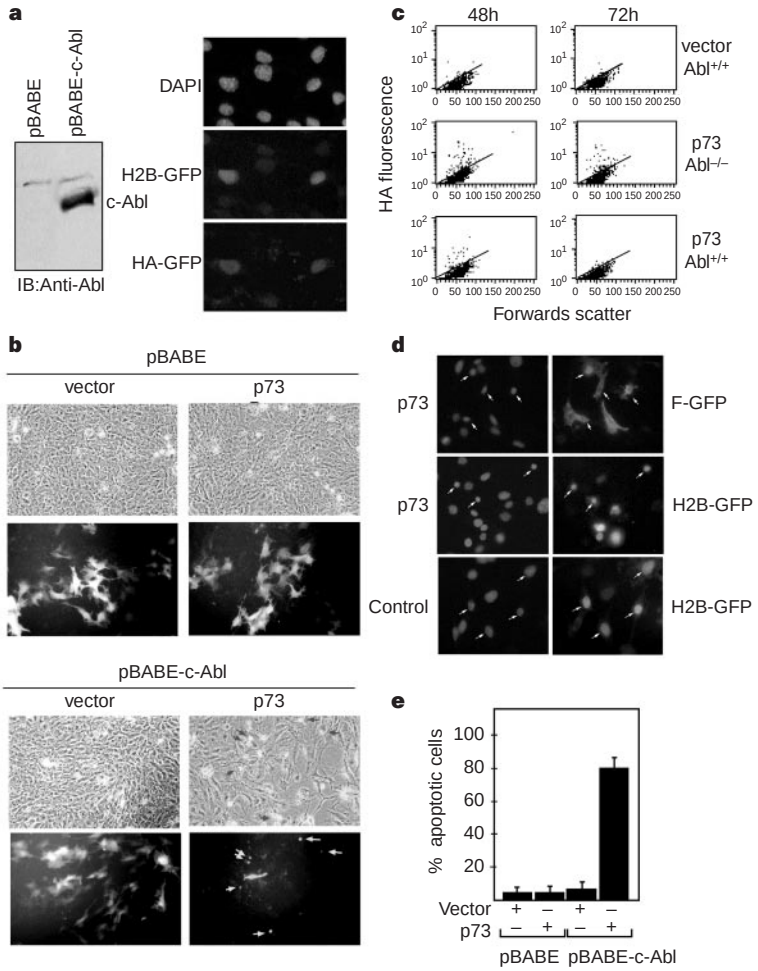

Figure 1 p73 induces apoptosis in a c-Abl-dependent manner. **a**, $Abl^{-/-}$ fibroblasts, infected with either a pBABE-Puro retrovirus or a pBABE-Puro-c-Abl retrovirus and selected with puromycin. Cell extracts were separated on 7.5% SDS–PAGE and immunoblotted with the 8E9 anti-Abl antibody. Also shown are pBABE cells co-transfected with H2B–GFP (GFP fused to histone 2B) and HA–p73. Cells were stained with DAPI to visualize nuclei and with anti-HA antibody to visualize p73. The transfected positive cells also express p73. **b**, Cells were transfected with 3 μg of either a control vector of HA–p73 expression plasmid with 0.5 μg pEGFP DNA (a commercial expression plasmid encoding GFP) using lipofectAMINE (Gibco-BRL). Cells were photographed 96 h post-transfection under UV (bottom) or phase microscope (top). Cells with apoptotic morphology are indicated by arrowheads. **c**, Kinetics of disappearance of HA–p73α-positive cells. Cultures of pBABE-Puro-c-Abl cells (top and bottom) or pBABE-Puro cells (middle) were transfected transiently with either empty vector (top) or HA–p73α expression plasmid (middle and bottom). At the indicated times after transfection, cells were fixed, stained with anti-HA antibody and analysed by FACS. The diagonal line represents an arbitrary division between HA–p73-positive cells (above line) and the rest of the cells in the culture. There is some nonspecific background staining (upper panels). **d**, pBABE-Puro-c-Abl cells growing on coverslips were transfected with either F-GFP (membrane-targeted GFP) or H2B-GFP (nucleus-associated GFP) with either p73 expression plasmid or the empty vector (control). Left, DAPI-stained fields; right, GFP fluorescence of the same fields. Note the shrunken condensed nuclei, indicating apoptotic morphology, in p73-transfected panels. **e**, Average percentage of apoptotic cells from five randomly chosen fields, with s.d.

this binding is mediated by a PxxP motif in p73 and the SH3 domain of c-Abl. We find that p73 is a substrate of the c-Abl kinase and that the ability of c-Abl to phosphorylate p73 is markedly increased by γ-irradiation. Moreover, p73 is phosphorylated *in vivo* in response to ionizing radiation. These findings define a pro-apoptotic signalling pathway involving p73 and c-Abl.

We investigated the relationship between p73 and c-Abl in the context of apoptosis using c-Abl nullizygous ($Abl^{-/-}$) mouse fibroblasts, stably transfected with either a c-Abl expression plasmid or the corresponding empty vector. As expected, the correct c-Abl protein was expressed in the former but not in the latter transfectants (Fig. 1a, left). Each type of cell was transfected transiently with an expression plasmid encoding haemagglutinin (HA)-tagged simian p73. Successfully transfected cells, identified using a co-transfected green fluorescent protein (GFP) expression plasmid, produced readily detectable amounts of HA–p73 (Fig. 1a, right). We then inspected these GFP-positive cells under a fluorescence microscope, without fixation, to assess the rate of apoptosis in the transfected cultures. Mouse fibroblasts usually appear flat and well attached, but they shrink and round up when undergoing apoptosis. When c-$Abl^{-/-}$ cells carrying only the empty vector (Fig. 1b, pBABE) were transfected transiently with p73, most of the transfectants retained their normal appearance (Fig. 1b). Hence, p73 is an inefficient inducer of apoptosis in these cells. In contrast, over-expression of p73 in cells reconstituted with c-Abl (pBABE–c-Abl) resulted in marked apoptosis, reflected by extensive rounding and shrinkage of the GFP-positive cells (Fig. 1b, right). When the HA–p73-transfected cells were monitored by fluorescence-activated cell sorting (FACS), the number of HA–p73-positive cells dropped sharply with time only in the presence of c-Abl (Fig. 1c). This observation was further confirmed by the analysis of fixed cultures; many of the GFP-positive p73 transfectants had shrunken, condensed nuclei, typical of apoptotic cells (Fig. 1d: arrows, upper two panels). This was seen with two different types of co-transfected

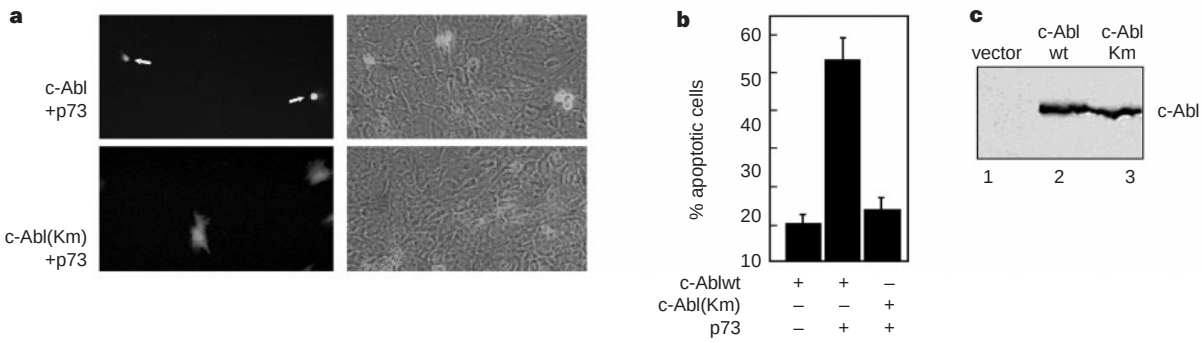

Figure 2 c-Abl and p73 collaborate to induce apoptosis in a kinase-dependent manner. **a**, $Abl^{-/-}$ fibroblasts were transfected with 1.5 μg of each of the indicated expression plasmids, with 0.3 μg pEGFP. The c-Abl(Km) plasmid drives c-Abl synthesis with a point mutation in the kinase domain. This mutant is inactive in kinase reactions (data not shown). Cells were photographed 72 h after transfection (arrowheads: apoptotic morphology). **b**, Average percentage of apoptotic cells from five randomly chosen fields. **c**, Protein extracts from cells transfected with the indicated plasmids were immunoblotted with the 8E9 anti-Abl antibody.

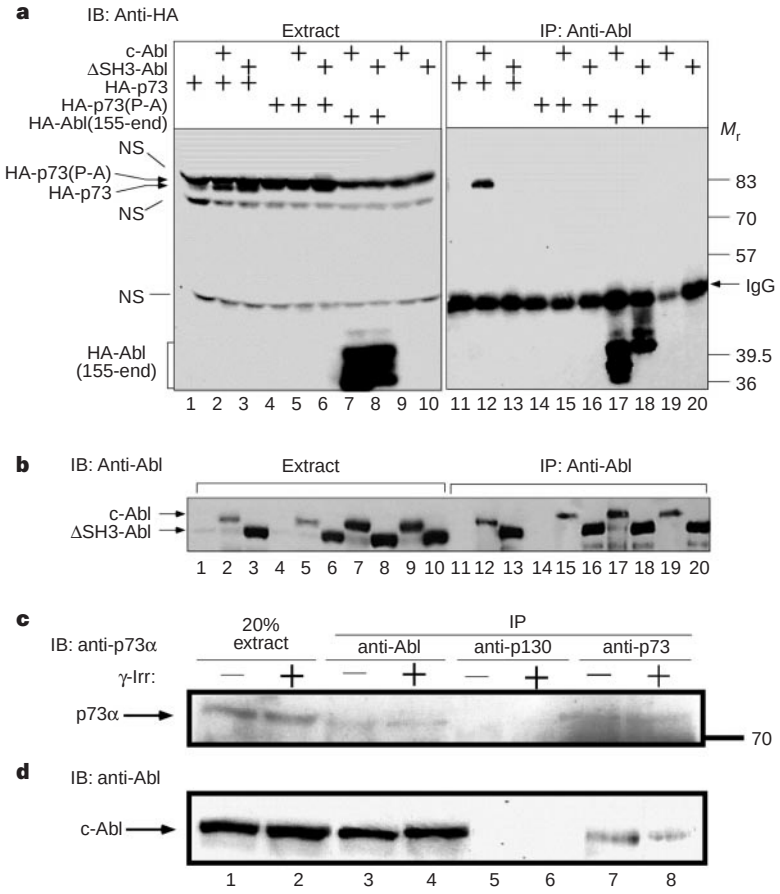

Figure 3 Interaction of p73 with c-Abl is mediated by the SH3 domain of c-Abl and a PxxP motif in p73. **a**, Cellular extracts were prepared from 293T cells transfected with 10 μg of the indicated expression plasmids. 90% of the extracts was immunoprecipitated with the K-12 anti-Abl antibody. The beads were extensively washed and bound proteins were eluted. 10% of the extracts (extract) and 50% of the eluted fractions (IP) were separated by 7.5% SDS–PAGE and immunoblotted with anti-HA antibody. The exposure times for the extract panel and the IP panel were 20 s and 5 min, respectively. The positions of HA–p73, its point mutant HA–p73(P–A), the c-Abl binding protein HA–Abi1(155–end) and a non-specific (NS) background band are indicated. The P–A mutant migrates more slowly than the wild type. **b**, The same blot, re-probed with the 8E9 anti-Abl antibody. The positions of intact c-Abl and its mutant lacking the SH3 domain (ΔSH3-Abl) are shown. **c**, MCF-7 cells were either exposed to 20 Gy of γ-irradiation (γ-Irr) or left untreated. Two hours later, cells were lysed and cleared lysates were immunoprecipitated (IP) with the indicted antibodies. The eluted proteins and 20% of the total extracts were separated by 7.5% SDS–PAGE and immunoblotted with anti-p73α antibody. **d**, The blot in **c**, re-probed with the 8E9 anti-Abl antibody.

GFP (Fig. 1d, F-GFP and H2B-GFP). In contrast, nuclear condensation was not seen in cells transfected transiently with GFP alone (Fig. 1d, control). The data are plotted in Fig. 1e.

Our results indicate that p73 and c-Abl collaborate to induce apoptosis. This conclusion is further supported by experiments in which both p73 and c-Abl were co-transfected into $c\text{-}Abl^{-/-}$ cells. Extensive apoptosis was induced when p73 was introduced with wild-type c-Abl, but not with a kinase-defective c-Abl mutant (c-Abl(Km), Fig. 2a, b), even though the two types of c-Abl proteins were produced in comparable amounts in the transfected cultures (Fig. 2c). Thus, the kinase domain of c-Abl is required for the induction of apoptosis in collaboration with p73.

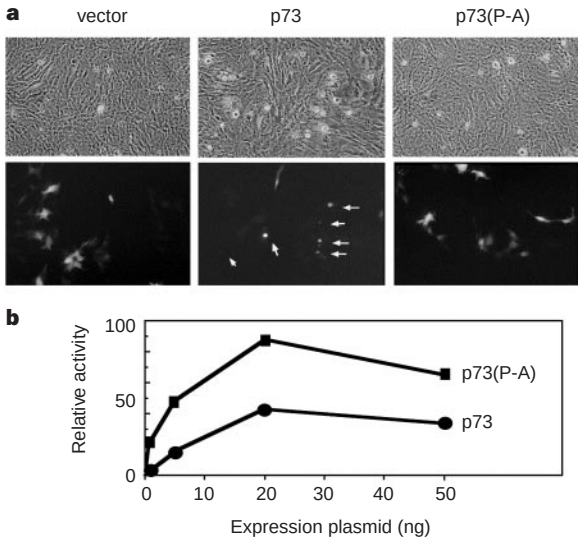

Figure 4 The PxxP motif of p73 is necessary for apoptosis but not for transcriptional activation. **a**, The c-Abl-reconstituted cells described in Fig. 1 were transfected with 3 μg of expression plasmid encoding either p73 or p73(P–A) with 0.5 μg of pEGFP. Cells were photographed 96 h after transfection. **b**, Hep3B cells were co-transfected with two reporter plasmids: one containing firefly luciferase under the *mdm2* promoter and the other containing renila luciferase under the SV40-promoter (SVRL, Promega), with the indicated amounts of either p73 or p73(P–A) expression plasmids. Cells were collected 36 h after transfection and analysed using the dual luciferase kit (Promega). Relative activity was calculated as 100 × firefly-luciferase/renila-luciferase. Similar results were obtained in three independent experiments.

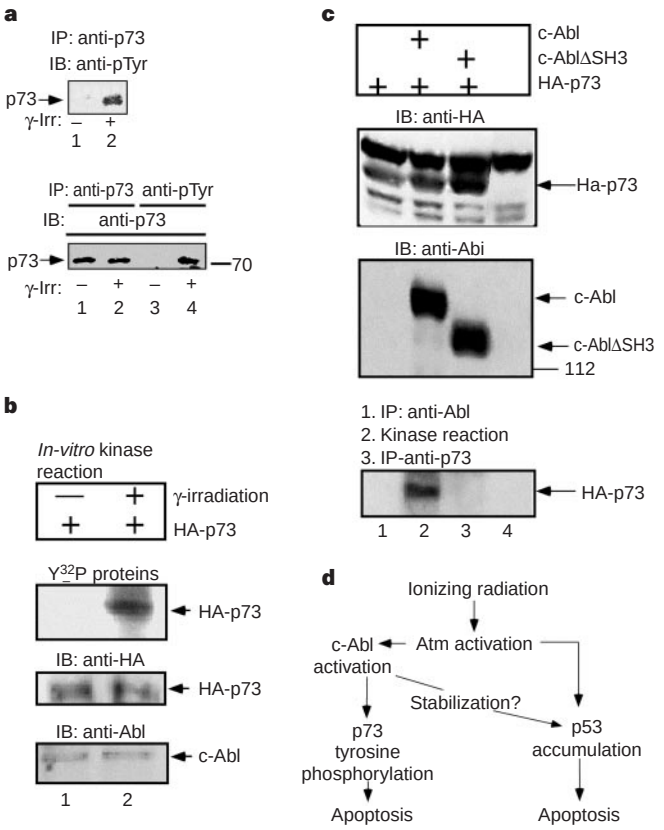

Figure 5 p73 is a substrate of the c-Abl kinase. **a**, MCF-7 cells were γ-irradiated and treated as in Fig. 3. Extracts were subjected to immunoprecipitation (IP) with anti-p73α antibody and the precipitated proteins were analysed by western immunoblotting (IB) using anti-pTyr antibody. In a reciprocal experiment, the anti-p73α antibody was used for IB (lower panel). **b**, MCF-7 nuclear extracts were prepared, either before or after γ-irradiation, and immunoprecipitated with the K-12 anti-Abl antibody. Immunoaffinity-purified HA-p73 protein, prepared from transiently transfected 293T cells, was added to the c-Abl-beads complex and incubated under kinase reaction conditions in the presence of [γ-^{32}P]ATP. The products were resolved by SDS–PAGE, blotted and treated with 1 M KOH for 2 h at 65 °C to detect solely phospho-tyrosines (upper panel). The quantity of c-Abl and HA–p73 in the reaction was monitored by the corresponding antibodies (IB, lower panels). **c**, 293T cells were transfected with the indicated expression plasmids and cellular protein extracts were prepared and analysed. Proper expression of the transfected plasmids was confirmed by immunoblotting with anti-HA and anti-Abl. Extracts were immunoprecipitated with the K-12 anti-Abl antibody and subjected to an *in vitro* kinase reaction for 1 h at 30 °C in the presence of [γ-^{32}P]ATP. The reaction was stopped and subjected to a second round of immunoprecipitation with the anti-HA antibody for 2 h at 4 °C. HA–p73 was eluted with 1 mg ml^{-1} HA peptide. The eluates were resolved on 7.5% SDS–PAGE, blotted onto a nylon membrane and alkaline-treated as in **b**. **d**, Proposed signalling cascades triggered by ionizing radiation. Activation of the Atm kinase results in c-Abl kinase activation, leading to p73 tyrosine-phosphorylation and p53 accumulation. The accumulation of p53 is activated in part through direct phosphorylation of p53 by Atm[12,22]. The activation of c-Abl may also contribute to p53 stabilization by protecting it against Mdm2-directed proteolysis[23].

We investigated whether p73 and c-Abl could associate with each other by using cells co-transfected transiently with expression plasmids for c-Abl and HA–p73. To facilitate the detection of protein–protein interactions, we used human 293 cells, which are more readily transfectable than mouse c-Abl$^{-/-}$ fibroblasts. As shown in Fig. 3a, HA–p73 was brought down by a c-Abl-specific polyclonal antibody, but only when wild-type c-Abl was co-expressed in the same cells (lanes 11, 12, 19). The same was true for Abi1, a c-Abl binding protein[4] serving as a positive control (lane 17). Hence, p73 and c-Abl can interact specifically in transfected cells.

Inspection of the p73 protein sequence revealed that the putative linker region, located between the DNA-binding domain and the predicted oligomerization domain, contains a consensus PxxP motif—a potential site of interaction with the SH3 domain of c-Abl[5,6]. The existence of such an interaction was investigated using a c-Abl mutant carrying a deletion that removes the SH3 domain (c-AblΔSH3), and a p73 mutant carrying a substitution from proline to alanine at residue 338 (P338A). Despite the fact that both mutants were expressed in transfected cells at levels comparable to those of the respective wild-type proteins (Fig. 3a, b, extract), none produced stable p73–c-Abl complexes (Fig. 3a, lanes 13–16). In contrast, the Abi1 polypeptide was brought down with either wild-type or SH3-domain-deleted c-Abl protein (lanes 17, 18), confirming that this interaction is independent of the c-Abl SH3 domain[4,7]. Hence, the specific interaction between c-Abl and p73 requires the SH3 domain of c-Abl and the PxxP motif of p73.

To study p73–c-Abl interactions in non-transfected cells, extracts of MCF7 cells were either γ-irradiated or left untreated, and analysed using co-immunoprecipitation. p73α was brought down by antibodies against c-Abl (Fig. 3c, lanes 3, 4), but not by control antibodies against p130 (lanes 5, 6). Specific co-precipitation was also seen in the reciprocal experiment, in which c-Abl was brought down by antibodies against p73 (Fig. 3d, lanes 7, 8). This indicates that c-Abl and p73α interact specifically under physiological conditions in non-overexpressing cells. Neither the total amount of p73 nor its interaction with c-Abl were affected by γ-irradiation (γ-Irr, Fig. 3c, d).

To test whether the induction of apoptosis by c-Abl and p73 depends on their physical interaction, c-Abl-reconstituted fibroblasts (pBABE–c-Abl, Fig. 1) were transfected with either wild-type p73 or the P338A mutant of p73. In agreement with the data described earlier, wild-type p73 induced apoptosis in many of the transfected cells (Fig. 4a, p73). In contrast, the P338A mutant did not (Fig. 4a, p73(P–A)). This mutant could, however, support transcription of a luciferase reporter gene driven by the p53/p73-

responsive *mdm2* promoter. In fact, it was even more potent in this respect than intact wild-type p73 (Fig. 4b). In the case of the p53 protein, deletion of the PxxP motif also results in loss of apoptotic capacity[8,9]. Importantly, although the deleted p53 can still activate many promoters, it fails to induce the promoter of the apoptosis-related *PIG3* gene[10]. Although the *PIG3* promoter does not appear to be a p73 target[11], p73(P–A) may therefore be defective for the activation of a limited subset of genes. Nevertheless, the overall transcriptional competence of p73(P–A) indicates that it retains many of its molecular interactions. Hence the defect in apoptosis is highly specific, implying a non-random relationship between the ability of p73 to bind c-Abl and its ability to induce apoptosis.

These findings raise the possibility that p73 is a substrate of the c-Abl kinase. The c-Abl kinase is activated by γ-irradiation[1], and endogenous p73α becomes phosphorylated on tyrosine after irradiation (Fig. 5a). To determine whether c-Abl may be responsible for this phosphorylation, we immunoaffinity-purified endogenous c-Abl from MCF7 cells and measured its ability to phosphorylate purified HA–p73 *in vitro*. HA–p73 was tyrosine-phosphorylated only when the c-Abl was purified from irradiated cells (Fig. 5b). Moreover, overexpressed c-Abl from transfected 293T cells could efficiently phosphorylate the co-precipitated p73α *in vitro* (Fig. 5c); as expected, this required the SH3 domain.

Collectively, our data support the existence of a signalling pathway that involves both p73 and c-Abl as mediators of apoptosis (Fig. 5d). Unlike p53, p73 protein levels do not increase following genotoxic stress[2]. However, c-Abl does respond to at least certain conditions of genotoxic stress, including ionizing radiation. Under such conditions, the kinase activity of c-Abl is induced, presumably through the action of the stress-activated[12] ATM protein kinase[13,14]. The facts that an intact kinase domain is essential for the cooperation of c-Abl with p73 in induction of apoptosis, and that p73 is a substrate of stress-induced c-Abl, indicate that p73 might be recruited into a stress-induced signalling complex through its association with c-Abl. □

Methods

Plasmid constructions and antibodies. To generate the pBABE-Puro–c-Abl plasmid, the retroviral vector pBABE-Puro was digested with *Eco*RI and *Bam*HI and filled in by the Klenow enzyme. The c-*abl* fragment insert was excised from pSP6–c-Abllb (ref. 15) with *Eco*RI and *Fsp*I, and ligated to the pBABE-Puro vector after filling in. For transfection experiments, we constructed pSG5–c-Abl by cloning the *Eco*R1–*Fsp*I c-Abllb complementary DNA fragment into Bluescript KS (Strategene) at the *Eco*RI–*Sma*I sites. The resulting plasmid was digested with *Eco*RI and *Bam*HI and the c-Abl fragment was inserted into the pSG5 vector (Strategene). We constructed the c-Abl-kinase-mutant expression plasmid pSG5-c-Abl(Km) using site-directed mutagenesis[16] with the oligonucleotide 5′-GACGGTGGCCGTGCACACCTTGAAGGAG-3′. The c-AblΔSH3 expression vector was constructed by digesting pSG5–c-Abl with *Hin*dIII and *Hin*CII and re-ligating the deleted plasmid in the presence of a linker oligonucleotide.

The expression plasmid for wild-type simian p73α (ref. 3) was a gift from W. J. Kaelin. The pSG5HA–p73α PxxA mutant was prepared by PCR using the primers 5′-ATGAGCCACCACAGGTGGGGAC-3′, 5′-CCCCCTGCCGTCGCCGCCCT-3′ 5′-AGGGCGGCGACGGCAGGGGG-3′ and 5′-GCTCTAGATCAGTGGATCTCGGCCTCCGTG-3′. The resulting PCR product was digested with *Xba*I and *Eco*RI and inserted back into the HA–p73 cDNA to form HA–p73(P–A). We constructed the expression plasmid pSG5HA–Abi1(155–end) by cloning the mouse cDNA coding for Abi1(155–end) from total RNA of an $Abl^{-/-}$ mouse cell line using RT-PCR (Promega) and the primers 5′-GCTCTAGAAGCGGAAGCCGAGAGAACAG-3′ and 5′-CGGGATCCTAATCAGTATAGTGCATGA-3′. The resulting fragment was digested with *Xba*I and *Bam*H1, and cloned into the pSG5–HA vector digested with *Nhe*I and *Bam*H1. We used the pEGFP expression plasmid (Clontech) for expression of the GFP under the cytomegalovirus promoter/enhancer. A plasmid encoding a histone 2B/GFP fusion protein was a gift from T. Kanda[17]. The F-GFP plasmid has been described[18]. All constructions and mutants were verified by enzymatic digestion and sequencing.

The antibodies used for western-blot analysis are the monoclonals 12CA5 anti-HA, 8E9 anti-Abl (Pharmingen), and the polyclonal K-12 anti-Abl (sc-131), anti-phosphotyrosine PY-99 and anti-p73α (Santa Cruz). The anti-HA monoclonal 11HA (Babco) was used for immunostaining of transfected cells[19].

Cell transfection and retroviral infection. Cell transfection was done either by using the calcium phosphate co-precipitation method[20], or with lipofectAMINE (Gibco-BRL) as recommended by the manufacturer. We generated high-titre retroviral supernatants by transfecting 293T cells with retroviral plasmids together with the Ψ-plasmid in the presence of 0.2 mM chloroquine, as described[21]. Cells were washed after 8 h and the medium was collected at intervals of 8–10 h for 38 h. Retroviral infection of the $Abl^{-/-}$ cell line was done by incubation of a 50%-confluent 10-cm dish with 2 ml of retrovirus-containing medium for 4 h together with 10 μg ml^{-1} Polybrene. After a further 24 h, cells were selected with 2.5 μg ml^{-1} puromycin (Sigma).

Co-immunoprecipitation experiments. 293T cells were transfected with 20 μg DNA per 10-cm dish, washed 12 h after transfection and collected after an additional 48 h. Cells were lysed in 300 μl lysis buffer containing 10 mM β-glycerophosphate, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, 0.5% N-P40, 1 M sucrose, 1 mM sodium vanadate, 1 mM DTT and a protease-inhibitor cocktail (Sigma), incubated for 10 min on ice and centrifuged at 13,000 r.p.m. for 10 min at 4 °C. Immunoprecipitation was carried out in 600 μl lysis buffer containing 200 μl cellular extract, 50 μl of 50% slurry protein A–Sepharose beads (Pharmacia Tech) and 2 μg of K-12 anti-Abl antibody for 2 h at 4 °C. The beads were then washed five times and the bound proteins were eluted by boiling in SDS-sample buffer and resolved by 7.5% SDS–PAGE.

Received 17 March; accepted 19 April 1999.

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Acknowledgements. We thank W. J. Kaelin for the p73α plasmid; T. Kanda for the H2B-GFP plasmid; W. Jiang and T. Hunter for E-GFP; S. P. Goff for the Abi plasmids; and R. Bernards for allowing some of the work to be performed in his laboratory. This work was supported by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (Y.S.), by the Ebner Family Biomedical Research Foundation at the Weizmann Institute of Science in Memory of Alfred and Dolfi Ebner (Y.S.), by the NCI (M.O.) and by Telethon (Italy) (G.B.).

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