

## p73 Is Regulated by Phosphorylation at the G<sub>2</sub>/M Transition\*

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**p73 is a p53 paralog that encodes proapoptotic (transactivation-competent (TA)) and antiapoptotic (dominant negative) isoforms. TAp73 transcription factors mediate cell cycle arrest and/or apoptosis in response to DNA damage and are involved in developmental processes in the central nervous system and the immune system. p73 proteins may also play a role in the regulation of cell growth. Indeed, p73 expression is itself modulated during the cell cycle and TAp73 proteins accumulate in S phase cells. In addition, the function of p73 proteins is also regulated by post-translational modifications and protein-protein interactions in different cellular and pathophysiological contexts. Here we show that p73 is a physiological target of the p34<sup>cdc2</sup>-cyclin B mitotic kinase complex *in vivo*. Both p73 $\beta$  and p73 $\alpha$  isoforms are hyperphosphorylated in normal mitotic cells and during mitotic arrest induced by microtubule-targeting drugs. p34<sup>cdc2</sup>-cyclin B phosphorylates and associates with p73 *in vivo*, which results in a decreased ability of p73 to both bind DNA and activate transcription in mitotic cells. Indeed, p73 is excluded from condensed chromosomes in meta- and anaphase, redistributes throughout the mitotic cytoplasm, and unlike p53, shows no association with centrosomes. Together these results indicate that M phase-specific phosphorylation of p73 by p34<sup>cdc2</sup>-cyclin B is associated with negative regulation of its transcriptional activating function.**

p73 is a nuclear protein that shares substantial sequence homology and functional similarities with p53 (1–3). Multiple TA<sup>1</sup> (transactivation-competent, pro-apoptotic, and anti-proliferative) p73 COOH-terminal splicing isoforms ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ ,  $\zeta$ ) exist (4, 5). In addition, dominant negative variants, which are

expressed from a second promoter, lack the amino-terminal transactivation domain, act as trans-repressors of p53- and p73-dependent transcription, and possess anti-apoptotic and pro-proliferative potential (6–8). This complex expression strategy of the p53 family of tumor suppressors may explain why the attempt to correlate p53 status with prognosis and response to anticancer treatments has proved of limited clinical impact in the past.

Although exogenously expressed TAp73 proteins transactivate endogenous targets of p53, several lines of evidence indicate that *in vivo* p53 and p73 may be differentially regulated and carry out specialized, non-overlapping functions. Indeed, p73- and p63-deficient mice exhibit specific phenotypes related to abnormal differentiation of brain and skin, respectively, but do not show defects related to spontaneous or x-ray-induced apoptosis during embryogenesis, nor do they develop spontaneous cancers (9–11). Second, p53 is induced in response to a variety of DNA-damaging agents and cellular stressors, whereas p73 is induced only by a subset of agents, including cisplatin, doxorubicin, and irradiation, in a p53-independent/*c-abl*-dependent manner (12–14). Finally, the specificity of p73 proteins role in the apoptotic response to DNA damage is further underlined by the recent evidence that p73 is required for p53-dependent apoptosis induced by DNA damage (15) and that blocking p73 function with a dominant negative or by specific small interfering RNA leads to chemoresistance of human tumor cells, irrespective of their p53 status (14). In addition, p73 mediates T-cell receptor activation-induced cell death (TCR-AICD), a p53-independent apoptotic process that occurs in mature peripheral T cells (16). Thus, p73 and p53 act through specific and distinctive pathways, although some of their functions clearly overlap.

p73 proteins are regulated both at the transcriptional level and by post-translational modifications in different cellular and pathophysiological contexts. The p73 gene has been reported to be transcriptionally regulated by the transcription factor E2F1 in apoptosis induced by E2F1 overexpression (17, 18), in response to DNA damage (19), and in physiological conditions during the S phase of the cell cycle (18). Specific phosphorylation and acetylation events contribute to the activation of the p73 gene product in response to DNA damage (12, 13, 20, 21) and bolster p73 apoptotic functions by potentiating the selective recruitment of p73 onto the promoters of apoptotic target genes *versus* genes involved in cell cycle arrest and re-entry (20).

A number of positive and negative regulators have been shown to interact with or modulate the activity of p73. Hmdm2 inhibits p73 transcriptional activity but does not affect its

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This work is dedicated to our late colleague and friend Franco Tatò.

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<sup>1</sup> The abbreviations used are: TA, transactivation-competent; IKK, I $\kappa$ B kinase; DAPI, 4,6-diamidino-2-phenylindole; CDK, cyclin-dependent kinase; CMV, cytomegalovirus; GST, glutathione *S*-transferase.

stability (22–25), and a subset of tumor-derived p53 mutants block p73 functions (26–30). On the other hand, the WW domain containing YES-associated protein (YAP) binds p73, but not p53, and potentiates its transcriptional activity toward apoptotic target genes (31). More recently, it has been also reported that p73 interacts physically with various cyclins *in vitro*, including cyclins A, B, D, and E, and that multiple p73 isoforms are phosphorylated by CDK1- and CDK2-containing complexes at threonine 86; however, the physiological relevance of these interactions *in vivo* has not yet been clearly established (32).

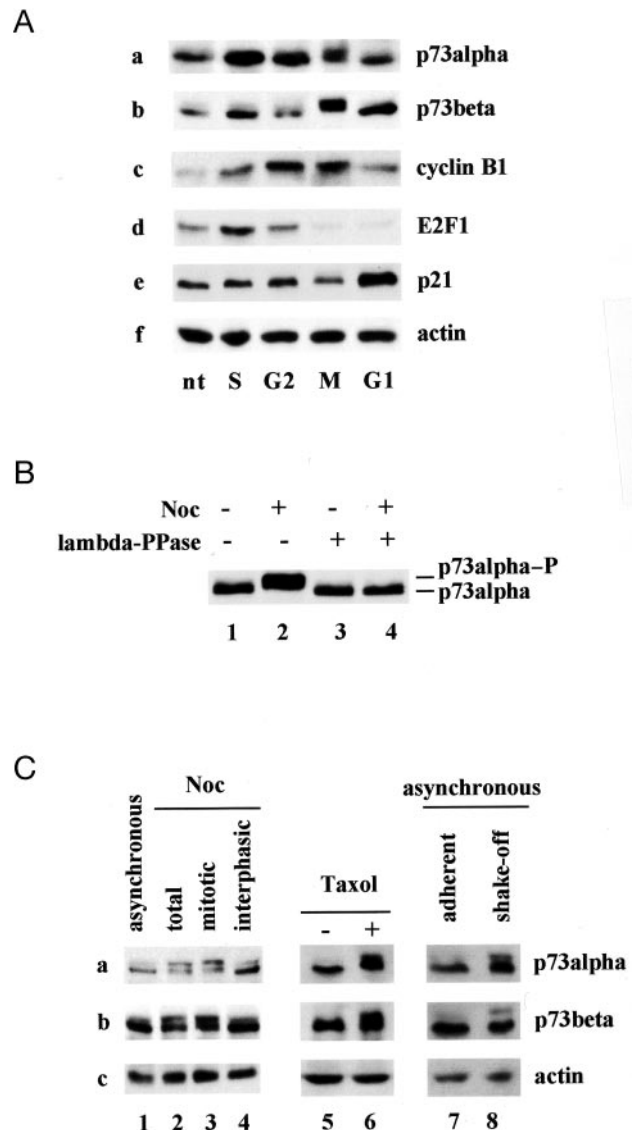
Here we show that p73 is a target of the p34<sup>cdc2</sup>-cyclin B mitotic kinase complex *in vivo*. Both p73 $\beta$  and p73 $\alpha$  isoforms are hyperphosphorylated during physiological mitosis and during mitotic arrest induced by microtubule-targeting drugs. p34<sup>cdc2</sup>-cyclin B phosphorylates and associates with p73 *in vivo*, which results in a decreased ability of p73 to both bind DNA and activate transcription in mitotic cells. This is associated with p73 detachment from condensed chromosomes and redistribution throughout the mitotic cytoplasm in meta- and anaphase.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfections**—Human HCT116(3)(6) (colon carcinoma), U2OS (osteosarcoma), MCF7 (breast cancer), and HeLa (cervix carcinoma) cells were cultured in Dulbecco's modified Eagle's medium plus antibiotics and 10% heated-inactivated fetal bovine serum. HCT-116(3) cells are a subclone of the HCT-116 colon carcinoma cell line in which chromosome 3 was reintroduced to restore the ability to activate *c-abl* in response to cisplatin (33). The human lung carcinoma cell line H1299 was maintained in RPMI medium supplemented with 10% fetal calf serum. H1299 ponasterone-inducible p73 $\alpha$  and wild-type p53 cell lines were described elsewhere (28, 34). Transient transfections were performed by either calcium phosphate precipitation or using the LipofectAMINE Plus reagent (Invitrogen). Samples for flow cytometry analysis of cell cycle distribution were prepared by fixing the cells in 70% ethanol (30 min, 4 °C), washing twice in phosphate-buffered saline plus 0.5% Tween 20, and stained with 50  $\mu$ g/ml propidium iodide. Samples were analyzed in a FACStar Plus (BD Biosciences) flow cytometer (10,000 events/sample) using the WinMDI software.

**Antibodies, Plasmids, Recombinant Proteins, and Reagents**—Antibodies directed against human p34<sup>cdc2</sup> (17), cyclin B1 (clone GNS1), p21 (clone H164), IKK $\alpha$  (clone C20), E2F1 (clone C20), actin (clone I19), and the HA tag (clone Y11) were purchased from Santa Cruz Biotechnology, Inc. The mouse monoclonal anti-p73 antibody (clone 1288, Imgenex Inc.) recognizes all known splicing variants of the human and mouse p73.<sup>2</sup> The polyclonal anti-p73 H79 antibody (Santa Cruz) was used in immunoprecipitation experiments. Murine anti- $\gamma$ -tubulin antibodies were from Dako, Inc. Secondary anti-mouse fluorescein isothiocyanate- and rhodamine-conjugated antibodies were from Sigma and Jackson ImmunoResearch, respectively. The pCDNAHA, pCDNAHA-p73 $\alpha$ , pCDNAHA-p73 $\beta$ , and pCDNAHA-p53 expression vectors and the p21-luc reporter plasmid were described previously (4, 13). The pCDNAHA-p73DD plasmid expresses a p73 miniprotein that acts as a selective dominant negative of p73-dependent, but not p53-dependent, transcription (Ref. 18 and data not shown). pCDNAHA-p73 $\alpha$ (T86A) was generated by PCR mutagenesis. The CMVneoHap34<sup>cdc2</sup> and CMVneoHap34<sup>cdc2</sup> dominant negative plasmids were kindly provided by Dr. van den Heuvel (Boston, MA) (35). CMV-neo-myc-cyclin B1 was kindly provided by G. Piaggio. Prokaryotic expression vectors for GST-p73 $\alpha$ , GST-p73 $\beta$ , GST-p73-(1–120), GST-p73-(1–321), GST-p73-(120–321), GST-p73-(321–636), GST-p73-(1–249), and GST-p73-(1–249)/T86A, were constructed by standard PCR techniques (primers available upon request) and verified by sequencing. GST fusion proteins were purified as described previously (28). Histone H1, nocodazole, paclitaxel (taxol), propidium iodide, hydroxyurea, and olomoucine were purchased from Sigma. Ponasterone A was from Alexis Biochemical, Inc.

**Immunoblotting, Immunoprecipitations, and Kinase Assays**—Immunoblotting and immunoprecipitation assays were performed as described (13). For p34<sup>cdc2</sup>-cyclin B1 kinase assay, immunoprecipitates and purified GST fusion proteins were incubated in kinase buffer (20  $\mu$ l)



**FIG. 1. p73 $\alpha$  and - $\beta$  are phosphorylated during mitosis.** *A*, mobility shift of p73 proteins in nocodazole-arrested cells. Hct116(3) cells were treated with hydroxyurea (2 mM) for 15 h and collected 1 and 6 h after wash-out to obtain cells enriched in S and G<sub>2</sub> phases, respectively (43). The percentage of S phase cells was evaluated by bromodeoxyuridine incorporation (data not shown). Mitotic cells were obtained after 16 h of nocodazole treatment (50 ng/ml); G<sub>1</sub>-enriched cultures were collected 6 h nocodazole after release. *nt*, not treated (asynchronously growing untreated cells). Extracts were analyzed by immunoblotting using antibodies directed against the proteins, as indicated on the right (also see “Experimental Procedures”). *B*, mitotic mobility shift of p73 is due to phosphorylation. Extracts from either nocodazole-arrested (*Noc*, lanes 2 and 4) or interphasic (lanes 1 and 3) Hct116(3) cells were incubated at 30 °C for 1 h in the presence (lanes 3 and 4) or absence of  $\lambda$ -phosphatase (*PPase*) (lanes 1 and 2). Cell extracts were then analyzed by immunoblotting using 1288 anti-p73 antibody. *C*, p73 is hyperphosphorylated in nocodazole- and taxol-arrested cells and in untreated mitotic cells. Hct116(3) cells were treated with either nocodazole (50 ng/ml, left panel) or taxol (1  $\mu$ M, middle panel) for 16 h. Nocodazole-arrested mitotic cells were separated from interphasic cells by vigorous shaking. Extracts prepared from asynchronously growing cells (lanes 1, 5, 7, and 8), total (lane 2), mitotic (lane 3), and interphase (lane 4) nocodazole-treated cells, taxol-treated cells (lane 6), and mitotic cells collected by shake-off from untreated asynchronously growing HCT116(3) cultures (lane 8) were analyzed by immunoblotting using anti-p73 (rows *a* and *b*) and anti-actin (row *c*) antibodies.

containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 100  $\mu$ M ATP, and 15  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP in the presence of 20 units of highly purified recombinant p34<sup>cdc2</sup>-cyclin B complex (New England Biolabs). The reaction was incubated for 30 min at 30 °C and

<sup>2</sup> Y. J. Y. Wang and A. Costanzo, unpublished observations.

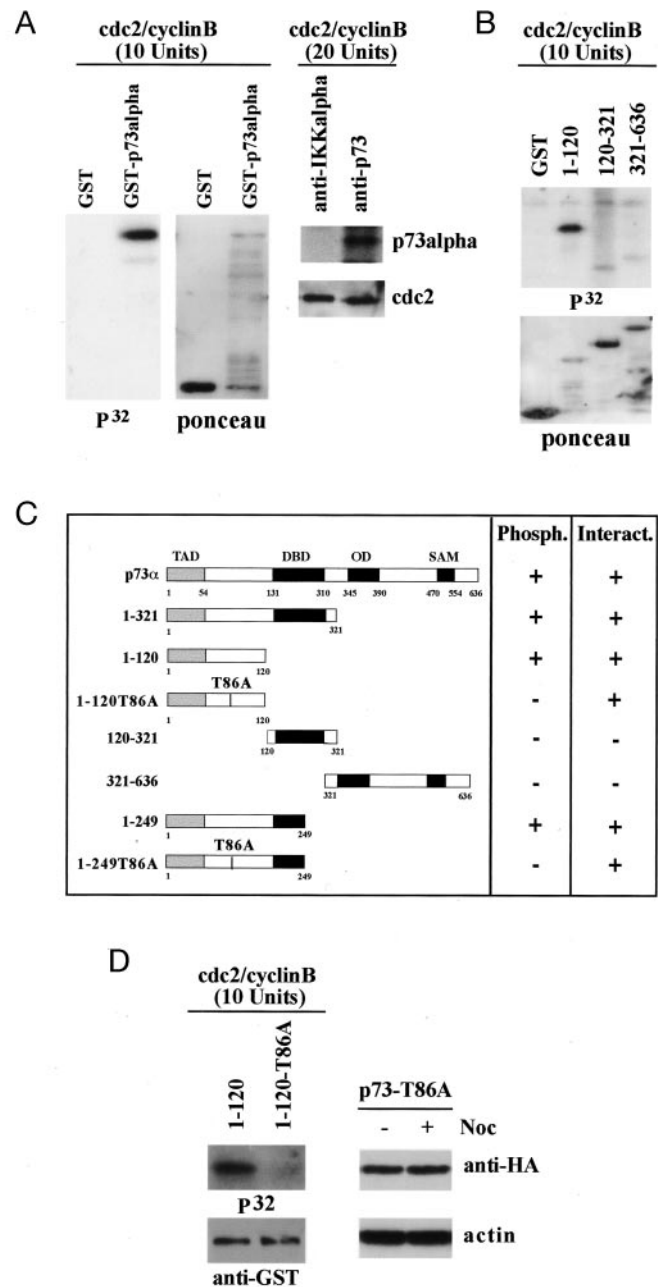
terminated by adding 10  $\mu$ l of 3 $\times$  SDS sample buffer. Samples were resolved through 10% SDS-PAGE, blotted on polyvinylidene difluoride membranes and analyzed by autoradiography. For pull-down assays, 2  $\mu$ g of either immobilized purified GST fusion proteins or wild-type GST were incubated for 2 h at 4  $^{\circ}$ C in the presence of 0.5  $\mu$ g of a 55-kDa full-length p34<sup>cdc2</sup> fusion protein produced in *Escherichia coli* (Santa Cruz). After 6 washes in radioimmune precipitation buffer, proteins were resolved by SDS-PAGE and probed with anti-p34<sup>cdc2</sup> antibody.

**Binding to Biotinylated Oligonucleotides**—200 ng of biotinylated oligonucleotides encompassing the p53-binding sites of the p21 promoter were immobilized to Dynabeads M-280 streptavidin (Dyna, AS, Oslo, Norway) and then incubated for 30 min at room temperature in the presence of *in vitro* translated (TNT, Promega) p73 $\alpha$  and p73 $\alpha$ -T86A proteins. After three washes, oligonucleotide-bound p73 proteins were incubated in 20  $\mu$ l of kinase buffer with (20 units) or without highly purified recombinant Cdc2-cyclin B complex (New England Biolabs). Where indicated the p34<sup>cdc2</sup>-cyclin B complex was preincubated with 0.5 mM olomoucine for 10 min at room temperature. After a 30-min incubation at 30  $^{\circ}$ C, oligonucleotide-bound p73 proteins were washed twice and resolved through 10% SDS-PAGE, blotted to polyvinylidene difluoride membranes, and analyzed using the 1288 anti-p73 antibody.

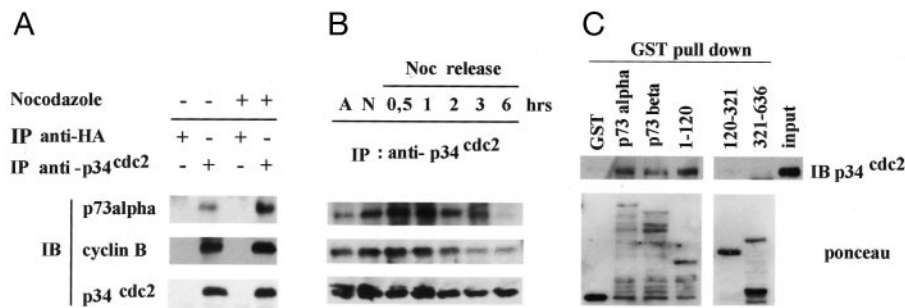
**Cytological Preparations and Microscopy**—Cells were fixed in 3.7% paraformaldehyde and processed for indirect immunofluorescence using antibodies against p73 (1:100, Imgenex) and p53 (1:200, Dako)  $\gamma$ -tubulin (3.5  $\mu$ g/ml in phosphate-buffered saline, 0.1% Tween 20, 5% fetal calf serum) followed by Texas Red- and fluorescein isothiocyanate-conjugated secondary anti-mouse IgG antibody (800-fold dilution in phosphate-buffered saline, 0.1% Tween 20, 5% fetal calf serum) before counterstaining in 0.1  $\mu$ g/ml DAPI. Images were analyzed using an Olympus AX70 microscope configured for epifluorescence and equipped with a cooled camera device (Photometrics).

## RESULTS AND DISCUSSION

**p73 $\alpha$  and - $\beta$  Are Phosphorylated during Mitosis**—As a first step toward extending our current knowledge of p73 regulation during the cell cycle (see the Introduction and references therein), we examined p73 steady state levels in cells that were drug-synchronized in different cell cycle phases. As shown in Fig. 1A, the amount of both p73 $\alpha$  and - $\beta$  isoforms increases in S phase-enriched cultures as compared with exponentially growing asynchronous cells (Fig. 1A, compare *nt* and *S* in rows *a* and *b*). The increase in p73 parallels the increase in E2F1 protein levels, consistent with the notion that p73 is regulated by E2F1 during G<sub>1</sub>/S progression (18). p73 protein levels decrease in cells progressing from S into the G<sub>2</sub> phase (Fig. 1A, compare lanes *S* and *G*<sub>2</sub> in rows *a* and *b*). In nocodazole-arrested M phase cells, p73 $\beta$  levels increase, and both p73 $\alpha$  and p73 $\beta$  isoforms display a slower electrophoretic mobility (Fig. 1A, lane *M* in rows *a* and *b*).  $\lambda$ -Phosphatase treatment shifted the migration of mitotic p73 $\alpha$  back to that of its interphase counterpart (Fig. 1B, compare lanes 3 and 4 with lanes 1 and 2), indicating that p73 proteins are modified by phosphorylation in nocodazole-treated cells. To establish whether p73 phosphorylation observed in nocodazole-arrested cells reflected a mitotic specific modification, we separated mitotic from interphase cells by vigorous shaking and found that p73 hyperphosphorylation was confined to mitotic cells and was absent in culture samples that remained adherent and were enriched in G<sub>2</sub> phase cells (Fig. 1C, left panel). Both p73 $\alpha$  and - $\beta$  displayed a similar mobility shift in taxol-treated cells (Fig. 1C, middle panel); thus, p73 phosphorylation was associated with mitotic arrest, irrespective of the drug specificity in the mechanism of action. Most importantly, p73 shift was also observed in mitotic cells collected by “shake-off” from an asynchronous population of untreated HCT116(3) cells (Fig. 1C, right panel). Similar results were obtained in human HeLa and breast cancer MCF7 cells (data not shown). Overall these results indicate that p73 $\alpha$  and - $\beta$  are subjected to mitosis-specific phosphorylation, both during the physiological mitotic division and during the mitotic arrest induced by microtubule-targeting drugs.



**FIG. 2. p73 protein associates with and is phosphorylated by p34<sup>cdc2</sup>-cyclin B complex.** A, p73 is phosphorylated by the p34<sup>cdc2</sup>-cyclin B complex *in vitro*. Left panel, GST-p73 $\alpha$  and GST were used as substrates for an *in vitro* kinase assay with recombinant p34<sup>cdc2</sup>-cyclin B complex. Middle panel, Ponceau Red staining of the same filter, showing the input GST proteins. Right panel, endogenous p73 $\alpha$  and IKK $\alpha$  proteins immunoprecipitated from HCT116(3) cells with the anti-p73 and anti-IKK $\alpha$  antibodies, respectively, were subjected to an *in vitro* kinase assay with 20 units of purified recombinant p34<sup>cdc2</sup>-cyclin B complex (New England Biolabs) (upper right). The amount of recombinant p34<sup>cdc2</sup> was evaluated by immunoblotting using anti-p34<sup>cdc2</sup> antibody (lower right). B, the amino-terminal region of p73 (residues 1–120) is targeted by p34<sup>cdc2</sup>-cyclin B phosphorylation. GST and GST-p73 deletion mutants (maps in panel C) were subjected to *in vitro* kinase assay with purified recombinant p34<sup>cdc2</sup>-cyclin B complex. Below, the same filter was stained with Ponceau Red to visualize the input GST proteins used. C, schematic representation of GST-p73 mutant structure, phosphorylation and physical interaction with p34<sup>cdc2</sup>-cyclin B. D, mapping of the phosphorylation target in p73. Left panel, *in vitro* kinase assay of GST-p73-(1–120) and GST-p73-(1–120)-T86A with 10 units of purified p34<sup>cdc2</sup>-cyclin B complex (upper panel). Input GST proteins were visualized by immunoblot (lower panel). Right panel, electrophoretic mobility of T86A p73 $\alpha$  protein exogenously expressed in Hct116(3) cells is not affected by nocodazole treatment (50 ng/ml).



**FIG. 3. p73 protein associates with the p34<sup>cdc2</sup>-cyclin B complex in mitosis.** **A**, p73 binds to p34<sup>cdc2</sup> *in vivo*, and this interaction increases in mitotic cells. Asynchronously growing or nocodazole-arrested Hct116(3) cells were detergent-solubilized and immunoprecipitated with either anti-p34<sup>cdc2</sup> (lanes 2 and 4) or anti-HA tag (lanes 1 and 3) antibodies. Co-immunoprecipitated proteins (IP) were analyzed by immunoblotting with antibodies to p73, cyclin B, and p34<sup>cdc2</sup>. **B**, the physical association between p73 and p34<sup>cdc2</sup>-cyclin B decreases during exit from mitosis. Nocodazole-arrested Hct116(3) cells were allowed to resume mitosis after nocodazole removal. Cell extracts from asynchronously growing (A) or nocodazole-arrested (N) or -released cells after increasing times (Noc release) were immunoprecipitated with anti-p34<sup>cdc2</sup> antibody, and the precipitates were analyzed by blotting using anti-p73, anti-cyclin B, and p34<sup>cdc2</sup> antibodies. **C**, identification of the p34<sup>cdc2</sup>-interacting region of p73. 0.5  $\mu$ g of purified recombinant p34<sup>cdc2</sup> (Santa Cruz) was incubated with GST alone, GST-p73 $\alpha$ , GST-p73 $\beta$ , and GST-p73 deletion mutants followed by immunoblot (IB) analysis with anti-p34<sup>cdc2</sup> antibody (upper panel). Bottom panel, the same filter was stained with Ponceau Red to visualize the input GST proteins in pull-down assays.

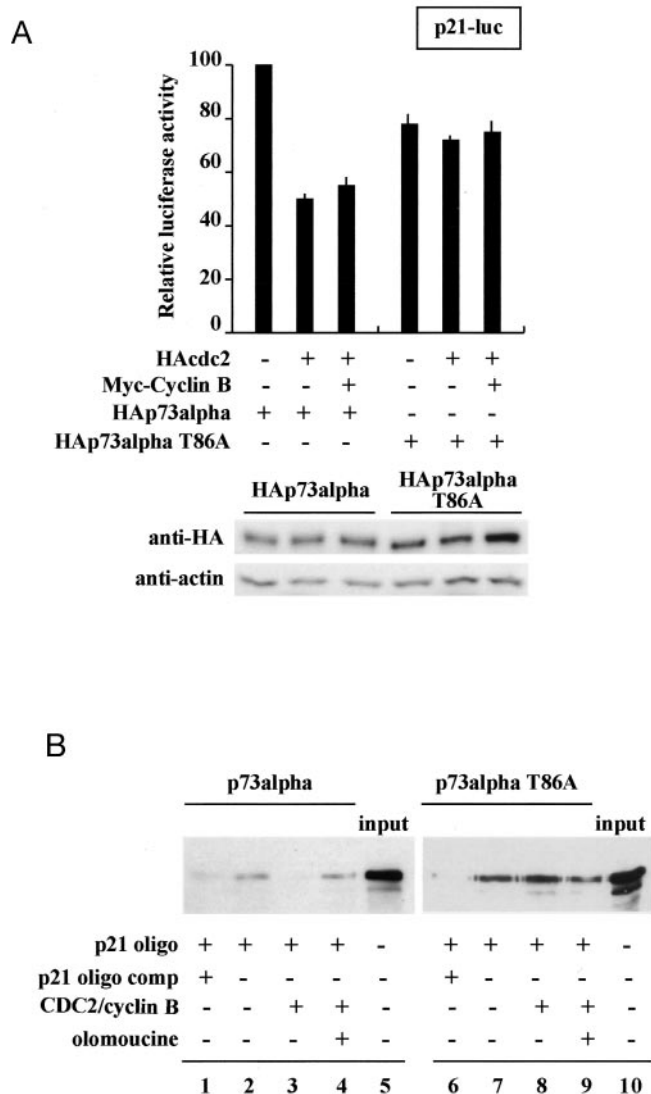
*p73 Is Phosphorylated by the p34<sup>cdc2</sup>-Cyclin B Complex in Vivo and in Vitro*—p34<sup>cdc2</sup>-cyclin B is the key regulator of mitosis and is the kinase complex responsible for the G<sub>2</sub>/M transition. A number of proteins involved in transcriptional regulation, including RNA PolIII, the basal factor TFIID (transcription factor IID), and several sequence-specific transcription factors, are subjected to mitosis-specific phosphorylation, which in most cases is performed by the cdc2-cyclin B kinase (see Ref. 36 for review). To test whether p34<sup>cdc2</sup>-cyclin B is involved in p73 mitotic phosphorylation, we first carried out an *in vitro* kinase assays using a highly purified p34<sup>cdc2</sup>-cyclin B complex. This complex efficiently phosphorylates histone H1, indicating that its activity is maintained *in vitro* (data not shown). p34<sup>cdc2</sup>-cyclin B complex phosphorylates a bacterially expressed GST-p73 $\alpha$  fusion protein (Fig. 2A, left panel). In addition, we found that endogenous p73 $\alpha$  protein immunoprecipitated from HCT116(3) cells acts as an efficient phosphorylation substrate of p34<sup>cdc2</sup>-cyclin B, contrary to IKK $\alpha$  protein immunoprecipitated from the same cultures (Fig. 2A, right panel). These results therefore identify p34<sup>cdc2</sup>-cyclin B as the candidate kinase that phosphorylates p73 *in vivo* during mitosis. By assaying several p73 $\alpha$  deletion mutants expressed as GST fusion proteins (depicted in Fig. 2C) in an *in vitro* phosphorylation assay, we found that the amino-terminal region of p73 (residues 1–120) is targeted by p34<sup>cdc2</sup>-cyclin B phosphorylation (Fig. 2B, left panel). This region contains a single TPEH sequence matching the consensus Ser/Thr-Pro-X-basic amino acid phosphorylation site for p34<sup>cdc2</sup> (37) with the potentially phosphorylated threonine in position 86. We found that a T86A substitution in the GST-p73 $\alpha$ (1–120) fusion protein abolished phosphorylation by p34<sup>cdc2</sup>-cyclin B (Fig. 2D, left panel), consistent with results recently reported by Gaiddon *et al.* (32). To assess the physiological relevance of these findings exogenous HA-tagged T86A-p73 $\alpha$ , which cannot be phosphorylated by p34<sup>cdc2</sup> *in vitro*, was expressed in cell cultures subjected to nocodazole synchronization. No electrophoretic variation was detected (Fig. 2D, right panel), indicating that no other phosphorylation event contributes to the M phase-specific pattern of p73 migration observed *in vivo* (see Fig. 1A). These results indicate that p73 is an *in vivo* target of phosphorylation by p34<sup>cdc2</sup>-cyclin B on threonine 86.

*p34<sup>cdc2</sup>-Cyclin B1 Complex Binds p73 in Mitosis*—Several p73 isoforms have been shown to interact with purified cyclin-Cdk complexes *in vitro* (32). Because *in vivo* p73 displays an obvious susceptibility to modifications during mitosis (see Fig. 1), we next asked whether p73 and the p34<sup>cdc2</sup>-cyclin B complex

do associate physically *in vivo*. As shown in Fig. 3A, p34<sup>cdc2</sup> immunoprecipitates from asynchronously growing HCT116(3) cells contained p73 $\alpha$ . It is noteworthy that the amount of co-associated p73 $\alpha$  was significantly higher in p34<sup>cdc2</sup> immunoprecipitates from nocodazole-arrested cells compared with asynchronously growing cultures (Fig. 3A), further indicating that p73 does associate with p34<sup>cdc2</sup>-cyclin B complex during mitosis. The association of p73 with p34<sup>cdc2</sup> was followed up during mitotic exit and re-entry into G<sub>1</sub> after release from nocodazole arrest (Fig. 3B). Levels of p34<sup>cdc2</sup>-cyclin B were down-regulated, as expected. In parallel, we observed a progressive reduction in the amount of p34<sup>cdc2</sup>-associated p73 $\alpha$  at 6 h after nocodazole release; when all cells were beyond mitosis and into G<sub>1</sub> phase, p34<sup>cdc2</sup>-p73 $\alpha$  association was barely detectable (Fig. 3B). To assess whether the association between p73 and p34<sup>cdc2</sup>-cyclin B observed *in vivo* was direct or mediated by other cellular proteins, we performed pull-down experiments using a panel of GST-p73 fusion proteins and a commercial 55-kDa full-length p34<sup>cdc2</sup> fusion protein produced in *E. coli*. p34<sup>cdc2</sup> efficiently binds to GST-p73 $\alpha$  and GST-p73 $\beta$  (Fig. 3C), and the association is maintained only with the GST p73(1–120) deletion mutant (Fig. 3C). Together these results demonstrate that p73 associates directly with p34<sup>cdc2</sup>-cyclin B complex through the amino-terminal region of p73 and the association is physiologically established *in vivo*.

*p34<sup>cdc2</sup>-Cyclin B-mediated Phosphorylation Interferes with p73 Functions*—Although mitosis is accompanied by a global repression of nuclear RNA synthesis in higher eukaryotes, increasing evidence indicate that some genes are stably transcribed even in the context of the highly condensed mitotic chromatin. Indeed, the human cyclin B1 gene itself is actively transcribed during mitosis (38). To assess the impact of p34<sup>cdc2</sup>-cyclin B-mediated phosphorylation on p73 transcriptional activity *in vivo*, we co-transfected the HA-p73 $\alpha$ , HA-p34<sup>cdc2</sup>, and Myc-cyclin B expression vectors together with a luciferase reporter gene driven by the p53–73-responsive p21 promoter. p73-mediated transcriptional activation of the p21 promoter was reduced by 50% when p73 was co-transfected with either HA-p34<sup>cdc2</sup> alone or with HA-p34<sup>cdc2</sup> and Myc-cyclin B (Fig. 4A). The nonphosphorylatable p73-T86A mutant retained a somewhat lower ability to activate p21 promoter-driven transcription, which was not affected by co-transfection of HA-p34<sup>cdc2</sup> alone or with Myc-cyclin B (Fig. 4A).

Transcriptional repression is thought to reflect both the increased condensation of mitotic chromatin (39) and phosphorylation events that inhibit the binding of transcriptional acti-



**FIG. 4. p34<sup>cdc2</sup>-cyclin B interferes with p73 functions.** **A**, p34<sup>cdc2</sup> reduces p73 transactivation. U2OS cells were transfected with p21/firefly luciferase reporter (0.5  $\mu$ g), the indicated expression vectors (1  $\mu$ g each), and 100 ng of the pRL-null vector encoding the *Renilla* luciferase. 24 h after transfection cells were lysed and assayed for luciferase activity using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized to the *Renilla* luciferase activity and expressed as -fold activation relative to the level obtained from co-transfection of the reporter with empty expression vector. *Error bars* represent two standard deviations calculated from three independent experiments. The levels of exogenously expressed proteins were assessed by immunoblotting using the appropriate anti-HA polyclonal antibody (*lower panel*). **B**, p73 $\alpha$  binding to a p21-specific p53/73RE oligonucleotide is abrogated by p34<sup>cdc2</sup>-cyclin B-mediated phosphorylation, whereas the binding of the nonphosphorylatable p73(T86A) mutant is not affected. *In vitro* translated p73 $\alpha$  and p73 $\alpha$ (T86A) were incubated with p21-specific biotinylated p53/73RE oligonucleotides prebound to streptavidin beads. Oligonucleotide-bound p73 proteins were subjected to *in vitro* kinase assay with either active (*lane 3* and *8*) or inactive (*i.e.* preincubated with 0.5 mM olomoucine (*lanes 4* and *9*)) purified p34<sup>cdc2</sup>-cyclin B complex; p73 proteins were revealed by immunoblotting using anti-p73 antibody.

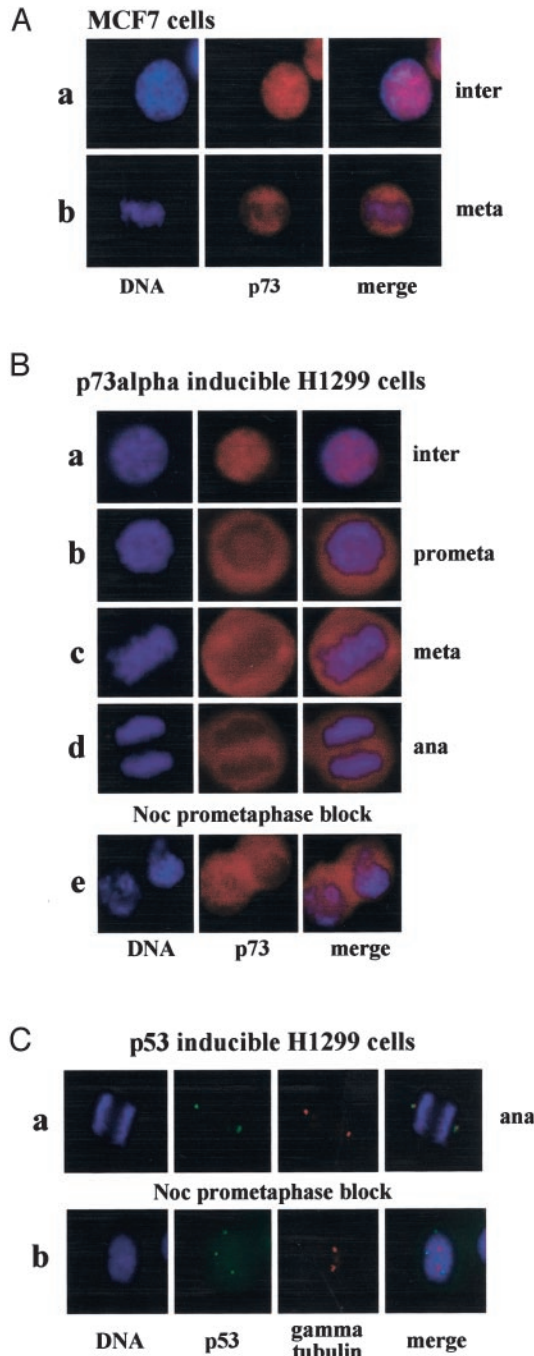
vators to their cognate DNA target (36). However, the mitotic dispersal of RNA polymerase II factors and sequence-specific transcription factors from the condensed mitotic chromosomes is not general. For example, AP2 (39) and NF-Y (38), serum response factor p67SRF (40), and a significant proportion of the TATA-binding protein (TBP) and TBP-associated factor 250 (TAFII250) (41) remain associated with the condensed and transcriptionally inactive mitotic chromatin. To assess whether

mitotic p34<sup>cdc2</sup>-cyclin B-dependent phosphorylation of p73 interferes with its DNA binding ability, we tested the effect of purified active p34<sup>cdc2</sup>-cyclin B on preassembled DNA-protein complexes containing *in vitro* translated p73 and a biotinylated p73-binding oligonucleotide from the p21 promoter. As shown in Fig. 4B, the amount of p73 $\alpha$  bound to its cognate site was drastically reduced in the presence of purified active p34<sup>cdc2</sup>-cyclin B (compare *lane 2* with *lane 3*). The amount of oligonucleotide-bound p73 $\alpha$  remained instead unchanged when the pre-assembled DNA-protein complexes were incubated with p34<sup>cdc2</sup>-cyclin B preincubated with 0.5  $\mu$ M olomoucine, a specific inhibitor of the p34<sup>cdc2</sup> mitotic kinase (Fig. 4B, compare *lanes 4* and *3*). Thus, the reduced affinity of p73 for its DNA target sites is indeed mediated by p34<sup>cdc2</sup> kinase activity. To further demonstrate that p34<sup>cdc2</sup>-cyclin B-mediated phosphorylation of p73 was responsible for its reduced DNA binding ability, the same experiment was performed using the *in vitro* translated p73-T86A mutant, which cannot be phosphorylated by p34<sup>cdc2</sup>-cyclin B. In these experiments, the amount of p73-T86A bound to p21-specific oligonucleotides was unchanged even in the presence of active p34<sup>cdc2</sup>-cyclin B (Fig. 4B, compare *lanes 7, 8, and 9*), consistent with the finding that Thr<sup>86</sup> is the key p73 phosphorylation site during mitosis.

To assess whether p34<sup>cdc2</sup>-cyclin B-dependent reduction in the DNA binding ability of p73 is reflected by displacement from mitotic chromatin *in vivo*, we studied p73 localization both in MCF7 (Fig. 5A) and H1299-derived p73 $\alpha$  ponasterone A-inducible cells (Fig. 5B). Interphasic cells display a homogeneous p73 nuclear staining that colocalizes with DAPI-stained chromatin and is excluded from nucleoli (Fig. 5, A, *row a*, and B, *row a*). In the metaphase and anaphase stages, both endogenous and inducible p73 are excluded from condensed chromosomes (Fig. 5, A, *row b*, and B, *rows c* and *d*). The mitotic localization of p73 differs from that of p53, which is associated with centrosomes in mitosis (Ref. 42 and Fig. 5C, *row a*).

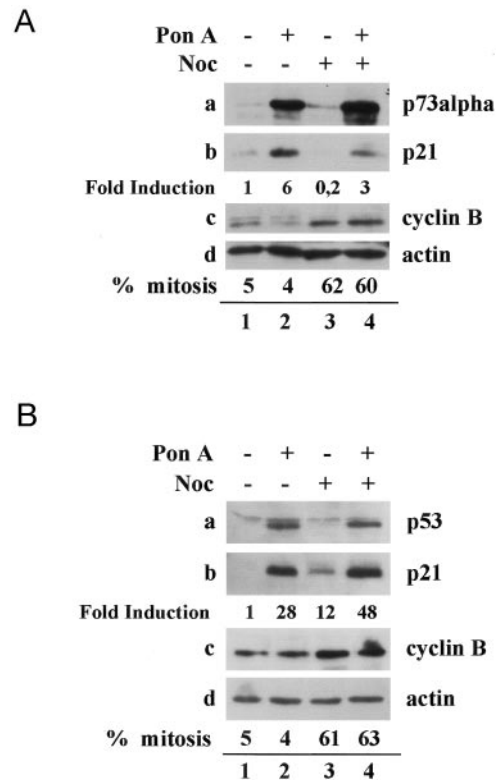
**Differential Behavior of p73 and p53 in Response to Spindle Damage**—The association of p53 with centrosomes during normal mitosis is disrupted by nocodazole treatment (Ref. 42 and Fig. 5C, *row b*) and is not re-established in cells that achieve mitotic exit after transient exposure to nocodazole. p53 delocalization and prolonged activation are responsible for the G<sub>1</sub> block that follows the removal of the spindle-damaging agent (42). We asked whether p73 localization would also be affected by nocodazole. As shown in Fig. 5B (*row e*), p73 remained excluded from chromatin in nocodazole-arrested prometaphase cells. The exclusion of p73 from chromatin during nocodazole induced mitotic block, together with its reduced DNA binding activity, is expected to result in a reduction of p73 transcriptional activity. Indeed, ponasterone A-induced p73 cells accumulated in mitosis by nocodazole, despite a level of p73 induction similar to that of untreated cells, showed a lower ability to up-regulate p21 (Fig. 6A). When the same experiment was performed in the p53 H1299-derived cell line, the induced p53 retained its intact transcriptional activity, as reflected by p21 protein levels in nocodazole-treated cells (Fig. 6B). The latter result suggests that some of the p53 delocalized from centrosomes is transcriptionally active in nocodazole-arrested mitotic cells. Together these findings suggest that p73 phosphorylation by *cdc2*-cyclin B and dispersal from mitotic chromatin contribute to down-regulate its transcriptional potential.

**Conclusions**—It is becoming increasingly clear that p53 and p73, despite their similarities in both primary amino acid sequence and DNA binding properties, execute partially overlapping, yet distinct functions (for review see Levrero *et al.* (2) and Strano *et al.* (3)). Differently from p53, p73 has been shown to be regulated along the cell cycle. p73 is a transcriptional target



**FIG. 5. p73 is displaced from mitotic chromosomes A and B.** Subcellular localization of p73 in interphase and mitotic cells. Asynchronously cycling MCF7 cells (A) and ponasterone A (2.5  $\mu$ M)-induced H1299-derived p73 $\alpha$  cells (B) were grown on glass coverslips for 24 h and then fixed and stained with anti-p73 antibody and rhodamine-conjugated secondary antibody. In row e, ponasterone A-induced H1299-derived p73 $\alpha$  cells were exposed to nocodazole (50 ng/ml) for 16 h. Cells were counterstained with DAPI to detect DNA. p73 is pseudocolored in red (middle panel), whereas DNA appears in blue (left panel). C, p53 colocalizes with centrosomes in normal mitosis from asynchronously cycling ponasterone A-induced H1299-derived p53-inducible cells (row a). Cells were processed for immunofluorescence to  $\gamma$ -tubulin (rhodamine-conjugated secondary antibody, pseudocolored in red) and p53 (fluorescein isothiocyanate-conjugated secondary antibody, pseudocolored in green) and counterstained with DAPI. After nocodazole (Noc) exposure for 16 h, the colocalization of p53 with  $\gamma$ -tubulin is lost (row b). ana, anaphase.

of transcription factor E2F1 during the S phase of the cell cycle, and TAp73 proteins accumulate in S phase cells (18). These results suggest that as yet unknown S phase-specific target



**FIG. 6. Differential behavior of p73 and p53 in response to spindle damage.** A, p73-induced p21 expression decreases after nocodazole treatment. H1299-derived p73 $\alpha$  inducible cells were either treated with ponasterone A (Pon A) (2.5  $\mu$ M) for 6 h (lanes 2 and 4) or mock-treated (lanes 1 and 3); nocodazole (Noc) (50 ng/ml) was then added to the culture medium for 18 h (lanes 3 and 4). Cell extracts were analyzed by immunoblotting using antibodies against the indicated proteins. The p21 signal was quantified by microdensitometry of autoradiographs of ECL-processed immunoblots and normalized relative to the p21 signal measured in asynchronously cycling cells (lane 1). B, p21 levels increase after ponasterone A induction of p53 in nocodazole-treated H1299-derived p53-inducible cells. Cell extracts were prepared and analyzed as described in A.

genes may be regulated by TAp73. *In vitro* p73 has also been shown to interact physically with and to be regulated negatively by various CDK2- and CDK1-containing complexes, although the physiological role of these interactions in the cell cycle has not yet been clearly established (32). Here we show that cyclinB-p34<sup>cdc2</sup>-dependent phosphorylation of both p73 $\alpha$  and p73 $\beta$  isoforms is physiologically relevant in G<sub>2</sub>/M and governs its subcellular localization and DNA binding features. Most importantly, p34<sup>cdc2</sup>-cyclinB-mediated phosphorylation of p73 on threonine 86 inhibits its transcriptional activity. At nuclear envelope breakdown, the endogenous phosphorylated p73 is excluded from condensed chromosomes and, unlike p53, disperses throughout the mitotic cytoplasm with no specific association with centrosomes. Taken together our results suggest that p73 phosphorylation by *cdc2*-cyclin B, and the ensuing dispersal from mitotic chromatin contributes to the down-regulation of its transcriptional potential. Instead, transcriptional inactivity of p53 in a normal mitosis is most likely ensured by its transient association with centrosomes. When p53-centrosome association is disrupted by nocodazole, p53 again becomes transcriptionally active, despite the persistence of chromatin condensation. p73 localization and transcriptional activity is not unaffected by nocodazole, suggesting that p73-dependent transcription has to be abrogated during physiological, as well as drug-induced, mitosis. We have also observed that after nocodazole removal, p73 can activate p21 in p53-null

cells<sup>3</sup> and that up-regulation of p21 peaks several hours after release from arrest, *i.e.* at a time when most cells have achieved mitotic exit and have reached the following G<sub>1</sub> phase. These results suggest that p73 can contribute to sustaining the post-mitotic checkpoint activated by transient spindle damage, as already established for p53. Besides this shared function common to both p53 and p73 during G<sub>1</sub> re-entry, several features reported here, *i.e.* p73 inhibitory phosphorylation at the G<sub>2</sub>-to-M transition by the p34<sup>cdc2</sup>-cyclin B complex, its lack of association with mitotic microtubules and centrosomes, and the differential behavior in response to nocodazole treatment, strongly suggest that p53 and p73 undergo specific and distinct cellular fates during mitotic division.

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<sup>3</sup> M. Fulco and P. Merlo, unpublished observations.

## **p73 Is Regulated by Phosphorylation at the G<sub>2</sub>/M Transition**

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