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An essential role for p73 in regulating mitotic cell death

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The molecular mechanisms regulating cell death during mitosis are poorly understood. We show here a critical role for p73, but not p53, in regulating mitotic cell death induced by various means. Prolonged mitotic arrest and the activation of spindle checkpoint are required for mitotic death, which occurs before mitotic exit and which can be ameliorated by accelerated mitotic exit. Absence or silencing of p73 expression abrogated mitotic death without accelerating mitotic exit, and was independent of BubR1 and Mad2, the loss of which promotes mitotic exit. However, the absence of p73 reduced mitotic death by compromising the expression of the proapoptotic BH3-only protein Bim and thereby affecting cytochrome *c* release and caspase activation. p73 was found to induce *bim* expression through direct binding to regulatory elements in intron 1. Congruently, mitotic cell death was rescued to similar extents by silencing either *bim* or *p73* expression. Taken together, the data show an important role for the p73–Bim axis in regulating cell death during mitosis that is independent of p53.
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Progression through the mitotic phase of the cell cycle seems to be the most vulnerable period in the life of a cell, during which it is susceptible to cell death induced by various insults.[1] On exposure to cellular insults leading to accumulation of cells in this phase of the cell cycle, checkpoint controls are activated that lead to the arrest of cells till the damage is corrected, and thus genomic integrity is preserved.[2,3] The spindle assembly checkpoint (SAC) that regulates this process has to be satisfied before the progression of cells out of mitosis.[4,5] The current belief is that prolonged arrest in mitosis, which is suggestive of the lack of efficient repair to maintain genomic integrity, signals the induction of an apoptotic program in mitosis – often referred to as mitotic cell death.[1,6] This default mechanism of death seems to have evolved to protect the organisms from accumulating genetic alterations.

Many cancer drugs that have been successful in eliminating cancer cells have exploited this mechanism. An example is paclitaxel (taxol), the commonly used microtubule-stabilizing drug that arrests cells in mitosis and leads to their death in what has also been termed as 'mitotic catastrophe'.[6] Although this process has been quite extensively described, the mechanisms regulating death in and around mitosis are not well understood. Many questions remain, such as what is the molecular timer that determines whether it is time for mitotic death to be initiated; what is the exact nature of the signaling cascades that are involved in activating this cell-death pathway; and so on.

p53, an important tumor-suppressor gene product, regulates multiple processes preventing the propagation of cancerous cells.[7] However, cell death during mitosis seems not to be regulated by p53, and consistently many chemotherapeutic drugs, including taxol, have been shown to induce p53-independent apoptosis.[8,9] In fact, absence of p53 was shown to enhance taxol-induced death, probably because of the lack of the G_1/S checkpoint control leading to accumulation of cells in the G_2/M phase, where they undergo mitotic death.[9]

p73 is a functional homolog of p53 that is expressed either as a full-length TAp73 form, which has a transactivation potential similar to p53 and is hence able to induce apoptosis on overexpression, or as the deltaN p73 (ΔNp73) form, which lacks the transactivation domain and hence has the ability to inactivate both p53 and p73 functions, thereby promoting cell viability.[10–12] Although overexpression of TAp73 led to apoptosis and its absence conferred resistance to some forms of cellular insults, including taxol,[13–15] *in vivo* data suggest that p73 may not be as strong a tumor suppressor as p53. *p73*$^{-/-}$ mice do not succumb to spontaneous tumors as early as *p53*$^{-/-}$ mice.[16] However, *p53*$^{+/-}$*p73*$^{+/-}$ mice had a higher tumor burden and metastasis formation compared with *p53*$^{+/-}$ mice, and *p63*$^{+/-}$*p73*$^{+/-}$ mice were also susceptible to spontaneous tumors.[17] Recent data showed that absence of TAp73 specifically led to the formation of spontaneous tumors in mice,[18] suggesting that p73 indeed could regulate tumor progression, albeit probably to a lesser extent compared with p53. The latter data, together with the findings that cells not expressing p63 and p73 are resistant to cell death,[13] indicate that p73 may have specific roles in regulating apoptosis that cannot be compensated by p53.

Whether p73 has any role in regulating mitotic death has not been carefully assessed. p73 is differentially expressed during the cell cycle, peaking during the S phase and decreasing thereafter during mitosis, during which it is

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phosphorylated by the p34^{cdc2}–cycin B complex, leading to inactivation of its transactivation function and exclusion from the condensed chromosomes.[19,20] This suggests that p73 protein may not be required during G_2/M transition. However, recent evidence points to a critical role for p73 in regulating the diploid status of cells. TAp73 knockout fibroblasts and cells in which TAp73α was overexpressed became polyploid.[18,21] Moreover, p73 has been suggested to regulate spindle assembly checkpoint by modulating BubR1 activity,[22] indicating that p73 is crucial in regulating the genomic integrity during the mitotic phase. Nonetheless, it is unclear whether it has any role in regulating death during mitosis.

We have therefore tested the hypothesis that p73 may regulate death during mitosis, and present data that indicate a crucial role for it in controlling mitotic death induced by various means, by the direct activation of Bim. p73, but not p53, is required for this process, highlighting a specific role for p73 in inducing death during mitosis. The data thus define an apoptotic pathway during mitosis that emancipates from p73, leading to Bim activation and finally resulting in activation of the mitochondrial death pathway.

Results

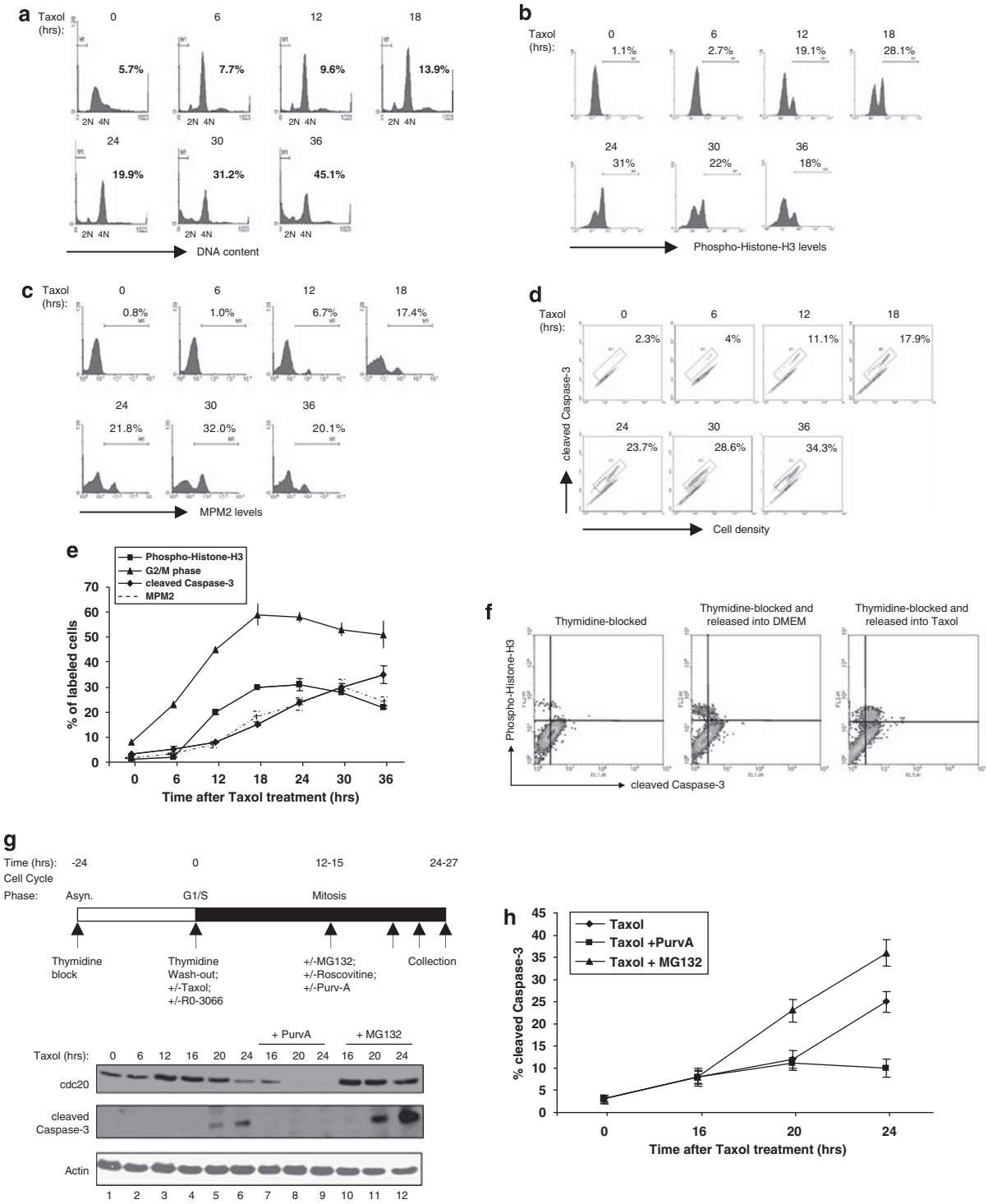
Taxol-induced cell death occurs during mitosis. We have used taxol as a prototype drug to investigate the molecular details of mitotic death. First, we examined the cell-cycle phase at which taxol-induced cell death occurred, by arresting cells at G_1/S phase with a single thymidine block for 24 h and then releasing them in taxol-containing medium without thymidine. Cells accumulated in the 4N population (G_2/M phase) starting from 6 h after release and peaked around 18–24 h (Figure 1a). The sub-G_1 population of cells, which indicates apoptotic cells, started appearing significantly from about 18–24 h (Figure 1a, gate M1). Parallel cultures were tested for phosphorylation of histone-H3 – representing cells in the mitotic phase – which started to accumulate from 12 h onward, peaking at 24 h (Figure 1b). In addition, we analyzed the status of MPM2 expression – indicative of cdc2 activity and of cells in mitosis – which started accumulating from about 12 h onward and peaked around 30 h (Figure 1c). Concomitant analysis of caspase-3 cleavage to determine cell death revealed the appearance of a cleavage product starting around 12 h and peaked at 36 h (Figure 1d). Together, the analyses indicate that taxol-induced caspase-3 cleavage is probably initiated during the mitotic phase of the cell cycle (Figure 1e). To determine whether cell death indeed occurred at the mitotic phase after taxol treatment, we co-stained cells with antibodies against both phospho-histone-H3 and cleaved caspase-3. As shown in Figure 1f, release from the thymidine block into taxol-containing medium led to cells that were stained for both of these markers, in contrast to cells that were only stained with phospho-histone-H3 antibody on release into medium (DMEM) alone. Thus, the data indicate that taxol-induced cell death indeed occurs in the mitotic phase.

We therefore investigated by several means whether it was essential for cells to be in the mitotic phase of the cell cycle to undergo taxol-induced cell death, or rather, for cells to die in a time-dependent manner after taxol treatment. To this end, first, taxol-treated cells were further treated with purvanalol A (PurvA) – an inhibitor of cdc2 activity[23] – 15 h after taxol treatment, at a time point when cells were already entering mitosis, to accelerate mitotic exit without accumulation in mitosis. Alternatively, cells were treated with the proteasomal inhibitor MG132, to block mitotic exit by preventing degradation of the targets of the anaphase-promoting complex, 15 h after taxol treatment, at a time point when cells were already in mitosis and were beginning to undergo apoptosis (Figure 1e). As expected, PurvA treatment resulted in the reduction of cdc20 accumulation, indicating exit from mitosis, in contrast to cells treated only with taxol, in which cdc20 was abundant (Figure 1g, compare lanes 7–9 with 4–6). In contrast, treatment with MG132 resulted in accumulation of cdc20 (Figure 1g, lanes 10–12). Caspase-3 cleavage, which occurred in cells treated only with taxol, was almost completely abrogated in the presence of PurvA, whereas it was further induced on MG132 treatment (Figure 1g, compare lanes 5–6, 8–9 and 11–12). Similar results were obtained on flow cytometric quantification of the amount of caspase-3 cleaved, in a time-course analysis (Figure 1h, $P = 0.0106$ at 24 h). To exclude the possibility that the lack of cleaved caspase-3 expression is due to loss of cellular viability on PurvA treatment, we analyzed cell death by propidium iodide (PI) exclusion assay. This analysis also revealed that PurvA treatment indeed protected cells from taxol-mediated death (percentage of cell death: untreated *versus* taxol *versus* taxol + PurvA → 7.1 *versus* 17.1 *versus* 10.6, $P = 0.009$) (Supplementary Figure 1a).

Next, we used roscovitine, another drug that inhibits cdc2 activity, as well as another selective cdk1 inhibitor, RO-3306, which was shown to arrest proliferating human cells at the G_2/M border,[24] thereby preventing entry into mitosis, to explore the effect of taxol-induced death. We added roscovitine 15 h after taxol treatment when cells were in mitosis already, similar to the PurvA experiments, to evaluate the effects of accelerated exit from mitosis. In contrast, we added RO-3066 when cells were released from G_1/S block, with the aim of preventing cells from entering mitosis. Similar to earlier observations with PurvA, treatment with roscovitine or RO-3306 at the various time points resulted in the reduction of taxol-induced cell death (percentage of cleaved caspase-3 of taxol-treated cells: DMSO *versus* MG132 *versus* roscovitine *versus* RO-3306 → 21.2 *versus* 38.3 *versus* 14.2 *versus* 15.8) (Figure 1i). Treatment with PurvA, roscovitine or RO-3066 similarly resulted in reduction in the number of MPM2-positive cells and consistently to less cells with sub-G_1 (indicative of apoptosis), in contrast to MG132 treatment (Supplementary Figures 1b and 2), indicating a strong correlation between reduction of cell death and less number of cells in the mitotic phase of the cell cycle, either because of prevention of entry into mitosis or because of accelerated exit from mitosis.

To further confirm whether cells indeed need to be maintained in mitosis after taxol treatment for cell death to occur, we delayed cells from entering mitosis by maintaining them in the presence of thymidine. Although cells released from thymidine underwent cell death on exposure to taxol, cells maintained in thymidine were significantly more resistant to cell death as measured by the accumulation of the sub-G_1

population (thymidine free *versus* +thymidine: 39.0 *versus* 6.1% at 28 h, $P = 0.003$) (Figure 1j). Histone-H3 phosphorylation was significantly affected in thymidine-blocked cells treated with taxol, in contrast to released cells (Figure 1k), indicating that thymidine blockage probably delayed mitotic entry. Cell cycle analysis confirmed that thymidine treatment delayed entry into the G_2/M phase significantly (Figure 1j). To determine the specificity of this effect to taxol, we repeated the experiment with a DNA-damaging agent, cisplatin (CDDP), that does not require cells to be specifically in mitotis to induce cell death. Exposure of cells to CDDP resulted in cell death to similar extents, regardless of whether they were blocked in thymidine (thymidine free *versus* +thymidine: 16.3 *versus* 15.1% at 28 h) (Supplementary Figure 3). Hence, the data



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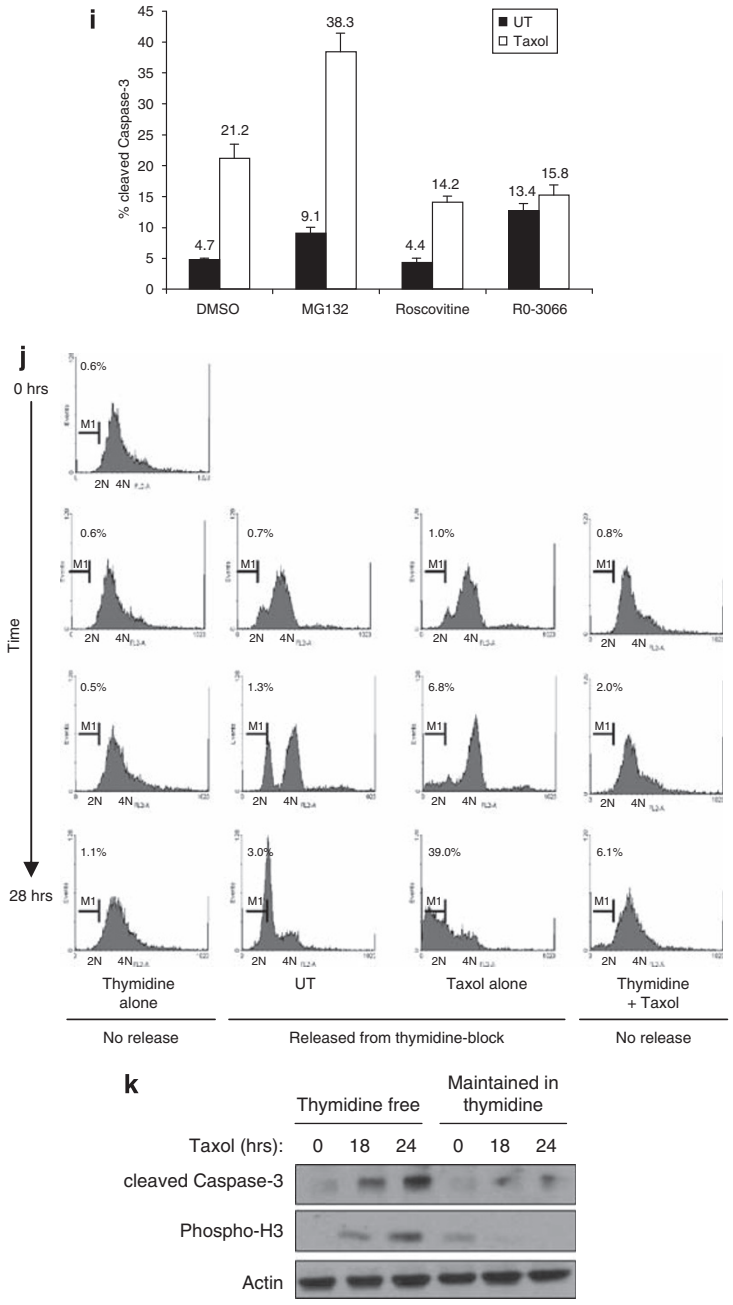


Figure 1 Taxol-induced cell death occurs before cells exit mitosis. (**a**–**e**) p53 null human H1299 cells were arrested at the G_1/S phase of the cell cycle by treatment with 2 mM thymidine for 24 h, after which the medium was replaced by the addition of 100 nM taxol. Cells were collected at the indicated time points and used for flow cytometric analysis of DNA content to determine cell cycle status (**a**); the amount of cells containing phosphorylated histone-H3 (**b**) or MPM2 (**c**), which reflects the numbers of cells in the mitotic phase of the cell cycle; and the number of cells expressing the cleaved caspase-3, reflecting cells undergoing apoptosis (**d**). The data from (**a**–**d**) are graphically illustrated, the results representing the mean ± S.D. (**e**) Gates M1 represent the apoptotic sub-G_1 population (**a**), or the phospho-histine-H3 (**b**) or MPM2 (**c**) positive cells, and the percentages are indicated in each panel. (**f**) Cells were blocked in thymidine for 24 h, and not released (blocked), or released into medium (DMEM) or taxol-containing medium, and were collected 18 h later for flow cytometric analysis of expression of phosphorylated histone-H3 and cleaved caspase-3. (**g**, **h**) The schematic shows the protocol for the treatment of cells with taxol in the presence or absence of the various inhibitors (top panel) (**g**). Taxol-treated H1299 cells were further treated with either 20 nM purvalanol A (PurvA) or 10 μM MG132 15 h post-taxol treatment when cells were in mitosis, and used for immunoblot analysis (**g**). Cell death was determined by percentage of cleaved caspase-3 (**h**). The results represent the mean ± S.D. from at least three independent experiments. (**i**) H1299 cells were similarly treated as indicated in the above schematic in (**g**), with the inclusion of 10 μM roscovitine 15 h post-taxol treatment (when cells were already in mitosis) or 10 μM RO-3066 at release from the thymidine block (when cells are released from the G_1/S block), and harvested 24 h from the time of taxol treatment for the percentage of cleaved caspase-3 analysis. (**j**, **k**) Taxol was added to H1299 cells in the absence or presence of thymidine, followed by flow cytometric analysis to determine the DNA profile over time (**j**). The percentage of dead cells (sub-G_1 population) is indicated. Parallel cultures were used for immunoblot analysis at the indicated time points (**k**). UT: untreated

show that taxol-treated cells forced to remain in mitosis by MG132 treatment underwent cell death, whereas exit from mitosis or prevention from entering mitosis reduced cell death.

Altogether, the data show that taxol-induced cell death occurs during but not before or after mitosis.

p73 is required for taxol-induced cell death. As the experiments were performed using cells lacking p53, we examined whether p73 is critical for mitotic death induced by taxol. Taxol treatment led to increased p73 protein expression in human p53-negative H1299 cells, which peaked between 6 and 12 hours (Figure 2a). Cleavage of caspase-9 and -3, indicative of initiation and execution of the apoptotic program, occurred around 20 and 28 h for caspase-9 and -3, respectively (Figure 2a, lanes 5–7). siRNA-mediated silencing of p73 expression markedly reduced cleaved caspase-9 and -3 products (Figure 2a, lanes 11–14). Moreover, p73 silencing also significantly reduced cell death on taxol treatment, as determined by both PI exclusion and cleavage of caspase-3 (percentage of dead cells – control *versus* p73 siRNA: 34 *versus* 20, $P=0.036$; percentage of cleaved caspase-3 product – control *versus* p73 siRNA: 27 *versus* 13.2, $P=0.009$) (Figure 2b). Several siRNA constructs against p73 were used that gave similar results in multiple cell lines (Supplementary Figure 4a and b).

We next compared the requirement of p53 *versus* p73 in taxol-mediated cell death. Although silencing of p53 in the p53-positive human U20S cells did not result in reduction of taxol-induced cell death, reduction of p73 expression resulted in a marked decrease in cell death (percentage of dead cells → control *versus p53 versus p73* siRNA: 11.1 *versus* 15.5 *versus* 6.7, $P=0.003$) (Figure 2c and d, right panel). Similar results were obtained for cleaved caspase-3 analysis (percentage of cleaved caspase-3 product – control *versus p73* siRNA: 10 *versus* 16 *versus* 4, $P=0.0007$) (Figure 2d, left panel). Further analysis was performed using mouse fibroblasts lacking p53 or p73 (Figure 2e). Although taxol treatment of wild-type fibroblasts resulted in an increase in cell death at 36 h, this effect was markedly reduced in $p73^{-/-}$ cells. By contrast, $p53^{-/-}$ cells were still sensitive to taxol (percentage of cell death on taxol treatment – wild-type *versus* $p53^{-/-}$ *versus* $p73^{-/-}$: 34 *versus* 27 *versus* 6.9, $P=0.029$ between wild-type *versus* $p73^{-/-}$ and $P=0.03$ between $p53^{-/-}$ *versus* $p73^{-/-}$) (Figure 2f). Consistent with these findings, caspase-3 cleavage was observed on taxol treatment only in $p53^{-/-}$ cells and not in $p73^{-/-}$ cells, even though p53 was upregulated in these cells (Figure 2g). It is interesting to note that even after prolonged exposure to taxol treatment $p73^{-/-}$ cells did not undergo death and remained arrested in the G_2/M phase, in contrast to $p53^{-/-}$ cells that were dead and also became polyploid (Supplementary Figure 5). Taken together, these results indicate that p73 is required for taxol-induced cell death in both human and mouse cells.

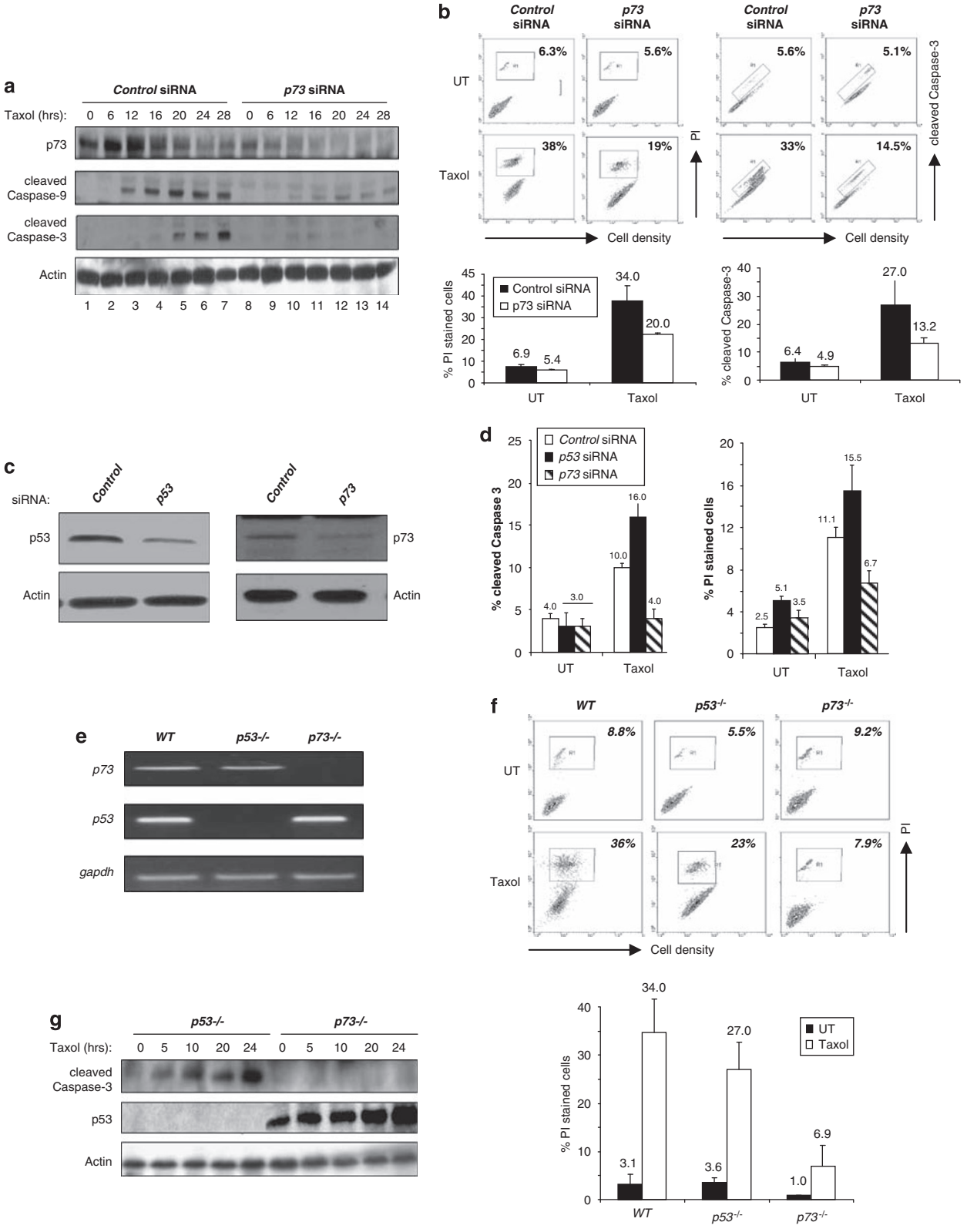
p73 is required for mitotic cell death induced by other means. Many drugs induce mitotic arrest. However, not all trigger the spindle checkpoint as taxol does. Nocodazole and MG132 treatment are known to activate and maintain the spindle checkpoint, whereas cytochalasin D treatment only affects the cytokinesis stage of mitosis and does not trigger the spindle checkpoint.[25–27] Hence, we examined whether mitotic arrest alone is sufficient to induce cell death. Although treatment with all these agents resulted in accumulation of cells with 4N DNA content (Figure 3a, upper panel), only nocodazole and MG132 treatment resulted in an increase in cell death, whereas cytochalasin D did not (percentage of cells with cleaved caspase-3 → untreated *versus* nocodazole *versus* MG132 *versus* cytochalasin D: 4 *versus* 30 *versus* 27 *versus* 8.8) (Figure 3a, lower panel), suggesting that a triggering of the checkpoint is important for cell death during mitosis.

Hence, we analyzed whether p73 would be required for mitotic cell death when the checkpoint is activated by other means. Firstly, nocodazole-mediated cell death was found to be dependent on p73, as silencing of p73 expression reduced cell death significantly, in contrast to silencing of p53 expression in U2OS cells (control *versus* p53 *versus* p73 siRNA → 19 *versus* 18 *versus* 9%, $P=0.018$ between control *versus* p73 siRNA and $P=0.045$ between *p53 versus p73* siRNA) (Figure 3b, upper panel). Similar data were obtained using $p73^{-/-}$ and $p53^{-/-}$ cells (percentage of dead cells for $p73^{-/-}$ *versus* $p53^{-/-}$: untreated – 5.1 *versus* 13.5 and nocodazole – 20.5 *versus* 46.4) (Figure 3b, lower panel). Next, we silenced the expression of mitotic polo-like kinase 1 (PLK1), which led to the accumulation of cells in mitosis and subsequent death (Supplementary Figure 6).[28] Cells were transfected with *PLK1* siRNA, blocked in G_1/S by thymidine block for 24 h before being released for a further 24 h to allow cell cycle progression. As expected, release into cell cycle resulted in accumulation of phosphorylated histone-H3 in *PLK1*-silenced cells, in contrast to control siRNA-transfected cells, regardless of p73 silencing (Figure 3c, compare lanes 2–3 and 5–6). Analysis of parallel cultures indicated that silencing of p73 resulted in a significant reduction of cell death and caspase-3 cleavage in the presence of *PLK1* siRNA (percentage of cleaved casapse-3 – control *versus p73* siRNA: 37 *versus* 23, $P=0.01$; percentage of dead cells – control *versus p73* siRNA: 22.5 *versus* 9.8, $P=0.012$) (Figure 3d). The data therefore suggest that p73 contributes to mitotic cell death induced by multiple means.

Protection from mitotic death on p73 silencing is not due to exit from mitosis through modulation of BubR1 and Mad2 expression. Our data indicate that activation of the spindle checkpoint, which occurs through the inhibition of APC function, is necessary for mitotic death. Silencing expression of BubR1 and Mad2, which are inhibitors of APC, was also shown to protect cells from taxol-induced death.[29] Thus, we investigated whether p73 could modulate the expression of BubR1 or Mad2 to regulate mitotic death. As shown in Figure 4a, reduction of BubR1 or Mad2 led to decreased taxol-mediated death, similar to the levels seen with p73 siRNA treatment (percentage of cleaved casapse-3 on taxol → control *versus p73 versus BubR1 versus Mad2* siRNA: 22.3 *versus* 13.5 *versus* 15.0 *versus* 12.1; % dead cells → 19.4 *versus* 8.5 *versus* 11.9 *versus* 7.0). However, although silencing of BubR1 and Mad2 resulted in reduced phosphorylated histone-H3, this was not the case on silencing of p73 (Figure 4b and c). p73 silencing did not also affect BubR1 and Mad2 levels or the phosphorylation

status of BubR1, as evidenced by the slower migrating band visible between 18 and 24 h after taxol treatment (Figure 4d), suggesting that reduction in p73 levels neither affects BubR1 or Mad2 expression nor leads to mitotic exit.

We further examined whether the mechanisms of p73-dependent mitotic death are different from those of BubR1 and Mad2-mediated death. To this end, cells in which BubR1, Mad2 or p73 expression was silenced were treated with



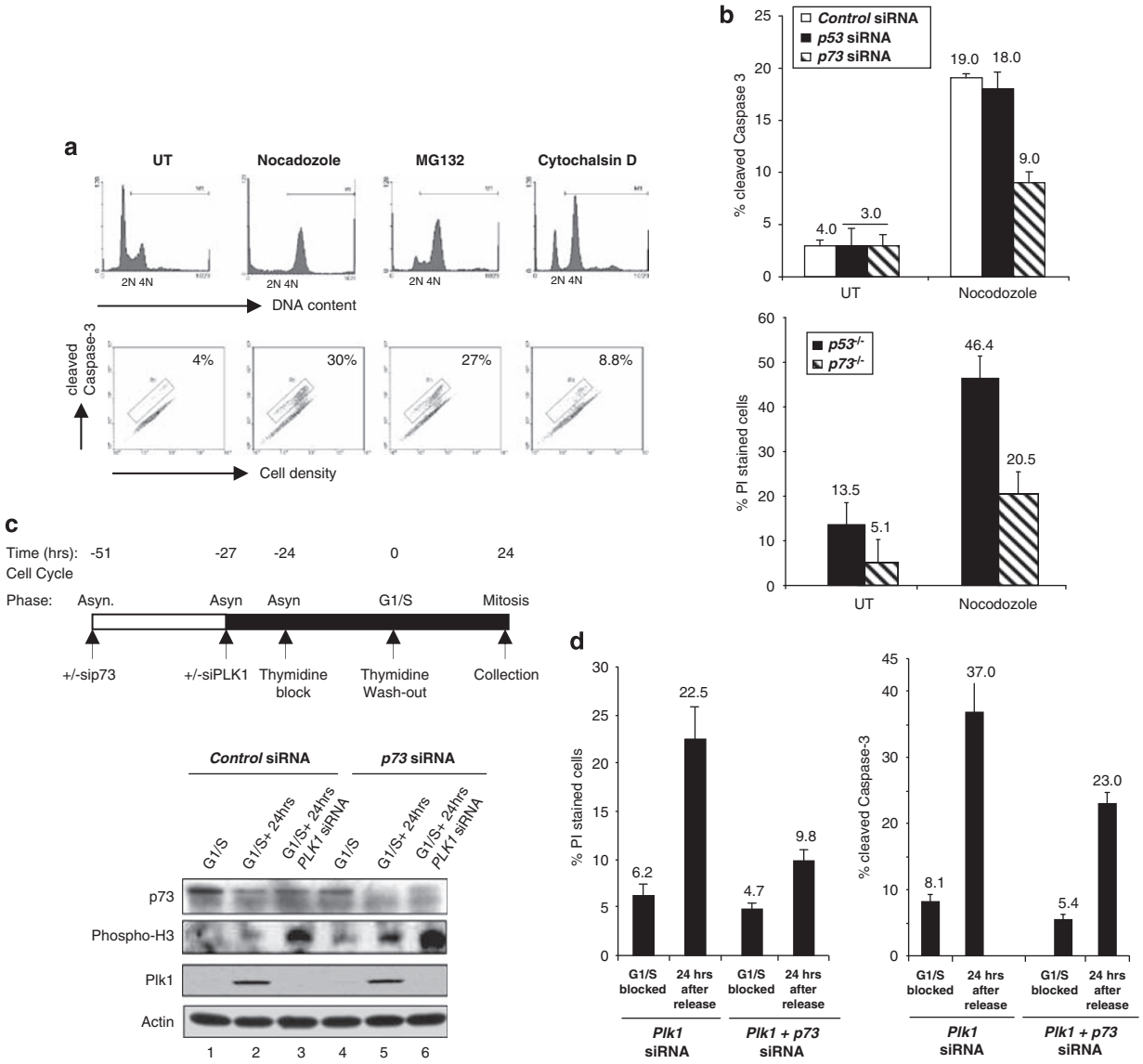


Figure 3 Mitotic death is dependent on p73 and on activation of the spindle checkpoint. (**a**) Asynchronous H1299 cells were treated with 1 μg/ml of nocodazole, 10 μM MG132 or 2 μM of cytochalasin D and collected 24 h later for DNA content analysis (upper panel) and to determine the percentage of cells with cleaved caspase-3 (lower panel). (**b**) U20S cells in which p53 or p73 expression was silenced using specific siRNA (upper panel), or *p53*$^{-/-}$ and *p73*$^{-/-}$ mouse fibroblasts (lower panel), were treated with 1 μg/ml of nocodazole for 30 h before determination of cell death. Experiments were performed in duplicates and at least twice, and mean ± S.D. is shown. (**c**, **d**) Schematic in (**c**) shows the setup of the experiments. Expression of *PLK1* was silenced in H1299 cells that were arrested at the G_1/S phase by the thymidine block for 24 h before release into the cell cycle. Control siRNA was used as indicated. In parallel cultures, p73 expression was silenced 24 h before silencing of PLK and subsequently released as shown in the schematic. All samples were used for immunoblot analysis (**c**) or for analysis of cell death by propidium iodide (PI) exclusion (left panel) and expression of cleaved caspase-3 (right panel) (**d**). Experiments were performed in duplicates and at least three times, and the mean ± S.D. is shown

Figure 2 p73 is required for taxol-induced cell death of human and mouse cells. (**a**) H1299 cells were transfected with control and *p73*-specific siRNA 24 h before arrest by the thymidine block for 24 h, subsequently released into 100 nM taxol-containing medium as described, and collected at various time points for immunoblot analysis. (**b**) Cell death was measured either by propidium iodide (PI) exclusion assay (left panel) or by caspase-3 cleavage (right panel), from the above samples at 24 h after taxol treatment, by flow cytometry. The percentage of dead cells is indicated. Experiments were performed in duplicates and at least three times, and representative results are shown with mean ± S.D. (**c**, **d**) p53 or p73 expression was silenced in human osteosarcoma U20S cells using specific siRNA (**c**), which were then treated with 100 nM taxol for 30 h before determination of the percentage of dead cells by PI (right panel) or caspase-3 cleavage (left panel) (**d**). (**e–g**) Reverse transcriptase (RT)-PCR analysis was performed to confirm the absence of p53 or p73 in the respective mutant mouse fibroblasts (**e**). Cells were treated with 100 nM taxol for 36 h and the percentage of dead cells was determined by PI exclusion. Experiments were performed in duplicates and at least three times, and representative data are shown together with the mean ± S.D. in the lower panel (**f**). Parallel cultures were collected at the indicated time points and used for immunoblot analysis (**g**)

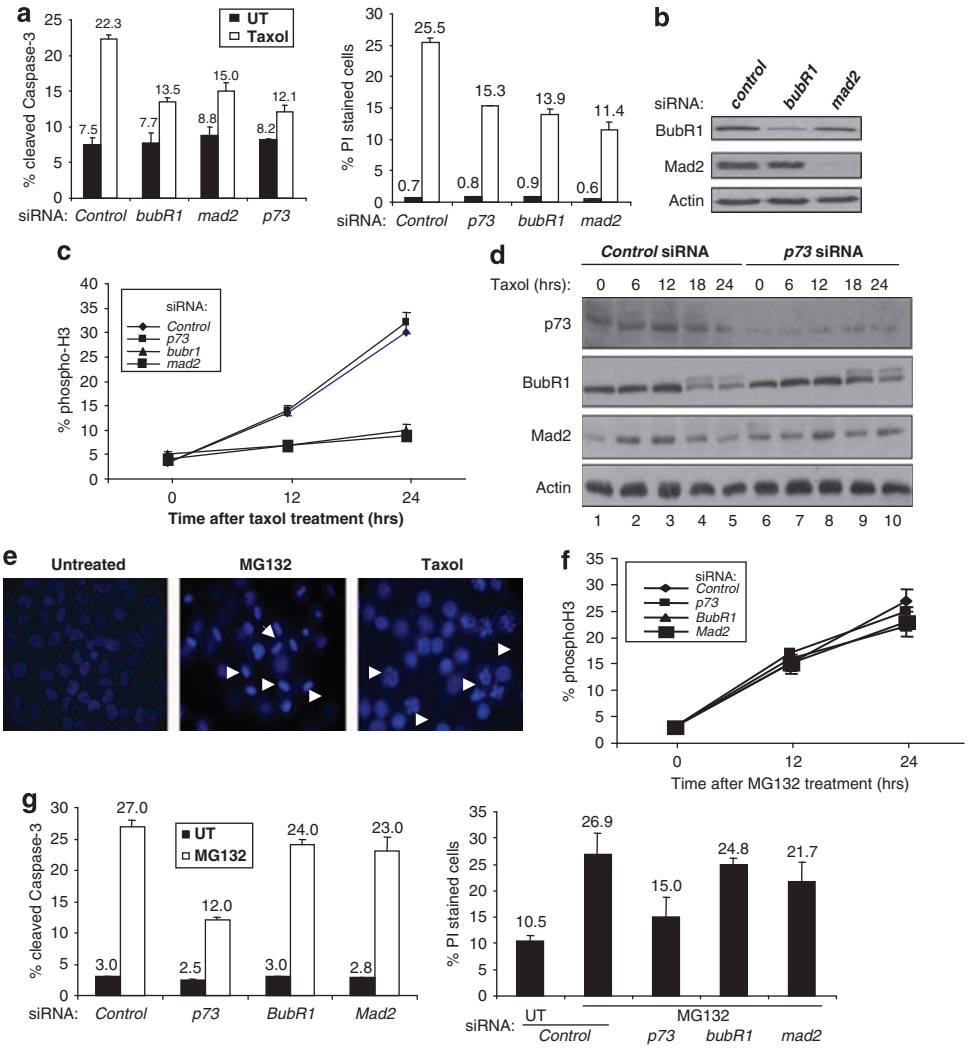


Figure 4 Mechanism of p73-mediated mitotic death is not related to activation of the components of the spindle checkpoint, BubR1 and Mad2. (**a**–**c**) Expression of BubR1, Mad2 or p73 was silenced in H1299 cells 30 h before taxol treatment. Cell death (**a**) or immunoblot (**b**) analysis was performed 24 h after taxol treatment. Parallel cultures were used to determine levels of phospho-histone-H3 by flow cytometry at the indicated time points (**c**). (**d**) Immunoblot analyses indicate that silencing of p73 expression 24 h before thymidine block and subsequent release into 100 nM taxol-containing medium does not affect BubR1 or Mad2 levels. (**e**) Immunofluoresence microscopy showing chromosomes from taxol- or MG132-treated cells in mitotic arrest. Taxol-treated cells exhibit aberrant chromosome formation, whereas MG132-treated cells exhibit aligned chromosomes (see arrowheads showing representative chromosomes). (**f**, **g**) Expression of p73, BubR1 or Mad2 was silenced in H1299 cells that were treated with 10 μM MG132 14 h after release from thymidine block to arrest cells in mitosis. The levels of phospho-histone-H3 (**f**) or cleaved caspase-3 (left panel) and propidium iodide (PI) exclusion (right panel) (**g**) were determined. Experiments were performed in duplicates and at least three times, and the mean ± S.D. is shown

MG132, which blocks the degradation of APC targets, therefore negating the effects of silencing BubR1 and Mad2 on mitotic exit. We reasoned that if p73 regulated BubR1 and Mad2 to regulate mitotic death, then silencing the expression of any one of the molecules would protect the cells from MG132-induced death. This hypothesis was thus tested. Cells were first treated with MG132 after release from thymidine block to arrest cells in mitosis, which resulted in chromosomes being aligned along a single plane with normal mitotic spindle, in contrast to taxol-treated cells, which have fragmented chromosome arrangement (Figure 4e, see arrowheads). Silencing BubR1 or Mad2 expression in MG132-treated cells did not result in mitotic exit, as determined by the lack of reduction of phosphorylated histone-H3 (Figure 4f). Importantly, BubR1 and Mad2 silencing in cells treated with MG132 did not result in reduction in cleavage of caspase-3, in contrast to silencing of p73 (percentage of cleaved caspase-3 after MG132 treatment → control *versus p73 versus BubR1 versus Mad2* siRNA: 27 *versus* 12 *versus* 24 *versus* 23) (Figure 4g, left panel). Similar results were obtained with PI exclusion analysis (percentage of dead cells → control *versus p73 versus BubR1 versus Mad2* siRNA: 26.9 *versus* 15.0 *versus* 24.8 *versus* 21.7) (Figure 4g, right panel). To confirm that MG132-mediated cell death occurred primarily in the G_2/M phase, we compared the rate of cell death in asynchronous cultures *versus* thymidine-blocked and -released cultures (where a large population of cells were entering mitosis), which indicated that cell death was very pronounced in the

latter population (percentage of cell death: asynchronous *versus* thymidine blocked and released → 15.6 *versus* 40.1) (Supplementary Figure 7). Taken together, the results indicate that the mechanism by which silencing of p73 protected cells from death was different from that seen when BubR1 and Mad2 are silenced, in which case cells are forced out of mitosis, prematurely leading to escape from mitotic death.

p73 is required for caspase activation and cytochrome *c* release. As caspase activation is a hallmark of apoptosis in many cellular systems,[30] we examined whether they were involved in mitotic death attributed to p73, by silencing the expression of various initiator caspases, such as caspase -2, -8 or -9. Silencing of all three caspases led to decreased caspase-3 cleavage, similar in extent to that found on silencing of p73 expression (percentage of cleaved caspase-3 after taxol treatment → control *versus p73 versus caspase-2 versus caspase-8 versus caspase-9* siRNA: 34 *versus* 19 *versus* 20 *versus* 22 *versus* 18) (Figure 5a, upper panel). Cell viability analysis also confirmed these results (percentage of dead cells → control *versus p73 versus caspase-2 versus caspase-8 versus caspase-9* siRNA: 19.4 *versus* 8.4 *versus* 9.0 *versus* 11.9 *versus* 9.9) (Figure 5a, lower panel). However, silencing of caspase-9 expression led to a reduction in the cleavage of both caspase-2 and -8 on taxol treatment (Figure 5b), whereas silencing of caspase-2 or -8 did not affect cleavage of caspase-9 (data not shown), suggesting that caspase-9 is upstream of both caspase-2 and -8 in this system. Together with the earlier observation that p73 silencing led to reduced caspase-9 cleavage (Figure 2a), the data suggest that p73 probably regulated cell death by acting upstream of caspase-9 by affecting the mitochondrial pathway.

We therefore examined whether p73 could affect cytochrome *c* release from mitochondria on taxol treatment. Although the absence of p53 did not affect taxol-mediated cytochrome *c* release into the cytosol, it was completely abrogated in *p73*$^{-/-}$ cells, although p53 was induced in these cells (Figure 5c). Cytochrome *c* release is dependent on Bax translocation to the mitochondrial membrane.[31] We therefore analyzed whether Bax translocation was affected in the absence of p73, by tracking Bax translocation using the N20 anti-Bax antibody, which is known to recognize activated Bax.[32] As shown in Figure 5d, Bax did not colocalize with the mitochondria in untreated cells. However, taxol treatment resulted in colocalization of Bax to the mitochondria in both *p53*$^{-/-}$ and *p73*$^{-/-}$ cells (Figure 5d, see arrowheads). To further confirm the status of Bax and cytochrome *c* in *p73*$^{-/-}$ cells, we analyzed the separated mitochondrial (pellet) and cytosolic (supernatant) fractions of the cellular lysates. ANT expression, which is detected only on the mitochondrial membrane, was monitored to determine the quality of fractionation. We noticed that Bax levels increased in the mitochondria of *p53*$^{-/-}$ and *p73*$^{-/-}$ cells on taxol treatment, concomitant to a decrease in the cytosolic fraction (Figure 5e). However, although cytochrome *c* was clearly released into the cytosol of *p53*$^{-/-}$ cells, this was not the case in *p73*$^{-/-}$ cells. Consistently, cytochrome *c* levels decreased in the mitochondrial fraction of *p53*$^{-/-}$ cells but not *p73*$^{-/-}$ cells (Figure 5e). Thus, the data together suggest that absence of p73 leads to defects in cytochrome *c* release without affecting Bax levels or its translocation to the mitochondria.

Bim is a direct transcriptional target of p73 regulating mitotic death. To explore the possible means by which p73 affected cytochrome *c* release and death during mitosis, we examined whether the transactivation property of p73 was required for its death-promoting function, by transfecting cells with the p73DD plasmid that acts as a dominant-negative inhibitor of p73 transcriptional activity, by binding to and inhibiting p73 function.[14] p73DD alone did not induce cell death unlike wild-type p73α. Taxol treatment of p73DD-transfected cells led to reduced cell death compared with vector or p73α-transfected cells (percentage of cleaved caspase 3 → vector *versus* p73α *versus* p73DD: – Taxol ► 4.8 *versus* 17 *versus* 4.9; + Taxol ►27.0 *versus* 38.0 *versus* 17.9, $P = 0.027$) (Supplementary Figure 8a). We further used the ΔNp73 plasmid lacking the transactivation domain. Expression of ΔNp73β resulted in reduction of taxol-induced cell death (percentage of cleaved caspase 3 → vector *versus* p73α *versus* ΔNp73β: – Taxol ► 1.3 *versus* 7.2 *versus* 2.8; + Taxol ► 15.6 *versus* 19.3 *versus* 11.3) (Supplementary Figure 8b). These preliminary data suggested that the transactivation property of p73 could be important for induction of cell death.

Thus, we analyzed whether the levels of several Bcl/BH3-only family members, which affect the ratio of anti-apoptotic and proapoptotic Bcl proteins and thereby regulate cytochrome *c* release, were altered in the absence of p73. No significant changes were observed in the levels of Bcl-2 and $Bclx_L$ in *p73*$^{-/-}$ cells (data not shown). However, although the levels of Puma and Bik were also not altered in *p73*$^{-/-}$ cells on taxol treatment, Bim expression was markedly reduced (Figure 6a). Absence of p73 also affected the increase of *bim* at the mRNA level on taxol treatment, in contrast to *p53*$^{-/-}$ cells (Figure 6b), suggesting that p73 could regulate *bim* transcription. Transient transfection of both p73α or p73β isoforms, as well as inducible expression of p73 in p53 null Saos2 cells, led to a strong induction of *bim*, indicating that p73 indeed contributed to the induction of *bim* mRNA (Figure 6c).

Search for p73 binding sites on the *bim* regulatory sequences revealed the presence of three canonical p53-responsive elements in intron 1 (Figure 6d), at positions 111601570 (RE1), 111605691 (RE2) and 111606633 (RE3), with reference to ENSG00000153094.[33] Chromatin immunoprecipitation (ChIP) experiments showed that the induction of p73 expression led to an increase in specific binding of p73 to RE1 and RE2 (Figure 6d). In contrast, we were unable to notice any binding of p73 onto RE3, although concurrent immunoprecipitations showed binding of p73 to the *mdm2* promoter (Figure 6d). The data therefore suggest that p73 is capable of binding directly to the p53 response elements *in vivo* on the *bim* regulatory locus, thereby controlling its expression.

As p73 was induced around the G_1/S boundary and disappeared as the cells enter the G_2/M phase (Figure 2a),[19] we next investigated whether p73 is capable

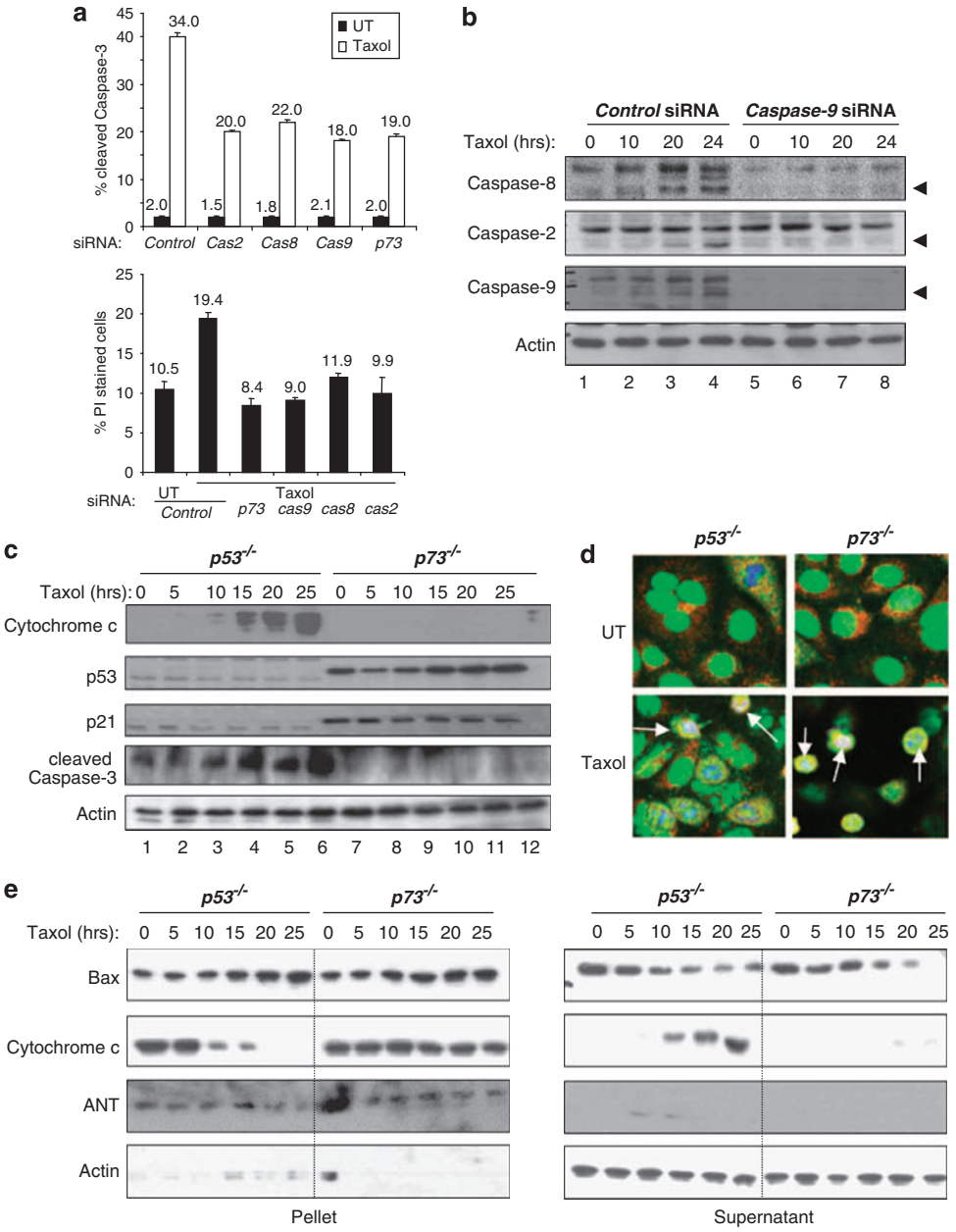


Figure 5 p73-dependent taxol-induced apoptosis occurs through the mitochondrial pathway. (**a**) H1299 cells were transfected with siRNA against caspase-2, -8 or -9, 30 h before taxol treatment and the percentage of cleaved caspase-3 (top panel) or propidium iodide (PI) exclusion (lower panel) was determined 24 h later. Experiments were performed in duplicates and at least three times, and the mean ± S.D. is shown. (**b**) Immunoblot analysis was performed to determine the activation (cleavage) of caspases-2, -8 or -9 in H1299 cells in which the expression of caspase-9 was silenced before taxol treatment. Cleaved caspase bands are indicated with arrowheads. (**c**) *p53*$^{-/-}$ and *p73*$^{-/-}$ fibroblasts were treated with taxol and the cytosolic fraction (which does not contain the mitochondrial fraction) was extracted by cellular fractionation for immunoblot analysis of cytochrome *c* release. (**d**) Active Bax translocation to mitochondria was monitored using confocal immunofluoresence in *p53*$^{-/-}$ and *p73*$^{-/-}$ fibroblasts 16 h after taxol treatment, using the N20 anti-Bax antibody, which recognizes active Bax. Green fluorescence indicates active Bax localization. Mitochondria were stained red using the mitotracker. Nuclear staining is shown in blue by DAPI staining. Active Bax colocalizing with the mitochondria is indicated by white arrows. (**e**) Cellular lysates of taxol-treated *p53*$^{-/-}$ and *p73*$^{-/-}$ fibroblasts were fractionated into mitochondrial (pellet) and cytosolic (supernatant) fractions and were used for immunoblot analysis. ANT and actin expression were monitored for quality of mitochondrial and cytosolic fractionation, respectively

of binding *in vivo* onto the *bim* regulatory sites RE1 and RE2. To this end, cells were first collected at various cell cycle stages (Figure 6e, right panel) and then analyzed for p73 binding. As shown in Figure 6f, p73 was bound onto both RE1 and RE2 sites at the G_1/S stage, where its expression was highest (Figure 6e), but not in the G_0/G_1 or G_2/M stages. Importantly, *bim* expression was also highest in the G_1/S phase, mirroring *p73* expression (Figure 6e, left panel). This indicated that p73 indeed regulated *bim in vivo* in a cell-cycle-dependent manner. Moreover, taxol treatment also led to p73 being bound to the RE1 and RE2 sites, unlike cisplatin (CDDP) – a DNA-damaging agent – which only led to binding

of p73 to the RE2 site (Figure 6g). These data together show that p73 is able to bind to the *bim* regulatory regions *in vivo* on taxol treatment and in a cell cycle-dependent manner, correlating with *bim* expression.

As p73 was unable to modulate Bim expression, and as Bim has been shown to be required for taxol-induced cell death,[34] we investigated whether mitotic death induced by other means that are p73-dependent also requires Bim. To this end, we silenced the expression of *PLK1*, which led to increased cell death that was p73-dependent (Figures 3d and 6h, upper panel). Silencing of *bim* expression also led to reduction of cell death, similar to the levels found when *p73* expression was silenced (percentage of dead cells → control *versus p73 versus bim* siRNA: −*PLK1* siRNA ► 8.8 *versus* 8.4 *versus* 8.2; +*PLK1* siRNA ► 18.0 *versus* 7.2 *versus* 8.0) (Figure 6h), supporting the notion that p73-mediated Bim expression is critical for modulating mitotic death. Moreover, silencing of *bim* expression also reduced taxol-mediated cell death, to similar extents as *p73* silencing (percentage of dead cells → control *versus p73 versus bim* siRNA: −taxol ► 11.3 *versus* 6.8 *versus* 5.8; +taxol ►24.4 *versus* 15.6 *versus* 16.2; $P = 0.05$) (Figure 6h, lower panel).

Finally, we examined whether Bim expression coincides with cleaved caspase-3 expression on taxol treatment *in vivo,* by microscopic analysis at the single-cell level. Untreated or

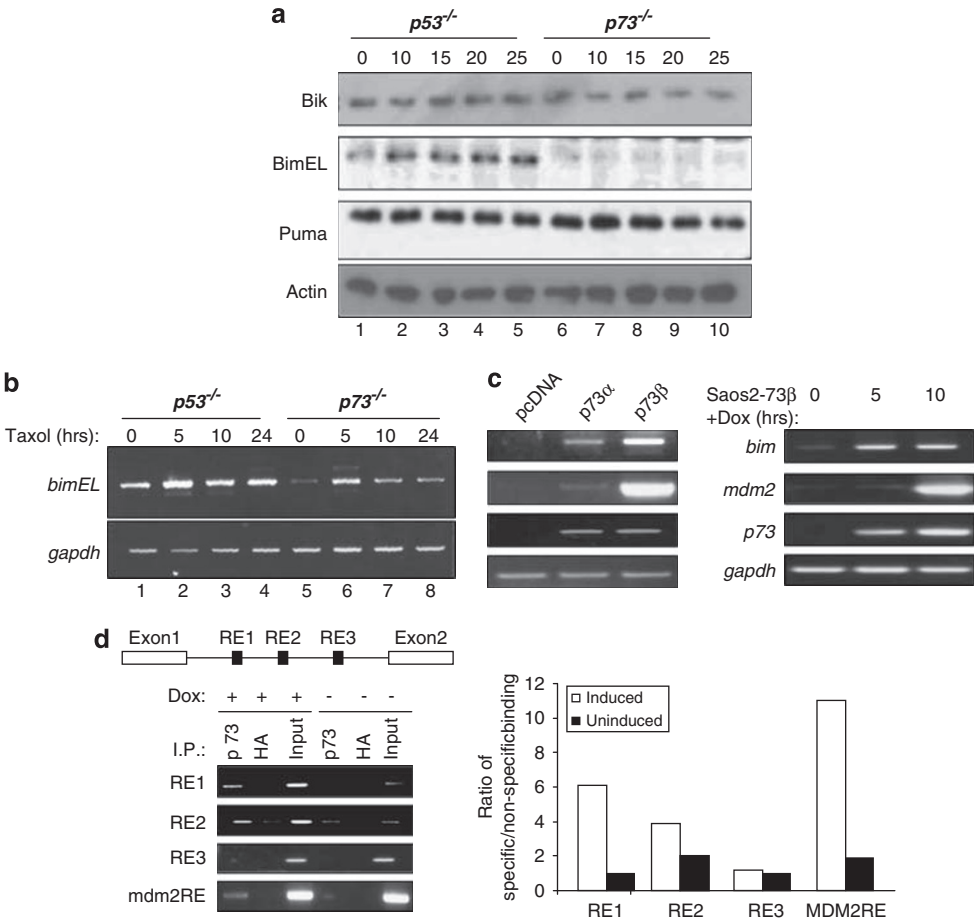


Figure 6 p73 transcriptionally regulates Bim to regulate mitotic death. (**a**, **b**) *p53*$^{-/-}$ and *p73*$^{-/-}$ fibroblasts were analyzed for the levels of various BH3-only family proteins after taxol treatment by immunoblot (**a**) or for *bim* levels by RT-PCR (**b**) analysis. (**c**) p73α and p73β were transfected into *p73*$^{-/-}$ fibroblasts and *bim* mRNA levels were analyzed 24 h later, together with that of the known target *Mdm2* (left panel). Similarly, *bim* expression was analyzed on induction of p73β expression in human Saos-2 doxycycline-inducible p73β cells for the indicated time periods (right panel). (**d**) Three canonical p53 consensus-binding sites (termed RE1, RE2, RE3) were detected in intron 1 of human *bim* genomic sequence. Chromatin immunoprecipitation (ChIP) experiments followed by PCR analysis were performed on the three REs using specific primers and cell lysates from Saos-2 doxycycline-inducible p73β cells. A known p73-binding site on *mdm2* intron 1 (Mdm2RE) was used as positive control. Input control is shown. To quantify the fold increase in the binding of p73 to *bim* promoter after induction of p73 expression, band intensity of anti-p73 ChIP lane was normalized against band intensity of anti-HA IP lane in both uninduced and induced sets for each RE1, RE2, RE3 and Mdm2 REs. The results are depicted graphically as a ratio of p73 *versus* HA intensity, determined in numerical values by the analysis software. (**e**, **f**) H1299 cells were collected at G_0/G_1 (serum starvation for 72 h), G_1/S (arrested with 2 mM thymidine for 24 h) or G_2/M (thymidine arrested and released for 15 h). Samples were used for RT-PCR (**e**) or ChIP analysis (**f**). Cell cycle status of the cells used is shown on the right panel (**e**). (**g**) H1299 cells were treated with 100 nM of taxol or 30 μM of cisplatin (CDDP) for 6 h after release from the thymidine block and samples were used for ChIP analysis. UT: untreated. (**h**) Bim knockdown, similar to p73 knockdown, reduces cell death caused by silencing of PLK1 or after taxol treatment in H1299 cells. Bim and p73 siRNA were transfected 12 h before PLK1 siRNA transfection (upper panel). Cells were maintained for 24 h after PLK1 siRNA transfection in thymidine before being released into normal medium, 24 h after which they were collected for analysis of cell death by PI exclusion assay. Similarly, cells were treated with taxol for 24 h instead of PLK silencing (lower panel). Experiments were performed in duplicates and at least twice, and the mean ± S.D. is shown. (**i**) H1299 cells were taxol-treated as described and collected 15 h later for immunofluorescence analysis with anti-Bim and anti-cleaved caspase-3 antibodies

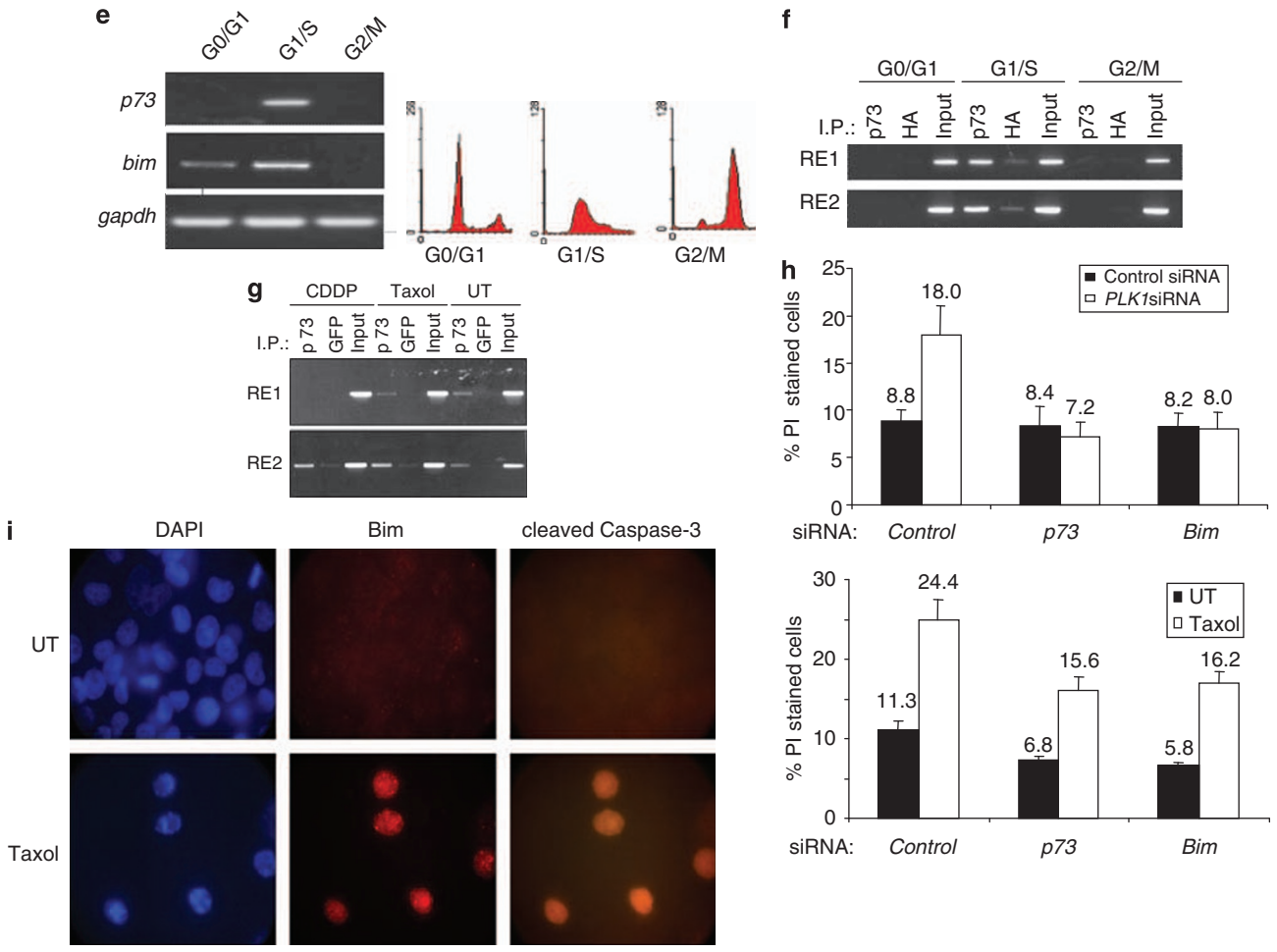


Figure 6 Continued

taxol-treated cells were fixed and stained with the respective specific antibodies, which revealed that the expression of both Bim and cleaved caspase-3 were induced on taxol treatment (Figure 6i). Importantly, both their expressions were colocalized in individual cells (Figure 6i, representative cells shown). These data therefore show that p73 regulates Bim expression, leading to cell death on taxol treatment.

Discussion

The data presented here show a critical regulatory role for p73 in controlling mitotic cell death that occurs during mitosis (and before its exit) and that is dependent on the activation of the spindle checkpoint. This highlights a specific role of p73, which occurs through the induction of Bim expression and the subsequent activation of caspases. These results therefore suggest the existence of a p73–Bim–caspase apoptotic axis that regulates the process of mitotic cell death independent of p53.

p73 and p53: cell cycle-specific roles in regulating apoptosis? Although p73 is required for death during mitosis, it should be noted that overexpression studies as well as exposure to various cytotoxic agents have indicated that cells can indeed undergo p73-dependent death, presumably at all other stages of the cell cycle.[10,14] Current knowledge does not allow us to conclude whether p73 is evolved for inducing apoptosis only during mitosis. However, the findings that the induction and abundance of p53 in p73 null cells do not contribute to Bim activation and subsequent mitotic death highlight the nonfunctionality of p53 in this process. Moreover, absence of p53 did not affect Bim expression, cytochrome *c* release and mitotic death. Besides, it is noteworthy that p73 is induced earlier on taxol treatment, peaking around 6–12 h after taxol treatment, compared with p53 levels that generally peak later at around 18 h. Hence, it is likely that p53 is upregulated in post-mitotic G_1 phase cells to arrest cells with compromised genomic integrity, and may have a role in inducing cell death that occurs after mitotic exit, in contrast to p73-dependent death that occurs during mitosis. It could therefore be expected that absence of p53 would lead to continual cycling of cells into mitosis on DNA damage attributed to the compromised DNA damage response pathway, where the damaged chromosomes may trigger mitotic checkpoint activation and arrest, resulting in subsequent p73-dependent apoptosis. Corroborating this suggestion is the finding that *p53*$^{-/-}$*p73*$^{-/-}$ MEFs are more resistant to death than single knockouts,

indicating a distinct apoptotic role for p73.[13] Thus, it can be concluded that p73-dependent apoptosis occurring during mitosis is independent of p53, and it is conceivable that p53 and p73 may have specific temporal roles in regulating apoptosis through the cell cycle.

p73-mediated mitotic death: priming for death that is dependent on the activation of the spindle checkpoint and prolonged mitotic arrest. A noteworthy and interesting observation is that p73 levels are not maintained but decrease during G_2/M transition (this report and Irwin *et al.*[19]), although the functional effects are realized in its absence during this phase of the cell cycle. Previous findings by Fulco *et al.*[20] also suggested that p73 is phosphorylated by $p34^{cdc2}$, leading to its inactivation. These observations, together with the fact that transcription is shut down during mitosis, suggest a possible paradox, whereby it appears that p73 is inactivated during mitosis but is functionally relevant to induce mitotic death. However, preliminary analysis indicated that p73 was bound to the *bim* regulatory sites during the G_1/S phase of the cell cycle, but not during the G_2/M phase, leading to *bim* upregulation (Figure 6e and f). Hence, the rise of p73 levels during the early cell cycle phases is probably sufficient to prepare cells for death during mitosis by the activation of the Bim-caspase pathway, in the event of spindle checkpoint activation. This suggests a priming role for p73 in regulating mitotic death, rather than transactivation of targets (such as Bim) during mitosis. This leads us to speculate that the mitotic death mechanism is a default one, initiated by p73 during the early phases of the cell cycle and triggered by activation of the spindle checkpoint and dependent on the extent of time spent in mitosis. Supportive evidence comes from the findings that complete prevention of mitotic exit by the addition of MG132 to taxol-treated cells hindered mitotic exit and potentiated cell death. Conversely, enforced exit of taxol-treated cells out of mitosis by knockdown of BubR1 and Mad2, or addition of the cdc2 chemical inhibitor PurvA or roscovitine, or prevention of mitotic entry by RO-3066 treatment or by thymidine –block, reduced the activation of the apoptotic program, together highlighting the requirement of cells to be in mitosis for a prolonged period of time for p73-mediated death. Besides mitotic arrest, our data suggest that the spindle checkpoint needed to be evoked for p73-dependent death. This was evident from treatment of cells with cytochalasin D – which blocks cells at late cytokinesis and does not induce the spindle checkpoint – that did not lead to cell death. Hence, in the absence of any trigger, cells continue out of mitosis without undergoing death.

The results therefore support the notion that a second signal is required for execution of p73-Bim-mediated death. It could therefore be envisaged that extending the length of the mitotic phase by perturbations to microtubules or spindle formation allows Bim, which is generally kept in an inactive state through physical binding to microtubules,[35] to be released to enhance apoptosis. Therefore, the increase in the length of the mitotic phase can be expected to allow the continuous release of Bim, thereby providing the opportunity for apoptosis to occur. Consistent with this idea, a recent report showed that cyclinB1/Cdk1-mediated phosphorylation of caspase-9 leads to inhibition of its default apoptosis-promoting activity during mitosis.[36] Extended time in mitosis led to the dephosphorylation of caspase-9 because of a decrease in cyclinB1, allowing the triggering of the default apoptotic mechanism. Interestingly, it has also been shown that Bim is actively degraded by the H-Ras pathway – which is activated by growth factors – thereby contributing to taxol resistance,[37] lending further support to the idea that the mitotic cell death pathway is indeed a default mechanism that is inactivated in the absence of any spindle checkpoint activation. Altogether, the data suggest an essential priming role for p73 in regulating mitotic cell death through the Bim-caspase-9 pathway.

p73 upregulates Bim to regulate caspase-dependent mitotic death. As abrogation of p73's transactivation property reduced taxol-mediated death, we surveyed for and found that Bim expression was dramatically reduced in the absence of p73 on taxol treatment, in contrast to *p53*$^{-/-}$ cells, and p73 expression led its induction. We identified regulatory sites in intron 1 of *bim*, to which p73 was able to bind, suggesting that p73 was sufficient to directly induce Bim expression to regulate mitotic death. Consistently, silencing of *bim* expression led to a reduction in mitotic death induced by *PLK1* silencing or by taxol treatment, to similar levels as when p73 expression was silenced (this report and Bouillet *et al.*[34]). The lethal effects of Bim overexpression on cells prevented us from reversing the taxol-resistant phenotype of *p73*$^{-/-}$ cells.[38] Nevertheless, the results together suggest that Bim is a direct transcriptional target for p73 in regulating mitotic death.

Downstream of Bim, p73-dependent death seems to occur through the caspase-9 cascade. Consistent with the notion that caspase-9 promotes mitochondria-mediated apoptosis, we found that cytochrome *c* release from the mitochondria, which is required for caspase-9 cleavage, was markedly affected in the absence of p73 but not in the absence of p53. A critical role for caspase activation in mitotic death has also been recently emphasized.[39] The data together show that p73 regulates cytochrome *c* release through Bim on taxol treatment, thereby modulating mitotic death that is caspase-dependent.

It is also worth noting that recent evidence has suggested a role for p73 in inducing cell death during mitosis through a caspase-independent pathway, termed caspase-independent mitotic death.[40] However, this process seems to occur specifically in cells in which the BubR1 activity is significantly reduced. Hence, it is possible that other pathways, which are also regulated by p73, do exit to regulate mitotic death.

Other roles for p73 in and around mitosis? A recent extensive study has convincingly shown that varied outcomes can occur in cells exposed to mitotic drugs, which include death in mitosis (as reported here), polyploidy and death in subsequent cell cycles.[39] The data presented here, together with those of Niikura *et al.*,[40] show that p73 is indeed required to regulate cell death during mitosis. However, evidence from *TAp73*$^{-/-}$ cells has indicated that absence of p73 leads to polyploidy on treatment with nocodazole,[18] implying a role in prevention of genomic

instability. Moreover, p73 was also shown to suppress polyploidy in the absence of p53.[41] Taken together, these data suggest that p73 is critical for cell death in mitosis, but in the event that cells escape mitosis, p73 has a role in preventing polyploidy. How exactly p73 regulates these processes coordinately requires further investigation.

In summary, we have shown that p73 has a crucial role in regulating apoptosis during mitosis, which occurs through the activation of Bim, eventually leading to the activation of the caspase-9 cascade. Our data suggest that mitotic arrest provides the necessary second signal that allows the manifestation of the priming effects of p73-mediated Bim activation. As p73 is often highly expressed in many cancers, activation of the mitotic death pathway, perhaps in combination with drugs that cause normal cells to be arrested in the G_1 phase of the cell cycle, may be a strategy for improved anticancer therapeutic response.

Materials and Methods

Details of the cell lines and reagents used, as well as the transfections, biochemical and chromatin immunoprecipitation procedures, cell death, cell cycle and immunofluorescence analysis, siRNA sequences, RT-PCR conditions and statistical analysis are provided in the supplementary section.

Conflict of interest

The authors declare no conflict of interest.

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1. Rieder CL, Maiato H. Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev Cell* 2004; **7**: 637–651.
2. Nigg EA. Mitotic kinases as regulators of cell division and its checkpoints. *Nat Rev Mol Cell Biol* 2001; **2**: 21–32.
3. Weaver BA, Cleveland DW. Decoding the links between mitosis, cancer, and chemotherapy: the mitotic checkpoint, adaptation, and cell-death. *Cancer Cell* 2005; **8**: 7–12.
4. Bharadwaj R, Yu H. The spindle checkpoint, aneuploidy, and cancer. *Oncogene* 2004; **23**: 2016–2027.
5. Castro A, Bernis C, Vigneron S, Labbe JC, Lorca T. The anaphase-promoting complex: a key factor in the regulation of cell cycle. *Oncogene* 2005; **24**: 314–325.
6. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell-death by mitotic catastrophe: a molecular definition. *Oncogene* 2004; **23**: 2825–2837.
7. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000; **408**: 307–310.
8. Debernardis D, Sire EG, De FP, Vikhanskaya F, Valenti M, Russo P *et al.* p53 status does not affect sensitivity of human ovarian cancer cell lines to paclitaxel. *Cancer Res* 1997; **57**: 870–874.
9. Vikhanskaya F, Vignati S, Beccaglia P, Ottoboni C, Russo P, D'Incalci M *et al.* Inactivation of p53 in a human ovarian cancer cell line increases the sensitivity to paclitaxel by inducing G2/M arrest and apoptosis. *Exp Cell Res* 1998; **241**: 96–101.
10. Jost CA, Marin MC, Kaelin Jr WG. p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 1997; **389**: 191–194.
11. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A *et al.* Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 1997; **90**: 809–819.
12. Melino G, De LV, Vousden KH. p73: Friend or foe in tumorigenesis. *Nat Rev Cancer* 2002; **2**: 605–615.
13. Flores ER, Tsai KY, Crowley D, Sengupta S, Yang A, McKeon F *et al.* p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* 2002; **416**: 560–564.
14. Irwin MS, Kondo K, Marin MC, Cheng LS, Hahn WC, Kaelin Jr WG. Chemosensitivity linked to p73 function. *Cancer Cell* 2003; **3**: 403–410.
15. Lin KW, Nam SY, Toh WH, Dulloo I, Sabapathy K. Multiple stress signals induce p73beta accumulation. *Neoplasia* 2004; **6**: 546–557.
16. Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J *et al.* p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* 2000; **404**: 99–103.
17. Flores ER, Sengupta S, Miller JB, Newman JJ, Bronson R, Crowley D *et al.* Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* 2005; **7**: 363–373.
18. Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC *et al.* TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* 2008; **22**: 2677–2691.
19. Irwin M, Marin MC, Phillips AC, Seelan RS, Smith DI, Liu W *et al.* Role for the p53 homologue p73 in E2F-1-induced apoptosis. *Nature* 2000; **407**: 645–648.
20. Fulco M, Costanzo A, Merlo P, Mangiacasale R, Strano S, Blandino G *et al.* p73 is regulated by phosphorylation at the G2/M transition. *J Biol Chem* 2003; **278**: 49196–49202.
21. Vernole P, Neale MH, Barcaroli D, Munarriz E, Knight RA, Tomasini R *et al.* TAp73alphs binds the kinetochore proteins Bub1 and Bub3 resulting in polyploidy. *Cell Cycle* 2009; **8**: 421–429.
22. Tomasini R, Tsuchihara K, Tsuda C, Lau SK, Wilhelm M, Ruffini A *et al.* TAp73 regulates the spindle assembly checkpoint by modulating BubR1 activity. *Proc Natl Acad Sci USA* 2009; **106**: 797–802.
23. O'Connor DS, Wall NR, Porter AC, Altieri DC. A p34(cdc2) survival checkpoint in cancer. *Cancer Cell* 2002; **2**: 43–54.
24. Vassilev TL, Tovar C, Chen S, Knezevic D, Zhao X, Sun H *et al.* Selective small-molecule inhibitor reveals critical mitotic functions of human CDK1. *Proc Natl Acad Sci USA* 2006; **103**: 10660–10665.
25. Carvalho A, Carmena M, Sambade C, Earnshaw WC, Wheatley SP. Survivin is required for stable checkpoint activation in taxol-treated HeLa cells. *J Cell Sci* 2003; **116**: 2987–2998.
26. Stukenberg PT. Triggering p53 after cytokinesis failure. *J Cell Biol* 2004; **165**: 607–608.
27. Meraldi P, Sorger PK. A dual role for Bub1 in the spindle checkpoint and chromosome congression. *EMBO J* 2005; **24**: 1621–1633.
28. Guan R, Tapang P, Leverson JD, Albert D, Giranda VL, Luo Y. Small interfering RNA-mediated Polo-like kinase 1 depletion preferentially reduces the survival of p53-defective, oncogenic transformed cells and inhibits tumor growth in animals. *Cancer Res* 2005; **65**: 2698–2704.
29. Sudo T, Nitta M, Saya H, Ueno NT. Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Res* 2004; **64**: 2502–2508.
30. Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell-death. *Pharmacol Ther* 2001; **92**: 57–70.
31. Green DR, Kroemer G. The pathophysiology of mitochondrial cell-death. *Science* 2004; **305**: 626–629.
32. Makin GW, Corfe BM, Griffiths GJ, Thistlethwaite A, Hickman JA, Dive C. Damage-induced Bax N-terminal change, translocation to mitochondria and formation of Bax dimers/complexes occur regardless of cell fate. *EMBO J* 2001; **20**: 6306–6315.
33. Sbisa E, Catalano D, Grillo G, Licciulli F, Turi A, Liuni S *et al.* p53FamTaG: a database resource of human p53, p63 and p73 direct target genes combining in silico prediction and microarray data. *BMC Bioinformatics* 2007; **8** (Suppl 1): S20.
34. Bouillet P, Metcalf D, Huang DC, Tarlinton DM, Kay TW, Kontgen F *et al.* Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* 1999; **286**: 1735–1738.
35. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell* 1999; **3**: 287–296.
36. Allan LA, Clarke PR. Phosphorylation of caspase-9 by CDK1/Cyclin B1 protects mitotic cells against apoptosis. *Mol Cell* 2007; **26**: 301–310.
37. Tan TT, Degenhardt K, Nelson DA, Beaudoin B, Nieves-Neira W, Bouillet P *et al.* Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell* 2005; **7**: 227–238.
38. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* 2005; **17**: 393–403.
39. Gascoigne KE, Taylor SS. Cancer cells display profound intra-and interline variation following prolonged exposure to antimitotic drugs. *Cancer Cell* 2008; **14**: 111–122.
40. Niikura Y, Dixit A, Scott R, Perkins G, Kitagawa K. BUB1 mediation of caspase-independent mitotic death determines cell fate. *J Cell Biol* 2007; **178**: 283–296.
41. Talos F, Nemajerova A, Flores ER, Petrenko O, Moll UM. p73 suppresses polyploidy and aneuploidy in the absence of functional p53. *Mol Cell* 2007; **27**: 647–659.

Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)