ING1b decreases cell proliferation through p53-dependent and -independent mechanisms

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Abstract **ING1b can stimulate cell cycle arrest, repair, senescence, and apoptosis. The actions of ING1b are attributed to its activation of the tumor suppressor p53. Here we investigate the more subtle effects of ING1b on the cell cycle and DNA damage responses in the absence of p53. To this end, we have generated isogenic cell lines that expressed ING1b and p53 either individually or in combination under the control of inducible promoters. A five- to 10-fold induction of ING1b over the endogenous protein in a p53-null H1299 background slightly impairs proliferation by increasing the doubling time by ~10%. Significantly, ectopic expression of ING1b enhanced the G_2/M DNA damage checkpoint induced by adriamycin. We demonstrated that the DNA damage-induced cell death mediated by the cooperation between ING1b and p53 was more prominent than by the individual proteins alone. In adriamycin-treated cells, p53 was stabilized and induced the expression of $p21^{CIP1/WAF1}$, but the expression of ING1b was not affected. The exact targets of ING1b in the p53-null background are not known, but we demonstrated that the transcriptional activities of other members of the p53 family, p63α and p73α, could be activated by ING1b. These data indicate that ING1 has a subtle antiproliferative effect even in the absence of p53, and ING1b enhances the DNA damage responses through p53-dependent and -independent mechanisms.**

Key words: Cell cycle; Checkpoint; DNA damage; ING1; p53; Tumor suppressor

1. Introduction

ING1 was first identified as a candidate tumor suppressor through a functional screening based on selection of gene fragments that can block the activity of tumor suppressors and cause transformation [1]. *ING1* encodes a 47-kDa protein (ING1a), an alternatively spliced 33-kDa protein (ING1b), and a 24-kDa protein (ING1c) from an internal initiation site [2,3]. The three isoforms have different N-terminal regions but share a common C-terminal PHD finger (a C4HC3-type zinc finger that is implicated in transcriptional regulation). ING1b and ING1c are the major isoforms expressed in human tissues [2], and there are indications that the different isoforms may have different functions [4].

ING1 has been implicated to play a role in cell cycle control, apoptosis, and senescence. ING1 is upregulated in the S phase and in senescent human fibroblasts, and antisense ING1 extends the proliferative lifespan of normal human fibroblasts [5]. ING1b is upregulated during apoptosis, and overexpression of ING1b inhibits cell growth and enhances apoptosis [1,6,7]. Conversely, expression of ING1 antisense constructs promotes transformation [1] and protects against apoptosis [6]. In this connection, the anti-apoptotic protein A1 can bind to mouse ING1 and inhibit the pro-apoptotic effects of ING1 [8].

The expression of ING1 is not regulated by DNA damage in fibroblasts, but ING1 is induced in keratinocytes and melanoma cell lines by UV irradiation [9,10]. After UV irradiation, ING1b shifts from the nucleoplasm to the nucleolus [11], and enhances p53-dependent repair [9] and apoptosis [12]. Possibly related to these functions, ING1b is found to associate with GADD45 [9] and with PCNA after UV irradiation [13].

The growth inhibitory and apoptosis-inducing functions of ING1 appear to involve the tumor suppressor p53 [14,15]. Physical interaction between ING1 and p53 was demonstrated by co-immunoprecipitation [14]. Moreover, the transcriptional activities of p53 are enhanced by ING1, but are eliminated by antisense ING1 constructs [14]. Our laboratory found that one mechanism by which ING1b stimulates the activity of p53 is by increasing the stability of p53 through disruption of the p53–MDM2 interaction [16]. Like ING1, other members of the ING family, ING2 [17,18] and ING3 [19], also negatively regulate cell growth and survival by activation of p53. But unlike ING1b, ING2 activates p53 by increasing the acetylation of p53 at Lys-382 [17]. Similarly, ING4 and ING5 enhance the acetylation of p53 at Lys-382 and induction of apoptosis in a p53-dependent manner [20]. Interestingly, the PHD domain of ING2 acts as a nuclear phosphoinositide receptor, and this interaction regulates the ability of ING2 to activate p53 and p53-dependent apoptotic pathways [21].

ING1 has been implicated in chromatin remodeling and transcriptional control. Three proteins in yeast that share significant sequence identity with ING1 (Yng1p, Yng2p, and Pho23p) are associated with histone acetyltransferase (HAT) complexes [22]. Yng1p and Yng2p are components of the NuA3 and NuA4 HAT complexes respectively [23–25]. In human cells, ING1b is associated with the Sin3-containing histone deacetylases (HDAC) through direct interaction with the Sin3 component SAP30 [26,27]. In another report, it was

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Abbreviations: ADR, adriamycin; CIS, cisplatin; DOX, doxycycline; HAT, histone acetyltransferase; HDAC, histone deacetylase

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found that while complexes containing human ING1b possess HAT activity, the isoform ING1a binds HDAC1 and inhibits histone acetylation [28].

Although *ING1* is rarely mutated, its expression and localization are altered in several cancers. Several missense mutations of *ING1* were found within the PHD finger domain and the nuclear localization motif in head and neck squamous cell carcinoma [29,30]. *ING1* expression is reduced in breast cancer [31,32], gastric cancer [33], and lymphoid malignancies [34]. Finally, the subcellular localization of ING1b is shifted from the nucleus to the cytoplasm in melanoma [35] and acute lymphoblastic leukemia [36].

The p53-dependent roles of ING1b in antiproliferation are well established. However, induction of ING1b after DNA damage does not require p53 [10], and it is not clear how ING1b affects cell proliferation in the absence of p53. Using isogenic cell lines, we have examined the effects of ING1b expression on cell cycle progression and DNA damage responses in the presence or absence of p53.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

2.2. DNA constructs

FLAG-ING1b in pUHD-P1 and p53 in pLINX were constructed as described previously [16]. The *Eco*RI–*Sal*I fragment from FLAG-ING1b in pUHD-P1 was ligated into pGEX-KG to produce GST-ING1b in pGEX-KG. The *Cla*I fragment from p53 in pLINX was put into pRevTRE2 (Clontech, Palo Alto, CA, USA). The *Bam*HI fragment containing the puromycin-resistant gene (a gift from Katsumi Yamashita, Kanazawa University, Japan) was put into *Bam*HI-cut FLAG-ING1b in pUHD-P1 to produce FLAG-ING1b in pUHD-P1/PUR. HA-tagged simian p73α in pcDNA3, p53 in pRcCMV, and p63α in pRc/CMV were obtained from sources as previously described [37].

2.3. Cell culture

H1299 (non-small cell lung carcinoma) [38] was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37°C in 5% CO_2. Cells were transfected with the calcium phosphate precipitation method [39]. UV treatment and cell-free extracts were as described previously [40]. Unless stated otherwise, cells were treated with the following reagents at the indicated final concentrations: adriamycin (ADR; 0.02 μg/ml), doxycycline (DOX; 1 μg/ml), G418 (100 μg/ml), hygromycin (100 μg/ml), nocodazole (0.1 μg/ml), and puromycin (1 μg/ml).

Inducible ING1b/H1299 cell lines were produced by cotransfection of FLAG-ING1b in pUHD-P1/PUR and pLINX constructs (a gift from Tony Hunter, Salk Institute, La Jolla, CA, USA) into H1299. The cells were then selected in medium supplemented with puromycin, G418, and DOX. Inducible p53/H1299 cell lines were produced by transfection of p53 in pLINX into H1299, followed by selection in medium supplemented with G418 and DOX. Inducible p53+ING1b/H1299 cell lines were produced by transfection of p53 in pRevTRE2 into ING1b/H1299, followed by selection in medium supplemented with hygromycin and DOX. After about 3 weeks of selection, individual colonies were isolated and expanded. The expression of FLAG-ING1b or p53 was analyzed after growing cells in medium in the presence or absence of DOX for 24 h. The established cell lines were propagated in the presence of DOX but without other antibiotic selection. The expression of ING1b and/or p53 was constantly monitored because they were frequently lost during passage.

2.4. Cell growth analysis and flow cytometry

Trypan blue exclusion analysis and flow cytometry analysis after propidium iodide staining were performed as described [41]. Cell growth was measured by seeding $\sim 10^4$ cells per 60-mm plate and counting the attached cells in the same randomly selected areas (2-mm-diameter circles fixed at the bottom of the plate) every 24 h using a light microscope. The population doubling time was estimated by plotting the log of cell number against time. For clonogenic survival assays, 500 cells were seeded per 10-cm plate either in the presence or absence of DOX. After 12 h, the cells were treated with the indicated concentration of ADR (also called doxorubicin) or cisplatin (CIS; cis-platinum(II)diammine dichloride) for 3 h. The cells were then washed and fresh medium (with or without DOX) was added and replenished every three days. After 2–3 weeks, colonies were fixed with methanol:acetic acid (2:1 v/v) and visualized by staining with 2% w/v crystal violet in 20% methanol.

2.5. Expression and purification of recombinant proteins and binding assays

Expression of GST-tagged proteins in bacteria and purification with glutathione (GSH)-agarose chromatography were as described previously [42]. Coupled transcription–translation reactions in the presence of [^{35}S]methionine in rabbit reticulocyte lysate were performed as instructed by the manufacturer (Promega, Madison, WI, USA). Binding assays were as described elsewhere [43].

2.6. Luciferase and β-galactosidase assays

Luciferase assays and β-galactosidase assays were performed exactly as described previously [16].

2.7. Antibodies and immunological methods

Monoclonal antibodies M2 against FLAG tag were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal antibodies YL1/2 against tubulin were gifts from Tim Hunt (Cancer Research UK, South Mimms, UK). Goat antibodies raised against a C-terminal peptide derived from ING1 (sc-7566), polyclonal antibodies against $p21^{CIP1/WAF1}$ (sc-397), monoclonal antibodies 4A4 against p63 (sc-8431), and monoclonal antibodies DO-1 against p53 (sc-126) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoblotting was performed as described previously [40].

3. Results

3.1. Establishing inducible expression of ING1b in p53-containing or -null backgrounds

An inducible expression system for FLAG-tagged ING1b under the repression of DOX was created in the H1299 (p53-null) background. Clones that had integrated the constructs were isolated (herein designated ING1b/H1299). Fig. 1A shows that several clones (#2 and #4) expressed FLAG-ING1b in the absence of DOX. Background expression seen in the presence of DOX is probably due to promoter leakage and endogenous ING1b. We estimated by densitometry that typically a five- to 10-fold induction of ING1b was obtained.

To create isogenic cell lines with or without p53, the p53 cDNA was introduced into both H1299 and ING1b/H1299. Fig. 1B,C shows that p53/H1299 and p53+ING1b/H1299 expressed p53 with similar kinetics when DOX was removed. As expected, ING1b was induced in the absence of DOX in p53+ING1b/H1299, but remained constant (endogenous ING1b) in p53/H1299. Immunoblotting for tubulin confirmed uniform loading of lysates and p53 and/or ING1b expression was specifically controlled by DOX. Thus isogenic cell lines that inducibly expressed p53, ING1b, or p53 and ING1b together were generated.

3.2. ING1b only slightly retards cell growth in the absence of p53

To see whether ectopic expression of ING1b affected proliferation of H1299 cells, cell growth was examined in the

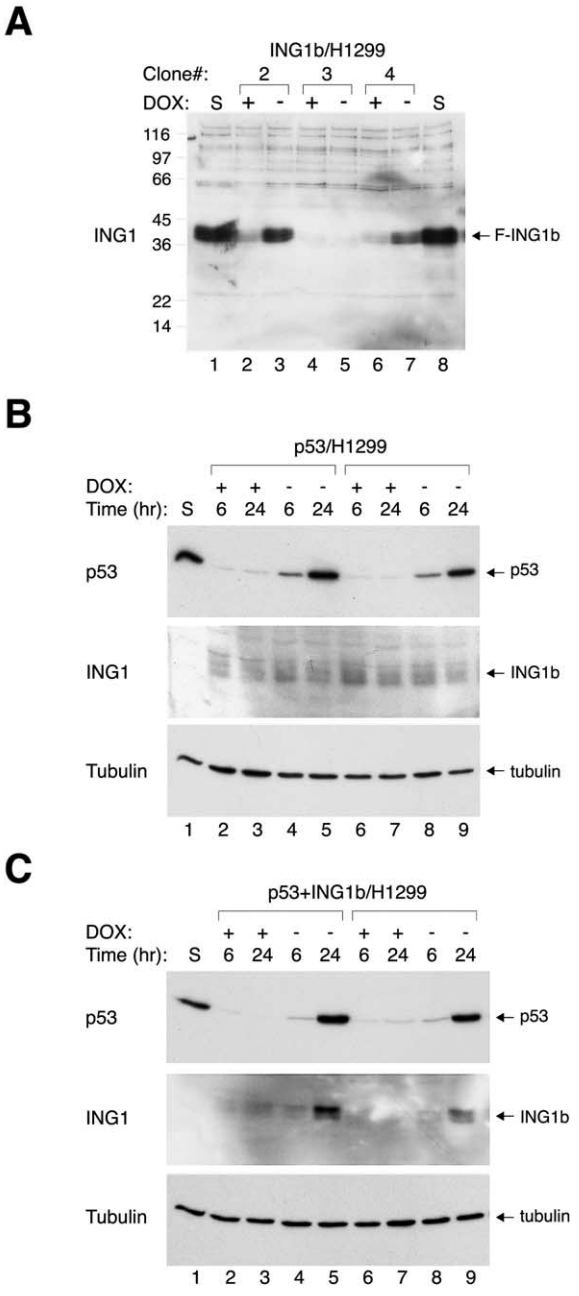


Fig. 1. Expression of ING1b and p53 in H1299 cells. A: ING1b/H1299 cells were generated as described in Section 2. Individual clones were isolated and grown in the presence (lanes 2, 4 and 6) or absence (lanes 3, 5 and 7) of DOX for 24 h. Cell-free extracts were prepared and were subjected to immunoblotting with antibodies against ING1. Standards of FLAG-ING1b from transiently transfected H1299 were loaded in lanes 1 and 8. The positions of the molecular size markers (in kDa) are indicated on the left. B: p53/H1299 cells were generated as described in Section 2, and were grown in the presence or absence of DOX for 6 and 24 h as indicated. Cell extracts were prepared and p53, ING1b, and tubulin were detected by immunoblotting as indicated. Standards of p53 from transiently transfected H1299 were loaded in lane 1. Results from two independent experiments are shown to indicate the consistency of protein induction. C: p53+ING1b/H1299 cells were treated exactly as in panel B.

presence or absence of DOX. We found that ING1b/H1299 grew only slightly slower than the parental H1299 if the expression of ING1b was suppressed (Fig. 2A). Cell proliferation was further reduced when ING1b was expressed. The doubling time increased about 10% when ING1b was expressed. The subtle decrease in growth rate by ING1b was further confirmed by trypan blue exclusion analysis (Fig. 2B). The growth of the parental H1299 cells was not affected by DOX (data not shown). Flow cytometry analysis indicated that the percentage of cells with a G_1 DNA content was decreased when ING1b was induced (Figs. 3B and 4B), suggesting that ING1b may cause a delay in S or G_2/M. These data indicate that expression of ING1b in the absence of p53 has a minor effect on cell proliferation in H1299 cells.

3.3. ING1b enhances ADR-induced damage responses

We next examined the effects of ING1b on the DNA damage responses triggered by various DNA-damaging agents. The chemotherapeutic agent ADR is a topoisomerase II poison that ultimately generates double-stranded DNA breaks. A relatively low dose of ADR was used in our experiments,

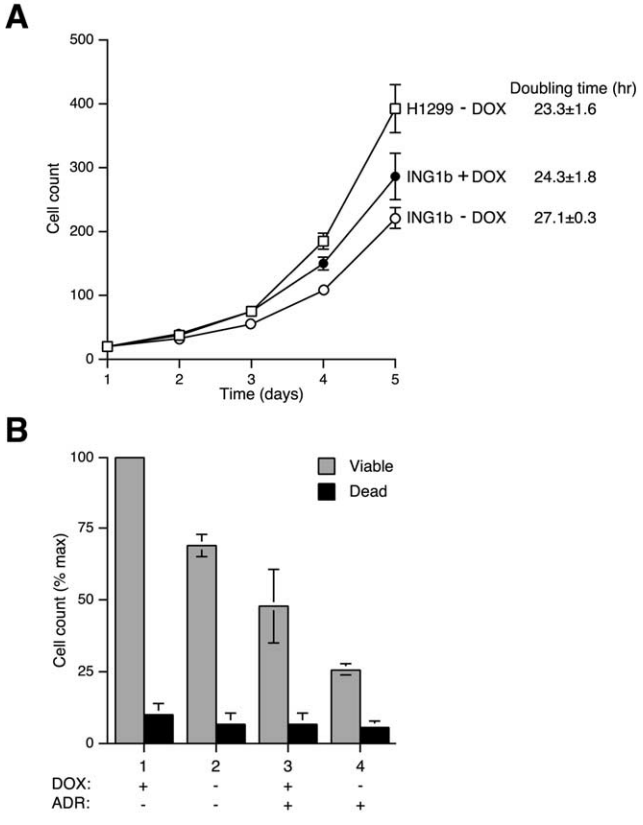
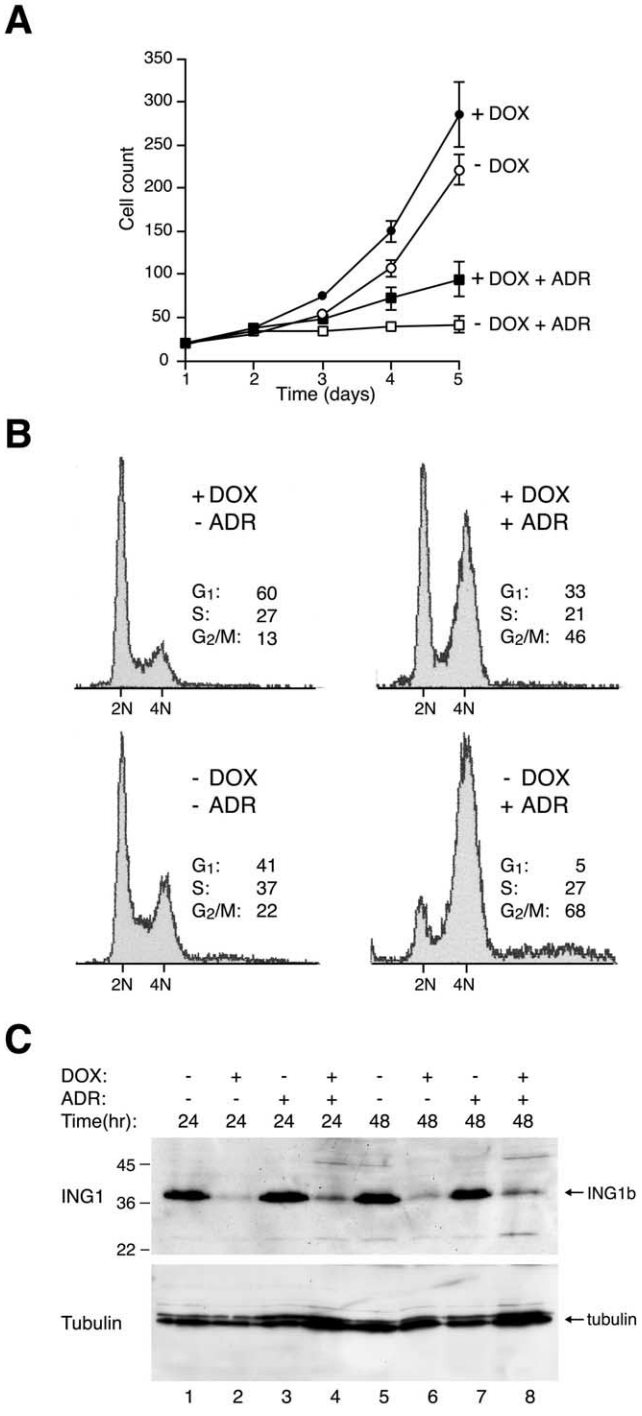


Fig. 2. Ectopic expression of ING1b decreases cell growth. A: H1299 or ING1b/H1299 cells were grown in medium in the presence or absence of DOX as indicated. The number of attached cells at different time points was counted as described in Section 2. The cell numbers are normalized to the same starting level, and the means and standard deviations of three independent experiments are shown. The mean population doubling time and standard deviations are shown on the right. B: ING1b/H1299 cells were grown in the presence or absence of DOX for 16 h as indicated. The cells were then treated with either control buffer or ADR for 24 h. The number of viable and trypan blue-stained dead cells was measured with a hemeocytometer after trypan blue staining. The means and standard deviations from three independent experiments are shown.

which was sufficient to cause a 50–70% reduction in population doubling time in the parental H1299 cells. ADR induced a similar reduction in proliferation of ING1b/H1299 when the expression of ING1b was suppressed by DOX (Fig. 3A). In contrast, proliferation was completely inhibited by ADR when ING1b was expressed. The enhancement of ADR-induced growth arrest by ING1b was also confirmed by trypan blue exclusion analysis (Fig. 2B). As expected, ADR treatment increased the population of cells in G_2/M (Fig. 3B). Remarkably, a prominent G_2/M phase arrest was reproducibly obtained when ADR was added in the presence of ING1b.



Taken together, these data show that overexpression of ING1b in a p53-null background enhanced the G_2/M arrest induced by ADR.

To verify that the expression of ING1b was not affected by ADR, cell-free extracts were prepared and ING1b was detected by immunoblotting. As expected, ING1b was induced in cells after removal of DOX (Fig. 3C, lanes 1, 2, 5 and 6). Importantly, the expression of ING1b was not affected by ADR (lanes 3 and 7), suggesting that the enhancement of ADR-mediated cell cycle arrest was not simply due to an increase in the ING1b level. Uniform loading of samples was confirmed by immunoblotting for tubulin.

We found that the enhancement of DNA damage response by ING1b was specific for some types of DNA-damaging agents and not extended to CIS and UV irradiation. The growth rate of ING1b/H1299 was equally suppressed by CIS treatment with or without the induction of ING1b (Fig. 4A). While a relatively low concentration of CIS (0.5 μg/ml) arrested H1299 predominantly in S and G_2/M, a higher concentration (50 μg/ml) triggered mainly G_1 arrest (Fig. 4B). Expression of ING1b did not affect the cell cycle arrest caused by either concentration of CIS. When H1299 was irradiated with UV and progression through the subsequent mitosis was blocked with nocodazole, there was only a modest increase in cells remaining in G_1 (Fig. 4C). This confirms that H1299 has a weak G_1 DNA damage checkpoint due to the lack of p53 pathway. Similar results were obtained with ING1b/H1299, indicating that ING1b did not enhance the UV-induced DNA damage responses. In mark contrast, re-introduction of p53 into H1299 (p53/H1299) restored the G_1 checkpoint after UV irradiation. Taken together, these data show that ING1b enhances the DNA damage responses to ADR but not to CIS or UV.

3.4. ING1b moderately reduces cell survival

We next compare the antiproliferative effects of ING1b in p53-containing and p53-null backgrounds. For the p53/H1299 cells, the doubling time increased about two-fold when p53 was expressed (Fig. 5A). The p53+ING1b/H1299 cells grew relatively slowly even in the presence of DOX, and proliferation was severely compromised when both recombinant proteins were expressed (Fig. 5A). These cells did not immediately enter apoptosis because the level of recombinant p53 expression was only comparable to the basal p53 level in other cell lines containing endogenous p53 (unpublished data).

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Fig. 3. ING1b enhances ADR-induced DNA damage responses. A: ING1b/H1299 cells were grown in medium in the presence or absence of DOX. Control buffer or ADR was added on day 1 and the number of cells was estimated every 24 h as described in Section 2. The means and standard deviations from three independent experiments are shown. B: ING1b/H1299 cells were grown in the presence or absence of DOX for 16 h. The cells were then treated with either control buffer or ADR for 24 h as indicated. Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry. The percentages of cells in different cell cycle phases are indicated. C: Expression of ING1b following ADR treatment. ING1b/H1299 cells were grown in the presence or absence of DOX for 16 h, and were then treated with control buffer or ADR for the indicated time. Cell-free extracts were prepared, applied onto sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with antibodies against ING1 or tubulin. The positions of the molecular size markers (in kDa) are indicated on the left.

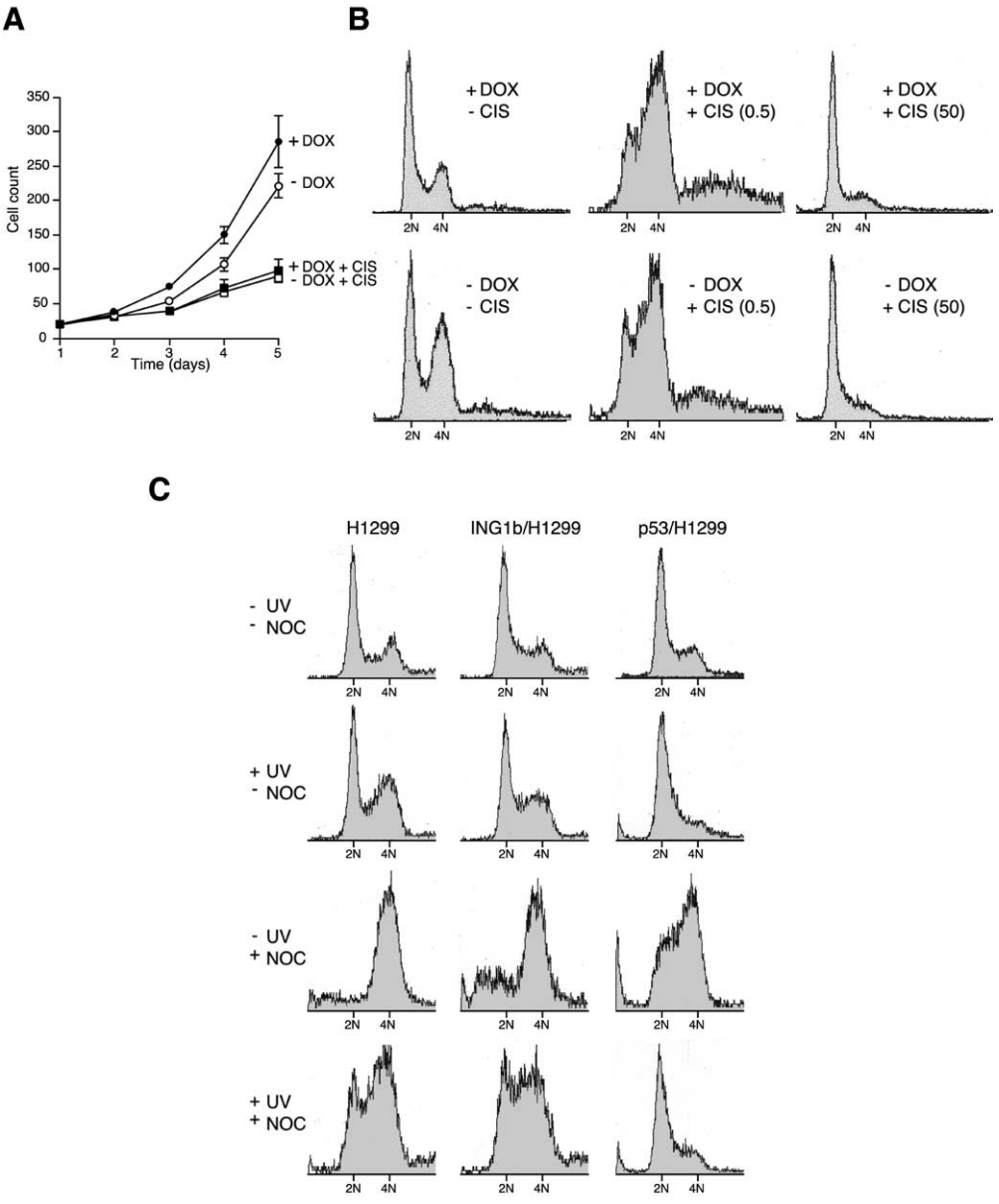

Fig. 4. ING1b does not affect DNA damage responses to CIS and UV. A: ING1b/H1299 cells were grown in medium in the presence or absence of DOX. Control buffer or CIS (0.5 μg/ml) was added on day 1 and the number of cells was estimated every 24 h as described in Section 2. The means and standard deviations from three independent experiments are shown. B: ING1b/H1299 cells were grown in the presence or absence of DOX for 16 h. The cells were then treated with either control buffer or CIS (0.5 μg/ml or 50 μg/ml) for 24 h as indicated. Cell cycle distribution was analyzed by propidium iodide staining and flow cytometry. The percentages of cells in different cell cycle phases are indicated. C: H1299, ING1b/H1299, or p53/H1299 cells were grown in medium without DOX for 24 h. The medium was removed and the cells were either mock treated or irradiated with 30 J/m^2 of UV. Medium was added back and the cells were either mock treated or treated with nocodazole to block the cells in G_2/M. After 24 h, the cells were harvested and processed for flow cytometry.

Proliferation of p53/H1299 and p53+ING1b/H1299 in the absence of DOX was completely inhibited when the cells were treated with ADR (Fig. 5B). It is significant that rather than the same cell number after ADR treatment (as in ING1b/H1299 cells in Fig. 3), there was a decrease in cell count in the p53-containing cells. This suggests that there was an increase in cell death in cells expressing p53. Loss of viability was further confirmed by trypan blue exclusion analysis (Fig. 5C). These data indicate that p53+ING1b/H1299 cells were more sensitive to ADR than p53/H1299, which in turn was more sensitive than ING1b/H1299 and H1299.

Induction of DNA damage in the p53 background was accompanied by a stabilization of p53. Fig. 5D shows that ADR and CIS increased the abundance of p53 in p53/H1299 and p53+ING1b/H1299, but not in control cells lacking p53. Moreover, one of the targets of p53, $p21^{CIP1/WAF1}$, was induced after DNA damage only in the presence of p53 (Fig. 5D). These results suggest that apart from p53 itself, the mechanisms that lead to the stabilization of p53 after DNA damage were intact in H1299.

Long-term growth potential of cells expressing ING1b and/or p53 was examined by clonogenic survival assays. There was a moderate reduction (~15%) in colony survival when ING1b/H1299 cells were grown in the absence of DOX in

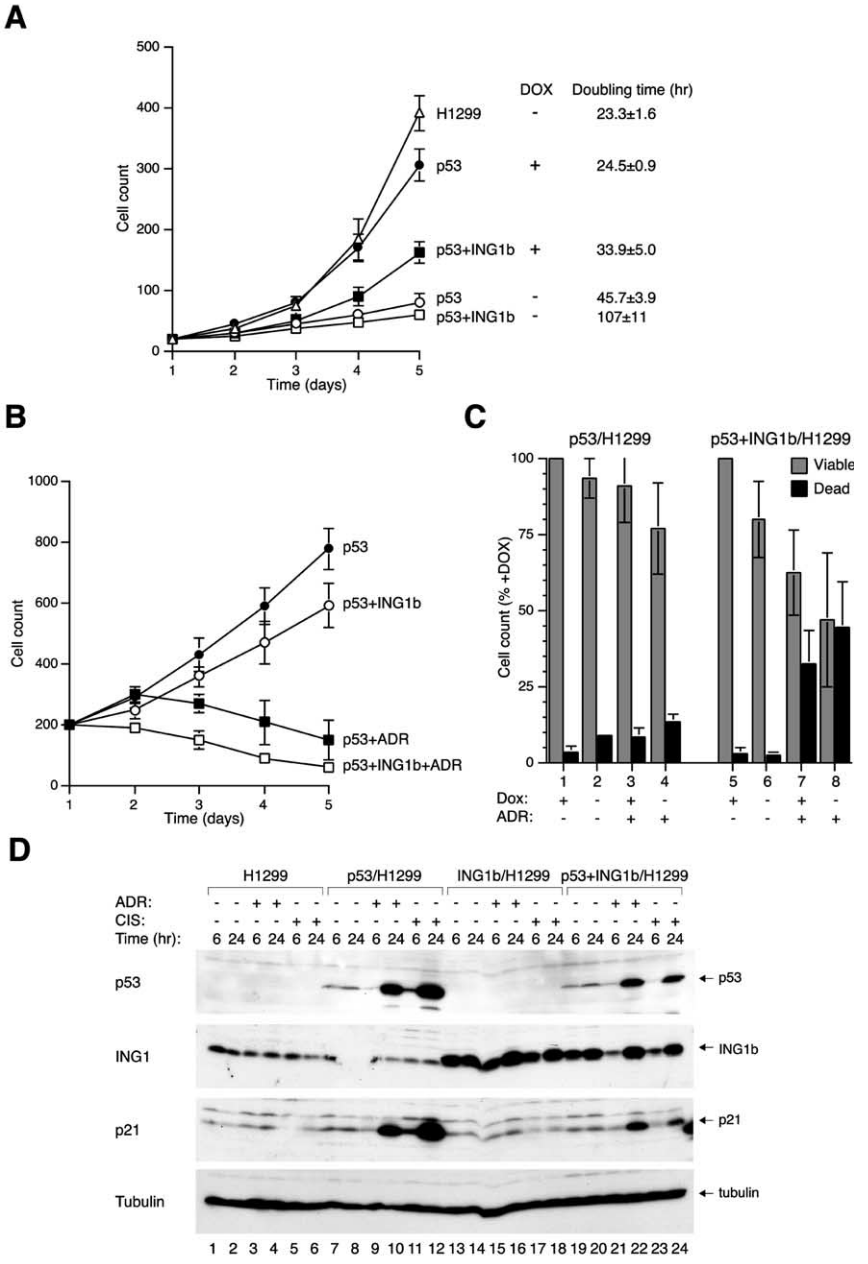

Fig. 5. ING1b and p53 enhanced ADR-induced cell death. A: p53/H1299 and p53+ING1b/H1299 cells were grown in medium in the presence or absence of DOX. The parental H1299 cells were grown under the same conditions in the absence of DOX. The number of attached cells was counted at different time points. The cell numbers are normalized to the same starting level, and the means and standard deviations of three independent experiments are shown. The mean population doubling time and standard deviations are shown on the right. B: p53/H1299 and p53+ING1b/H1299 cells were grown in medium without DOX. Control buffer or ADR was added on day 1, and the number of attached cells was counted at different time points. The means and standard deviations of three independent experiments are shown. C: p53/H1299 (lanes 1–4) and p53+ING1b/H1299 (lanes 5–8) cells were grown in medium with or without DOX for 16 h. The cells were then treated with control buffer or ADR for 24 h and were analyzed by trypan blue staining. The means and standard deviations of viable and the trypan blue-stained cells from three independent experiments are shown. D: Activation of p53 and $p21^{CIP1/WAF1}$ by DNA damage. H1299, p53/H1299, ING1b/H1299, and p53+ING1b/H1299 cells were grown in the absence of DOX and treated with buffer, ADR (0.2 μg/ml), or CIS (5 μg/ml) for either 6 or 24 h as indicated. Cell-free extracts were prepared and p53, ING1, $p21^{CIP1/WAF1}$, and tubulin were detected by immunoblotting.

comparison to cells that were continuously incubated in DOX (Fig. 6, lanes 1 and 2). DOX had no effect on clonogenic survival in the parental H1299 cells (data not shown). A 50–60% reduction of the number of colonies occurred when p53/H1299 and p53+ING1b/H1299 were maintained in the absence of DOX. More detailed examination of the plates under light microscopy indicated that the decrease in colony formation was likely to reflect cell cycle arrest or cell death rather than slower growth. These data suggest that expression of ING1b had a small but significant effect on clonogenic survival.

In a variation of the above experiments, p53/H1299 cells were grown in the absence of DOX for three days, and were then switched back to DOX-containing medium for the remaining period of the experiment. Interestingly, a similar decrease in the colony number was observed as if the cells

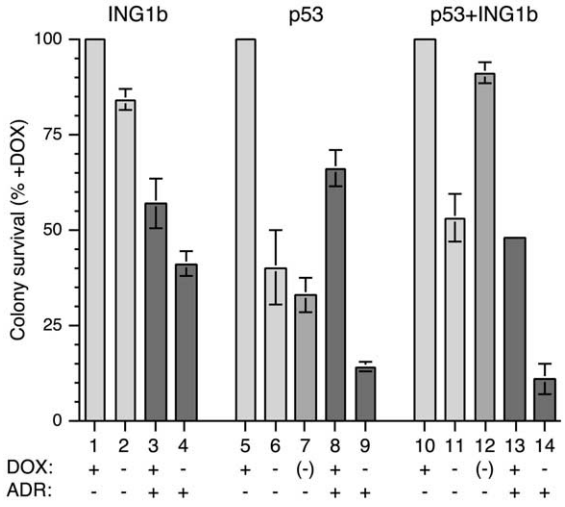

Fig. 6. ING1b and p53 affect long-term survival of untreated and ADR-treated cells. ING1b/H1299, p53/H1299, and p53+ING1b/H1299 were seeded at 500 cells per plate in the presence of DOX. The cells were treated with control buffer or ADR for 3 h. The cells were then washed and maintained in medium with or without DOX until colonies were visible. For samples in lanes 7 and 12, cells were grown in the absence of DOX for three days before DOX was added back for the rest of the experiment. The number of colonies was expressed as a percentage of that grown in the presence of DOX and without ADR treatment. The means and standard deviations of three independent experiments are shown.

were grown continuously in the absence of DOX (Fig. 6, lane 7). These data suggest that when p53 is expressed transiently, cells are not able to recover to re-enter the cell cycle. Intriguingly, p53+ING1b/H1299 could fully recover after growing in medium without DOX for three days (lane 12). One possibility is that cells may enter a reversible cell cycle arrest state when p53 and ING1b are expressed together. A less interesting possibility is that the expression of p53 in p53+ING1b/H1299 was not as robust as in p53/H1299, especially when the expression of recombinant proteins decreased rapidly as the passage number increased (see Fig. 5D, for example).

Although ING1b sensitized cells to short-term G_2/M cell cycle delay induced by ADR (Fig. 3), ING1b alone only had a small impact on the long-term survival after ADR treatment (Fig. 6, lanes 3 and 4). In contrast, the presence of p53 had a decisive effect on clonogenic survival following ADR treatment (lanes 8, 9, 13 and 14). These data suggest the possibility that the interplay between p53, ING1b, and DNA damage may govern whether the cell enters reversible cell cycle arrest or cell death response pathways.

3.5. *ING1b can activate p53-related transcription factors*

One possible explanation of the growth inhibitory effects of ING1b in the absence of p53 is that ING1b may activate other members of the p53 family. To see whether ING1b could enhance the activities of p53-related proteins, their transactivation of a MDM2 promoter-luciferase reporter was analyzed in the presence or absence of ING1b (Fig. 7A). As expected, the transcriptional activity of p53 was strongly activated by cotransfection with ING1b. We found that the transcriptional activities of p73α and p63α were also enhanced by ING1b, albeit not as robust as p53.

To see whether ING1b could physically interact with p53-related proteins, a GST-ING1b fusion protein was expressed in *Escherichia coli* and purified (Fig. 7B). As expected, [^{35}S]-labeled p53 produced in rabbit reticulocyte lysates was retained by GST-ING1b, but not by control GST (Fig. 7C). A slightly weaker interaction between p73α and GST-ING1b was detected, but the background p73α signal was higher. Similarly, recombinant p53 was co-immunoprecipitated with FLAG-ING1b when the two proteins were co-expressed in H1299 cells (Fig. 7D). Although binding between p63α and FLAG-ING1b was detected by co-immunoprecipitation, the binding was considerably weaker than that between p53 and ING1b.

Taken together, these data show that the transcriptional activities of p63α and p73α were enhanced in the presence of ING1b. However, both the transcriptional activation and interaction between p63α/p73α and ING1b were not as robust as that between p53 and ING1b.

4. Discussion

ING1 has been implicated to play roles in cell cycle arrest, senescence, and apoptosis. The execution of these antiproliferative functions of ING1 is mainly attributed to its collaboration with p53. In agreement with the published data that introduction of p53 into H1299 cells induces apoptosis [44], it is not surprising that the p53/H1299 cells described here were more sensitive to DNA damage than the parental cells. Cells expressing ING1b and p53 together grew slower and were more prone to cell death than cells expressing p53 or ING1b individually. It was difficult to assess whether ING1b conferred any additional long-term sensitivity to DNA damage because cells expressing p53 alone were already very sensitive to ADR. Our laboratory has recently shown that transient expression of ING1b increases the cytotoxic effects of p53 in H1299 cells [16]. We showed that one contributing mechanism involves the inhibition of interaction between p53 and MDM2 by ING1b, leading to the stabilization and activation of p53 [16]. Unexpectedly, here we show that ectopic expression of ING1b in H1299 cells induced a slight cell cycle delay in the absence of p53 or stress. Moreover, ING1b alone markedly accentuated the transient cell cycle arrest induced by ADR, but not by CIS or UV. In our experimental setup, ING1b was expressed at about five- to 10-fold more than the endogenous protein. It would be interesting to see how this induced level of ING1b compares to the variation of ING1b seen in some cancers, and to the p53-independent induction of ING1b triggered by UV [10].

The cell cycle delay induced by ING1b appears to be transient, since clonogenic survival after ADR treatment was not significantly affected by ING1b. One possibility is that ING1b-expressing cells were able to efficiently arrest the cell cycle after damage, thus they are able to re-enter the cell cycle after the DNA is subsequently repaired. In contrast, the propensity of activated p53 to trigger apoptosis after DNA damage may result in the more potent reduction in survival in p53/H1299 and p53+ING1b/H1299 cells.

It is conceivable that the cell cycle delay triggered by ING1b in the absence of p53 involves a similar pathway as p53-dependent arrest. In H1299 cells, expression of ING1a, ING1b, or ING1c in the absence of p53 does not transactivate p53-responsive promoters like that of MDM2 [16] or $p21^{CIP1/WAF1}$ (our unpublished data). A possible explanation of the cell cycle delay induced by ING1b without p53 is that

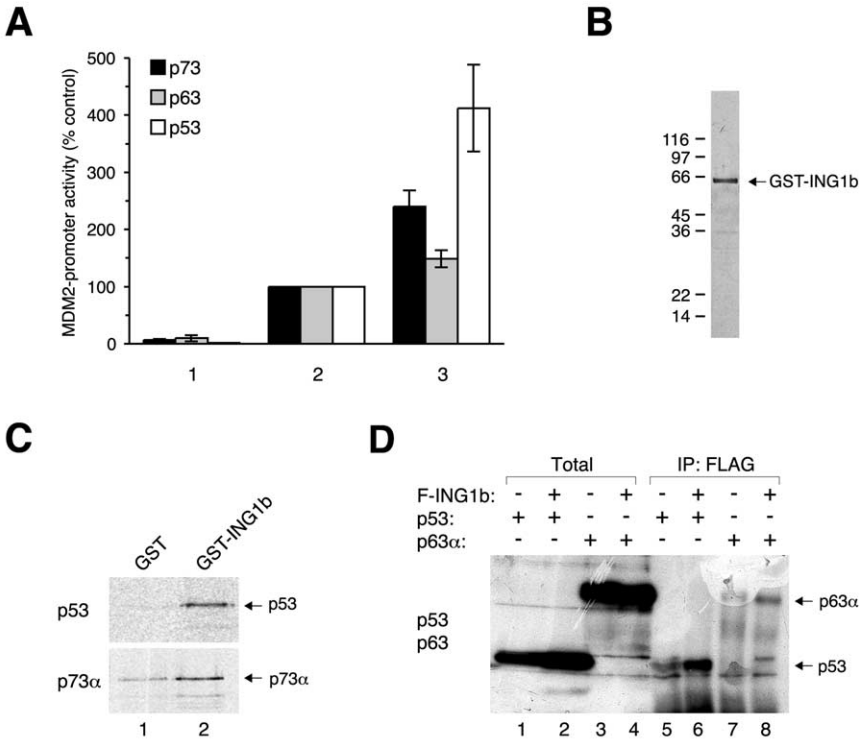

Fig. 7. ING1b can activate p53-related proteins. A: Activation of the transcriptional activities of p53-related proteins by ING1b. H1299 cells were transfected with plasmids expressing an MDM2 promoter-luciferase reporter and β-galactosidase. Control reactions without cotransfection of other plasmids are shown in experiment 1. Plasmids expressing p73α, p63α, and p53 were cotransfected with control plasmids (experiment 2) or ING1b (experiment 3) as indicated. Cell extracts were prepared, and the luciferase and β-galactosidase activities were determined. The luciferase activities were normalized with the β-galactosidase activities to correct for transcriptional efficiencies and plotted as a percentage of p53/p63α/p73α alone. The means and standard deviation of three independent experiments are shown. B: Purification of recombinant GST-ING1b. GST-ING1b was expressed in *E. coli* and purified as described in Section 2. The purified protein was applied onto SDS–PAGE and detected by Coomassie blue staining. The positions of molecular mass standards (in kDa) are indicated. C: ING1b can interact with p53 and p73α. Reticulocyte lysate-expressed p53 and p73α were incubated with either purified GST or GST-ING1b. The GST fusion proteins were captured with GSH-agarose and unbound proteins were washed off. The coprecipitated p53 and p73α were detected by SDS–PAGE and PhosphorImagery. D: ING1b can interact with p53 and p63α. H1299 cells were transfected with plasmids encoding p53 (lanes 1, 2, 5 and 6) or p63α (lanes 3, 4, 7 and 8) with control vector (odd-numbered lanes) or FLAG-ING1b (even-numbered lanes). Cell-free extracts were prepared at 24 h after transfection and 100 μg was subjected to immunoprecipitation with anti-FLAG immune serum. The retained proteins were detected by immunoblotting using antibodies against p53 and p63.

ING1b may activate other members of the p53, like p63 and p73. Indeed we found that ING1b can enhance the transcriptional activities of p63α and p73α using the MDM2 promoter (Fig. 7). Although not as well established as p53, there are indications that both p63 and p73 are involved in DNA damage responses [45–48]. We have shown that ING1b interacts with the N-terminal region of p53 [16]. It is noteworthy that the N-terminal regions are well conserved among members of the p53 family.

Another possible explanation of the enhancement of ADR-induced responses by ING1b may be related to its association with histone acetylation and chromatin remodeling. Acetylation of histones increases accessibility of chromatin templates, while deacetylation is frequently linked with chromatin condensation and gene silencing. The exact role of ING1 in histone acetylation is not very clear since ING1 has been reported to associate with both HAT and HDAC. The *Saccharomyces cerevisiae* ING1-related protein Yng2p is associated with HAT activities and components of HAT [22–25]. Human *ING1* is able to complement most of the yeast *yng2* deletion phenotypes, including the swollen, multibudded morphology and abnormal DNA distribution, and complexes containing human ING1b (but not ING1a) also possess HAT activity [28]. However, human ING1b was found to be associated with the mSin3-containing HDAC [26,27]. It is possible that ING1b modulated the responses to DNA-damaging agents like ADR by global or local chromatin remodeling. It is interesting that the enhancement of cell cycle arrest by ING1b is specific for ADR but not for CIS or UV. This may be due to the fact that ADR (a topoisomerase II inhibitor) and CIS/UV (DNA-crosslinking agents) inflict different types of DNA damages. The responses to the exact type of DNA damage may be sensitive to the possible role of ING1b in chromatin remodeling.

In conclusion, we have shown that ING1b can reduce cell proliferation with p53-dependent and -independent mechanisms. The enhanced sensitivity of both p53-negative and -positive cancer cells to ADR after ING1b expression suggests that ING1b may be an attractive target for cancer therapies, which already has been explored in some studies [15,49].

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