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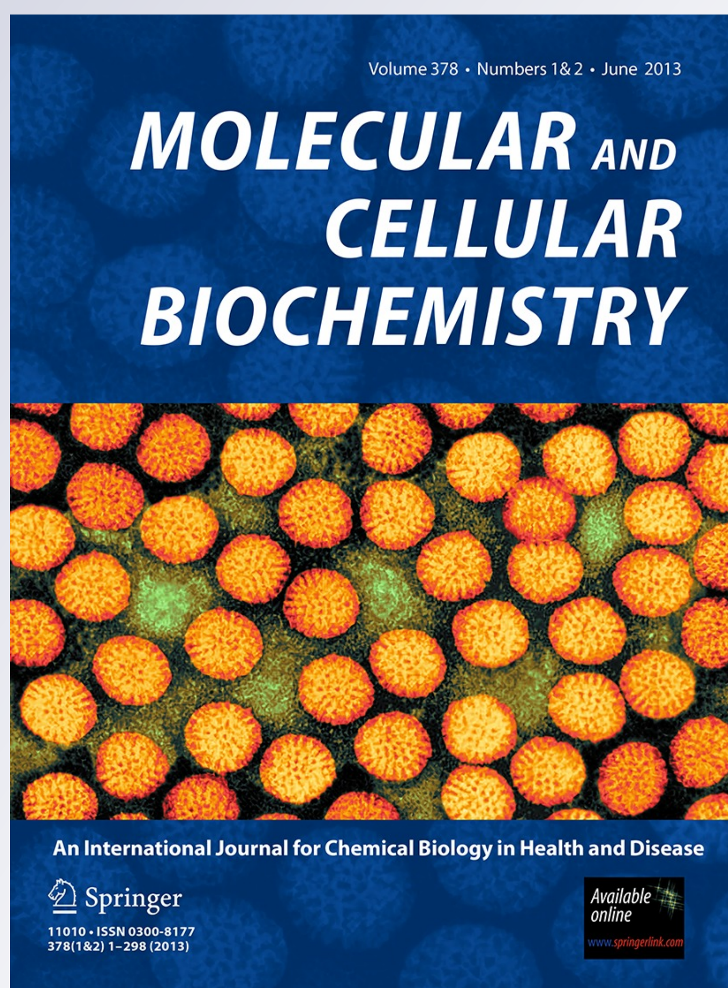

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Inhibitor of growth 1 (ING1) acts at early steps of multiple DNA repair pathways

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Abstract ING proteins are tumor suppressors involved in the regulation of gene transcription, cell cycle arrest, apoptosis, and senescence. Here, we show that ING1b expression is upregulated by several DNA-damaging agents, in a p53-independent manner. ING1b stimulates DNA repair of a variety of DNA lesions requiring activation of multiple DNA repair pathways. Moreover, *Ing1*$^{-/-}$ cells showed impaired genomic DNA repair after H_2O_2 and neocarzinostatin treatment and this defect was reverted by overexpression of ING1b. Two tumor-derived ING1 mutants failed to promote DNA repair highlighting the physiological importance of the integrity of the PHD domain for ING1b DNA repair activity and suggesting a role in the prevention of tumor progression. Ing$^{-/-}$ cells showed higher basal levels of γ-H2AX and, upon DNA damage, γ-H2AX increase was greater and with faster kinetics compared to wild-type cells. Chromatin relaxation by Trichostatin A led to an exacerbated damage signal in both types of cells, but this effect was dependent on Ing1 status, and more pronounced in wild-type cells. Our results suggest that ING1 acts at early stages of the DNA damage response activating a variety of repair mechanisms and that this function of ING1 is targeted in tumors.

Keywords ING1 · DNA repair · p53 · Chromatin structure · Genotoxic stress · DNA damage response · Checkpoint kinase 1

Abbreviations

ING1	Inhibitor of growth 1
DDR	DNA damage response
NCS	Neocarzinostatin
UDS	Unscheduled DNA synthesis
PHD	Plant homeodomain

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Introduction

The inhibitor of growth (ING) family includes several sequence-related proteins (ING1 to ING5 in human and mice) that participate in essential cellular functions linked to preventing tumor initiation and progression, such as cell division, apoptosis, senescence or migration [1–3]. Impaired function of ING proteins due to mislocalization, reduced expression or point mutations has been described in different types of human tumors [4, 5], highlighting the role of this protein family in the suppression of tumorigenesis. ING proteins exert their functions primarily through the regulation of gene expression, mediated by chromatin-modifying activities. ING proteins specifically recognize the chromatin mark H3K4me3 (trimethylation of lysine 4 of histone H3), a

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distinctive mark of active promoters, via their conserved PHD domain [6]. In addition, ING proteins are components of diverse protein complexes with histone acetylase or deacetylase activities [7, 8]. It is well-established that the connection of ING proteins with chromatin is exploited for the regulation of specific gene targets, in cooperation with transcription factors, such as p53 or NFkappaB [9–12]. In addition to this gene-specific function, there is also evidence that ING proteins can play a role in general regulation of chromatin structure and genome maintenance. In particular, it has been shown that ING1 could play a role in the formation of constitutive heterochromatin during replication [13] and in heterochromatin formation during oncogene-induced senescence [9, 14].

In order to protect and preserve the integrity of the genome, eukaryotic cells have developed accurate DNA repair pathways involving a coordinated network of DNA repair and epigenetic factors. The DNA damage response (DDR) includes sensing and signaling of DNA lesions, DNA repair, and the processes that restore chromatin structure [15]. ING1 can bind to proliferating cell nuclear antigen (PCNA) in a DNA damage-inducible manner [16] and is required for the Rad18-mediated PCNA monoubiquitination during lesion bypass [17]. As PCNA is an essential factor for DNA replication and repair, ING1 may act to couple these processes to chromatin remodeling. Also, a role for ING1 in nucleotide excision repair (NER) has been suggested, based on overexpression or silencing experiments. Overexpression of ING1b has been reported to enhance NER of UV-damaged DNA, in host-cell reactivation assays and radioimmunoassay [18]. This effect is possibly linked to its ability to bind XPA [19]. Two mutations at codons 102 and 260 of *ING1b* gene from human cutaneous melanoma biopsies, displayed reduced NER phenotype similar to that for a truncated ING1b mutant lacking the PHD domain [20]. Recently, it was suggested that p33ING1b may be a downstream target of the ATM/ATR response cascade to genotoxic stress [21]. However, it is not clearly understood whether ING proteins are involved in other DNA repair pathways as base excision repair (BER), homologous recombination repair (HRR) or non-homologous end joining (NHEJ). Also, further investigation should be conducted to verify the effect of different genotoxics in the regulation of ING1 expression.

Here, we investigated the physiological role of ING1 in genome maintenance, using cells genetically deficient for the *Ing1* locus and specific mutant versions of the p33ING1 protein. Our results show that ING1 function is required for the efficient repair of DNA lesions via a variety of repair mechanisms, supporting the notion that ING1 acts at early stages of the DDR and that this function is targeted in tumors. In addition, this study shows that physiological levels of ING1 are needed for cells to respond properly at the early steps of the DDR.

Materials and methods

Cell lines, plasmids, and transfections

BHK-21, HCT116, H1299, HeLa cells, and mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's modified Eagle medium supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin–streptomycin, 100 non-essential amino acids, and 2 glutamine at 37 °C in a 5 % CO_2 humidified atmosphere. MEFs derived from mice with targeted *Ing1* alleles (g/g MEFs) were previously described [14]. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. 10^6 cells were seeded in 35 mm plates and transfected with 2.5 μg of Ing1b expression plasmids and 0.5 μg of pBabePuro. Transfected cells were selected with 1 μg/ml puromycin (Sigma) for 60 h. An Ing1b antisense oligonucleotide (ODN) complementary to bases +646 to +667 of the human *Ing1b* cDNA (5′-TCACGGTTCTCGTTGTTGC GCT-3′) was transfected at a final concentration of 200 nM. When indicated, cells were treated with Trichostatin A (TSA) for 5 h, or SB-218078 for 1 h before DNA damage.

Mutant Ing1 expression plasmids were generated using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) [22] and mutations confirmed by sequencing [9].

Cell treatments

Cells were seeded at 50–60 % confluence and 24 h after plating, growth medium was removed and cells were irradiated in open dishes with the corresponding UV dose, 254 nm (range 240–280 nm) at room temperature using a Philips ultraviolet lamp (TUV15WG15T8) calibrated to deliver 0.25 mJ/cm^2/s. Alternatively, cells were irradiated with 5 Gy of IR delivered by a γ-irradiator (MARK 1, Shephard and Associates) that uses a ^{137}Cs probe.

When indicated, cells were treated with 100 μM hydrogen peroxide (H_2O_2), 50 ng/ml neocarzinostatin (NCS) or 10, 50 or 100 μM cisplatin (Cis) added to the culture medium.

RNA extraction and northern blot analysis

Total RNA was isolated from cultures as previously described [23]. In brief, 10 μg of total RNA were denatured, electrophoresed in 1 % glyoxal/agarose gels, and transferred to nylon membranes (Hybond-N+, GE Healthcare). Membranes were sequentially hybridized with the indicated [^{32}P]-labeled probes and radioactivity was detected using a PhosphorImager (Fujifilm BAS-1800II). Probe sequences were as follows: Ing1b: same antisense ODN used in transfections, p21^{Cip1} : 5′-TCTGTCATGCTGGTCTGCCGC

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CG-3′, Bax: 5′-TGGACGCATCCTGAGGCACC-3′ and β-tubulin: 5′-GCCCCTATCTGGTTGCCGCACTG-3′.

Western blot analysis

Total cell lysates and western blot analysis were carried out as previously described [24]. Antibodies used were γ-H2AX (1:1000, 05-636 Upstate), ING1 (1:200, C-19, Santa Cruz and LG1, 1:1000), Actin (1:1000, C4 sc-47778, Santa Cruz). Secondary antibodies were from Santa Cruz.

Unscheduled DNA synthesis

Cells grown in 35-mm plate to 80–90 % confluence were washed with PBS and growth medium was replaced with arginine-free medium containing 0.1 % FBS (arrest medium). Cells were further incubated for 48 h with arrest medium renewal at 24 h. It was determined that, under these conditions, semi-conservative DNA synthesis is completely inhibited. Cells were then treated with 100 μM H_2O_2, 50 ng/ml NCS or irradiated with 8 mJ/cm^2 UV and further cultured in arrest medium containing 10 μCi/ml [^{3}H]-thymidine for 8 h. Cells were washed three times with cold PBS, harvested and collected at 3,000×*g* for 5 min. Cells were lysed with 5 % TCA for 30 min and centrifuged at 10,000×*g* for 10 min. Cell pellets were washed twice with cold water and resuspended in 1 M NaOH. The incorporated radioactivity was determined by scintillation counting. Unscheduled DNA synthesis was expressed as dpm/μg of protein.

Immunofluorescence

Wild-type or g/g MEFs were seeded in glass chamber slides (40,000 cells per well in eight-well LabTek Chambers) and treated the following day with TSA for 5 h. Cells were then irradiated (5 Gy) and processed for immunofluorescence at indicated time points as previously described [25] using anti-γ-H2AX (05-636, Upstate, dil 1:500) and Alexa 594-conjugated anti-mouse antibodies (Molecular Probes, dil 1:500). Fluorescence was captured with a Leica TCSSP2 DMIRE 2 confocal microscope and images analyzed with Image J 1.44b software.

Results

Ing1b expression is upregulated following DNA damage

ING1b, the major isoform expressed from the *Ing1* locus, is a candidate tumor suppressor protein that cooperates with p53 in the cellular response to DNA damage. To study its role in the DDR, we examined *Ing1b* expression levels in several cell lines treated with different DNA-damaging agents. Northern blot analysis from HeLa cells showed that the *Ing1b* gene was strongly induced by UVC irradiation, peaking at 2 h following treatment and returning to basal levels between 12 and 24 h (Fig. 1a). The p53 target gene *p21* displayed a similar pattern of induction. *Ing1b* mRNA levels were also upregulated in HeLa cells by UV irradiation, cisplatin or ionizing radiation (IR) in a dose-dependent manner (Fig. 1b) and in wild-type (wt) MEFs treated with H_2O_2 or NCS (Fig. 1c). Strikingly, in these MEFs, *p21* mRNA expression was significantly induced by H_2O_2 but not by NCS. Finally, treatment with UV, NCS or H_2O_2 greatly increased *Ing1b* mRNA levels in human colorectal carcinoma HCT116 cells, with a maximal expression 4 h following DNA damage (Fig. 1d, left panel). Notably, we observed a similar response in the isogenic p53$^{-/-}$ HCT116 cell line, in which *Ing1b* was also induced by all the DNA-damaging treatments (Fig. 1d, right panel). As expected, the p53 target genes *Bax* and *p21* were upregulated by these treatments in the wt but not the p53-null HCT116 cells.

We next examined ING1b response at the protein level. We observed a modest increase in ING1b in MEFs at 4 h following treatment with H_2O_2 or NCS (2.5- and 1.5-fold respectively) (Fig. 1e). Interestingly, in spite of the strong induction at the mRNA level in both wt and p53$^{-/-}$ HCT116 cells, ING1b expression was not significantly affected by NCS or H_2O_2 treatment, showing a disconnect between mRNA and protein levels in these cells (data not shown). Finally, in p53-null H1299 cells, all treatments upregulated ING1b protein levels (Fig. 1f).

Taken together, these results show that the *Ing1b* gene is induced by a variety of DNA-damaging agents in different cell types and that this regulation is independent of p53.

ING1b enhances repair of a variety of DNA lesions

ING1b was shown to become upregulated by UV and to enhance DNA repair in host-cell reactivation assays and radioimmunoassay following UV [26]. The role of endogenous ING1 in DNA repair in response to other types of damage was then assessed in wild-type and Ing1 deficient MEFS, where the Ing1 locus has been inactivated by the integration of a βgeo cassette, hereafter designated as g/g MEFS [14]. Genomic DNA repair was measured through unscheduled DNA synthesis (UDS) assays performed in conditions in which semi-conservative DNA synthesis is completely inhibited. Overexpression of ING1b significantly stimulated genomic DNA repair in MEFs treated with H_2O_2 or NCS, measured as an increase in [^{3}H]-thymidine incorporation (Fig. 2a, b). Conversely, downregulation of ING1b in wt MEFs using an antisense

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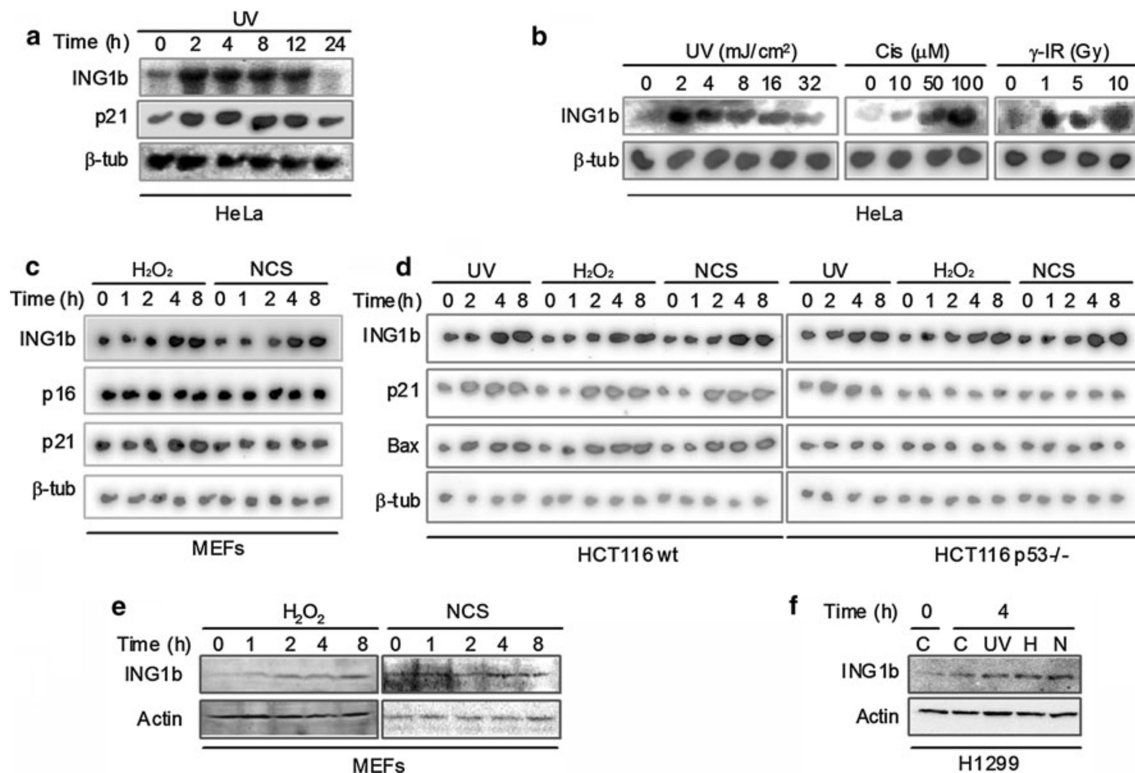

Fig. 1 ING1 expression is positively regulated by DNA damage. **a** HeLa cells were irradiated with 8 mJ/cm^2 UV and harvested at the indicated time points following damage or **b** treated with increasing doses of UV, cisplatin or γ-IR and ING1 mRNA level was analysed by northern blot 8 h after the damage. **c** MEFs or **d** HCT116 cells were irradiated with 8 mJ/cm^2 UV or treated with 100 μM H_2O_2 or 50 ng/ml neocarzinostatin (NCS) and harvested at the indicated time points following damage. The expression of ING1b, p16, p21, and Bax mRNA was assessed by Northern blotting. β-tubulin (β-tub) was used as a loading control. Each panel is representative of two independent experiments. **e** MEFs were treated with 100 μM H_2O_2 or 50 ng/ml NCS and ING1b protein level was determined in total cell lysates by western blotting along time. **f** H1299 cells were damaged with UV, H_2O_2 (H) or neocarzinostatin (N) as in (**c**) and (**d**). ING1b protein level was determined in total cell lysates by western blotting 4 h later

ODN resulted in an impaired DNA repair activity of H_2O_2 or NCS-induced lesions. Finally, g/g MEFs displayed a reduced ability to repair DNA lesions caused by both DNA-damaging treatments compared to wt MEFs, (Fig. 2a, b). Ectopic overexpression of ING1b restored the ability to repair DNA both in MEFs transfected with *Ing1b* antisense ODN and in g/g MEFs to levels similar to those observed in wt cells (Fig. 2a, b). Overexpression of ING1b in HeLa cells also improved genomic DNA repair after UV damage as revealed by UDS assays (Online Resource 1).

Taken together, our results extend the previous observation of a role for ING1b in the repair of UV-induced DNA lesions to other types of lesions and highlight the role of endogenous ING1b in this cellular process.

Two tumor-derived ING1b mutants fail to stimulate DNA repair

All members of the ING family contain unique amino-terminal regions and a highly conserved carboxy-terminal plant homeodomain (PHD). The *Ing1* gene has been found mutated or silenced in several types of human cancer [5]. Given its role as a type II tumor suppressor and to address the physiological relevance of ING1b DNA repair activity in tumorigenesis, we generated two tumor-derived ING1b mutants and characterized their ability to promote DNA repair. The missense mutations, C215S and K270N were described in patients with head and neck and esophageal squamous cell carcinoma and map to the PHD domain of ING1b [27, 28]. We previously determined that aminoacid substitution from Cysteine (TGC) to Serine (TCC) at codon 215, disrupts the three-dimensional structure of the ING1 PHD domain, that becomes essentially unfolded in solution [9]. Expression plasmids encoding the ING1b mutants were transfected into HeLa cells that were then UV irradiated and wt MEFs that were treated with H_2O_2 or NCS. The mutant ING1b proteins were expressed at levels comparable to those obtained with a wt ING1b expression construct as assessed by Northern and Western blot analysis (Fig. 2c). Surprisingly, not only did the tumor-derived ING1b mutants fail to promote DNA repair following UV irradiation, H_2O_2, or NCS treatment but they also appear to

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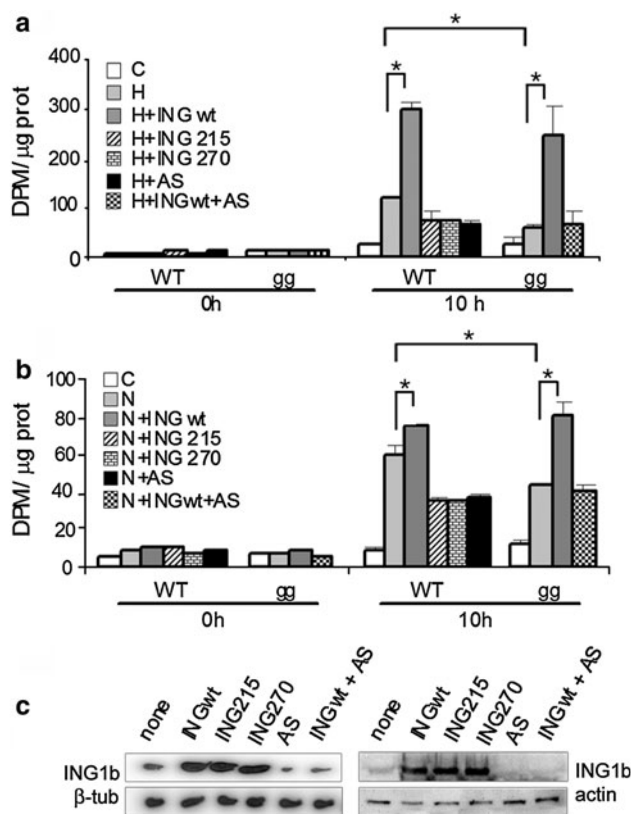

Fig. 2 ING1b improves DNA repair in response to a variety of genotoxic agents. Wild-type or g/g MEFs were transfected with a vector encoding wild-type ING1b (ING wt) or mutants ING1bC215S (ING 215), ING1bK270N (ING 270) or antisense oligonucleotide for ING1b (AS) along with pBabePuro. Twenty-four hours after transfection regular medium was substituted for arginine-free medium containing 1 % FBS and cells were incubated for additional 60 h in the presence of puromycin. Resistant cells were treated with: **a** 100 µM H_2O_2 (H) or **b** 50 ng/ml neocarzinostatin (N) and incubated with 10 µCi [^{3}H]thymidine for 10 h. Cell lysates were tested for genomic DNA repair by UDS assay. *Bars* represent the mean ± SE of three experiments performed in triplicate. Student *t* test was used to compare samples (*$P < 0.05$); DPM (desintegrations/min). **c** Expression level of ING1b mRNA (*left*) and protein (*right*) was tested by northern and western blots in wt MEFs transfected as in (**a**) and (**b**)

behave as dominant negative proteins, reducing the amount of [^{3}H]-thymidine incorporation to that observed in cells treated with the *Ing1b* antisense ODN (Fig. 2a, b).

These results suggest that the integrity of the PHD domain is required for the ability of ING1b to promote DNA repair. Moreover, the data implies that this activity of ING1b might be critical for its function as a tumor suppressor as it needs to be over-ridden as cells progress to a cancerous state.

ING1b acts at early steps of the DDR

Phosphorylated histone H2AX (γ-H2AX) is a mark found at sites of DNA double-strand breaks and participates in the first layer of the recruitment of DNA repair proteins to DNA breaks [29, 30]. To further explore the action of ING1b in the DDR, we treated wt and g/g MEFs with H_2O_2 and the kinetics of γ-H2AX protein levels were analyzed by Western blot. g/g MEFs showed higher levels of γ-H2AX compared to wt MEFs. Shortly after the genotoxic insult, phosphorylated H2AX levels increased in both genotypes. However, γ-H2AX increase was significantly more intense and occurred with faster kinetics in g/g compared to wt MEFs (Fig. 3a). For early time points, this was more evident when Δγ-H2AX/Δt values were plotted (Fig. 3b). At approximately 60 min following DNA damage, variations of γ-H2AX levels with time were similar in both cellular types, where they appeared constant for up to 240 min. Considering the level of γ-H2AX as a result of phosphorylation and dephosphorylation dynamics, it is understandable that Δγ-H2AX/Δt value was near zero. Absolute levels of γ-H2AX remained elevated in g/g MEFs, and did not return to basal level as in wt MEFs, even after 4 h (Fig. 3a). We also analyzed γ-H2AX kinetics at shorter time points. g/g MEFs displayed higher basal levels of γ-H2AX compared to the wt cells. More importantly, H2AX phosphorylation increased rapidly and over a more extended period of time following H_2O_2 treatment in g/g MEFs to levels that were significantly higher than those observed in wt MEFs (~ninefold, at 12 min). In contrast, wt MEFs displayed slower kinetics compared to their *Ing1*-deficient counterparts, with γ-H2AX signal reaching a plateau 12 min following treatment (Fig. 3c). Here again, Δγ-H2AX/Δt values showed that the appearance of γ-H2AX occurs more rapidly in g/g MEFs (Fig. 3d).

Similar results were obtained when both types of MEFs were treated with IR. Upon IR treatment, phosphorylation of H2AX increased to levels which were ~2, 5 times greater in g/g MEFs than those observed in wt cells at 0.5 h and the γ-H2AX signal remained elevated for a longer period of time (Fig. 4a, b).

These results suggest that *Ing1* deficiency results in an exacerbated early response to DNA damage. The elevated γ-H2AX levels observed in g/g MEFs compared to wt MEFs were maintained over a more prolonged period of time, a sign that the DDR in g/g MEFs is somehow deregulated and that physiological levels of ING1 are required to maintain its normal signaling.

It has been reported that chromatin remodeling into a more open state correlates with an increase in the total amount of γ-H2AX and the size of foci within the damaged region, suggesting that the DDR is amplified in the context of open chromatin [31, 32]. Since ING1b has been reported to interact with various chromatin modifying complexes, associated with histone deacetylase (HDAC) or histone acetyltransferase (HAT) activities [33–35] we hypothesized

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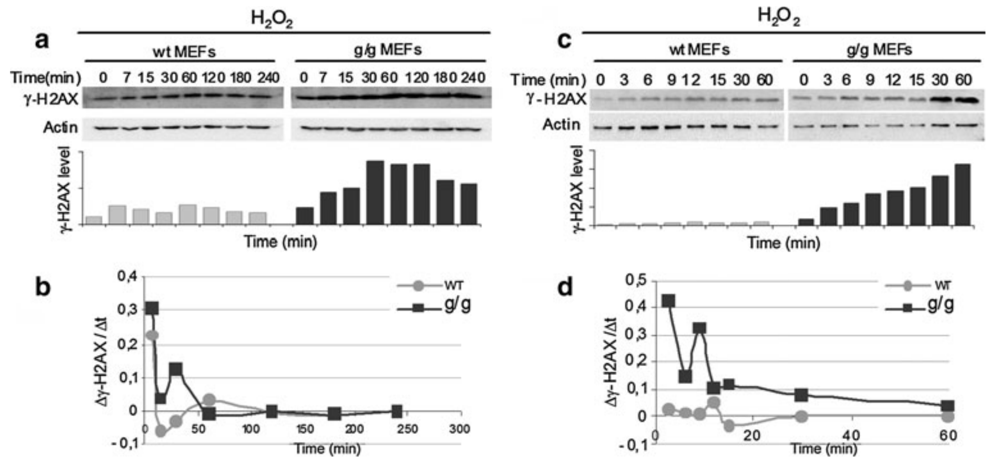

Fig. 3 ING1 participates in early steps of the DNA damage response. **a** and **c** Wild-type or g/g MEFs were treated with 100 μM H_2O_2 for indicated time points and γ-H2AX level was determined in total cell lysates by western blot. **b** and **d** Difference in γ-H2AX level between two consecutive time points relative to that time interval in minutes was calculated for each interval an expressed as Δγ-H2AX/Δt. Images and data belong to one representative experiment from three with similar results

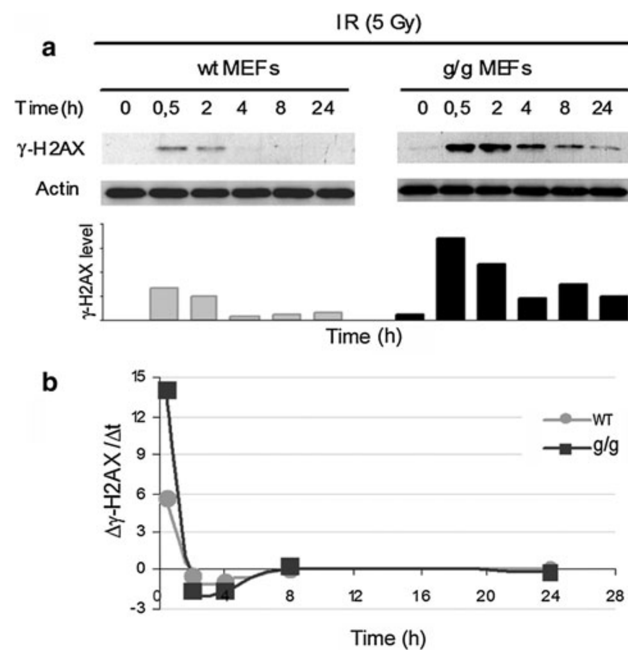

Fig. 4 ING1 deficiency generates an exacerbated early response to DNA damage. **a** Wild-type or g/g MEFs were treated with 5 Grays of γ-IR and at indicated time points γ-H2AX level was determined in total cell lysates by western blot. **b** Difference in γ-H2AX level between two consecutive time points relative to that time interval in minutes was calculated for each interval expressed as Δγ-H2AX/Δt. Images and data belong to one representative experiment from three with similar results

that the differences observed in γ-H2AX levels could be due to variations in the structure of chromatin in the *Ing1*-deficient MEFs compared to wt MEFs. To examine this possibility, we analyzed γ-H2AX levels in wt and g/g MEFs treated with the HDAC inhibitor trichostatin A (TSA) for 5 h before exposure to 5 Gy of IR. An exacerbated γ-H2AX signal was observed in wt MEFs following treatment with TSA, similar to that observed in cells lacking ING1b. Interestingly, the effect of 0.1 μM TSA on γ-H2AX levels was additive to the deficiency of *Ing1* at 30 min (Fig. 5a), with the relative effect being greater on wt MEFs. TSA treatment resulted in an ~eightfold increase in γ-H2AX levels 30 min after irradiation in wt MEFs compared to a ~twofold in irradiated g/g MEFs. Although the differences were more modest, we observed similar results when γ-H2AX was examined by immunofluorescence in wt and g/g MEFs (Fig. 5b).

HDAC inhibition by treatment with TSA appears then to mimic the absence of ING1b, resulting in a more dramatic increase in H2AX phosphorylation following DNA damage by IR. These results suggest that ING1 might play a role in the remodeling of chromatin during the DDR.

ING1b requires a functional Chk1 kinase to repair genomic DNA

The cellular response to DNA damage is coordinated primarily by the ATM/ATR and Chk1/Chk2 kinases. We analyzed a potential role for Chk1 on ING1b DNA repair activity. We performed UDS assays in wt MEFs treated with H_2O_2 that were preincubated or not for 1 h with the Chk1 inhibitor SB218078. As shown before, ING1b significantly improved the repair of DNA damage caused by H_2O_2. This activity was however greatly inhibited in the presence of a specific Chk1 inhibitor (Fig. 6), demonstrating a requirement for this kinase for ING1b DNA repair ability.

ING1b has been reported to become phosphorylated on Ser126 by Chk1 upon doxorubicin treatment or UVB irradiation [21]. This post-translational modification was suggested to increase the ING1b half-life, stabilizing the protein [21]. Of note, ING1b overexpression did not overcome the effect of Chk1 inhibition, suggesting that stabilization of the ING1b protein might not be the sole mechanism of activation by Chk1.

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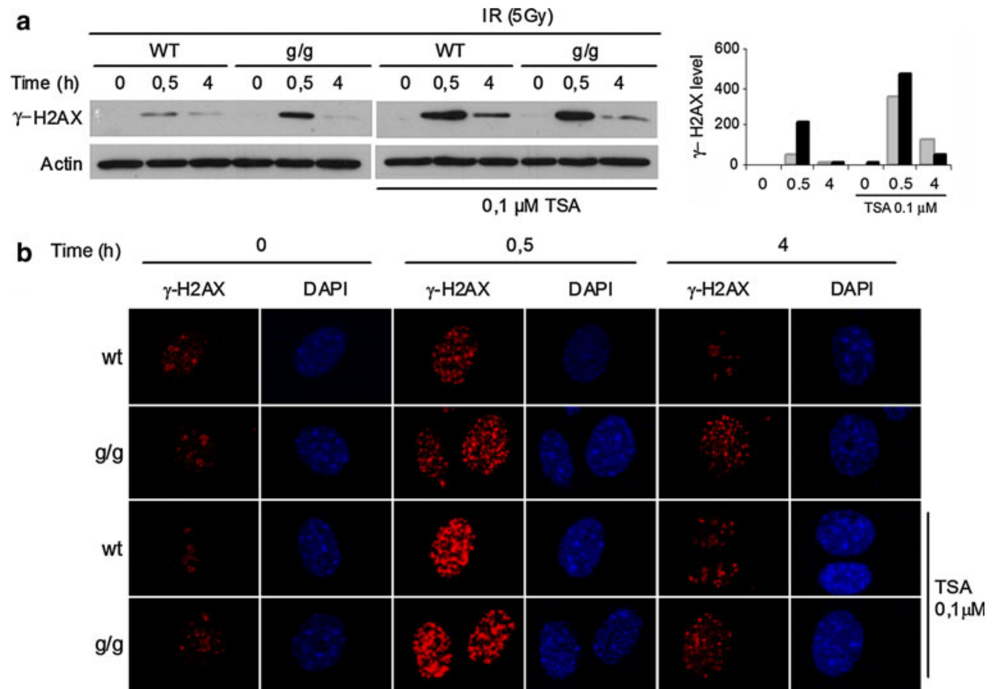

Fig. 5 Chromatin relaxation and lack of Ing1 cooperate in potentiating γ-H2AX signal after IR damage. **a** Wild-type or g/g MEFs, previously treated (or not) for 5 h with TSA (0.1 μM) were damaged with 5 Grays of γ-IR. At indicated time points γ-H2AX level was determined in total cell lysates by western blot. **b** Immunofluorescence analysis with anti-phospho-H2AX antibody (*red*) in wild-type or g/g MEFs treated as in (**a**). Images belong to one representative experiment from three with similar results. (Color figure online)

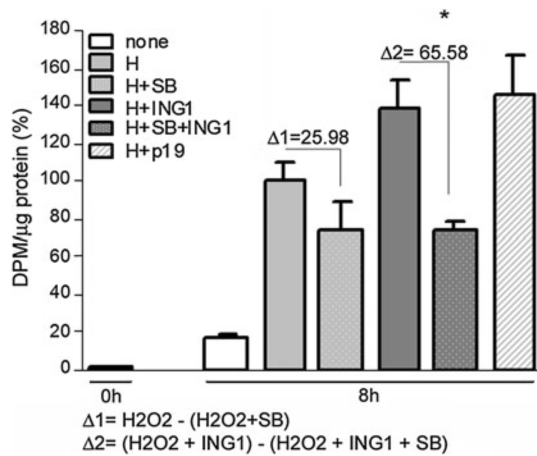

Fig. 6 ING1b requires functional Chk1 kinase to repair genomic DNA. Wild-type MEFs were transfected with a vector encoding wild-type ING1b (ING wt) along with pBabePuro or pBabePuro alone as in Fig. 1. Twenty-four hours after transfection regular medium was substituted for arginine-free medium containing 1 % FBS and cells were incubated for additional 60 h in the presence of puromycin. Resistant cells were treated with 100 μM H_2O_2 (H) and incubated with 10 μCi [^{3}H]thymidine for 8 h. When indicated cells were incubated with Chk1 inhibitor SB218078 for 1 h before damage. Cell lysates were tested for genomic DNA repair by UDS assay. p19INK4d (p19) was used as a control protein known to improve genomic DNA repair in UDS assays. *Bars* represent the mean ± SD from three experiments performed in triplicate, expressed as percentage of radioactivity incorporated per μg of protein, with respect to the value of cell lysate treated with H2O2 that was set to 100. Student *t* test was used to compare samples (*$P < 0.01$)

Taken together, these results indicate that ING1b requires a functional Chk1 kinase to repair genomic DNA in an efficient manner.

Discussion

ING proteins are type II tumor suppressors whose activity is found impaired in several types of human cancer through mutation or gene silencing. They have been linked to the p53 pathway, and ING1b, for instance, can physically interact with the tumor suppressor protein p53 and cooperates with p53 in transcriptional activity and regulation of cell proliferation [36], apoptosis, and senescence [37–40]. ING proteins have also been shown to associate with many chromatin remodeling complexes, among other proteins [7, 33, 35, 41].

The results reported here represent new insight on the role of ING1b in the maintenance of genomic integrity. Our results show that *Ing1b* mRNA and, to a lesser extent, ING1b protein levels become upregulated in a variety of cell types in response to multiple types of DNA damage. Regulation of ING1b expression by DNA damage is not universal and has proved to be cell type and stimulus specific: it is induced by treatment with doxorubicin in MMRU cells but not in human fibroblasts, MEFs or HCT116 cells [21, 29] and our own data, not shown); it also becomes upregulated in MMRU cells by etoposide [21] but fails to respond to UVB in mice dermal fibroblasts [26] or in C3ABR normal lymphoblastoid cells [38]. Notably, although ING1b has been linked to the p53 pathway, the induction of Ing1 mRNA or protein was observed both in p53 wild-type and null cells (Fig. 1). This is consistent with data supporting p53-independent functions of Ing1, such as the phenotype of Ing1 knockout mice [1, 42–44], or the involvement of ING1b in TSA-induced

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apoptosis and caspase-3 signaling in p53-deficient glioblastoma cells [45]. Similarly, genetic studies of tumors have failed to show a consistent correlation between ING1 and p53 status [46]. Our results also show a disconnect between Ing1 mRNA and protein levels following DNA damage suggesting the existence of additional cellular mechanisms that regulate ING1b expression. Understanding the regulation of ING1 mRNA and protein levels under normal and stress conditions is especially important as manipulation of the expression of *Ing* genes could potentially be used for therapeutic purposes in human cancer.

It has been reported that $Ing1^{-/-}$ MEFs are more sensitive to UVB treatment and that *Ing1* deficiency resulted in hypersensitivity to total body gamma irradiation in mice [42]. The mechanism by which ING1b acts during the general DDR remains however to be elucidated. In this report, we examined the role of ING1b in cells exposed to several different DNA-damaging agents using UDS assays. H_2O_2 is produced in the cells by several physiological processes and is widely used exogenously as an oxidative agent that leads to a wide spectrum of DNA lesions. The BER pathway is believed to be the major mechanism for repairing deaminated bases and bases with oxidative damage generated by ROS [47]. Neocarzinostatin is a very potent DNA-damaging drug that generates single- and double-strand breaks (DSB) in the DNA and the most critical lesion caused by IR is also DSB. In eukaryotic cells, these types of lesions activate mainly the recombination repair pathways: NHEJ and HRR [48, 49]. Finally, DNA lesions caused by UV irradiation, mostly cyclobutane pyrimidine dimers and 6–4 photoproducts, are repaired via the NER pathway. Here, we show that overexpressed ING1b was able to stimulate DNA repair in cells treated with a variety DNA-damaging agents, suggesting that it can stimulate virtually all DNA repair pathways: BER, NER, NHEJ, and HRR. We also show that cells with targeted *Ing1 locus* have impaired genomic DNA repair activity when they are subjected to these treatments. These results are consistent with previous reports showing that overexpression of ING1b enhances NER of UV-damaged DNA, in host-cell reactivation assays and radioimmunoassay [18], in a process possibly linked to its ability to bind XPA [19], and that cells lacking ING1b expression are defective in recovering from UV-induced stalled replication [17]. Moreover, our data extend this observation to other types of DNA lesions involving other DNA repair pathways. The most parsimonious explanation for these results is that ING1b must act at the earliest stages of the DDR to be able to stimulate DNA repair through any of these pathways.

In this report, we examined the ability to promote DNA repair of two new cancer-associated mutations of ING1, C215S, and K270 N, found in patients with head and neck and esophageal squamous cells carcinoma, respectively. These mutations map within or close to the PHD domain, a module that is conserved among all members of the ING family and found predominantly on chromatin-associated proteins [50]. The PHD domain of ING1 specifically binds to histone H3 trimethylated at lysine 4 (H3K4me3) [51]. Different mutations within the PHD fingers of numerous proteins are present in patients with immunodeficiency or autoimmune syndromes [52–54], with many of these alterations occurring at zinc-coordinating residues. Using UDS assays, we show that the tumor-derived ING1b mutants were greatly impaired in their DNA repair activity in cells treated with H_2O_2 or NCS (Fig. 2). Moreover, they appeared to exert a dominant negative effect on endogenous ING1, as they reduced the level of DNA repair to that observed in cells with ablated ING1 (Fig. 2a, b).

Similar results have been shown with a mutant defective for H3K4Me3 recognition that was shown to be inactive in HCR assays in UV irradiated cells [51]. Finally, mutations found in melanomas within the PHD of p33ING1b (N260S) and within a region that interacts with SAP30 (R102L) were deficient for DNA repair through NER [20], however it has been questioned whether these ING1 variants represent *bona fide* tumor-derived alterations[55]. Taken together, these results support the concept that the integrity of the PHD domain is critical for the DNA repair function of ING1b. The data suggests that the ability of ING1b to interact with chromatin through the PHD domain is central to its ability to stimulate the multiple pathways of DNA repair and to function in cells as a tumor suppressor.

Phosphorylation of the histone variant H2AX at Ser139 by the PI3K-related kinases ATM, ATR, and DNA-PK is a key event during the DDR, both required for the assembly of DNA repair complexes at the sites of damage and for signaling to checkpoint proteins. Here, we show that *Ing1*-deficient MEFs had higher basal levels of γ-H2AX compared to wt MEFs, and that they accumulated phosphorylated H2AX more intensely and with faster kinetics upon DNA damage (Figs. 3, 4). These results identify a novel role for ING1 in early steps of the DDR. We propose that ING1, apart from improving the DNA repair of different types of DNA lesions originated by diverse genotoxic agents, would play a moderating role to trigger appropriate signals in early steps of the DDR. Also, our results with the inhibitor of HDACs TSA suggest that the effect of ING1 is related to histone acetylation in this system. Kuo and colleagues have suggested that ING1b facilitates NER by inducing global histone H4 acetylation and promoting chromatin accessibility [19], and more recently that ING1b regulates histone H4 acetylation and Rad18-mediated PCNA-ubiquitination upon replication stalling to facilitate lesion bypass [17]. Certainly, it is known that ING proteins are components of various HAT

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and HDAC complexes [7, 33, 35, 41]. Interaction of ING1b with HDACs also appears to be important to keep the physiological balance of the DDR, at least in MEFs after IR. Moreover, these results reinforce the concept that subtle differences in the epigenetic configuration may be responsible for variations in the strength of DDR when cells are treated with genotoxic agents [32] and highlight the multifunctional role of ING1b as it can bind many effector proteins with varied functions through different structural domains [3].

Finally, we show that Chk1 kinase activity was necessary for ING1b to repair genomic DNA. ING1b was found to be phosphorylated on Ser126 by Chk1 under DNA-damaging conditions [21]. This phosphorylation is reported to alter the half-life but not the sub-cellular localization of ING1b [21], suggesting that Ser126 phosphorylation may influence ING1b activity by regulating its stability. We show that overexpressing ING1b was not enough to improve DNA repair, when Chk1 is inhibited. Therefore, it is possible that phosphorylation on Ser126 may have additional effects, in addition to stimulating its stability, that enable ING1b to interact with proteins involved in DNA repair pathways. Collectively, our results, together with previous reports, show the essential role of ING proteins at different steps in the response to DNA damage and highlight their importance in the maintenance of genome stability.

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