The Inhibitor of Growth1 (ING1) tumor suppressor is the founding member of a family of five genes (ING1–5) with conserved plant homeodomains (PHDs). ING proteins are implicated in the regulation of cellular senescence, chromatin remodeling, differentiation, DNA damage response, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.

Cell Death and Disease (2013) 4, e788; doi:10.1038/cddis.2013.321; published online 5 September 2013

Subject Category: Cancer

Received 24.3.13; revised 23.7.13; accepted 29.7.13; Edited by M Agostini

P Bose1,3, S Thakur1,3, S Thalappilly1, BY Ahn1, S Satpathy1, X Feng1, K Suzuki1, SW Kim1 and K Riabowol*1,2

The ING family of tumor suppressors acts as readers and writers of the histone epigenetic code, affecting DNA repair, chromatin remodeling, cellular senescence, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.

Cell Death and Disease (2013) 4, e788; doi:10.1038/cddis.2013.321; published online 5 September 2013

Subject Category: Cancer

Received 24.3.13; revised 23.7.13; accepted 29.7.13; Edited by M Agostini

The Inhibitor of Growth1 (ING1) tumor suppressor is the founding member of a family of five genes (ING1–5) with conserved plant homeodomains (PHDs). ING proteins are implicated in the regulation of cellular senescence, chromatin remodeling, differentiation, DNA damage response, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.

Received 24.3.13; revised 23.7.13; accepted 29.7.13; Edited by M Agostini

P Bose1,3, S Thakur1,3, S Thalappilly1, BY Ahn1, S Satpathy1, X Feng1, K Suzuki1, SW Kim1 and K Riabowol*1,2

The Inhibitor of Growth1 (ING1) tumor suppressor is the founding member of a family of five genes (ING1–5) with conserved plant homeodomains (PHDs). ING proteins are implicated in the regulation of cellular senescence, chromatin remodeling, differentiation, DNA damage response, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.

Received 24.3.13; revised 23.7.13; accepted 29.7.13; Edited by M Agostini

The Inhibitor of Growth1 (ING1) tumor suppressor is the founding member of a family of five genes (ING1–5) with conserved plant homeodomains (PHDs). ING proteins are implicated in the regulation of cellular senescence, chromatin remodeling, differentiation, DNA damage response, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.

Received 24.3.13; revised 23.7.13; accepted 29.7.13; Edited by M Agostini

The Inhibitor of Growth1 (ING1) tumor suppressor is the founding member of a family of five genes (ING1–5) with conserved plant homeodomains (PHDs). ING proteins are implicated in the regulation of cellular senescence, chromatin remodeling, differentiation, DNA damage response, cell cycle regulation and apoptosis. The best characterized member of the ING family, ING1, interacts with the proliferating cell nuclear antigen (PCNA) in a UV-inducible manner. ING1 also interacts with members of the 14-3-3 family leading to its cytoplasmic relocalization. Overexpression of ING1 enhances expression of the Bax gene and was reported to alter mitochondrial membrane potential in a p53-dependent manner. Here we show that ING1 translocates to the mitochondria of primary fibroblasts and established epithelial cell lines in response to apoptosis inducing stimuli, independent of the cellular p53 status. The ability of ING1 to induce apoptosis in various breast cancer cell lines correlates well with its degree of translocation to the mitochondria after UV treatment. Endogenous ING1 protein specifically interacts with the pro-apoptotic BCL2 family member BAX, and colocalizes with BAX in a UV-inducible manner. Ectopic expression of a mitochondria-targeted ING1 construct is more proficient in inducing apoptosis than the wild type ING1 protein. Bioinformatic analysis of the yeast interactome indicates that yeast ING proteins interact with 64 mitochondrial proteins. Also, sequence analysis of ING1 reveals the presence of a BH3-like domain. These data suggest a model in which stress-induced cytoplasmic relocalization of ING1 by 14-3-3 induces ING1-BAX interaction to promote mitochondrial membrane permeability and represent a paradigm shift in our understanding of ING1 function in the cytoplasm and its contribution to apoptosis.
This occurs, in part, by the PHD of ING proteins reading the ‘histone code’ in a methylation-dependent manner. Binding of ING PHDs to trimethylated histones recruits the HDAC complexes to the promoters of proliferation-promoting genes, thus leading to gene repression in response to damage-inducing stimuli. This interaction has also been shown to be important for the DNA repair and apoptotic functions of ING1. ING1 interacts with members of the 14-3-3 family, and phosphorylation of Ser199 of ING1 is necessary for this interaction. However, although the ING1-14-3-3 interaction has been shown to be necessary for the cytoplasmic localization of ING1, the significance of this nuclear–cytoplasmic relocation is not fully understood.

The mammalian apoptosis machinery consists of two partially distinct pathways: the intrinsic and the extrinsic pathways. The extrinsic pathway is heavily influenced by the FAS death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily, whereas the intrinsic pathway involves the mitochondria as a central factor. When stimulated, the intrinsic pathway leads to the release of cytochrome c from the mitochondria and formation of the apoptosome consisting of cytochrome c, APAF-1 and pro-caspase 9 (reviewed in Letai and Brenner and Mak). Both the extrinsic and intrinsic pathways converge with the cleavage and activation of pro-caspase-3 and imminent cell death.

Death-inducing stimuli, including DNA damage, growth-factor deprivation, oncogene activation and cell signaling pathway perturbations, are communicated through the intrinsic apoptotic pathway. The B-cell CLL/lymphoma 2 (BCL-2) family of proteins are important regulators of this apoptotic pathway. BCL-2 family proteins regulate the permeabilization of the outer mitochondrial membrane in response to apoptosis-inducing signals and share homology in 4 α-helical BCL-2-homology (BH) regions (BH1–4), through which they also interact physically. BCL-2-associated X protein (BAX) and BCL-2-antagonist/killer (BAK) share only the BH1–3 domains and are pro-apoptotic members of the BCL-2 family. Another class of pro-apoptotic BCL-2 family members contains the BAX Homology domain 3 (BH3) domain alone. Prominent members of this class include BH3-interacting domain death agonist (BID), BCL-2 agonist of cell death (BAD), BIM (Bcl-2 Interacting Mediator of cell death), NOXA and PUMA (P53-Upregulated Modulator of Apoptosis). The BH3-only proteins are activated through incompletely understood signaling mechanisms; however, once activated, BH3-only proteins induce the activation of BAX and BAK. BAX and BAK are absolutely essential for death induced by BH3 proteins, as the deletion of BAX and BAK leads to a profound inhibition of apoptosis in most tissues.

p53 and ING1 have been shown to functionally cooperate in the activation of apoptosis and physical interactions between the two proteins have been reported. Ectopic expression of ING1 can activate p53 target genes such as p21WAF1 and BAX in a number of transformed cell lines; however, gene array studies performed with normal human diploid fibroblasts did not reveal significant activation of these genes in response to ING1 overexpression. As ING1 is a primarily nuclear protein that has been reported to relocalize to the cytoplasm, and p53 has been reported to be effective in inducing apoptosis when targeted to the mitochondria, we investigated whether ING1 localizes to the mitochondria in response to apoptosis-inducing stimuli. Our data suggest a mechanism through which ING1 induces apoptosis by translocation from the nucleus to the mitochondria where interaction between a BAX BH3 motif and a putative ING1 BH3 domain stabilizes BAX, promoting its pro-apoptotic function.

**Results**

**ING1 translocates to a mitochondrial locale after different forms of stress.** Immunofluorescence analysis of ING1 localization showed that a pool of ING1 translocated to the cytoplasm after UV and IR treatments in primary HS68 fibroblast cells (Figure 1a). As the distribution of ING1 resembled mitochondrial localization, we tested whether ING1 colocalized with the mitochondria. Initial time course experiments showed that by 6 h after UV or IR treatment, significant colocalization occurred (Figure 1b). As conditions for isolating mitochondria had been optimized in the MCF7 epithelial breast cancer cell line that harbors wild-type p53, translocation was examined in these cells. Translocation to the cytoplasm began 3 h after UV and IR treatments; however, maximum colocalization of ING1 and mitochondria was observed 6 h post treatment (Figure 1c). To examine subcellular localization by an independent method, nuclear, cytosolic and mitochondrial fractions with and without UV treatment were prepared from MCF7 cells and immunoblotted with anti-ING1, anti-mtHSP70 (mitochondrial marker), tubulin (cytoplasmic marker) and lamin A (nuclear marker) antibodies. Purity of the fractions was confirmed by the markers and a significant amount of ING1 appeared in the mitochondrial fraction after UV treatment (Figure 1d, lane 8 versus lane 4).

**ING1 translocation to the mitochondria is independent of p53.** Initial studies indicated that ING1 effectively blocked the growth of cells with functional p53 and a physical interaction between ING1 and p53 and functional interdependence was later reported. ING1 also enhanced levels of p53 acetylation on lysine 382 affecting p53 stability under stress, possibly by inhibiting hSIR2 that can deacetylate p53. However, in contrast to reports suggesting an obligatory link between ING1 and p53, a more recent study in knockout mice has indicated that the two proteins can function independently.

As contrasting reports exist regarding the possible interdependence of ING1 and p53, we asked whether p53 was required for ING1 translocation to the mitochondria in response to UV using H1299 p53 null cells. Figure 2a shows that ING1 translocation to the mitochondria after UV treatment can occur independently of p53. To further confirm that ING1 translocation to the mitochondria was independent of p53, we knocked down p53 in MCF7 cells using short hairpin RNA (shRNA) (Figure 2b). As shown by the arrows in Figure 2c, p53-depleted MCF7 cells showed no difference in ING1 translocation to the mitochondria after UV treatment when compared with cells having significant levels of p53, suggesting that the lack of dependence on p53 was not cell-type-specific.
The ability of ING1 to induce apoptosis correlates with the degree of mitochondrial translocation. Examination of the ability of an adenoviral ING1 expression construct to induce apoptosis in a panel of 11 breast cancer cell lines showed that sensitivity varied widely as estimated by sub G1 DNA content, 48 h after infection (Figure 3a). To ask whether sensitivity to ING1-induced apoptosis might correlate with the degree of mitochondrial localization after stress, we selected the SKBR3 and MDA-MB468 cell lines that showed high sensitivity to ING1-induced apoptosis, and the BT474 and T47D lines that were significantly less sensitive to ING1-induced apoptosis and examined the degree of ING1 translocation after UV treatment. Figure 3b shows typical fields of the four cell lines before and after UV treatment, and Figure 3c summarizes the relationship between the degree of localization and sensitivity to ING1-induced apoptosis. As seen in Figures 3b and c, SKBR3 and MDA-MB468 cells showed high degrees of ING1 translocation to the mitochondria, whereas T47D and BT474 cells showed significantly less colocalization of ING1 with mitochondria following UV treatment. Thus, the degree of ING1 translocation to the mitochondria correlated very well with its ability to induce apoptosis in the tested cell lines.

**ING1 interacts with BAX.** As ING1 has been reported to increase BAX levels and BAX is known to act at the mitochondrial membrane to induce apoptosis, we next asked whether ING1 colocalized with BAX. Immunofluorescence analysis showed that ING1 and BAX induce colocalization with mitochondria following UV treatment (Figure 4a). To further ask whether a physical interaction might occur between ING1 and BAX, we performed reciprocal co-immunoprecipitation–western assays in the absence and presence of UV treatment. We found that BAX is indeed...
present in ING1 but not in control immunoprecipitates (Figure 4b); however, contrary to expectations, the interaction appeared to be constitutive under the conditions used in IP-western analysis. As a pool of ING1 appears to relocalize to the mitochondria and bind to BAX, this suggested that ING1 binds to and possibly activates BAX for induction of apoptosis. As our results indicated that ING1-induced apoptosis might be BAX-dependent, we tested this by investigating the ability of ING1 to induce apoptosis in the absence of BAX. A significant decrease in ING1-induced apoptosis was observed in HCT116 BAX−/− cells relative to wild-type HCT116 cells after UV treatment (Figure 4c).

Mitochondrially targeted ING1 is more effective in inducing apoptosis than wild-type ING1. To directly test the possibility that mitochondrial localization of ING1 is necessary for its ability to optimally induce apoptosis, we investigated whether targeting ING1 to the mitochondria was more effective in inducing apoptosis than wild-type (nuclear) ING1. Figure 5a shows representative micrographs of wild-type and mitochondrially targeted ING1 in HEK293 cells. Wild-type ING1 localized exclusively to the nucleus on overexpression. Although nuclear localization persisted for mitochondrially targeted ING1, there was a remarkable increase in cytoplasmic localization with the targeted construct. As previously reported for p53, ING1 targeted to the mitochondria induced apoptosis at earlier time points compared with wild-type ING1 as assayed by levels of cleaved Caspase3 and PARP (Figure 5b). Similar results were obtained in MDA-MB468 breast cancer cells where fractionation experiments confirmed effective mitochondrial targeting (Figure 5c) and an increased ability to induce apoptosis compared with wild-type ING1 (Figure 5d). Thus, affinity for mitochondria directly affects the ability of ING1 to induce apoptosis.

ING proteins interact with several mitochondrial proteins. Homologs of human ING proteins exist throughout
the plant and animal kingdoms. The yeast ING proteins, YNG1, YNG2 and YNG3 (Pho23) exhibit sequence homology with the human ING proteins at their C-terminal regions that affect histone acetylation. As members of the ING family of tumor suppressors show significant sequence conservation from yeast to humans, we previously used available yeast interactome data to predict mammalian counterparts of yeast ING-interacting partners. In silico analysis of ING ‘interologs’ indeed showed that ING proteins in yeast interact with 64 mitochondrial proteins with human counterparts (Supplementary Table 1). This list does not include BAX because it is absent in the yeast proteome. Functional classification of the ING-interacting mitochondrial proteins mined from our previous studies is shown in Table 1. ING1 also contains a region showing high homology with BH3 domains. Visual inspection of the ING1 sequence revealed a short sequence adjacent to the Ser199 residue responsible for 14-3-3 binding that shows a high degree of homology to previously characterized BH3 domains. Supplementary Figure 1 indicates that this sequence has overlapping binding sites for 14-3-3 and BH3 domains, suggesting that nuclear to cytoplasmic translocation of ING1 may be coupled through a common sequence element.

Figure 3 Translocation of ING1 to the mitochondria correlates well with its ability to induce apoptosis. (a) Differential sensitivity of breast cancer cell lines to ING1-induced apoptosis. Eleven breast cancer cell lines were tested for their ability to undergo ING1-induced apoptosis. BT20, BT474, BT578, HS578T, MCF7, MCF10A, MDA-MB435S, MDA-MB468, SKBR3, T47D and ZR-75-1 breast cancer cells were infected with Ad-GFP or Ad-GFP-ING1 constructs. Apoptosis was estimated with propidium iodide staining for sub-G1 DNA content. SKBR3 and MDA-MB468 cells that responded well to ING1-induced apoptosis and T47D and BT474 cells that were significantly less responsive to ING1-induced apoptosis were chosen for further analysis. Results were obtained using an MOI of 10, 48 h after infection. Data are presented from three independent experiments. (b) Immunofluorescence analysis of ING1 translocation to the mitochondria of SKBR3, MDA-MB468, BT474 and T47D cell lines. All four cell lines were either left untreated or treated with 60 J/m² of UVC and fixed after 6 h. MitoTracker was added to the media 0.5 h before fixation and cells were stained for ING1 and DNA. (c) Quantification of ING1 translocation to the mitochondria in relation with responsiveness to ING1-induced apoptosis. Translocation of ING1 to the mitochondria was quantified based on immunofluorescence analysis of SKBR3, MDA-MB-468, T47D and BT474 cells. A total of 100 cells were analyzed for each cell line in a blind experimental protocol in three independent experiments by two individuals. Data are presented in relation to the SKBR3 cell line, in which the maximum ING1 mitochondrial translocation (represented as ++++ based on visual estimation of immunofluorescence images) and the maximum relative responsiveness to ING1-induced apoptosis (represented as +++ based on visual estimation of Figure 3a) were observed. ING1 protein levels were quantified using western blotting and scanning densitometry (data not shown).
Discussion

Previous studies regarding the function of ING1 in apoptosis have focused on nuclear functions.\textsuperscript{12,13,21,31} As ING1 has been previously implicated in the mitochondrial or intrinsic apoptotic pathway\textsuperscript{21} and an ING1-interacting protein has been shown to localize to the mitochondria,\textsuperscript{38} we asked whether it has a function in apoptosis directly through the mitochondria. Immunofluorescence analysis and biochemical fractionation studies showed that ING1 translocates to the mitochondria after treatment of cells with various apoptosis-inducing stimuli. Reciprocal IPs were performed by immunoprecipitating MCF7 cell lysates either with rabbit IgG (nonspecific IP) or with anti-BAX antibody in the presence or absence of UV treatment. Data are presented from four independent experiments (**P = 0.0002).

**Figure 4** BAX is required for ING1-induced apoptosis. (a) Immunofluorescence analysis of BAX-ING1 colocalization in MCF7 cells. Cells were either left untreated or were treated with 60 J/m\textsuperscript{2} of UVC and co-stained for mitochondria, ING1 and BAX. (b) Association between endogenous ING1 and BAX in MCF7 cells. MCF7 cell lysates were immunoprecipitated either with anti-GST antibody (non-specific IP) or with anti-ING1 antibody in the presence or absence of UV treatment. Reciprocal IPs were performed by immunoprecipitating MCF7 cell lysates either with rabbit IgG (nonspecific IP) or with anti-BAX antibody in the presence or absence of UV treatment. (c) Flowcytometry analysis of the dependence of ING1-induced apoptosis on cellular BAX levels. HCT116 wild-type and HCT116 BAX\textsuperscript{−/−} cells were transfected with the above mentioned plasmids and treated with UV 48 h after transfection. Cells were stained with Annexin V and 7-AAD 6 h after UV treatment and were analyzed using flowcytometry within 30 min. Data are presented from four independent experiments (**P = 0.0002).
translocating to the mitochondria in response to apoptosis-inducing stimuli. To further understand the significance of this translocation, we asked whether the ability of ING1 to induce apoptosis in several breast cancer cell lines correlated with its ability to colocalize with the mitochondria after treatment with apoptosis-inducing stimuli. The degree of translocation of ING1 to the mitochondria correlated well with susceptibility to ING1-induced apoptosis. Further investigation of the mechanism by which ING1 might act via the mitochondria led us to ask whether ING1 interacts with pro-apoptotic proteins in the mitochondria. A clear UV-inducible colocalization of ING1 with BAX at the mitochondria was seen by using indirect immunofluorescence. Reciprocal co-immunoprecipitation confirmed that ING1 and BAX physically interacted, although the interaction was not UV-inducible under the conditions we used. This may be because of many factors, including UV-induced exposure of epitopes or the association of ING1 with BAX after the disruption of cells for immunoprecipitation; differential localization of ING1 and BAX would not be maintained in cell lysates. Consistent with ING1 interacting directly with BAX, we identified a BH3 domain in ING1 through which it could theoretically bind BAX and other BH3 domain containing proteins (Supplementary Figure 1). As this region is adjacent to the Ser199 residue that defines the
ING1-14-3-3-binding site, it suggests a mechanism of ING1 relocalization from the nucleus and subsequent targeting to BAX and the mitochondria (Figure 5e). Although ING1 association with and activation of BAX are the simplest interpretations of our results, we have not shown that BAX interacts with ING1 only at the mitochondria or that ING1 is released from 14-3-3 when it translocates to the mitochondria, therefore other possibilities remain; however, a dependence upon p53 has been ruled out.

Our *in silico* analysis indicates that yeast ING proteins interact with several mitochondrial proteins in yeast and a majority of the interacting partners are conserved in humans. This conservation of interactors across species suggests that ING1 serves evolutionarily conserved and biologically significant functions at the mitochondria, one of which is in the induction of apoptosis. Although we did not identify mitochondrial translocation sequences in ING1, functional classification of mitochondrial ING-interacting proteins showed that ING interacts with a variety of mitochondrial transport proteins, which might provide a potential mechanism for its transport to the mitochondria. Mitochondrial interaction data coupled with our observation that protein levels of ING1 remain unchanged in MCF7 cells when exposed to apoptosis-inducing stimuli (Supplementary Figure 2) also support the idea that change in subcellular localization is an important mechanism by which ING1 function in apoptosis is regulated. These data parallel closely the effects of p53 on the process of apoptosis and may help explain why in some studies ING1 appears to affect apoptosis independent of p53, although other reports suggest an interdependence of these two tumor suppressors.

**Materials and Methods**

**Cell lines and Plasmids.** Normal diploid Ha68 fibroblasts were cultured in low-glucose Dulbecco's Modified Eagles Medium (DMEM) (Thermo Scientific, Logan, UT, USA) supplemented with 10% fetal bovine serum, MC7, MDA-MB468, BT474 and T47D epithelial breast cancer cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum. SKBR3, HTC116 wild-type and HTC116 BAX-/- (gift from Dr. Bert Vogelstein)39 cells were grown in McCoy's 5A medium (Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum. All cells were grown at 37 °C with 5% CO2.

**Materials and Methods**

**Cell lines and Plasmids.** Normal diploid Ha68 fibroblasts were cultured in low-glucose Dulbecco’s Modified Eagles Medium (DMEM) (Thermo Scientific, Logan, UT, USA) supplemented with 10% fetal bovine serum. MC7, MDA-MB468, BT474 and T47D epithelial breast cancer cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum. SKBR3, HTC116 wild-type and HTC116 BAX-/- (gift from Dr. Bert Vogelstein)39 cells were grown in McCoy’s 5A medium (Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum. All cells were grown at 37 °C with 5% CO2. Except for MC7 cells, which have wild-type p53, all the other cells lines possess mutations in the p53 gene.

The mitochondria-targeted ING1 plasmid was generated by cloning the ING1b cDNA into the pDaRed2-Mito Vector. The cDNA was amplified with the help of PCR using pCI-ING1b as a template (forward primer: 5'-CAGGGATCCCGTGAGTCCTGGAACGGG-3', reverse primer: 5'-CCCGGATCCGGTGTGGAATCCCTCTTTTGGT-3'). The insert was ligated in frame into the BamHI restriction site on the vector such that the fusion product contained ING1b with N-terminal mitochondrial-targeting sequence and C-terminal dsRed. The construct was verified by sequencing. Western blotting with lysates from HEK293 cells transfected with the constructs showed an increase in the relative molecular mass of the fusion product. Mitochondrial localization was verified using immunofluorescence after transfection of HEK293 cells. mCherry-ING1b was constructed by cloning full-length ING1b cDNA cut using XhoI and NcoI restriction enzymes and mCherry cDNA at the NcoI site of pCI vector. pCI-ING1b and pLEX-mCherry were used as templates for PCR for amplifying the inserts. Adenoviral constructs (Ad-GFP and Ad-GFP-ING1) used in this study have been previously described, and the conditions for infections have been optimized.23,60

**Table 1** Functional classes of mitochondrial proteins interacting with yeast ING proteins. All proteins were previously determined to interact with ING proteins were using TAP-tagging and LC-MS-MS in an interactome analysis. A total of 57 were categorized based upon their localization and function within the mitochondria.

| Function                      | Number of mitochondrial proteins |
|-------------------------------|----------------------------------|
| Metabolism                    | 21                               |
| Mitochondrial transport       | 14                               |
| Mitochondrial translation     | 10                               |
| DNA repair and metabolism     | 5                                |
| Protein modification          | 3                                |
| RNA metabolism                | 2                                |
| Uncharacterized               | 9                                |

**Indirect immunofluorescence.** Cells were grown in 6-cm dishes on coverslips. Twenty-four hour after plating, cells were treated with 60 J/m2 of UV in or with 2 Gy of ionizing radiation. Cells were then labeled with Mitotracker Deep Red (Life Technologies, Burlington, ON, Canada) at 5.5h and fixed at 6 h post UV treatment with cold 95% methanol at room temperature (RT). Cells were washed with phosphate-buffered saline (PBS) and incubated with primary antibodies for 1 h. Cells were then washed in PBS and incubated with secondary antibody for 0.5h. Cells were washed in PBS, stained with 4′,6-Diamidino-2-phenyldi-

**Subcellular fractionation and western blotting.** The subcellular fractionation protocol has been previously described.23,60 Brefeld, cells were lysed using RIPA buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.5% (v/v) NP-40, protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Nuclear, cytoplasmic and mitochondrial fractions were obtained using a Qproteome mitochondria extraction kit and a Qproteome nucleus extraction kit (Qiagen, Toronto, ON, Canada) according to the manufacturer’s protocol.

Cells were washed three times in PBS and collected by scraping in PBS. Pellets were collected by centrifugation at 2500 × g for 5 min and the appropriate amount of Laemmli’s sample buffer was added to the cell pellets. Aliquots corresponding to 2 × 106 cells were loaded on 12.5% polyacrylamide gels, and electrophoresis was performed at a constant 100 V. Separated proteins were transferred to nitrocellulose membranes and immunoblotting was performed by incubation with either rabbit anti-BAX (1:1000 dilution) or mouse monoclonal anti-ING1 (hybridoma supernatant) after blocking in 5% non-fat milk. After several washes in PBS with 0.5% Tween-20, membranes were incubated with 1:5000 dilution of the corresponding secondary antibody conjugated with horseradish peroxidase for 30 min. Membranes were washed with PBS-Tween and exposed to an X-ray film.
Identification of orthologs. Yeast ING-interacting partners were identified from data in reference\(^7\) and previously partially analyzed in reference.\(^3\) BLAST searches were performed to identify human counterparts of the yeast ING-interacting mitochondrial proteins. The results from BLAST search were confirmed using homologene (NCBI) and genecard databases, and biological functions were assigned.

Conflict of Interest

The authors declare no conflict of interest.
53. Wei MC, Zong WX, Chang EH, Lindsten T, Panoutsakopoulou V, Ross AJ et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001; 292: 727–730.

54. Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P et al. p53 has a direct apoptogenic role at the mitochondria. Mol Cell 2003; 11: 577–590.

55. Green DR, Kroemer G. Cytoplasmic functions of the tumor suppressor p53. Nature 2009; 458: 1127–1130.

56. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 1997; 139: 1281–1292.

57. Krogan NJ, Cagney G, Yu H, Zhong G, Guo X, Ignatchenko A et al. Global landscape of protein complexes in the yeast Saccharomyces cerevisiae. Nature 2006; 440: 637–643.

58. Gordon PM, Soliman MA, Bose P, Trinh Q, Sensen CW, Riabowol K. Interspecies data mining to predict novel ING-protein interactions in human. BMC Genomics 2006; 9: 426.

59. Zhang L, Yu J, Park BH, Kintzer KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. Science 2000; 290: 989–992.

60. Thakur S, Feng X, Qiao Shi Z, Ganapathy A, Kumar Mishra M, Atadja P et al. ING1 and 5-azacytidine act synergistically to block breast cancer cell growth. PLoS One 2012; 7: e43671.

61. Ahn BY, Trinh DL, Zajchowski LD, Lee B, Elwi AN, Kim SW. Tid1 is a new regulator of p53 mitochondrial translocation and apoptosis in cancer. Oncogene 2010; 29: 1155–1166.

62. Boland D, Olneck V, Bonnetif P, Veye D, Pann E, Riabowol K. A panel of CAB antibodies recognize endogenous and ectopically expressed ING1 protein. Hybridoma 2000; 19: 161–165.

Cell Death and Disease is an open-access journal published by Nature Publishing Group. This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/3.0/