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HSP70 Induction by ING Proteins Sensitizes Cells to Tumor Necrosis Factor Alpha Receptor-Mediated Apoptosis▽

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ING proteins affect apoptosis, growth, and DNA repair by transducing stress signals such as DNA damage, binding histones, and subsequently regulating chromatin structure and p53 activity. p53 target genes, including the p21 cyclin-dependent kinase inhibitor and Bax, an inducer of apoptosis, are regulated by ING proteins. To identify additional targets downstream of $p33^{ING1}$ and $p32^{ING2}$, cDNA microarrays were performed on phenotypically normal human primary fibroblasts. The 0.36% of genes affected by ING proteins in primary fibroblasts were distinct from targets seen in established cells and included the HSP70 heat shock gene, whose promoter was specifically induced >10-fold. ING1-induced expression of HSP70 shifted cells from survival to a death pathway in response to tumor necrosis factor alpha (TNF-α), and $p33^{ING1b}$ protein showed synergy with TNF-α in inducing apoptosis, which correlated with reduced NF-κB-dependent transcription. These findings are consistent with previous reports that HSP70 promotes TNF-α-mediated apoptosis by binding I-κB kinase gamma and impairing NF-κB survival signaling. Induction of HSP70 required the amino terminus of ING1b but not the plant homeodomain region that was recently identified as a histone binding domain. Regulation of HSP70 gene expression by the ING tumor suppressors provides a novel link between the INGs and the stress-regulated NF-κB survival pathway important in hypoxia and angiogenesis.

The ING1 (*in*hibitor of *g*rowth *1*) type II tumor suppressor was initially identified by combining subtractive hybridization between normal and cancerous breast epithelial cells with an in vivo biological screen (16), and it was subsequently shown to be downregulated in a broad variety of human cancers (14). The ING family consists of five genes (ING1 to ING5), several of which generate multiple isoforms. For example, ING1 encodes at least three isoforms ($p47^{ING1a}$, $p33^{ING1b}$, and $p24^{ING1c}$), of which $p33^{ING1b}$ is well conserved and the major isoform expressed in human cells (14). Of the other INGs, $p32^{ING2}$ shares the most sequence homology (23) and functional similarities with $p33^{ING1b}$ (12). Functional links have been found between the ING family of proteins and cellular processes important in tumorigenesis, including apoptosis, cell cycle regulation, senescence, and the DNA damage response. One major pathway through which the ING proteins exert these effects is through physically and functionally interacting with integral components of histone acetyltransferase- and histone deacetylase chromatin-modifying complexes (8, 26, 31, 33, 43, 51, 56) as well as binding preferentially to methylated histones through their plant homeodomains (PHD) (37, 44, 50). This has the effect of targeting these complexes to regulate local acetylation and deacetylation levels, which alters gene expression (15). The PHD (21) and/or a polybasic region downstream of the PHD (28) has also been shown to bind rare stress-induced phospholipids and to induce ING proteins to activate p53. It is interesting to note that the BRCA1 protein also impinges upon chromatin remodeling through interactions with BARD1 (3, 27, 39, 61), so both the ING1 and BRCA1 tumor suppressors may contribute to the emergence of cancers through similar mechanisms. Consistent with ING proteins altering chromatin structure, the expression of p21 and Bax is upregulated by $p33^{ING1b}$, $p32^{ING2}$, and $p47^{ING3}$ proteins in a number of cancer cell lines (29, 40, 41). Although a recent cDNA microarray analysis using the transformed mouse mammary epithelial cell line NMuMG did not register p21 or Bax as a target of ING1, it did identify more than a dozen other genes whose expression was altered in response to $p33^{ING1b}$, including cyclin B1 and the DEK proto-oncogene (54).

Heat shock proteins (HSPs) are a large family of evolutionarily conserved proteins which function as molecular chaperones (5). These specialized proteins play important roles in cellular defense mechanisms against protein aggregation and misfolding by binding nonnative states of other proteins and assisting them in reaching a correctly folded and functional conformation. They are also involved in protein translocation across membranes to different organelles for final packaging, degradation, or repair through their unfoldase activities (20, 22). More than a dozen HSPs, such as HSP27, -40, -60, -70, -90, and -110, etc., have been identified so far, and they are named according to their molecular mass (5, 63). Among them, HSP70 is one of the most conserved, and it is also the best characterized. In most mammalian cells there are two prominent isoforms of HSP70, an abundant constitutive member called HSP73 and the highly stress-inducible HSP72. HSP70 isoform expression is enhanced by a number of different stress factors, including heat, cold, glucose, alcohol, heavy metals, and ischemia (38). Expression of HSP70 protects cells from stress (46) and is generally thought to play cytoprotective roles through antagonizing components of apoptotic pathways, such as apoptosome formation and caspase complex activation (53).

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Thus, HSPs are believed to be involved in the proteasome-mediated degradation of apoptosis-regulatory proteins in addition to having previously identified chaperoning functions (45, 52). However, some studies show that elevated HSP70 inhibits cellular proliferation (34) and promotes T-cell receptor/CD3- and Fas/APO-1/CD95-mediated Jurkat T-cell apoptosis (32). The heat shock response also sensitizes primary endothelial cells to apoptosis through the inhibition of NF-κB activity (11), and the expression of HSP70 correlates with susceptibility to apoptosis in acute myeloid leukemia cells (6). As seen in studies of endothelial cells, elevation of HSP70 to a significant level favored tumor necrosis factor alpha (TNF-α)-mediated apoptosis via inhibiting the NF-κB survival pathway (45). This finding is consistent with several studies reporting that HSP70 blocks NF-κB activation and NF-κB-dependent gene expression through inhibition of both I-κB kinase (IKK) activation and subsequent degradation of I-κB (35, 36, 46, 49, 62).

To examine the mechanism by which ING proteins affect apoptosis in a genetically normal model, we tested the effect of altering ING levels on global gene expression by cDNA microarray analysis in the Hs68 strain of primary human diploid fibroblasts. A subset of genes was specifically and reproducibly affected by overexpression of the related $p33^{ING1b}$ and $p32^{ING2}$ proteins, including the 70-kDa heat shock protein HSP70. ING proteins also sensitized cells to TNF-α-mediated apoptosis by impairing the NF-κB signaling pathway via upregulation of HSP70 expression, and this effect was dependent upon the amino terminus, but not the PHD, of ING1.

MATERIALS AND METHODS

Cell culture. Human primary diploid fibroblasts (Hs68 [ATCC CRL-1635]) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Normal human embryo lung fibroblasts (WI38; Coriell Institute, Camden, NJ) were maintained in minimum Eagle's medium supplemented with 10% FBS. The mean population doubling level for Hs68 cells used in experiments was between 30 and 35, and that for WI38 cells was between 20 and 25. At this in vitro "age," cells remain competent for growth and traverse the cell cycle freely in response to mitogens. Immortalized HeLa cells (ATCC CCL-2) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, and HCT-116 human colon carcinoma cells (ATCC CCL-247) were grown in McCoy's 5a media with 1.5 mM L-glutamine and 10% FBS. All cells were maintained at 37°C in 95% air and 5% CO_2, and culture medium (Gibco BRL) was changed every 2 or 3 days. All cell lines and strains tested negative for mycoplasma.

Generation of adenoviral constructs and infection of cells. Adenoviral constructs were generated using a modified "pAdEasy" system (24). ING1a, ING1b, ING2, and p53 were subcloned into pAdTrack-CMV, which contains a separate enhanced green fluorescent protein (GFP) expression cassette, and were recombined with pAdEasy-1 in *Escherichia coli* BJ5183. Recombinant clones were screened and subsequently verified by a series of enzymatic digestions. Recombinants were reamplified in XL1-Blue (Clontech), linearized by PacI (NEB), and transfected into 293 cells for packaging. Viral clones were plaque purified, selected for expression, amplified, and purified by $CsCl_2$ gradient centrifugation. Plaque assays were routinely performed to ensure accurate viral titers. Optimized adenoviral infections were done at multiplicities of infection of 100 for young Hs68 cells and WI38 cells and of 10 for HeLa and HCT116 cells, giving >95% infectivity as monitored by GFP expression. No toxicity was observed when adenoviruses were used at these multiplicities of infection.

Analysis of cDNA microarrays. Hs68 cells were infected either with GFP alone or with GFP-ING adenoviruses. At 24 h after infection, cells were harvested and total RNA was extracted using TRIzol (Invitrogen). DNase treatment preceded the confirmation of RNA quality by examining the 260/280 absorbance ratio on a UV spectrometer (Pharmacia), by visual inspection after 1% agarose gel electrophoresis, and by PCR with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers by use of RNA as the template and genomic DNA as a control. Reverse transcription generated cDNA from 10 μg of total RNA by use of a FairPlay microarray labeling kit (Stratagene). An indirect labeling method provided with the above-mentioned kit (Stratagene) was used to generate Cy3 and Cy5 (Amersham Biosciences) fluorescence-labeled cDNA. The labeled cDNA samples were then purified to remove uncoupled fluorescent dye and subsequently combined together with yeast tDNA (Stratagene) and hybridized to 14,000 human oligonucleotide chips (Southern Alberta Microarray Facility) by incubating at 37°C under a humidified condition for 18 h. After hybridization, arrays were washed for 3 to 4 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.2% sodium dodecyl sulfate at room temperature (RT), for 5 min with 0.2× SSC at RT, and with 0.05× SSC for 5 min at RT. Slides were quickly dried and scanned using a fluorescence laser microarray scanning device (Virtex). Three independent replicates, including one dye reversal experiment, were performed for each ING gene assay. Replicate data were first quantitated by Array-Pro software (Media Cybernetics) and then transferred to GeneTraffic bioinformatic software (Iobion Informatics Company) for further data normalization, annotation, and management.

Primers and reverse transcription-PCR (RT-PCR). Hs68, WI38, or HCT116 cells were harvested 24 h after infection, total RNA was extracted using TRIzol (Invitrogen), and RNA quality was confirmed as described above. cDNA was made from 1 to 5 μg total RNA immediately after DNase treatment by use of a two-step reverse transcription method as described below. First, RNA treated with DNase was denatured with 1 μl of 500-ng/ml random primer (Amersham) and 1 μl of 10 mM deoxynucleoside triphosphate mixture (Amersham) at 70°C for 10 min and quickly chilled on ice. Then, 2 μl of 5× Superscript II buffer, 2 μl of 0.1 M dithiothreitol, and 1 μl of 32-U/μl RNase inhibitor (Invitrogen) were added, and the samples were incubated at RT for 10 min before the addition of 400 U of Superscript II (Invitrogen) to each tube. After incubation at 42°C for 1 h, cDNA samples were heated to inactivate enzymes at 70°C for 15 min and were subsequently used for PCR to estimate relative levels of HSP70, 25-kDa brain-specific protein (p25), mitotic kinesin-related protein/M-phase phosphoprotein (MPP), DNA polymerase theta (POLQ), clathrin heavy chain 2 (clathrin), L-type, calcium channel alpha-2/delta subunits (CANCLB), and ING1b and ING2 by comparison to internal controls (GAPDH or β-actin) by use of the primer-dropping method (59). PCR primers used were as follows: HSP70, 5′-CGACCTGAACAAGAGCATCA-3′ (forward) and 5′-TCGTCCTCCGCTTTGTACTT-3′ (reverse); p25, 5′-ATCGGAGGGTGCTGGTGAGG-3′ (forward) and 5′-GGTGCCTGCGTGCTTGTAGC-3′; MPP, 5′-TACGGGCTAACCAATTCAGG-3′ (forward) and 5′-AGGCAACACTCTGGTGCTTT-3′ (reverse); POLQ, 5′-TCAAAAGGCATAGCTCTCCT-3′ (forward) and 5′-CCTCAAACATAGGTGTAACCAG-3′; CANCLB, 5′-GCATTGGAACTGGGACTTGT-3′ (forward) and 5′-TTCCGAATCCTTCATTTTGC-3′ (reverse); Clathrin, 5′-TGTGAATTATGCAACCAACG-3′ (forward) and 5′-CCCCTCAGCAGAAAGATCC-3′; ING1b, 5′-GAAGATCCAGATCGTGAGCC-3′ (forward) and 5′-GAGACCTGGTTGCACAGACA-3′ (reverse); ING2, 5′-AAAATCGGGCAAGACAAATG-3′ (forward) and 5′-GAAGCTTCCCTTTCCTGCTT-3′ (reverse); GAPDH, 5′-GTCAGTGGTGGACCTGACCT-3′ (forward) and 5′-AGGGGTCTACATGGCAACTG-3′ (reverse); and β-actin, 5′-GAACCCTAAGGCCAACCGTGA-3′ (forward) and 5′-AGGAAGAGGATGCGGCAGTGG-3′ (reverse). Aliquots of PCR products equalized to give equivalent signals from the internal control mRNAs (GAPDH/β-actin) were electrophoresed through 2% agarose gels (Ultrapure; Pharmacia), stained with 0.2 μg/ml ethidium bromide, and analyzed by computerized densitometric scanning of the images by use of Kodak imaging software normalized using internal controls.

Transfection and dual luciferase reporter assays. HeLa or Hs68 cells were seeded into 24-well tissue culture plates (8×10^4 cells/well) 16 to 18 h prior to transfection. Cells were first cotransfected with either pHSE-luc reporter plasmid (a generous gift from K. Yoshihara) or NF-κB-dependent luciferase reporter construct, and with the pRL-TK (Promega) control vector to control for transfection efficiency, by use of Lipofectamine 2000 (Invitrogen). Sixteen to 18 h after transfection, cells were infected with GFP, GFP-ING, or GFP-p53 adenoviruses. Positive control experiments using heat shock were performed by incubating cells at 42°C for 30 min and then transferring back to 37°C for recovery. Twenty-four h after infection or heat shock, luciferase activities were measured by use of a luminometer (Berthold Technology Junior LB 9509) with the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. All experiments were performed in triplicate, and statistical data analyses were performed using Microsoft Excel software. TNF-α (Sigma) treatments began 12 h after infection and continued for another 24 h.

Western blotting. Hs68, WI38, HCT116, or HeLa cells were infected with the adenoviral constructs indicated. In some experiments, cells were treated with TNF-α (Sigma) either at 25 ng/ml for 15 min or at 50 ng/ml for 24 h (unless otherwise indicated) 12 or 24 h after infection. At the end of the TNF-α incu-

TABLE 1. Genes with increased expression in response to p33^{ING1b}[a]

Gene ID	Fold change from expt:						MFC	Gene name or characteristic
	1	1R	2	2R	3	3R		
L38518	18.88	20.05	21.36	19.53	29.44	30.55	23.3	Sonic hedgehog (*Drosophila*) homolog (SHH)
AL137576	42.19	34.2	7.34	7.77	22.4	24.12	23	Hippocampus abundant transcript 1 (HAT1)
AB017016	2.8	3.28	17.9	18.1	30.98	27.54	16.8	25-kDa brain-specific protein (p25)
AL117496	13.3	12.22	2.09	2.08	1.1	3.4	7.43	MPP
AF058925	4.29	4.25	5.89	5.46	9.19	12.96	7.01	Janus kinase 2 (JAK2)
M11717	7.81	8.32	5.86	5.62	5.27	5.02	6.32	Human heat shock protein gene (HSP70)
NM 006596	2.62	2.36	7.45	7.74	8.57	8.63	6.23	POLQ
M93284	1.74	1.57	18.33	17.98	3.74	3.68	7.84	Pancreatic lipase-related protein 2 (PLRP2)
AF151079	13.06	12.47	1.81	1.81	4.58	4.52	6.38	Hypothetical protein MGC874
U61167	2.25	2.4	4.56	3.9	9.25	9.49	5.31	SH3 domain protein 1B
AC005764	2.38	2.15	3.54	3.8	8.48	9.38	4.96	Hypothetical human protein most similar to AF037204
AL137472	2.26	1.17	5.54	4	6.52	7.32	4.91	Hypothetical protein DKFZp434C0923
M21305	1.08	1.03	7.34	7.16	9.51	7.47	5.6	Hypothetical protein similar to hornerin
AL161960	1.5	1.65	4.44	5.51	7.4	7.57	4.68	Chromosome 21 open reading frame 97
AK000188	2.05	1.78	1.84	2.2	9.71	10.86	3.42	Acetyl coenzyme A synthetase 2
NM 005346	4.19	3.86	3.4	3.41	2.17	2.28	3.22	Heat shock 70-kDa protein 1B
L13266	9.71	9.9	2.54	2.71	−1.24	−1.02	3.77	Glutamate (NMDA) receptor subunit zeta 1
U80456	−1	−1.24	2.05	4.73	4.71	4.78	2.34	Single-minded (*Drosophila*) homolog 2 (SIM2)
AC005559	7.15	7.27	2	2.21	−1.25	−1.26	2.69	*Homo sapiens* chromosome 19, cosmid F18382
X60188	2.31	2.37	1.94	2.12	2.17	2.26	2.2	ERK-1/p44-MAPK3[b]
NM_006076	2.58	2.74	1.67	1.38	2.44	2.59	2.23	HIV-1 Rev-binding protein-like protein[c]
Y10262	1.02	1.06	2.14	3.29	3.35	3.74	2.43	*H. sapiens* EYA3 gene
D87468	2.11	1.69	2.1	2.7	2.07	2.06	2.12	Activity-regulated cytoskeleton-associated protein

[a] Three independent replicates, including dye reversal experiments (R), were performed for each ING gene in the current microarray screening study. All replicate data were quantitated by Array-Pro software (Media Cybernetics) and transferred to GeneTraffic bioinformatic software (Iobion Informatics Company) for further data normalization, annotation, and management. On the 14,000 human oligonucleolide chips, each gene is spotted in duplicate. In total, six relative ratios (Cy3/Cy5 or Cy5/Cy3), identified as changes (*n*-fold), were measured for every gene on the chip from which the mean fold change (MFC) was calculated. The selection criteria for inclusion of genes up- or down-regulated by the p33^{ING1b} and p32^{ING2} proteins were a ≥2× MFC in at least two out of three independent replicates or four out of six relative ratios. Genes that are underlined were chosen to be verified by RT-PCR.

[b] ERK-1, extracellular signal-regulated kinase 1; MAPK3, mitogen-activated protein kinase 3.

[c] HIV-1, human immunodeficiency virus type 1.

bations, cells were lysed in sodium dodecyl sulfate loading buffer, and samples were electrophoresed and blotted with anti-HSP72/73 monoclonal (Stressgen), anti-I-κB polyclonal, anti-I-κB phospho-32/36 monoclonal (Cell Signaling), anti-poly-(ADP-ribose) polymerase, anti-cIAP2, anti-actin polyclonal, anti-FLIP, anti-GFP monoclonal (Santa Cruz), anti-ING1 monoclonal, or anti-ING2 polyclonal (SACRI Antibody Services) antibodies.

Apoptosis assays and cell viability. For microscopic visualization of chromatin condensation and fragmentation, Hs68 cells on coverslips were fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 5 min, permeabilized for 5 min in 0.5% Triton X-100 in PBS, and stained with 1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole) in PBS. Coverslips were then mounted in 1 mg/ml paraphenylenediamine in PBS-90% glycerol. Digital imaging was performed using a 14-bit cooled charge-coupled-device camera (Princeton Instruments) mounted on a Leica DMRE immunofluorescence microscope. Annexin V kits (Roche) were used according to the manufacturer's instructions to identify apoptotic cells by use of a FACScan flow cytometer in combination with BD Lysis II software (Becton-Dickinson). The viability of cells was assessed by trypan exclusion assay. The floating dead cells in the medium and the cells that remained attached to the plates were collected by trypsinization and counted using a hemacytometer in the presence of 0.4% trypan blue reagent (Sigma). All the experiments were done in triplicate, and statistical data analysis was performed using Microsoft Excel software.

RESULTS

Oligonucleotide microarray analyses. To determine if p33^{ING1b} and the closely related p32^{ING2} (14, 23) could reproducibly regulate gene expression, we performed a high-throughput gene expression analysis with normal Hs68 fibroblasts infected by replication-defective recombinant adenoviruses encoding these tumor suppressors. The cDNA probes were fluorescently labeled with Cy3 or Cy5 dye by use of an indirect labeling method in order to minimize the biased incorporation of the different Cy dyes in reverse transcription reactions. Reciprocal labeling experiments, i.e., labeling with Cy5 and Cy3, were also included to preclude problems of interpretation due to dye bias. Fluorescence-labeled cDNA probes were combined and hybridized to a single array containing 14,000 genes printed in duplicate. Detection of hybridized probes was achieved by laser excitation of the individual fluorescent markers followed by scanning using a confocal laser scanning microscope. In the current study, +2 and −2 were set as the cutoff values for mean fold change (MFC). Tables 1 and 2 list the genes that were regulated by the p33^{ING1b} protein. In total, 22 (0.15%) and 30 (0.21%) genes displayed relatively higher and lower expression levels, respectively, when cells overexpressed p33^{ING1b}. Table 3 lists the genes upregulated by p32^{ING2}. Despite identical assay conditions in the same primary cell type, only eight genes were upregulated by p32^{ING2}, and no reproducibly or significantly downregulated gene targets were revealed, suggesting independent cellular roles for these related ING family members. However, both the p33^{ING1b} and p32^{ING2} proteins induced both isoforms of the 70-kDa heat shock protein (HSP70). Neither the p21 nor the Bax genes that were previously reported to be induced by ING1 (17) were sufficiently induced to be detected in Hs68 fibroblasts by use of this method.

Confirmation of array hits by RT-PCR. Hits were confirmed by the primer-dropping method (59), in which the target gene of interest is amplified in the linear range using gene-specific primers together with the internal control GAPDH or β-actin in the same PCR. RT-PCR confirmed that all microarray hits

TABLE 2. Genes decreased in expression by $p33^{ING1b}$[a]

Gene ID	Fold change from expt:						MFC	Gene name or characteristic
	1	1R	2	2R	3	3R		
M76559	−10.9	−13.77	−13.42	−13.93	−25	−34.6	−19	L-type, calcium channel alpha-2/delta subunit precursor
AB037771	−5.36	−6.36	−3.08	−3.73	−3	−2.12	−3.9	KIAA1350 protein
AB033025	−4.43	−5	−3	−3.12	−2.7	−2.36	−3.4	KIAA1199 protein
AB008822	−3.47	−3.14	−2.56	−2.47	−3.5	−4.25	−3.2	*Homo sapiens* gene for osteoclastogenesis inhibitory factor
X78924	−3.8	−4.23	1.09	1.03	−5	−4.51	−2.6	Zinc finger protein 266
U04636	−3.21	−3.75	−1.78	−1.96	−2.5	−2.72	−2.7	Human cyclooxygenase 2 (hCox-2) gene
X61598	−5.55	−5.44	−1.38	−1.49	−1.9	−2.51	−3.1	Collagen-binding protein
NM_000165	−5.03	−4.87	−2	−2.03	−1.5	−1.61	−2.8	Gap junction protein, 43 kDa
NM_016125	−1.16	−1.19	−1.95	−3.09	−5	−5.28	−2.9	PTD016 protein
X14420	−4.05	−4.11	−1.96	−1.96	−1.8	−1.75	−2.6	Collagen III, alpha-1 polypeptide
X78925	−3.9	−3.36	1.04	1.04	−4.2	−4.01	−2.2	Zinc finger protein 267
L20861	−3.9	−3.3	−1.69	−1.66	−2.2	−2.38	−2.5	WNT-5A protein precursor
AB011084	−3.18	−3.05	−1.68	−1.76	−2.2	−2.03	−2.3	Armadillo repeat protein ALEX2
L27560	−4.87	−5.01	−1.62	−1.59	−1.4	−1.54	−2.7	IGF-binding protein 5
M31166	−4.37	−3.97	−1.35	−1.43	−1.8	−2.07	−2.5	Pentaxin-related gene, rapidly induced by IL-1 beta[b]
AL050031	−3.28	−3.78	−1.29	−1.2	−2.4	−2.47	−2.4	Phospholipase C, epsilon 1
AL137718	1.05	−1.17	−1.11	−1.15	−12	−6.8	−3.6	Diaphanous homolog 3 (*Drosophila*)
AB029000	−3.66	−3.93	−1.62	−1.82	−1.6	−1.47	−2.3	Sulfatase
U78722	−4.71	−4.55	1.23	1.26	−2.7	−2.52	−2	Zinc finger protein 165
J03464	−3.78	−3.66	−1.86	−1.68	−1.4	−1.4	−2.3	Alpha 2 type I collagen
NM_007034	−2.47	−1.8	−1.38	−1.48	−3	−3.12	−2.2	DnaJ-like HSP40
U08021	−3.53	−3.56	−1.75	−1.73	−1.5	−1.49	−2.3	Nicotinamide *N*-methyltransferase
X68686	−2.53	−2.01	1.02	−1.17	−3.7	−3.82	−2	*H. sapiens* ZNF11A gene
AK001700	−2.53	−2.1	−1.53	−1.44	−2.5	−2.69	−2.1	Solute carrier family 38 (amino acid transporter), member 2
AL080111	−2.99	−2.99	−1.38	−1.33	−2.4	−1.96	−2.2	Never in mitosis gene a-related kinase 7
AF050127	−3.3	−3.39	−1.47	−1.29	−1.9	−1.85	−2.2	Hypoxia-inducible factor 1 alpha subunit (HIF1A) gene
J04177	−3.91	−4.35	−1.51	−1.8	−1.4	−1.38	−2.4	Collagen, type XI, alpha 1
AK001015	−2.29	−2.63	−1.7	−1.68	−1.8	−2.41	−2.1	BAG family molecular chaperone regulator 2
AB023194	−2.74	−2.85	−1.69	−1.79	−1.8	−1.62	−2.1	KIAA0977 protein
D83174	−3.12	−3.02	−1.32	−1.38	−1.8	−2.02	−2.1	Heat shock protein 47

[a] Assays and criteria are as described for Table 1.
[b] IL-1 beta, interleukin-1 beta.

were significantly regulated by $p33^{ING1b}$ and/or $p32^{ING2}$ in Hs68 cells (Fig. 1A). For example, both $p33^{ING1b}$ and $p32^{ING2}$ induce HSP70, while only $p33^{ING1b}$ induces the mitotic M-phase phosphoprotein MPP. HSP70 was not induced by the infection of Hs68 cells with the control GFP-expressing adenoviral construct that was used to coexpress the INGs with GFP under separate promoters (Fig. 1A). These data indicate that the increase in HSP70 in cells coexpressing ING1b or ING2 together with GFP is due to a specific effect of the ING proteins and not to GFP or to adenovirus infection.

Upregulation of HSP70 gene expression by ING1 and ING2. To further confirm whether the HSP70 gene was a bona fide downstream target of the INGs, another primary cell strain, WI38, and the transformed cell line HCT116 were examined. As shown in Fig. 1B and C for both WI38 and Hs68 primary cells, HSP70 gene expression was markedly increased by $p33^{ING1b}$ and $p32^{ING2}$. Induction was less dramatic in HCT116, since this cancer line constitutively expresses high levels of HSP70, as previously reported (38). The other major splicing isoform of the ING1 gene, $p47^{ING1a}$, weakly induced

TABLE 3. Genes increased in expression by $p32^{ING2}$[a]

Gene ID	Fold change from expt:						MFC	Gene name or characteristic
	1	1R	2	2R	3	3R		
AB012853	85.6	50.77	147.9	170.02	120.41	226.79	114	p32ING2/ING1L
M11717	16.6	15.61	6.91	6.8	18.61	20.29	11.5	Heat shock 70-kDa protein 1 (HSP70)
NM005346	14.5	13.5	4.32	4.41	11.45	10.51	9.19	Heat shock 70-kDa protein 1B (HSPA1B)
S66793	11.6	10.72	3.67	3.94	5.85	7.31	7.48	X-arrestin (S-antigen homolog)
U41763	5.91	5.63	2.86	2.98	5.53	4.23	4.35	Clathrin heavy chain 2 (clathrin)
AF151079	5.39	6.24	2.72	2.91	2.45	2.46	4.32	Hypothetical protein MGC874
AF060865	5.93	8	1.91	2.33	1.97	2.55	4.54	Zinc finger protein 205
AL133640	2.3	2.25	1.84	1.82	2.87	2.66	2.05	Polycystic kidney and hepatic disease 1 (autosomal recessive)-like 1
M93284	30.2	23.44	8.82	9.41	48.62	54.27	18	Pancreatic lipase-related protein 2 (PLRP2)

[a] Assays and criteria were as described for Table 1. This ING protein (ING-2) did not significantly decrease the expression of any of the 14,000 genes assayed for on the digonucleotide chips used.

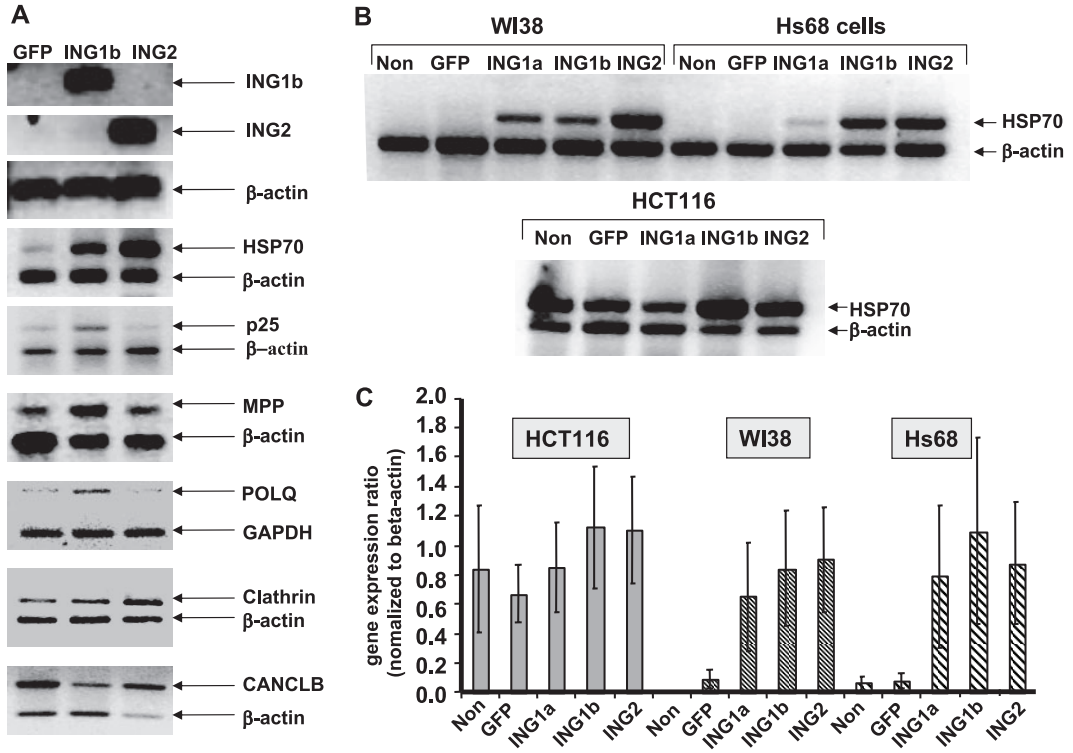

FIG. 1. Verification of array hits by primer-dropping RT-PCR. (A) Confirmation of microarray targets. The genes that were reproducibly and significantly regulated in microarray analyses were further tested by primer-dropping RT-PCR (59). Hs68 cells were infected with GFP, GFP-p33^{ING1b}, or GFP-p32^{ING2} adenovirus and harvested 24 h later, and total RNA was extracted and treated with DNase. Duplex RT-PCR was then carried out to detect HSP70, 25-kDa brain-specific protein (p25), MPP, POLQ, L-type, CANCLB, and clathrin heavy chain 2 (Clathrin) by use of sequence-specific primers for each gene and either β-actin primer or GAPDH primer as an internal control. (B) HSP70 analyses. Primary WI38 and Hs68 fibroblasts and immortalized HCT116 cells were infected with the indicated adenoviral constructs, and 24 h later total RNA was extracted. Primer-dropping RT-PCRs were used to detect mRNA expression levels of HSP70 in the linear range of the reaction using sequence-specific priming with β-actin primer as an internal control. Non, mock infected. (C) Summary of HSP70 induction data. The graph shows the average level of HSP70 gene expression (normalized to β-actin) from three independent primer-dropping RT-PCR experiments and is plotted ± standard deviation.

HSP70 in primary fibroblasts but not in HCT116 (Fig. 1B and C). Levels of p47^{ING1a}, p33^{ING1b}, and p32^{ING2} were routinely checked by Western blotting to verify the expected expression levels in response to adenoviral infection (data not shown).

To determine whether higher levels of HSP70 mRNA resulted in increased protein levels, lysates of control and infected WI38, Hs68, and HCT116 cells were analyzed. As shown in Fig. 2A, p33^{ING1b} and p32^{ING2} strongly induced levels of HSP72 and HSP73 in primary fibroblasts compared to the control, whereas p47^{ING1a} was less effective. In contrast, although modest effects were seen at the mRNA level (Fig. 1C), none of the ING proteins tested markedly affected HSP70 protein levels in HCT116 (Fig. 2B), which is most likely due to the constitutively high levels of HSP70 expression previously noted for these cells (38). In addition, only a single HSP70 protein band was detected in this highly mutated line. β-Actin was used as a loading control (Fig. 2A and B), and GFP blots were used to confirm that adenovirus infection gave equal levels of GFP expression in different samples (data not shown).

Since the ING proteins have been reported to activate p53 as a transcription factor, we asked whether the ING proteins induce HSP70 expression through p53. We found that overexpression of p53 in the same viral expression construct does not have a significant effect upon HSP70 levels in either primary cells or cancer cells (Fig. 2B and data not shown).

To further test for possible effects of p53 on HSP70 expression and to compare the effects of the ING proteins to those of heat shock treatment by use of an alternative assay and different cell types, an HSP70 promoter-driven luciferase reporter construct was cotransfected with control (GFP), ING, or p53 expression construct into HeLa and Hs68 cells. This reporter construct contains the HSP70 promoter, including the heat shock element responsible for inducing the expression of HSP70 in response to stress, upstream of the luciferase gene. As seen in Fig. 2C, the ING proteins induced HSP70 expression as effectively as heat shock in HeLa cells and nearly as effectively in Hs68 cells. Consistent with previous reports, we found that overexpression of the p53 protein inhibited HSP70 transcription in both cell types (1). Together, our results suggest that the ING proteins induce HSP70 by a mechanism independent of p53.

Dose-dependent expression of HSP70 depends upon the amino terminus of ING1 and is independent of the PHD. Next, we investigated the effect of increasing concentrations of the ING1b expression plasmid on the induction of the HSP70 promoter-driven reporter in primary fibroblasts. As shown in

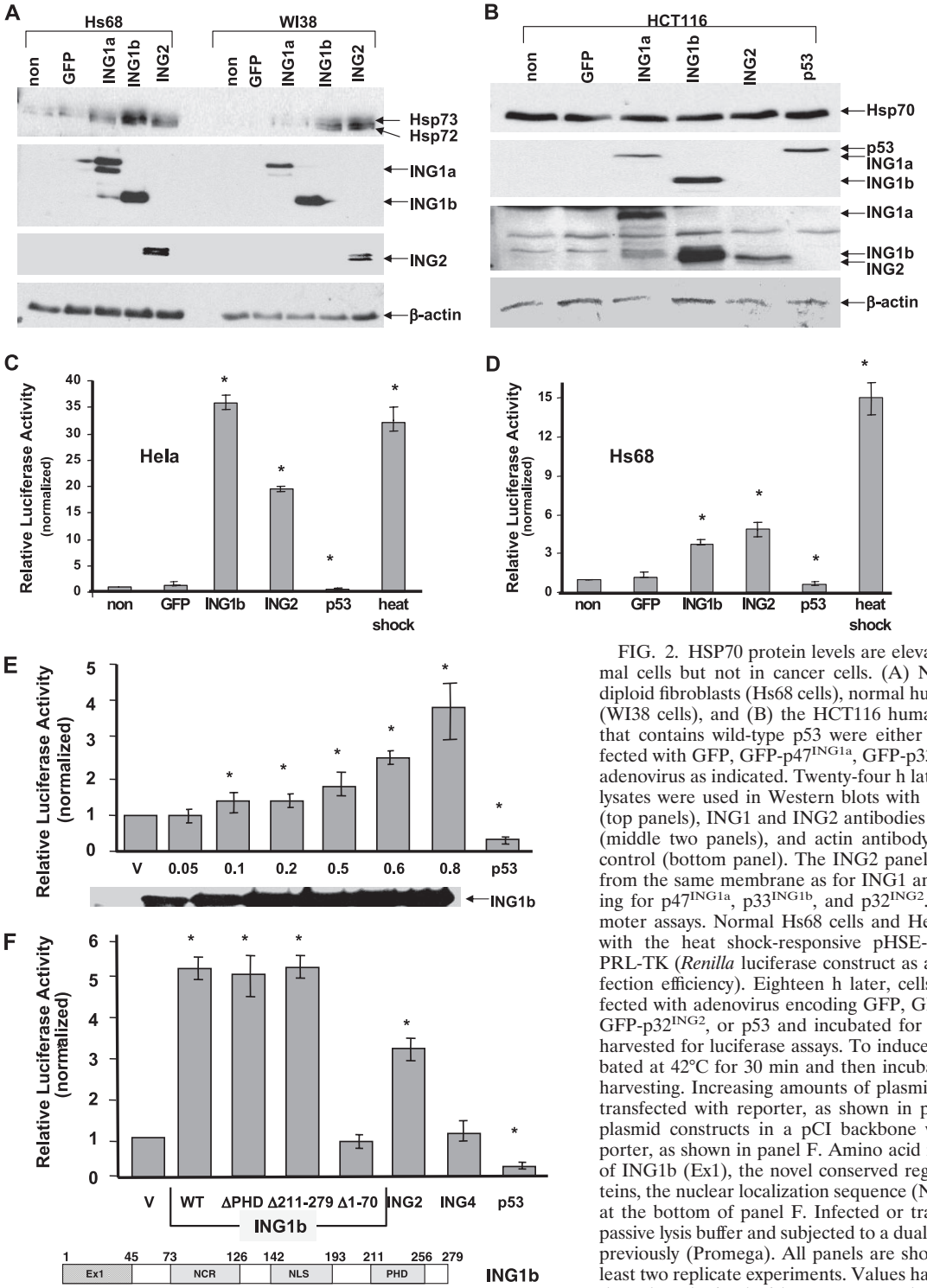

FIG. 2. HSP70 protein levels are elevated by ING proteins in normal cells but not in cancer cells. (A) Normal human skin primary diploid fibroblasts (Hs68 cells), normal human embryo lung fibroblasts (WI38 cells), and (B) the HCT116 human colon carcinoma cell line that contains wild-type p53 were either mock infected (non) or infected with GFP, GFP-p47^{ING1a}, GFP-p33^{ING1b}, GFP-p32^{ING2}, or p53 adenovirus as indicated. Twenty-four h later, cells were harvested, and lysates were used in Western blots with HSP70 antibody (Stressgen) (top panels), ING1 and ING2 antibodies (SACRI Antibody Services) (middle two panels), and actin antibody (Santa Cruz) as a loading control (bottom panel). The ING2 panel for HCT116 was generated from the same membrane as for ING1 and shows simultaneous staining for p47^{ING1a}, p33^{ING1b}, and p32^{ING2}. (C to F) HSP70 gene promoter assays. Normal Hs68 cells and HeLa cells were cotransfected with the heat shock-responsive pHSE-luc reporter construct and PRL-TK (*Renilla* luciferase construct as an internal control for transfection efficiency). Eighteen h later, cells were mock infected or infected with adenovirus encoding GFP, GFP-p47^{ING1a}, GFP-p33^{ING1b}, GFP-p32^{ING2}, or p53 and incubated for a further 24 h before being harvested for luciferase assays. To induce heat shock, cells were incubated at 42°C for 30 min and then incubated at 37°C for 24 h before harvesting. Increasing amounts of plasmid encoding ING1b were cotransfected with reporter, as shown in panel E, while the indicated plasmid constructs in a pCI backbone were cotransfected with reporter, as shown in panel F. Amino acid residues demarcating exon 1 of ING1b (Ex1), the novel conserved region (NCR) of the ING proteins, the nuclear localization sequence (NLS), and PHD are indicated at the bottom of panel F. Infected or transfected cells were lysed in passive lysis buffer and subjected to a dual luciferase assay as described previously (Promega). All panels are shown as a representative of at least two replicate experiments. Values have been equalized relative to the value obtained with non or control vector (set at 1) and are the averages of three independent determinations with standard deviations shown by the error bars. The differences seen between non/vector and all of the other groups (indicated as *) are significant at the level of $P < 0.01$ as estimated by analysis of variance. V, vector; WT, wild type.

Fig. 2E, ING1b produced a dose-dependent increase in HSP70 promoter activity. Next, we carried out experiments to map out the determinants in ING1b that specify the induction of the HSP70 promoter. We tested the abilities of several deletion constructs of ING1b that are stably expressed to induce the HSP70 promoter. Interestingly, these data showed that the PHD region, which binds methylated histones (amino acids 211 to 256), and the adjacent polybasic region, which binds phospholipids (amino acids 256 to 279), were not required for induction (Fig. 2F). In contrast, deletion of the first 70 amino

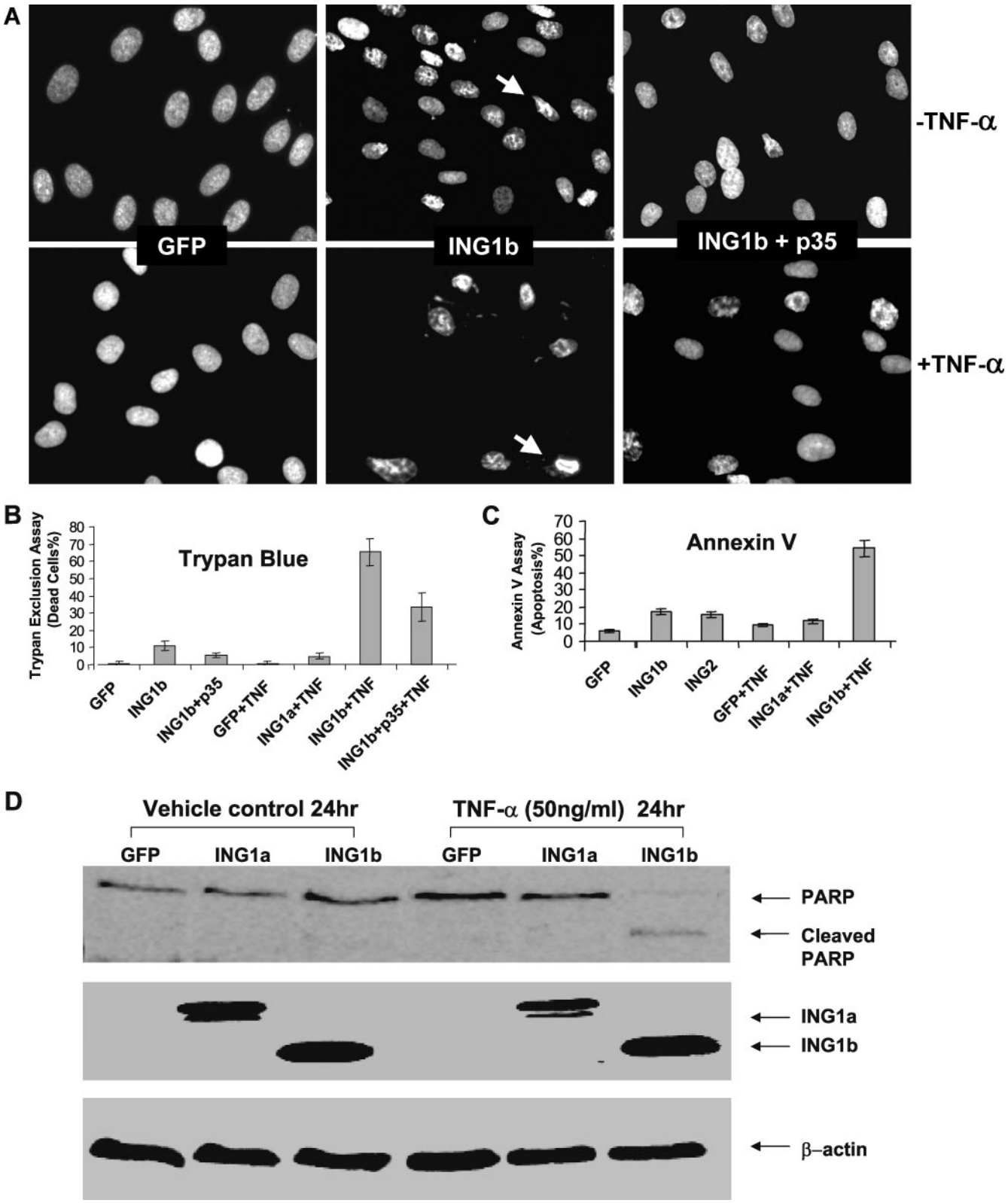

FIG. 3. $p33^{ING1b}$ and TNF-α act synergistically to induce apoptosis. (A) Hs68 cells infected with the indicated adenoviral expression constructs were incubated for 12 h and subsequently treated either with vehicle (upper panels) or with TNF-α (50 ng/ml) (lower panels) for another 24 h. Cells were then fixed, stained by DAPI, and observed under the fluorescence microscope. (B to D) Hs68 cells were infected with the indicated expression constructs and 12 h later were treated with vehicle or with TNF-α (50 ng/ml) for another 24 h. Cells were harvested and subjected to trypan exclusion assay to assess viability (B), annexin V assay (Roche) (C), or Western blotting with PARP antibody (D). Lower panels show control Western blots to verify that ING expression levels and protein loads were similar.

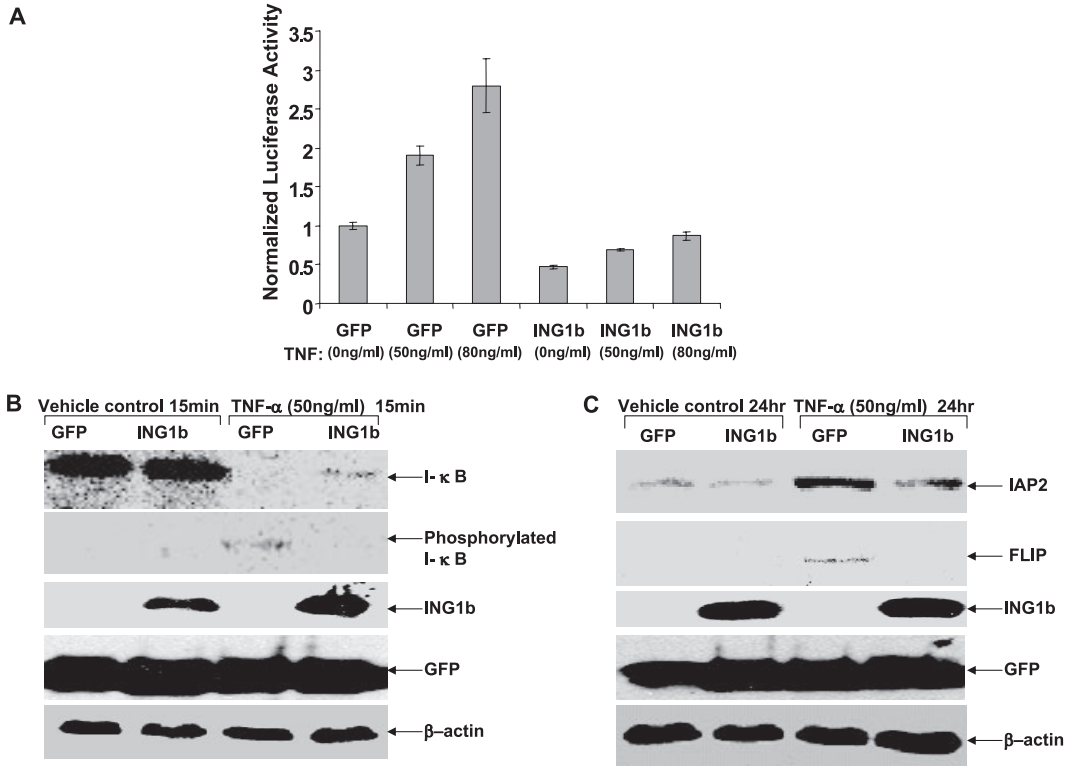

FIG. 4. p33^{ING1b} expression blocks TNF-α-induced NF-κB activity. (A) Hs68 cells were cotransfected with NF-κB-Luc reporter construct and PRL-TK (*Renilla* luciferase construct as an internal control) and 12 h later were infected with GFP or with GFP-p33^{ING1b} adenovirus as indicated. Twelve hours postinfection, cells were treated with vehicle or with TNF-α (50 or 80 ng/ml) as indicated for another 24 h. Cells were then lysed and subjected to a dual luciferase assay (Promega). (B) HeLa cells were infected with GFP or with GFP-p33^{ING1b} adenovirus as indicated for 24 h and then treated with vehicle or with TNF-α (50 ng/ml) for 15 min. Cells were quickly lysed and subjected to Western blotting with I-κB, phosphorylated I-κB, ING1, GFP, and β-actin antibodies. (C) Hs68 cells were infected with GFP or with GFP-p33^{ING1b} adenovirus as indicated for 18 h and then treated with vehicle or with TNF-α (50 ng/ml) for another 24 h. Cells were lysed and subjected to Western blotting with IAP2, FLIP, ING1, GFP, and β-actin antibodies.

acids completely blocked the ability of ING1b to induce HSP70 (Fig. 2F). This region of the ING1b protein contains a PCNA-interacting protein domain as well as a partial bromodomain (14). ING2, which shares partial homology with ING1b in the amino terminus (23), was somewhat effective in inducing the HSP70 promoter, whereas ING4, which is divergent in this region, did not. Together these data suggest that the conserved N-terminal regions of ING1b and ING2 are required for these ING proteins to induce HSP70 expression.

Induction of apoptosis by TNF-α is enhanced by p33^{ING1b}. Since HSP70 was recently reported to promote TNF-α-mediated apoptosis via interference with NF-κB signaling (45), we asked whether the ING proteins combined with TNF-α treatment would more efficiently induce apoptosis as a result of the concerted upregulation of HSP70 expression and the subsequent disruption of NF-κB signaling. In these studies, we used the ING1b protein, since it produced a consistent induction of HSP70 in all of the different assays used. Consistent with previous reports (57), p33^{ING1b} alone induced a certain amount of apoptosis/cell death in Hs68 cells, as evidenced by characteristic DAPI staining (chromatin condensation and/or fragmentation in Fig. 3A), trypan blue exclusion (Fig. 3B), annexin V membrane exposure (Fig. 3C), and PARP cleavage (Fig. 3D). When cells overexpressing p33^{ING1b} were treated with TNF-α (Fig. 3A to D), much higher levels of apoptosis were noted with all assays. This effect appears to be synergistic, since when combined, p33^{ING1b} and TNF-α result in levels of apoptosis higher than the sum resulting from the use of both agents individually. For example, the annexin V data indicate that 18% of cells are apoptotic in response to p33^{ING1b} and 10% are apoptotic after TNF-α treatment but that when combined, approximately 60% of Hs68 primary human diploid fibroblasts, which are generally resistant to apoptosis, initiate programmed cell death (Fig. 3C). In contrast to these effects, TNF-α treatment led to a much lower degree of apoptosis in the ING1a-expressing cells (Fig. 3B and C). Together, these data indicate that enhanced apoptotic response to TNF-α stimulation by ING1b correlates with its ability to induce HSP70.

The p35 protein is a broad-spectrum caspase inhibitor that inhibits the activation of all caspases except caspase 9 (53). Therefore, it primarily inhibits the receptor-mediated apoptosis pathway as opposed to the mitochondrion-mediated pathway. To test if p33^{ING1b} operated solely through the receptor-mediated pathway, we overexpressed both p33^{ING1b} and p35 (a kind gift from D. Larocque) in Hs68 cells and treated them with TNF-α. As shown in Fig. 3A and B by use of independent assays, p35 reduced apoptosis in TNF-α-treated ING1b-expressing cells by approximately half. These data are consistent

with p33^{ING1b} having effects upon the receptor-mediated pathway via the TNF-α pathway and through effects upon the mitochondrial apoptosis pathway, perhaps by inducing the p53 target gene Bax.

The p33^{ING1b} protein sensitizes cells to TNF-α-mediated apoptosis by inhibiting NF-κB signaling. As noted previously, HSP70 promotes TNF-mediated apoptosis by binding IKK-γ and impairing NF-κB survival signaling. To test whether p33^{ING1b}-induced HSP70 accumulation affected apoptosis by inhibiting this pathway, we transfected cells with an NF-κB-responsive promoter driving luciferase expression and asked whether the expression of p33^{ING1b} would interfere with the ability of TNF-α to induce NF-κB activity. As shown in Fig. 4A, reporter activity was increased in a TNF-α dose-dependent manner as expected. Overexpression of p33^{ING1b} in Hs68 cells reproducibly inhibited NF-κB-dependent gene activation in both the absence and the presence of TNF-α, consistent with p33^{ING1b} inhibiting NF-κB survival signaling in response to TNF-α. In addition, when overexpressed, p33^{ING1b} reduced the TNF-α-dependent phosphorylation and subsequent degradation of I-κB (Fig. 4B). Moreover, induction of the antiapoptotic NF-κB target genes IAP2 and FLIP by TNF-α was inhibited more by the expression of GFP plus p33^{ING1b} than by GFP alone (Fig. 4C). Blotting for GFP confirmed that cells were uniformly infected with adeno-GFP and adeno-GFP-ING1b, and β-actin served as a protein loading control (Fig. 4B and C). These data suggest that the expression of p33^{ING1b} interferes with the ability of TNF-α to induce NF-κB activity, thus switching from a pathway of survival to an apoptotic pathway, as diagrammed in the model shown in Fig. 5.

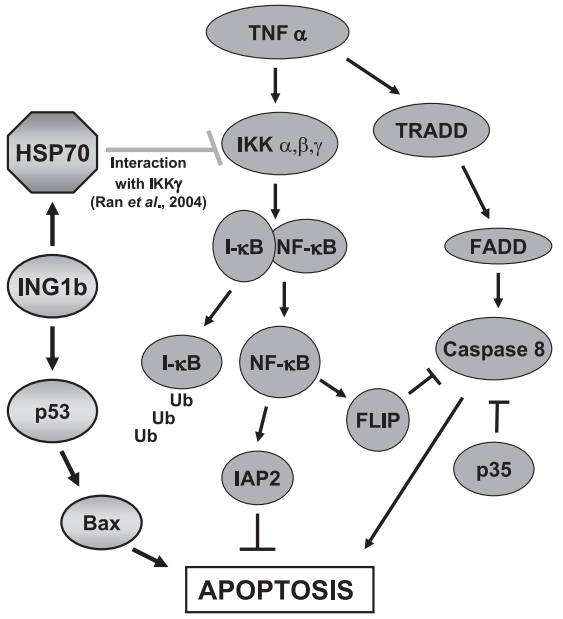

FIG. 5. Model linking HSP70 induction to p33^{ING1b}-induced apoptosis. Previous studies have linked HSP70 to TNF-α-mediated apoptosis by impairing NF-κB survival signaling through binding IKK-γ. p33^{ING1b} has also been implicated in the p53-inducible expression of the proapoptotic Bax member of the Bcl2 family. In this study, we show that p33^{ING1b} induces significant levels of HSP70, that it inhibits NF-κB activity, and that it acts synergistically with TNF-α to induce apoptosis. The p33^{ING1b} protein, therefore, is proposed to induce apoptosis by at least two mechanisms: by activating p53 and inducing signaling through the mitochondrial pathway and by inhibiting the NF-κB survival pathway through the induction of HSP70.

DISCUSSION

This study demonstrates that in normal primary fibroblasts, the p33^{ING1b} and p32^{ING2} proteins increase the levels of a partially overlapping set of genes, while p33^{ING1b} is unique in negatively regulating a distinct subset of genes. Both of these related ING gene products increase the levels of HSP70 mRNA by severalfold, and HSP70 expression appears to correlate well with p33^{ING1b}-induced apoptosis, as assayed by several independent methods. Induction of HSP70 occurs independently of p53, and overexpression of p33^{ING1b} acts synergistically with TNF-α to induce apoptosis, as assayed by chromatin condensation, trypan blue dye exclusion, annexin V staining, and cleavage of PARP. Induction is not a result of adenoviral effects, since the same virus overexpressing GFP, GFP plus p53, GFP plus an amino-truncated ING1b, or GFP plus ING4 did not induce HSP70. Finally, p33^{ING1b} inhibited the ability of TNF-α to induce NF-κB transcriptional activity, supporting the idea that p33^{ING1b}-induced expression of HSP70 promotes apoptosis by inhibiting the NF-κB survival signaling pathway.

The ING1 family of type II tumor suppressors regulates gene expression through modulating chromatin structure via interacting with histone acetyltransferase and histone deacetylase complexes (14) and may direct them to specific chromatin locales by binding methylated histones via their PHD regions (37, 44, 50). Moreover, p53 target genes such as p21^{WAF1} and Bax, which regulate cell cycle progression and apoptosis, have previously been identified as downstream targets of p33^{ING1} and p32^{ING2}. However, in our study these genes were not identified either by microarray analysis or by RT-PCR. This may be due to the fact that the studies noted above were carried out with different transformed cell lines in which multiple signaling pathways are altered to various degrees, while in this study we used normal Hs68 human primary diploid fibroblasts that have no known mutations in genes encoding tumor suppressors or cell cycle regulators or in proto-oncogenes. A recent microarray study done with the transformed epithelial mouse cell line NMuMG identified 25 genes (1.1% of 2,304 genes examined) whose expression levels were significantly altered upon a decrease in p33^{ING1b} expression, but the set did not include p21^{WAF1}, Bax, or HSP70 (54). In our microarray analysis, the expression levels of 22 (0.15% of 14,000 genes) and 30 (0.21% of 14,000 genes) genes were significantly up- and downregulated, respectively, in response to p33^{ING1b}. The percentages of significant hits were comparable in spite of the different cell types and methodologies used in the two studies. However, the gene targets identified in these two microarray analyses did not overlap, which likely reflects profound cell type differences (immortalized mouse epithelial cells versus normal human fibroblasts) and/or microarray assay differences (cDNAs versus oligonucleotides and the noncomprehensive natures of both chip sets used).

In our analysis, the upregulation of HSP70 was both robust and reproducible in response to p33^{ING1b} in at least two normal fibroblast strains and in two immortalized cell lines. The

expression of several other heat shock genes, including those encoding HSP10, -27, -60, -75, -90, -105, and -110, which were included on our array chips, were not affected by the overexpression of ING proteins, suggesting that induction is not a result of a general stress reaction. Indeed, genes encoding HSP40 and HSP47 were downregulated by $p33^{ING1b}$. HSP40 is a cochaperone protein that regulates complex formation between HSP70 and client proteins, thus facilitating protein folding and enhancing cell survival in response to stresses such as heat shock (13). HSP47 is a collagen-specific chaperone residing in the endoplasmic reticulum (42) that is essential for collagen biosynthesis. The downregulation of HSP40 by $p33^{ING1b}$ and the lack of induction of other HSPs suggest that the upregulation of HSP70 by ING proteins may have downstream effects differing from the cellular protective roles of HSP70 (46). This is consistent with our results showing that the overexpression of ING proteins did not have any effects in protecting cells from the adverse effects of heat shock (data not shown).

It has been known for some time that cancer cells exposed simultaneously to heat and chemotherapeutic agents or radiation die at a higher rate than do cells treated with chemotherapeutic agents or radiation alone, which is the rationale for the use of hyperthemia in combination with chemotherapy and radiotherapy to treat cancer in the clinic (9, 10, 11). Some ING proteins have also been implicated in chemosensitivity and radiosensitivity (4, 7, 19, 47, 48, 55, 64). It is possible, therefore, that the upregulation of HSP70 by ING proteins is one mechanism by which ING proteins confer chemosensitivity and radiosensitivity, consistent with a link to the arm of the TNF-α pathway that impinges on NF-κB-regulated gene expression.

Several groups have now reported that $p33^{ING1b}$ and $p32^{ING2}$ induce apoptosis in p53-dependent or p53-independent manners in different cell types (25, 41), but the underlying mechanisms remain unclear. Here we find that ING proteins sensitize cells to TNF-α-mediated apoptosis through upregulation of HSP70, which inhibits NF-κB survival signaling as outlined in the model shown in Fig. 5. Thus, in addition to a previous report linking $p33^{ING1b}$ to a p53-associated mitochondrial apoptotic pathway (7), this study supports the contention that ING proteins also have a significant impact upon p53-independent receptor-mediated apoptosis through NF-κB signaling pathways.

HSP70 mRNA expression is regulated, at least in part, by the direct effects of ING1 on the HSP70 promoter. This idea is supported by previous reports showing ING binding to promoter regions of genes (29) and by the results shown in Fig. 2C, in which the cotransfection of ING is shown to activate HSP70 promoter-driven reporter constructs in a dose-dependent manner. However, the Hs68 primary fibroblasts show approximately three- to fourfold-higher reporter activity in response to the ING proteins (Fig. 2C), but HSP70 mRNA and protein levels increase to a much greater degree (~10- to 15-fold [Fig. 1B and C]). Furthermore, small interfering RNA against ING1 did not markedly reduce HSP70 expression (data not shown) or affect the ability of TNF-α to induce apoptosis, indicating that the upregulation of HSP70 promoter activity by ING1, while contributing to induction to some degree, may not be the dominant mechanism responsible for the ability of ING proteins to induce HSP70. Another mechanism that might contribute to the ING-specific induction of HSP70, but not that of other stress-inducible proteins, might be related to ING protein activation of a pathway that impinges upon HSP70. Since the ING proteins have been linked to the transduction of stress signals via binding rare phosphatidylinositol monophosphates (21, 28), it is possible that the overexpression of ING in the absence of additional stimuli may produce activated forms of ING protein, initiating a stress response. The mechanism by which this response is generated could be through the formation of aggresomes by ING proteins, which is known to induce HSP70 expression (30), and in many cases is linked directly to the induction of apoptosis (2), as is seen upon ING1 overexpression (Fig. 3). This idea is supported by the observation that the amino terminus of ING1b that tends to form aggregates (data not shown), but not the more hydrophilic downstream domains containing the nuclear localization sequence, PHD, and polybasic regions of ING1b, is effective in inducing HSP70 expression (Fig. 2F).

Traditionally, HSP70 has been thought to protect cells by interfering with the mitochondrial apoptotic pathway (20, 53); however, a growing body of evidence shows that heat shock or elevated HSP70 can also promote receptor-mediated apoptosis (32, 60). In particular, HSP70 can enhance cell death when it is overexpressed in cells that are also exposed to TNF-α via inhibiting the NF-κB signaling cascade (45). This is reasonable considering the fact that it is well established that NF-κB signaling is negatively regulated by HSP70 (22, 36, 49, 62). In this study, we show that $p33^{ING1b}$ inhibits TNF-α-induced NF-κB-dependent gene transcription (Fig. 4), suggesting that $p33^{ING1b}$ disrupts NF-κB signaling through the upregulation of HSP70.

Another member of the ING family of tumor suppressors, named ING4, has also recently been shown to inhibit NF-κB activity, resulting in reduced expression of NF-κB target genes (18). In that study, evidence consistent with a physical interaction between ING4 and the p65 subunit of NF-κB, obtained primarily by use of overexpression studies, was presented, although links to HSP70 and TNF-α signaling (58) were not examined. Given these data, it is possible that ING1b and ING4 negatively regulate NF-κB activity through mechanisms involving direct binding to p65 and via HSP70 induction. Alternatively, ING1 and ING4 may inhibit the NF-κB signaling pathway by distinct mechanisms. Our finding that ING2 overexpression also leads to HSP70 induction but that ING4 does not (Fig. 2 and 3), together with the conserved features of the ING proteins (23), suggests that ING1b and ING2 downregulate the NF-κB pathway by use of a common mechanism related to the amino termini of these proteins. Testing if $p33^{ING1b}$ directly interacts with components of the NF-κB pathway will further refine the model presented in Fig. 5 and provide a better understanding of how the ING family of type II tumor suppressors exert their effects upon apoptosis and cell growth control.

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