Novel Splice Variants of ING4 and Their Possible Roles in the Regulation of Cell Growth and Motility*

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The ING4 gene is a candidate tumor suppressor gene that functions in cell proliferation, contact inhibition, and angiogenesis. We identified three novel splice variants of ING4 with differing activities in controlling cell proliferation, cell spreading, and cell migration. ING4_v1 (the longest splice variant), originally identified as ING4, encodes an intact nuclear localization signal (NLS), whereas the other three splice variants (ING4_v2, ING4_v3, and ING4_v4) lack the full NLS, resulting in increased cytoplasmic localization of these proteins. We found that one of the three ING4 variants, ING4_v2, is expressed at the same level as the original ING4 (ING4_v1), suggesting that ING4 variants may have significant biological functions. Growth suppressive effects of the variants that have a partial NLS (ING4_v2 and ING4_v4) were attenuated by a weaker effect of the variants on p21WAF1 promoter activation. ING4_v4 lost cell spreading and migration suppressive effects; on the other hand, ING4_v2 retained a cell migration suppressive effect but lost a cell spreading suppressive effect. Therefore, ING4_v2, which localized primarily into cytoplasm, might have an important role in the regulation of cell migration. We also found that ING4_v4 played dominant-negative roles in the induction of p21WAF1 promoter activation and in the suppression of cell motility by ING4_v1. In addition, ING4 variants had different binding affinities to two cytoplasmic proteins, protein-tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), α1, and G3BP2a. Understanding the functions of the four splice variants may aid in defining their roles in human carcinogenesis.

The ING4 (inhibitor of growth family member 4) was identified in our laboratory as a candidate tumor suppressor gene (1) that is down-regulated in glioblastoma cells (2) and head and neck squamous cell carcinoma (3). ING4 suppresses cell growth (1, 4, 5), suppresses the loss of contact inhibition (6), inhibits angiogenesis (2), and down-regulates the stability of hypoxia-inducible factor (HIF)3-α (7) through a physical interaction with HIF prolyl hydroxylase (HPH)-2C (8), resulting in repressed HIF activation in a chromatin-dependent manner. More recently, it was reported that ING4 acetylates histone H4 lysine 5, 8, and 12 with a histone acetyltransferase, HBO1/ MYST2 (MYST2 is MOZ, YBF2/SAS3, SAS2 and TIP60 histone acetyltransferase 2) (9), and interacts with methylated histone H3 (10). Thus, ING4 seems to play several major roles in cells like other ING family proteins (11) that are conserved from yeasts to vertebrates (12).

In this study, we describe three novel splice variants of ING4, ING4_v2 (a 3-bp skip form), ING4_v3 (a 9-bp skip form), and ING4_v4 (a 12-bp skip form), besides ING4_v1 (the originally enrolled ING4, the longest form). These variants are produced from the alternative use of two splice donor sites at the end of exon 4 and two splice acceptor sites at the start of exon 5 of the ING4 gene, although one of the three variants, ING4_v4, was previously attributed to a common deletion mutation (6). The alternative RNA splicing of the ING4 pre-mRNA causes a partial loss of an NLS and affects nuclear localization of the proteins. We show that the small deletion in the ING4 protein leads to functional differences between the ING4 variants. Growth suppressive effects of the variants that have the partially missing NLS were attenuated by weaker activity on p21WAF1 induction. In addition, ING4_v4 showed attenuated suppressive effects on cell spreading and migration compared with the original ING4 (ING4_v1). On the other hand, ING4_v2 only lost the suppressive effect on cell spreading, suggesting an important role for ING4_v2 on the regulation of cell migration. ING4_v4 played dominant-negative roles on these ING4_v1 effects. In addition, we found that ING4_v1 and ING4_v2, but not ING4_v4, have a binding affinity to Liprin α1 that may play a role in the regulation of focal adhesion disassembly (13, 14). In addition, only ING_v1 has a binding affinity to G3BP2a, which is involved in Ras signaling, NF-κB signaling, and the ubiquitin proteasome system (15–17). These differences may affect the function of ING4 splice variants.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank/EMBL Data Bank with accession number(s) AB197695, AB197696, and AB197697.

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3 The abbreviations used are: HIF, hypoxia-inducible factor; NLS, nuclear localization signal; Liprin α1, LAR-interacting protein α1; G3BP2a, Ras-GTPase activating protein SH3 domain-binding protein 2; PHD, plant homeodomain; NF-κB, nuclear factor of κB; HPH-2, HIF prolyl hydroxylase 2; ESE, exonic splicing enhancer.
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EXPERIMENTAL PROCEDURES

Tissues and Cell Lines—cDNA from various tissues (Human Multiple Tissue cDNA Panels 1 and 2) were obtained from BD Biosciences. RKO, U-118 MG, M059K, DBTRG-05MG, NCI-H82, A549, LNCap.FGC, PC-3, SW480, HCT 116, 293, 293T, U-2 OS, and MRC-5 cells were obtained from ATCC (Manassas, VA). All cell lines were grown as a monolayer in appropriate media, supplemented with 10% fetal bovine serum, as follows: Dulbecco’s modified Eagle’s medium for RKO, U-118 MG, M059K, 293, 293T, U-2 OS, and MRC-5 cells; Eagle’s minimal essential medium for DBTRG-05MG; RPMI 1640 for A549 and NCI-H82, LNCap.FGC; F12 medium for PC-3; McCoy’s 5a medium for HCT 116; and Leibovitz’s L-15 medium for SW480.

Construction of Minigene Plasmids—All sequences for cloning were amplified by PCR using KOD-Plus (Novagen, Madison, WI) and verified by DNA sequencing. A partial ING4 gene from exon 3 to exon 6 and its point mutants were cloned into pcDNA 3.1 (+) (Invitrogen) using the HindIII and XbaI site. Expression plasmids for two major ING4 splice variants (ING4_v2 and ING4_v4) were derived from the original ING4 plasmid (pFLAG-CMV-2 and pcDNA3.1/Hygro) as a template.

Transfection of Plasmids and Isolation of RNA—The plasmids were transfected into cells by Lipofectamine reagent (Invitrogen). After 24 h of transfection, cells were harvested, total RNAs were isolated from the cells with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Then 5–10 µg of the total RNAs were reverse-transcribed using the SuperScript III first-strand synthesis system (Invitrogen) according to the manufacturer’s protocol.

Separation of Each ING4 Variant by PCR-Restriction Fragment Length Polymorphism—The transcribed artificial cDNAs from the series of minigenes were amplified using a forward primer at a vector-specific region (T7 promoter) between the transcriptional starting site and exon 3 of ING4 (5’-TAATAC-GACTCCTATATTGCC-3’) and a reverse primer at exon 5 of ING4 (5’-ACTTCTTCTGGAGGCTTCTGT-3’). A nested PCR was performed on the first PCR using a forward primer at exon 4 (5’-CCAAACAACATCGGGAGGCT-3’), combined with a reverse primer at exon 5 (5’-ACTTCTTCTGGGCACTGTTG-3’). One µl of the gel-purified first PCR product was used as a nested PCR template. The nested PCR product was digested by HaeIII (New England Biolabs, Beverly, MA), and stained with SYBR Gold (Molecular Probes, Eugene, OR).

Real Time PCR Analysis—Total ING4 was detected using variant common primers (5’-CCGGACTCAAAAGGAGAA-3’ and 5’-GAGGGGATCCCTACTGAGA-3’) and a variant common probe (5’-TTCGATGAGGAATGCCCCACAGACTG-TAMRA-3’). For specific detection of ING4_v1, a variant-specific forward primer (5’-CCAGGACAGAAAGCCTAGAC-3’), a variant common reverse primer (5’-GAGGGCATCCCTACTGAGA-3’), and a variant common probe (5’-TTCGATGAGGAATGCCCCACAGACTG-TAMRA-3’) were designed. For specific detection of ING4_v2, a variant common forward primer an ING4_v2-specific reverse primer (5’-TTCTCTTTTTGATCAGGCCTC-3’), and an ING4_v1-and ING4_v2-specific probe (5’-FAM-ATGACAGCTCTCTACGAAAA-TAMRA-3’) were designed. For specific detection of ING4_v3 and ING4_v4, variant-specific probes (ING4_v3: 5’-FAM-TCCAGAAAGGCGCG- GACTCA-TAMRA-3’ and ING4_v4: 5’-FAM-TCTTCCAGCAAAGGCGGACTCA-TAMRA-3’), a variant common forward primer (5’-AGAGAAACAGATTGCTAGACG-ACTATG-3’), and a variant common reverse primer (5’-TTTGAAACGACGACGACGAC-3’) were designed. The variant-specific PCR products were continuously measured by means of an ABI PRISM 7700 sequence detection system (Applied Biosystems) during 40 cycles. The annealing temperature was modified for each variant-specific amplification (ING4_v1 and ING4_v3, 64 °C; ING4_v2 and ING4_v4, 66 °C). β2-Microglobulin RNA was used for normalization using pre-developed TaqMan assay reagent (4310886E; Applied Biosystems). The annealing temperature for the amplification of total ING4 and B2M was 60 °C.

Western Blotting—Plasmid constructs were transfected into cells using Lipofectamine reagent (Invitrogen). After 24 h of transfection, cells were washed with phosphate-buffered saline, harvested in Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, and 50 mM Tris-HCl, pH 8.0) with Proteinase Mixture Set III (Calbiochem), and sonicated. The extracted proteins were separated by SDS-PAGE. The FLAG-tagged proteins were detected by anti-FLAG antibody (Sigma).

Fractionation of Proteins—Cells were transfected with different plasmids that express FLAG-tagged ING4_v1, ING4_v2, and ING4_v4. 24 h after transfection, the cellular proteins were fractionated by the nuclear/cytoplasm fractionation kit (BioVision Research Products, Mountain View, CA) and the same amount of proteins from nuclear and cytoplasmic fractions was applied into a gel. Immunoblotting was performed by a standard protocol. FLAG-tagged proteins were detected by anti-FLAG antibody (Sigma). Fractionation was evaluated by a nuclear marker, anti-Lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Indirect Immunofluorescence Assay—Cells were cultured on coverslips in 6-well plates and transfected with different untagged ING4 plasmid constructs using Lipofectamine Reagent (Invitrogen). After 24 h, cells were fixed, permeabilized, and incubated with anti-ING4 antibody (Rockland, Gilbertsville, PA), followed by a secondary antibody (fluorescein isothiocyanate-conjugated donkey anti-goat IgG from Jackson ImmunoResearch, West Grove, PA). Then the slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 1.5 µg/ml 4,6-diamidino-2-phenylindole. The fluorescent images were obtained using a fluorescent microscope (Axioplan 2 imaging; Carl Zeiss, Thornwood, NY).

Colony Formation Assay—Three cancer cell lines, RKO, U-118 MG, and U-2 OS were used for the experiments. Cells were plated on 10-cm dishes (5 × 10^5 cells/dish), cultured for 12 h at 37 °C, and then transfected with 5 µg of pcDNA3.1/Hygro (6.6 kb) as a control, and 5.7 µg of pcDNA3.1/Hygro-ING4_v1 (6.35 kb), pcDNA3.1/Hygro-ING4_v2 (6.35 kb), and pcDNA3.1/Hygro-ING4_v4 (6.35 kb). Cells were cultured in the selection medium (hygromycin B; Invitrogen) as follows:
Immunoprecipitation was performed using FLAG M2 resin from Dr. Derek Kennedy (University of Queensland, Australia). 24 h after transfection, the cells were lysed in Nonidet P-40-based lysis buffer (0.1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, and protease inhibitor mixture; Calbiochem). The area of the colonies (pixels) in each dish was calculated by Photoshop CS (Adobe, San Jose, CA). The data are shown as the average and standard deviation of three independent experiments. Statistical analysis was carried out by both Scheffe’s F test and Student’s t test.

**Reporter Gene Assay**—Cells were plated into 12-well plates and transfected with 0.1 μg of pEYFP-Nuc that encodes the enhanced yellow-green variant of the *Aequorea victoria* green fluorescent protein gene (BD Biosciences) and with 0.5 μg of WWW-Luc-p21 using Lipofectamine 2000 (Invitrogen) and with the indicated amount of pcDNA3.1/Hygro empty vector, pcDNA3.1/Hygro-ING4_v1, pcDNA3.1/Hygro-ING4_v2, or pcDNA3.1/Hygro-ING4_v4. The cells were lysed in cell culture lysis reagent (Promega, Madison, WI), and the lysates were collected to measure the p21 promoter activity 48 h after transfection. The transcriptional efficiency was examined by detecting a signal from enhanced yellow fluorescent protein (the fluorescence excitation maximum is 513 nm and the peak of the emission spectrum is 527 nm), and the promoter activity (the peak of the emission spectrum of luciferase is 562 nm) was examined by the Bright-Glo luciferase assay system (Promega). The activities of enhanced yellow fluorescent protein and luciferase were quantified with an FLX800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT).

**Cell Spreading Assay**—RKO cells transiently transfected with ING4 expression plasmids were plated on glass cover-slips in medium depleted of serum. Following overnight adherence at 37 °C, the starved cells were stimulated with 5% fetal bovine serum to observe the cellular response in membrane spreading. At designated time points, cells were fixed and stained on F-actin by rhodamine-phalloidin. Quantitative data of cell spreading, the percent of the cells that have filopodia and/or lamellipodia (%), were derived from the equation % S = (S/T) × 100%, where S is the number of cells containing filo/lamellipodia, and T is the total number of cells counted.

**Modified Boyden Chamber Migration Assay**—RKO cells transiently transfected with ING4 expression plasmids were subjected to a transmembrane cell migration assay containing 8 μM pore size polystyrene membranes coated with FluoroBlok materials in the migration chamber (BD Biosciences). By following the manufacturer’s protocol, the transverse cells were stained with calcein AM (Molecular Probes, Eugene, OR) in the lower chamber, and the magnitude of activated fluorescence was read by fluorescence spectrometry (Victor II; PerkinElmer Life Sciences).

**Immunoprecipitation**—Cells were transfected with different plasmid vectors that express FLAG-tagged or untagged ING4_v1, ING4_v2, or ING4_v4, and a plasmid that expresses FLAG-tagged G3BP2a that was a generous gift from Dr. Derek Kennedy (University of Queensland, Australia). 24 h after transfection, the cells were lysed in Nonidet P-40-based lysis buffer (0.1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, and protease inhibitor mixture; Calbiochem). Immunoprecipitation was performed using FLAG M2 resin (Sigma). Each precipitate was washed, and proteins were eluted in sample buffer. Immunoblotting was performed by standard protocols. FLAG-tagged ING4_v1, ING4_v2, ING4_v4, and G3BP2a were detected by rabbit anti-FLAG antibody (Sigma). Liprin α1 was detected by chicken anti-liprin α1 antibody (Genway, San Diego, CA). Untagged ING4_v1, ING4_v2, and ING4_v4 were detected by anti-ING4 antibody (Rockland, Gilbertsville, PA).

**RESULTS**

**Identification of ING4 Variants by EST Search**—We identified three novel ING4 variants, ING4_v2, ING4_v3, and ING4_v4 (Fig. 1A, DDBJ accession numbers are AB197695, AB197696, and AB197697, respectively) by EST search using the BLAST program (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/). The originally enrolled ING4 is ING4_v1 (DDBJ accession number AF156552). The expression composition of the variants enrolled as EST fragments (ING4_v1, 34%; ING4_v2, 49%; ING4_v3, 2%; and ING4_v4, 13%) was almost the same as the normal fibroblast cell line, MRC-5, and a cancer cell line, H82, by reverse transcription-PCR and subsequent TA cloning and sequencing (data not shown). ING4_v1 (original ING4) and ING4_v2 were expressed at a high level with a similar ratio (both variants were expressed around 40–50% among the four variants), whereas the ratio of ING4_v4 and ING4_v3 was ~10% and less than 10%, respectively. Because ING4_v4 was reported previously as a 12-bp common deletion mutation (6), we examined the genomic DNA sequence of the ING4 gene in H82 that was reported to have a mutation (6), and did not detect any mutations or polymorphisms (data not shown). These variations occurred only at the cDNA level, suggesting they were presumably splicing variants.

**Determination of Alternative Usage of Splice Donor and Acceptor Sites for the Production of the Splice Variants by Minigene Experiment**—To prove our assumption, we analyzed the ING4 gene sequence and identified two potential alternative splice donor sites, D1 and D2, at the end of exon 4 and two potential alternative splice acceptor sites, A1 and A2, at the start of exon 5 (Fig. 1A). The consensus sequences for the splice donor and acceptor sites are MAGGTRAGT and YYYYYYYYNCAGR, respectively. D1 (AAGGAAAA) matched 67% with the consensus sequence, whereas D2 (AAG-A-GTGGAG) matched 89% with the consensus sequence but includes an extra ‘A’ base between MAG and GTRAGT. A1 (CCTCTCTCACTAGA) matched 92% with the consensus sequence, whereas A2 (CTGCCCTAGAAGG) matched 85% with the consensus sequence. It was predicted that ING4_v1 might be a spliced product from D1 to A1, and ING4_v2, ING4_v3, and ING4_v4 might be the spliced products from D1 to A2, D2 to A1, and D2 to A2, respectively.

To prove usage of the potential splice sites, a “minigene” approach was utilized (Fig. 1B). The plasmid contains a partial ING4 gene from exon 3 to exon 6, including the introns, a TATA box, a transcriptional starting site, and a T7 promoter just before ING4 exon 3, and a poly(A) signal just after ING4 exon 6. The expected artificial pre-mRNA should undergo alternative RNA splicing with cellular splicing machinery. Along with the wild-type minigene, we also constructed five
mutant minigenes with a destroyed D1, D2, or A2 site or a combination of D1A2 and D2A2 site mutations to examine the usage of each splice site.

Each minigene was transfected into cells, and total RNA was extracted from the cells 24 h after transfection for cDNA preparation by reverse transcription. We amplified the artificial

FIGURE 1. Determination of splicing donor and acceptor sites. A, partial genomic DNA structure and cDNA sequence of the variable region of four ING4 splicing variants. Upper and lowercase letters show exon and intron sequence, respectively, based on ING4_v1. Two splice donor sites (D1 and D2) and two splice acceptor sites (A1 and A2) are shown. The blue squares show 9 bp between D1 and D2, and the yellow squares show 3 bp between A1 and A2. The variations are at the exon 4 and 5 boundaries, and the missed nucleotides are indicated by lines. ING4_v1 is enrolled as the original ING4. ING4_v2, ING4_v3, and ING4_v4 lack nucleotides 392–394, 383–391, and 383–394, respectively. EST hit numbers are as annotated on December 2004. B, ING4 minigene structure. Partial genomic DNA of ING4 (exon 3–6) was cloned downstream of a TATA box and a transcriptional starting site of pcDNA 3.1(–/H11001). Between the transcriptional starting site and exon 3, there is a T7 promoter. Each D1, D2, and A2 as well as D1A2 or D2A2 double mutations are shown. Red letters show the nucleotides that were changed from the original nucleotide to G to destroy the splice site. We performed nested PCR to separate each splicing variant. The location of primers for the PCR was indicated under the minigene construct. After nested PCR, we digested the fragment by Haell, and these are indicated above the minigene construct. C, results of the minigene expression. The nested PCR product was digested by Haell, separated by electrophoresis using 9% polyacrylamide gel, and stained by SYBR Gold. Haell digestion creates an 82-bp fragment specific for ING4_v1, a 167-bp fragment specific for ING4_v2, a 73 bp fragment for ING4_v3, and a 70-bp fragment for ING4_v4. Lanes 1–4, the Haell fragments from each variant-containing plasmid were used as positive controls. Lanes 5–10, the Haell fragments from each ING4 minigene. Lane 11, mixture of all positive controls. WT, wild type.
cDNAs from the minigenes using a forward primer at the T7 promoter (vector specific region) and a reverse primer at exon 5 of ING4 (Fig. 1B), and reamplified it by a nested PCR using a forward primer at exon 4 and the same reverse primer. Subsequent HaeIII digestion of the nested PCR products allowed us to distinguish each variant by electrophoresis. The HaeIII restriction enzyme that recognizes the GGCC sequence creates an 82-bp fragment specific for ING4_v1, a 167-bp fragment specific for ING4_v2, a 73-bp fragment for ING4_v3, and a 70-bp fragment for ING4_v4 (Fig. 1B). Fig. 1C shows the result of the minigene experiment. For positive controls, each variant-containing plasmid was used (Fig. 1C, lane 1–4). All four variants were made by alternative splicing from the wild-type minigene of ING4 (Fig. 1C, lane 5). As predicted, only ING4_v3 and ING4_v4 were generated from the D1 mutant (Fig. 1C, lane 6). ING4_v1 and ING4_v2 were generated from the A2 mutant (Fig. 1C, lane 7). ING4_v1 and ING4_v2 were generated from the D2 mutant (Fig. 1C, lane 8). Only ING4_v3 (Fig. 1C, lane 9) and ING4_v1 (lane 10) were generated from the D1A2 mutant and D2A2 mutant, respectively. This result clearly shows the alternative usage of two splice donor and acceptor sites. ING4_v4 (the 12-bp skip type) was attributed to a common deletion mutation at 379–390 (6), although we show here that the skip region of ING4_v4 is 383–394. The variation is the result from an AAAG sequence duplication of 379–382 and 391–394, and 391–394 is correct. Our data indicate that it is one of the three novel splice variants derived by RNA splicing from D2 to A2 (Fig. 1A).

**Expression Level of the ING4 Variants in Various Tissues**—Although it was reported that ING4 expression is ubiquitous in various tissues (2), the expression levels of each of the ING4 variants have not been examined. Therefore, we designed variant-specific TaqMan primer and probe sets along with a variant-nonspecific primer and probe set. First, a suitable annealing temperature for the specific amplification of each variant was determined (supplemental Fig. S1). Then the expression level of the total and each splice variant was examined. The variants were expressed ubiquitously among all tissues and cell lines we examined (supplemental Fig. S2). The expression pattern of all the variants was similar. We did not observe the expression of each variant in specific tissues or cell lines. Among the tissues that we examined, all the variants were expressed at the highest level in the brain and testis, and the next highest level in the pancreas. In the other tissues, all variants were expressed at lower levels. Among the cell lines that we examined, all the variants were expressed at the highest levels in a glioblastoma cell line, M059K, and embryonic kidney cell lines 293 and 293T. In the rest of the cell lines, all variants were expressed at lower levels.

**Subcellular Localization of the ING4 Variants**—So far, several putative motifs, a coiled-coil domain, two NLSs, an endoplasmic reticulum membrane retention signal, and a PHD finger motif were predicted by computer searches (supplemen-
Attenuated Growth Suppression of ING4 Variants—The overexpression of ING4 variants was conducted using two p53 wild-type cancer cell lines, U-2 OS and RKO, and a p53-mutated cell line, U-118 MG. We confirmed equal expression levels of all of the splice variants by Western blotting (data not shown). Cell growth was suppressed by ING4_v1 effectively in all cell lines we examined, but the variants, ING4_v2 and ING4_v4, that have a partial NLS were less effective (Fig. 3, A and B). We did not observe a significant p53 dependence. The ING4 variant effects on the p21<sub>WAF1</sub> promoter were further examined by a reporter assay. Fig. 4A shows that ING4_v1 overexpression could activate the p21<sub>WAF1</sub> promoter (1.5-fold when compared with the vector control), but both ING4_v2 and ING4_v4 were weaker inducers (1.2- and 0.8-fold when compared with the vector control, respectively). Equal expression levels of all of the splice variants were confirmed by Western blotting (data not shown). In addition, we found ING4_v4 could block ING4_v1-mediated p21<sub>WAF1</sub> induction (Fig. 4B), suggesting a dominant-negative effect of ING4_v4 on ING4_v1.

ING4 Splice Variants Lose the Capacity to Suppress Cell Spreading and Migration—Because we recently found that ING4_v1 interacts with several cytoplasmic proteins that might associate with cell migration and spreading under some conditions, we examined the activity of the two major splice variants (ING4_v2 and ING4_v4) in cell spreading and cell migration assays in comparison with ING4_v1. We found that in the cell spreading assay, both ING4 variants (ING4_v2 and ING4_v4) lost the capacity to suppress actin filament polymerization and the consequent membrane spreading (Fig. 5, A–C). In the modified Boyden chamber assay, we found that ING4_v4 completely lost the capacity to suppress cell migration, whereas ING4_v2 retained the capacity (Fig. 6A). These results regarding cell migration were further confirmed by the scratch assay (supplemental Fig. S4). We confirmed equal expression levels of all of the splice variants by Western blotting (data not shown). In light of the loss-of-function characteristic of ING4_v4, which suppressed neither cell spreading nor cell migration, we performed a competition assay between ING4_v1 and ING4_v4. ING4_v4 inhibited the filopodia/lamellipodia formation by ING4_v1 (Fig. 5D) and also completely abrogated the migration suppression activity conducted by ING4_v1 (Fig. 6B), suggesting a dominant-negative regulation of ING4_v4 on ING4_v1.

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**DISCUSSION**

Alternative RNA splicing plays a major role in modulating gene function by expanding the diversity of expressed mRNA transcripts (18–21). Alternative splicing determines cell fate in numerous contexts, such as sexual differentiation in *Drosophila* and apoptosis in mammals, and aberrant regulation of alternative splicing has been implicated in human diseases (22–24). Alternative RNA splicing is controlled by multiple splicing factors whose expression and status are tightly regulated in animal development, cell cycle, and cell differentiation. Although intron removal from a pre-mRNA by RNA splicing was initially thought to be controlled by intron splicing signals (25), exon sequences have been found recently to also regulate RNA splicing, polyadenylation, export, and nonsense-mediated RNA decay in addition to their coding function (26). The regulation of alternative RNA splicing by exon sequences is largely attributed to the presence of two major cis-acting elements in the regulated exons, the exonic splicing enhancer (ESE) and suppressor or silencer (26).

In this study, in addition to the original *ING4* (*ING4_v1*) (1), we describe three novel splice variants of ING4, *ING4_v2*, *v3*, and *v4*, that are produced from two alternative splice donor sites at the end of exon 4 and two alternative acceptor sites at the start of exon 5. Although *ING4_v4* was reported to be a common deletion-type mutation (6), we proved that the deletion was one of the four splice variants of *ING4* by the minigene analysis. Interestingly, although *ING5* has a similar sequence with *ING4*, no ESTs corresponding to *ING4* splice variants have been enrolled, probably because small sequence differences between *ING4* and *ING5* in the region of exon 4 and exon 5 make the *ING5* missing the splicing signals seen in the *ING4* (supplemental Fig. S5A). These ING4 variants were expressed ubiquitously in all tissues and cell lines examined. All variants were expressed at high levels in the same tissues and cell lines, suggesting that the four splice variants are generated from the same pre-mRNA at the same ratio by cellular splicing machinery. In addition, several stimulations failed to change the composition of the variants. Serum starvation induced expression levels of all splice variants, on the other hand, serum stimulation suppressed expression levels of all splice variants in WI-38 and MRC5 normal fibroblast cells (supplemental Fig. S6A). There was no significant difference between the variants, although this observation might be very important for understanding the mechanism of cell cycle regulation by ING4. Treatment of several cell lines with DNA-damaging reagents, adriamycin and etoposide, also failed to differentially induce type-specific expression of the variants, although both adriamycin and etoposide induced expression levels of all splice variants examined in a particular cell line, U-118 MG cells, at a similar ratio (supplemental Fig. S6B). Considering the presence of putative ESE-like motifs (26) in exons 4 and 5 of the *ING4* gene, we overexpressed SF2/ASF, one of the ESE-binding proteins (27–29), in cells to examine whether the protein might induce type-specific expression of the variants, and we did not observe the specific induction of any variants (data not shown). Although the exact mech-
Functional Analysis of Novel Splice Variants of ING4

Because of the relatively low expression level of ING4_v3, we focused on the two major novel splice variants, ING4_v2 and v4, for further functional analysis. We tried to analyze endogenous ING4 splice variants by making small interfering RNA but failed, because of cross-reactions between the variants. Separation of each endogenous ING4 variant by Western blotting also failed, because of the small difference of molecular weight between the splice variants. Therefore, we applied an overexpression approach to seek the functions of the splice variants. The two variants, ING4_v2 and ING4_v4, that have a partially deleted NLS were primarily distributed in the cytoplasm, whereas ING4_v1 with an intact NLS was displayed primarily in the nucleus, suggesting that the novel ING4 variants might function primarily in the cytoplasm and lose their function in the nucleus. We performed a colony formation assay and found that the variants with a partial NLS exhibited an attenuated growth suppressive effect when compared with ING4_v1, which was associated with their ability to induce p21\(^WAF1\) promoter activity. ING4 interacts with p53 in modulating cell cycle arrest and apoptosis (1, 4), but we did not observe a p53 dependence in the colony formation assay. We also observed that ING4_v1 modestly activated p21\(^WAF1\) promoter activity. There are several reports regarding a p53-independent mechanism of p21\(^WAF1\) activation (30, 31); a variety of transcriptional factors, including STATs, E2Fs, AP2, C/EBP\(\alpha\), C/EBP\(\beta\), and GAX, and other tumor suppressors, including BRCA1, transforming growth factor-\(\beta\), and Wnt-1, regulate p21\(^WAF1\) transcription. ING4 could activate one of these p53-independent pathways as well as the p53-dependent pathway under some conditions. It was reported that ING4_v1 acetylates histone H4 (9), and its PHD finger motif interacts with methylated histone H3 (10). In addition, several binding partners of ING4_v1 in nucleus have been determined (9).

Before, the nuclear ING4_v1 may have multiple functions.

Recently, we found that ING4_v1 interacts with several cytoplasmic proteins, including Liprin\(\alpha\) and G3BP2 by protein pulldown and subsequent mass spectrometry analysis, suggesting important roles of cytoplasmic ING4_v1 similar to ING2 that have been reported to change subcellular localization dynamically between the cytoplasm and the nucleus under some conditions through its PHD finger motif that has a high identity with that of ING4 (32). Liprin\(\alpha\) is a cytoplasmic protein necessary for focal adhesion and intracellular vesicle transport (13, 14). G3BP2 has been implicated in Ras signaling, NF-\(\kappa\)B signaling, and the ubiquitin proteosome pathway (15–17). In addition, G3BP2 is overexpressed in human breast cancer tissue (33). Because we are interested in the function of Liprin\(\alpha\) in focal adhesion and the association between the Ras signaling pathway and G3BP2, we are investigating the functions of ING4_v1 in cell migration and cell spreading. Because
G3BP2 retains IκBα/NF-κB complexes in the cytoplasm, ING4_v1 might also be retained in the cytoplasm by G3BP2 that is overexpressed in cancer (33).

We observed dominant-negative effects of ING4_v4 on ING4_v1 in p21^{WAF1} promoter activation, cell spreading, and cell migration. Because ING4_v1 and ING4_v4 neither affect their localization when they overexpressed in same cells (supplemental Fig. S7; ING4_v1 localizes in the nucleus, whereas ING4_v4 localizes in the cytoplasm) nor make heterodimers or homodimers (data not shown), it seems that the mechanisms of the dominant-negative effects are not a physical interaction between these two variants. Cytoplasmic ING4_v4 may anchor partner nuclear proteins (9) of ING4_v1 in the cytoplasm.

ING4 has also been shown to inhibit angiogenesis through an interaction with NF-κB (2) and to regulate HIF through an interaction with HPH-2/PHD2, a family member of the prolyl hydroxylases (7). NF-κB shuttles between the cytoplasm and nucleus dynamically (34), and HPH-2/PHD2 is expressed both in the cytoplasm and the nucleus (7). Our report suggests that there are many chances for spatial interaction between ING4 and their partners both in the cytoplasm and nucleus. The balance among the four splice variants of ING4 and their subcellular localization may modulate the recently reported functions in controlling the cell cycle checkpoint, cell contact inhibition, and angiogenesis.

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