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A novel ING2 isoform, ING2b, synergizes with ING2a to prevent cell cycle arrest and apoptosis ☆

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Abstract

We identified a novel inhibitor of growth family member 2 (ING2) isoform, ING2b, which shares exon 2 with ING2a, but lacks the N-terminal p53 binding region. Contrary to ING2a, ING2b's promoter has no p53 binding sites. Consistently, activation of p53 led to suppression of ING2a, leaving ING2b unaffected. Through isoform-specific targeting, we showed that ING2a knockdown suppressed cell growth only in the presence of p53, ING2b knockdown had no effect on cell growth, and knockdown of both induced cell cycle arrest and apoptosis independently of p53. ING2a and ING2b have compensatory roles that protect cells from cell cycle arrest and apoptosis and may be involved in development of chemotherapeutic resistance.

Keywords

ING2; ING2a; ING2b; p53; Isoform

1. Introduction

ING2 shares the highest amino acid homology with ING1 among the inhibitor of growth (ING) protein family members [1]. So far, five isoforms of *ING1* that have different abilities on apoptosis and cell proliferation have been reported [2]. However no isoforms of ING2 have been reported despite the similarity observed between ING1 and ING2.

ING2 binds to H3K4me3 with mSin3A-HDAC1 complex [3–5]. Wang et al. [6] showed that a leucine zipper-like motif at the N-terminal end of ING2 is critical for its association with p53 and modulation of p53-mediated chromatin remodeling. Recently, Kumamoto et al. [7] showed that the *ING2* promoter has two p53 binding sites through which p53 down-regulates expression of ING2. Moreover, suppression of ING2 induced p53 dependent senescence in

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normal human fibroblasts. Thus ING2 seems to have crucial roles in transcriptional regulation of various genes.

Here, we report a novel ING2 isoform, ING2b. We found different transcriptional regulation of *ING2a* and *ING2b* by p53, and feedback between p53 and ING2a, but not ING2b. Using siRNA that targeted ING2a or ING2b specifically, or both, we found that ING2a and ING2b have compensatory roles that protect cells from cell cycle arrest or apoptosis. Because concurrent knockdown of ING2a and ING2b induced cell cycle arrest or apoptosis in cancer cells regardless of p53 status, our results may encourage the development of new therapeutic approaches directed to overcome clinical resistance to chemotherapy.

2. Materials and methods

2.1. Tissues and cell lines

cDNA from various tissues (Human Multiple Tissue cDNA Panel 1 and 2) were obtained from BD Bioscience (Palo Alto, CA). All cell lines were obtained from ATCC (Manassas, VA) except for the hTERT-immortalized cell line, NHF-hTERT, and isogenic cell line pairs of lung adenocarcinoma A549. NHF-hTERT was derived by infecting the primary cell strain GM07532 (Coriell Cell Repositories) with a telomerase-expressing retrovirus [7]. Isogenic cell line pairs of wild type (wt) p53-expressing A549 cells featuring a short-hairpin shRNA sequence against human p53 (A549 p53KD) or a scrambled (A549 SC) sequence were established in our laboratory [8] and cultured in media containing 1 μg/μl puromycin (Sigma, St Louis, MO).

2.2. Determination of the transcriptional starting site of ING2b

We determined the transcriptional starting site of *ING2b* by 5′ RLM-RACE (FirstChoice® RACE-Ready cDNA: human testis and placenta, Ambion, Austin, TX). We used adapter-specific outer and inner primers, an *ING2b*-specific outer and inner primers (5′-GGAAAGAGGTTGGAAACATCA-3′ and 5′-TGCAAACCCCGATTCGGACT-3′, respectively). We performed the first and second PCR according to manufacturer's protocol. The second PCR product was separated in 2% agarose gel. A unique, distinct PCR band was extracted, and its sequence was determined.

2.3. Plasmids and reagents

ING2b cDNA was amplified by PCR using KOD-Plus polymerase (Novagen, Madison, WI) and cloned into pFLAG-CMV-6c (Sigma). Lipofectamine Reagent (Invitrogen) was used for all plasmid transfections, according to manufacturer's instructions. The following antibodies were used for Western blotting or immunofluorescence: anti-FLAG monoclonal M2 (Sigma); anti-p53 monoclonal antibody DO-1 (Santa Cruz); anti-β-actin mouse monoclonal AC-15 (Sigma).

2.4. Indirect immunofluorescence assay

Cells were cultured on coverslips in 6-well plates and transfected with plasmid constructs. After 24 h, FLAG-tagged proteins were visualized as previously described [9] using Alexa Fluor 488 phalloidin probe for detecting F-actin (Molecular probes, Invitrogen) and anti-FLAG M2 antibody.

2.5. Detection of endogenous ING2b by TaqMan-PCR

For specific detection of *ING2a* and *ING2b*, *ING2a* and *ING2b*-specific forward primers (5′-GACATGCAGAGGAACGTGTCT-3′ and 5′-TGGATCAGGACGGCGATCAG-3′, respectively), *ING2a* and *ING2b* common reverse primer (5′-

GAGAAGCTGCTGTAGACGTTTC-3′, and *ING2a* and *ING2b* common probe (5′-FAM-CTTATATTTTTCGTAGACATCATCAATTTCCTTTAACGTT-TAMRA-3′) were designed. The PCR products were followed real-time using ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) for 40 cycles. β2-*microglobulin* was used for normalization (4310886E, Applied Biosystems).

2.6. Nutlin-3a and adriamycin treatment

Nutlin-3a was a generous gift from Dr. Lyubomir Vassilev (Hoffmann-La Roche Inc., Nutley, NJ). Nutlin-3a was dissolved in DMSO and added at 10 μM. For control, cells were treated with DMSO alone. Adriamycin (Sigma) was used at 1 μg/ml. Cells were harvested after exposure to the drugs for 24 h and total RNA was isolated and reverse-transcribed by a general method using Trizol Reagent (Invitrogen) and SuperScriptIII First-Strand Synthesis System (Invitrogen).

2.7. siRNA interference

We designed siRNAs that can selectively knock down *ING2a* or *ING2b*. We also designed one siRNA sequence that targets both *ING2a* and *ING2b* (*ING2* common siRNA). The targeting sequences of these siRNAs are as follows: *ING2a*-specific siRNA targeting sequence; 5′-CAACAAAUAUCAAGAAACGUU-3′, *ING2b*-specific siRNA targeting sequence; 5′-GGUGAUGUUUCCAACCUCUUU-3′, and *ING2* common siRNA targeting sequence; 5′-ACAGUGUUUCCAAGAUCCTGCUGAA-3′. The non-specific siRNA sequence used was 5′-AAUUCUCCGAACGUGUCACGU-3′ (Qiagen, Valencia, CA). These siRNAs were transfected at a final concentration of 20 nM using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. The siRNA transfection efficiency under these conditions was 100%, as evaluated by a fluorescent siRNA (data not shown).

2.8. Growth assay

Cells were transfected with siRNAs twice, on the first and fourth days. Cell numbers were counted using Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD) on the sixth day.

2.9. Flow cytometry and TUNEL assay

Cells were transfected with siRNAs twice, on the first and fourth days. After 6 days, flow cytometry and TUNEL assay was performed as previously described [10].

3. Results

3.1. Identification of a novel isoform of ING2, ING2b

We identified a novel *ING2* isoform, *ING2b* (GenBank Accession No: AB196793), by EST search using the BLAST program (NCBI). We renamed the original *ING2*, as *ING2a* in this report (DDBJ accession number: AF053537). The transcriptional starting site of *ING2b* was determined by 5′-RLM-RACE in the intron 1 of *ING2a* using full length cDNAs from testis and placenta. The predicted molecular weights of ING2a and ING2b are 33 kDa and 28 kDa, respectively. In contrast to the *ING2a* promoter that has two p53 binding sites through which p53 suppresses its expression [7], we did not find any apparent p53 binding sites on the *ING2b* promoter. Instead, a HSF1 and HSF2 binding site, a C-Rel binding site, a SP1 binding site, five MZF1 binding sites, and a p300 binding site were predicted in the promoter region by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Among these binding sites, the HSF1 and HSF2 binding site and the C-Rel binding site are unique in the *ING2b* promoter (Fig. 1A). *ING2a* and *ING2b* share exon 2 (Fig. 1A), but differ in their N-terminal regions, which are encoded by exons 1a and 1b, respectively (Fig. 1B). Exon 1b of *ING2b*

encodes 18 unique amino acids and exon 1a of *ING2a* encodes 58 unique amino acids (Fig. 1B). No motif was predicted in the unique 18 amino acids of ING2b. The N-terminal region of ING2b does not show any homology with the N-terminal region of ING1 isoforms. ING2a has a leucine zipper domain and an intact coiled-coil domain in its unique N-terminal region. These domains are absent in ING2b. Mouse *Ing2b* sequence was also identified (Supplemental Fig. S1, DDBJ Accession No: AB433625). Mouse *Ing2b* is transcribed from intron 1 of *Ing2a* and encodes a truncated 20.3 kDa protein that does not have a unique N-terminal sequence, leucine zipper domain, or coiled-coil domain.

3.2. Subcellular localization of ING2b

After we checked expression of FLAG-tagged ING2a and FLAG-tagged ING2b from the plasmid vectors by Western blotting (Fig. 1C), subcellular localization of the two isoforms are examined by immunofluorescence. The majority of ING2b localized at the nucleus, similarly to ING2a (Fig. 1D). Both ING2a and ING2b possess two nucleolar-targeting sequences, a RRQR motif and a KKKK motif in their first and third NLS, respectively [11], and localized at nucleoli (Fig. 1B).

3.3. Expression level of ING2b in various tissues and cell lines

We examined expression level of ING2a and ING2b in normal tissues and various cell lines by qRT-PCR, using two unique forward primers on exon 1a or 1b, and a common reverse primer and a probe on exon 2. Efficiency of amplification using the same number of plasmid molecules as templates was almost the same with either primer set (Supplemental Fig. S2). Both genes were expressed ubiquitously in all tissues and cell lines examined, although *ING2a* was expressed predominantly (Fig. 2). We did not see any correlation between p53 status and basal expression level of either *ING2a* or *ING2b*.

3.4. p53 Dependent suppression of ING2a, but not ING2b

We examined how p53 activation might differentially regulate *ING2a* and *ING2b*. We induced endogenous p53 stabilization through nutlin-3a, an inhibitor of MDM2, or adriamycin, a DNA intercalating drug used for cancer chemotherapy. A pair of isogenic A549 cells with different p53 status was exposed to these agents. The A549 transfectants bearing scramble shRNA, A549 SC, served as a control for normal level of p53 expression. The A549 transfectants bearing p53 shRNA, A549 p53KD, have very low expression of p53, and p53 is not efficiently stabilized upon exposure to nutlin-3a or adriamycin (Supplemental Fig. S3A and B). We also treated four other p53 wild type cell lines and three p53-mutant cell lines with nutlin-3a. *ING2a*, but not *ING2b*, was down-regulated upon exposure to nutlin-3a or adriamycin (Fig. 3A and B) only in cells bearing wt p53, indicating that *ING2b* is not a direct transcriptional target of p53. As expected, neither gene was down-regulated by the treatment when wt p53 was absent (A549 p53KD), or in p53-mutant cell lines. We further examined the expression level of *ING2a* and *ING2b* in young and senescent MRC5 fibroblasts. During senescence p53 becomes post-translationally modified by acetylation (Supplemental Fig. S3C) and is activated. We found that *ING2a* was down-regulated in MRC5 fibroblasts undergoing senescence, but *ING2b* was not changed (Fig. 3C).

3.5. Effect of ING2a and ING2b knockdown on cell growth

Selectivity of *ING2a*-specific and *ING2b*-specific siRNAs was evaluated by qRT-PCR using a panel of cancer cell lines (Fig. 4A and B). Each siRNA led to reduced expression of the cognate sequence, while leaving expression of the other mRNA largely unaffected. A siRNA designed to target a shared exon 2 sequence (*ING2* common siRNA) suppressed expression of both *ING2a* and *ING2b*. We then examined the effect of such siRNAs on cell growth, cell cycle and apoptosis of a panel of cancer cell lines with different p53 status. We found that *ING2a*

siRNA required the presence of wild type p53 for effective growth suppression, while *ING2b* siRNA did not show any growth suppressive activity (Fig. 5A). This growth suppressive effect was not mediated by stabilization of p53, since the total amount of p53 protein was not changed by ING2a knockdown (Fig. 5B). *ING2* common siRNA suppressed cell growth both in the p53 wild type cells and in the p53-mutant cells. Thus, down-regulation of ING2 may circumvent the resistance of p53-mutant cells to cell death. Under the microscope, we observed a large percentage of dead cells upon treatment with *ING2* common siRNA in U-2 OS and U-118 MG cells, while M059K and A549 cells simply stopped dividing. Such phenotypes became apparent after the second consecutive siRNA transfection. These observations were confirmed by TUNEL assay (Supplemental Fig. S4). There was no relationship observed between these phenotypes and p53 status. We also examined the cell cycle in these cells after 6 days of transfection (Supplemental Fig. S5). Although the result was not very clear, we observed a tendency of the ING2 common siRNA to increase the subG1 population compared with the ING2a siRNA in all the cell lines that we examined. However, the subG1 population was increased more than 20% only in U-2 OS and U-118 MG cells by the ING2 common siRNA transfection, concordant with the result of the TUNEL assay. We removed dead cells that had died during the first few days by changing cell culture media, thus we measured the cell cycle only in the surviving cells on the 6th day. We think that this is the reason why the cell cycle of ING2a siRNA transfected cells and that of ING2b siRNA transfected cells were not so different.

4. Discussion

Recently, we identified two p53 binding sites in the promoter region of *ING2* (*ING2a* in this article) [7]. We found that p53 down-regulates *ING2a* expression through direct binding to these sites when senescence is induced by mutlin-3a in normal human fibroblasts. In the present study, we identified a novel ING2 isoform, ING2b. The *ING2b* promoter does not possess p53 binding sites, suggesting differential regulation of the two isoforms. When p53 was induced by nutlin-3a or adriamycin treatment, expression of *ING2a* was significantly down-regulated in the presence of p53, while expression of *ING2b* was not affected, suggesting that only *ING2a* is a p53 transcriptional target. We further found that *ING2a*, but not *ING2b*, was down-regulated in normal human fibroblasts during replicative senescence. Activation of p53 is one of the hallmarks of replicative senescence, and the differential regulation of *ING2a* and *ING2b* may be important in determining cell fate.

We utilized siRNAs designed to selectively target ING2a or ING2b or both to address their specific functions. The ING2a siRNA suppressed cell growth only in the presence of wild type p53, on the other hand, the *ING2b* siRNA did not have any effect on cell growth. The *ING2* common siRNA suppressed cell growth more effectively than *ING2a* siRNA and independently of p53 status, suggesting that both ING2a and ING2b suppress cell cycle arrest or apoptosis and compensate for each other's function. We propose a negative feedback loop model to explain these findings (Supplemental Fig. 6). We think that even though p53 is activated by *ING2a* knockdown, the presence of ING2b can still prevent cells from apoptosis and cell cycle arrest, and only moderate suppression of cell growth is induced under *ING2a* knockdown in a p53 wild type background. A molecular mechanism underlying how ING2a knockdown activates p53 is still unknown. It has been recently reported that ING2a binds to p53 through its N-terminal unique region [6]. The significance of this binding is still unclear. ING2a may hasten p53 degradation, or abrogate p53 activity through binding or modification. The amount of p53 was not changed by ING2a knockdown in our present result, suggesting that ING2a does not contribute to p53 degradation. Therefore, we think that the ING2a binding itself may inhibit p53 activity, or modify p53 and convert it into an inactive form. If this is the case, ING2a knockdown can enhance p53 activity, and impede cell growth. In p53-mutant cells, cell survival is equally dependent on ING2a and ING2b function, and each may

compensate for the other's absence. In this case, abrogation of both genes is necessary for induction of apoptosis or cell cycle arrest, through a p53-independent mechanism. This data is encouraging for the development of therapeutic approaches directed to diminish chemotherapeutic resistance in the clinic. Although further analysis will be required, we found that the growth suppressive effect of the combination of adriamycin and *ING2* siRNA is larger than that of the adriamycin or *ING2* siRNA transfection alone (Supplemental Fig. S7), supporting a possibility of *ING2* siRNA as a new generation of cancer therapeutic agent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.febslet.2008.10.024.

Abbreviation

ING, the inhibitor of growth.

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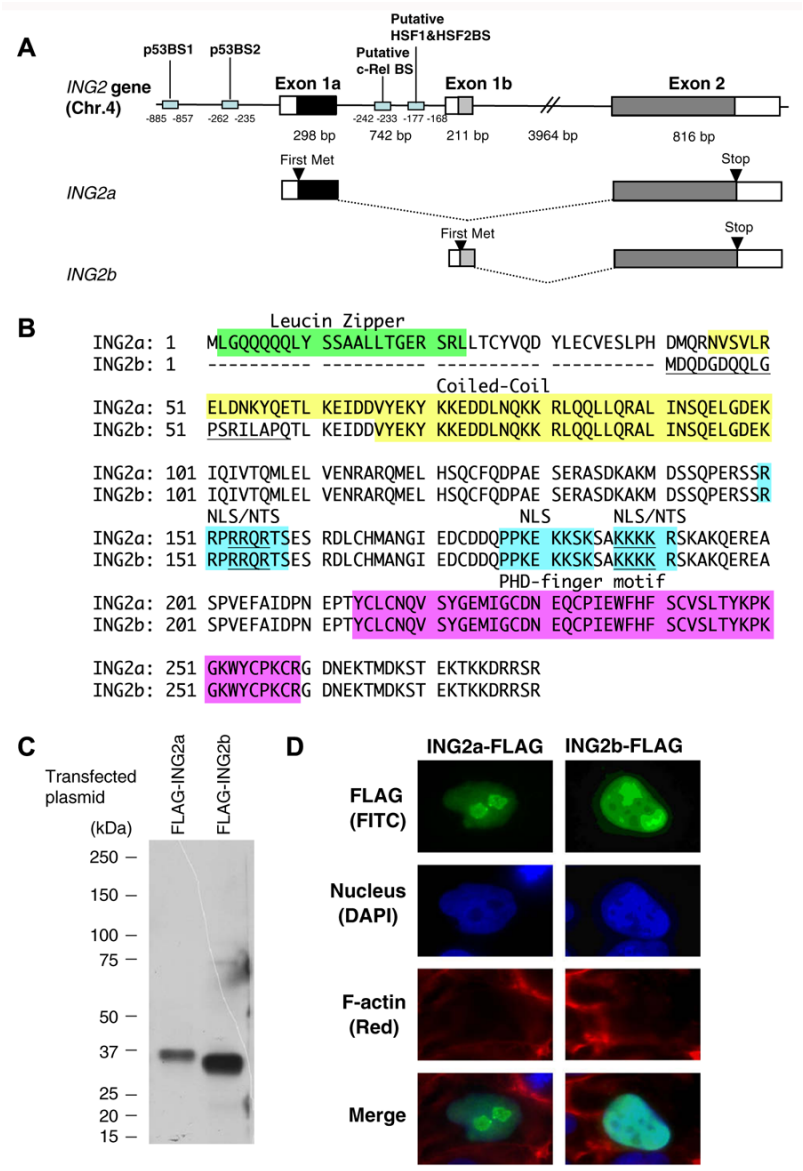

Fig. 1.

Gene structure, amino acid sequence, and subcellular localization of ING2a and ING2b. (A) Genomic and mRNA structure of *ING2a* and *ING2b*. *ING2a* promoter possesses two p53 binding sites. In the *ING2b* promoter region, a HSF1 and HSF2 binding site and a c-Rel binding site were uniquely predicted. (B) Amino acid sequence and domains of ING2a and ING2b. (C) Expression of ING2-FLAG and ING2b-FLAG from the vectors were detected by Western blotting using anti-FLAG antibody. (D) Subcellular localization of exogenous ING2a-FLAG and ING2b-FLAG visualized by anti-FLAG M2 antibody. Alexa Fluor 488 phalloidin was used for detecting F-actin, and DAPI for nucleus.

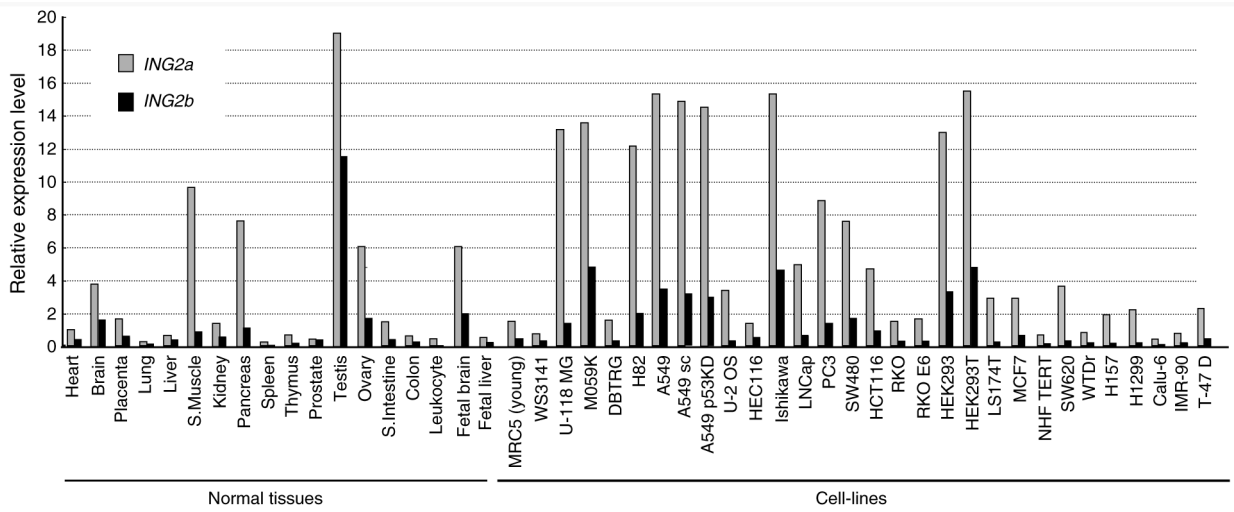

Fig. 2.

Expression levels of *ING2a* and *ING2b* in various tissues and cell lines detected by TaqMan-PCR. The amplification efficiency of ING2a and ING2b primer sets was the same between *ING2a* and *ING2b* (see Supplemental Fig. S1). β2-*microglobulin* was used for normalization.

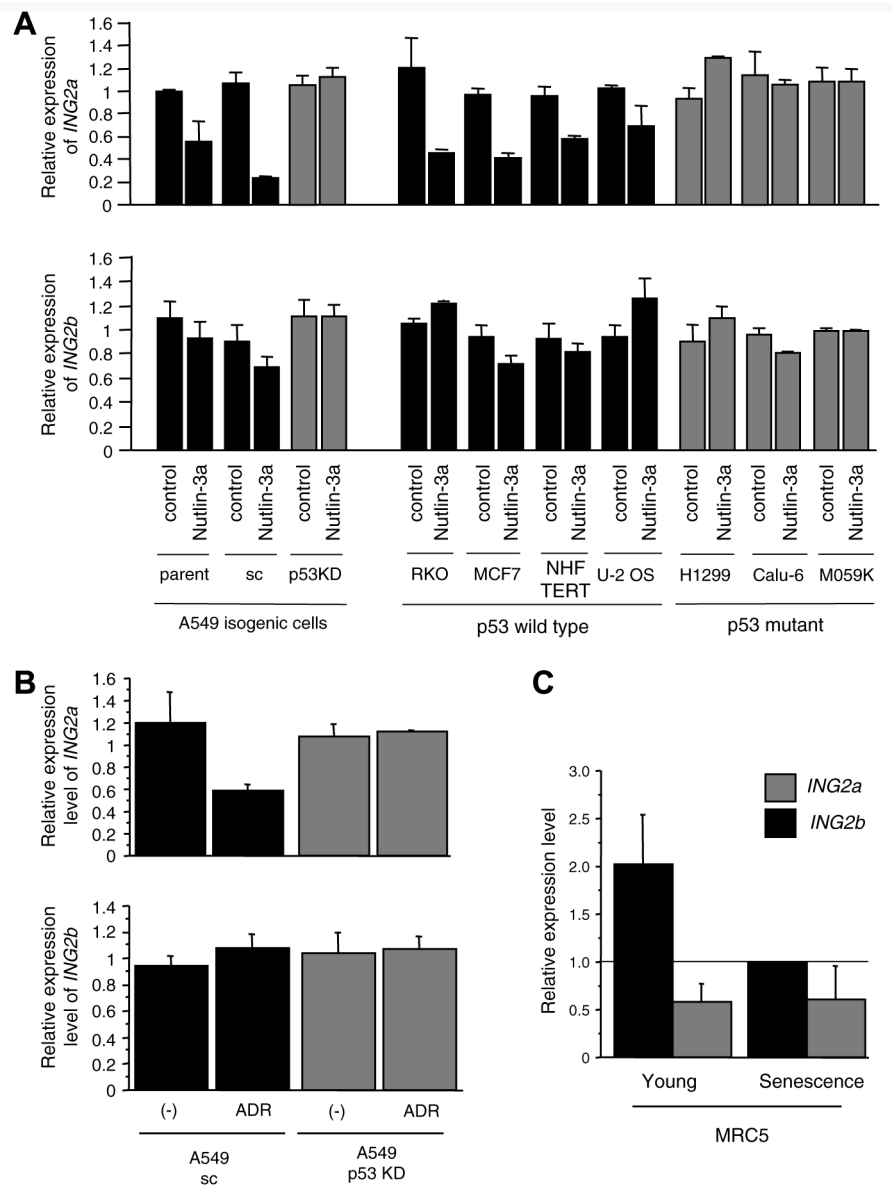

Fig. 3.

p53 dependent down-regulation of ING2a, but not ING2b. Expression level of *ING2a* and *ING2b* was majored by TaqMan-PCR. β2-*microglobulin* was used for normalization. Each experiment was repeated three times. (A) Cells were treated with 10 μM nutlin-3a for 24 h. Solvent, DMSO, was used as a control. (B) Cells were treated with 1 μg/ml adriamycin (ADR) for 24 h. (C) Expression of *ING2a* in senescent MRC5 human fibroblasts.

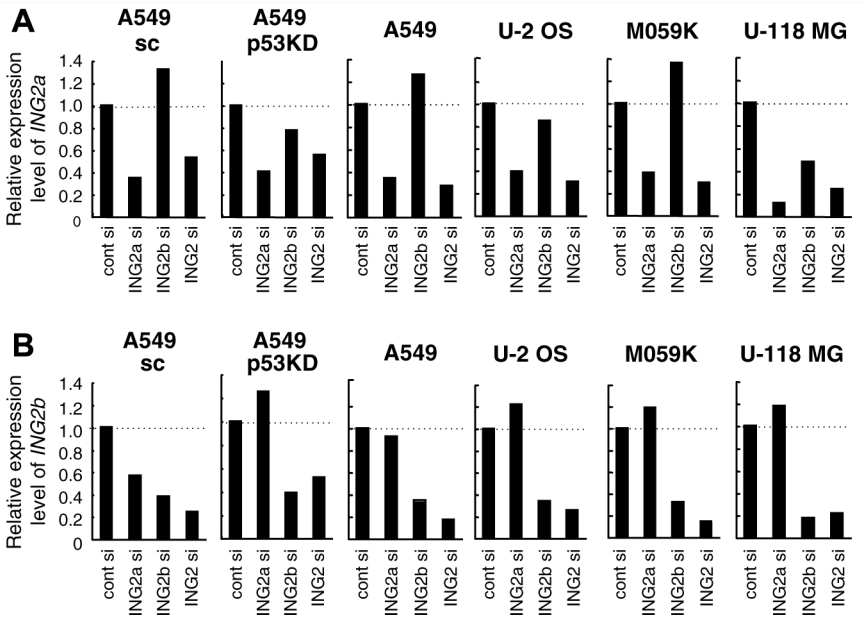

Fig. 4.

Specific knockdown of *ING2a* and *ING2b* by siRNAs. Cells were transfected with control siRNA, *ING2a* siRNA (ING2a si), *ING2b* siRNA (ING2b si), and ING2 common siRNA (ING2 si) on the first and fourth day, and harvested on the sixth day. Expression level of *ING2a* and *ING2b* was examined by TaqMan-PCR. β2-*microglobulin* RNA was used for normalization. (A) Expression level of *ING2a* in A549 isogenic cells with different p53 status and four other cell lines. (B) Expression level of *ING2b* in the six cell lines.

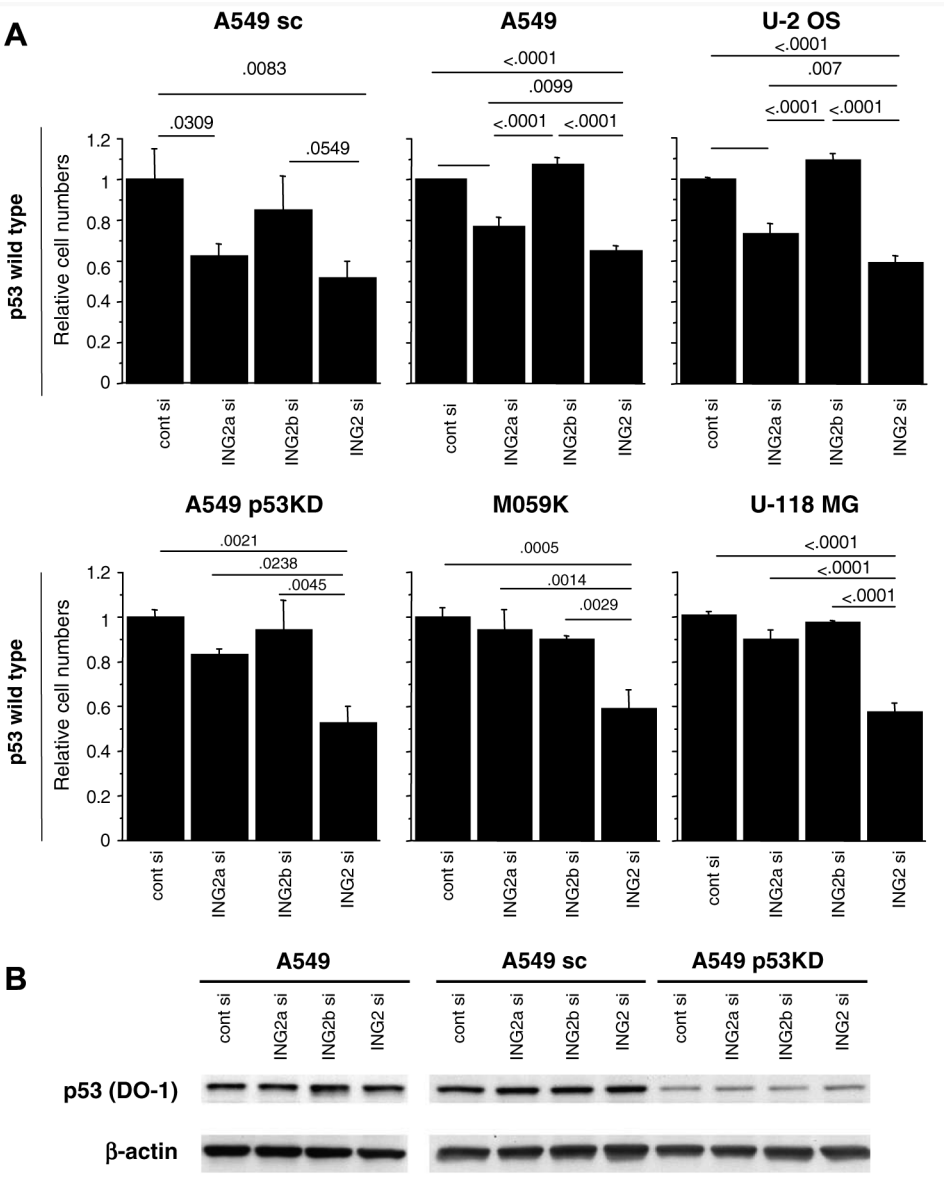

Fig. 5.

Effect of knockdown of ING2a, ING2b, or both on cell growth. (A) Cells were treated with the same condition shown in Fig. 4. Cell growth was measured by cell counting kit-8 on the sixth day after the first transfection. Relative cell numbers were calculated compared with the average of cells treated with control siRNA as 1.0. Data are shown as the average with the SD from three independent experiments. Statistical analysis was carried out by Scheffé's *F*-test (asterisk shows $P < 0.01$). (B) Expression of p53 was examined by Western blotting in A549 parental and isogenic cells using anti-p53 antibody (DO-1) on the sixth day. β-actin was used for loading control.