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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.**
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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 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).

☆ The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with Accession Nos. AB196793 (human ING2b) and AB433625 (mouse ING2b).

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Abbreviation: ING, the inhibitor of growth

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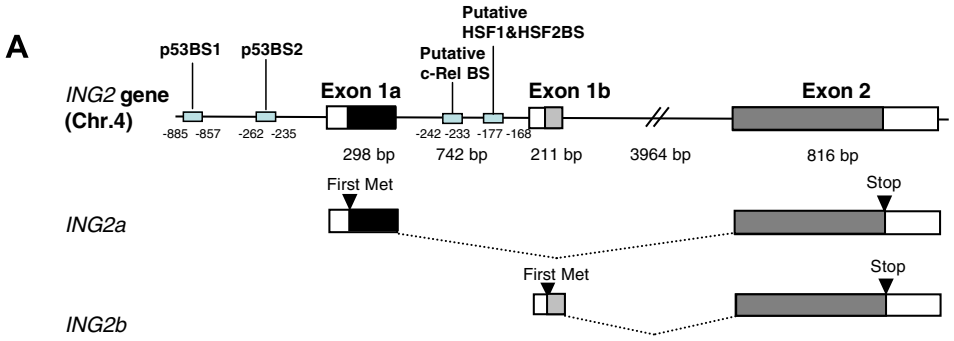
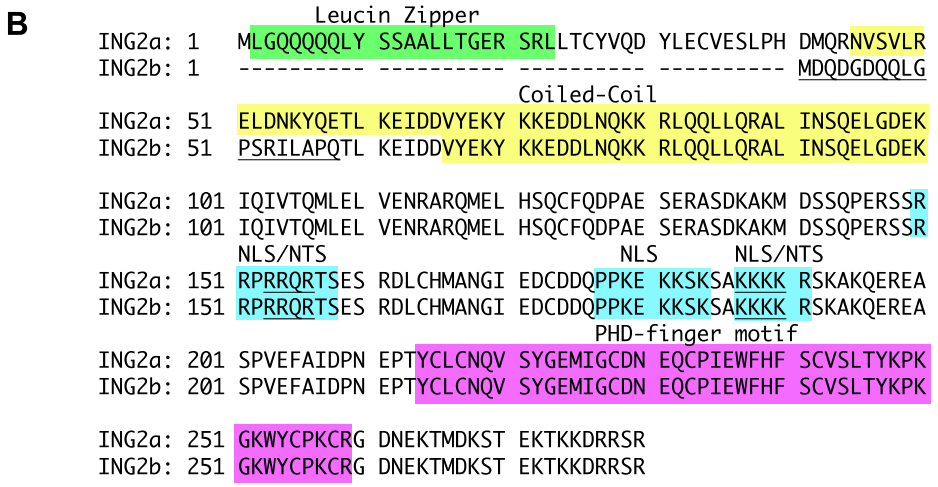
2.4. Indirect immunofluorescence assay

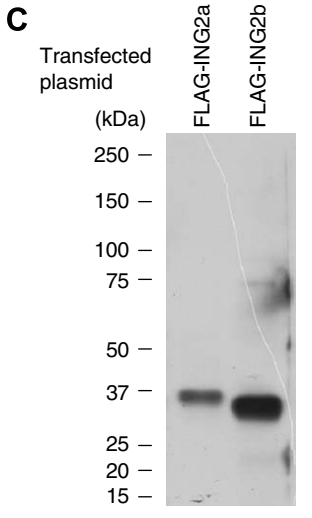
Cells were cultured on coverslips in 6-well plates and transfected with plasmid constructs. After 24 h, FLAG-tagged proteins were visualized as previously described [9] using Alexa Fluor 488 phalloidin probe for detecting F-actin (Molecular probes, Invitrogen) and anti-FLAG M2 antibody.

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′-GAGAAGCTGCTGTAGACG-TTTC-3′), and *ING2a* and *ING2b* common probe (5′-FAM-CTTA-







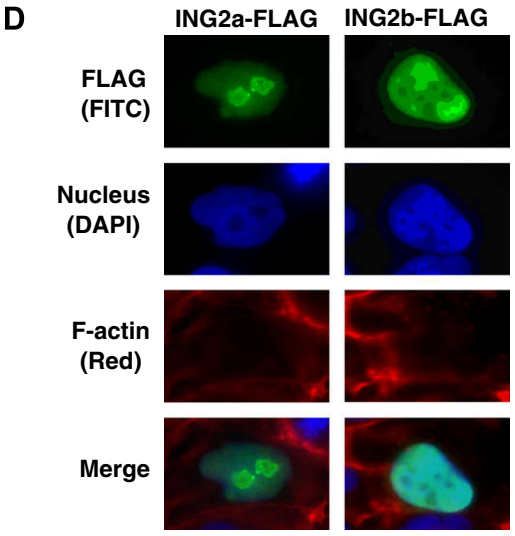


Fig. 1. Gene structure, amino acid sequence, and subcellular localization of ING2a and ING2b. (A) Genomic and mRNA structure of *ING2a* and *ING2b*. *ING2a* promoter possesses two p53 binding sites. In the *ING2b* promoter region, a HSF1 and HSF2 binding site and a c-Rel binding site were uniquely predicted. (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.