

Review

Inhibitor of Growth (ING) Family: An Emerging Molecular Target for Cancer Therapy

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Abstract: ING1 gene, the founding member of the ING tumor suppressor family, was originally identified through subtractive hybridization between normal mammary epithelial cells and breast cancer cell lines, and subsequent *in vivo* selection of genetic suppressor element that displayed oncogenic features. Soon after identification of ING1, four additional members of the ING family (ING2-5) were cloned and all the gene products contain a highly conserved plant homeodomain (PHD) finger motif in the carboxy (C)-terminal end, that plays important role for their function. Furthermore, ING family members contain nuclear localization signals and N-terminal sequences important in the interaction with histone acetyltransferase (HAT) and histone deacetyltransferase (HDAC) that regulate gene promoter activity within chromatin. Although exact functions of ING family genes have not been clarified, the gene products are involved in transcriptional regulation, apoptosis, cell cycle, angiogenesis and DNA repair through p53-dependent and -independent pathways. Chromosomal deletion and decreased expression of each ING family member gene in various cancer types strongly suggested products of these genes as tumor suppressor factors. Rare mutation but frequent allelic loss and epigenetic changes have been shown in ING family genes, suggesting them as a class II tumor suppressor gene. This review summarizes the known biological functions of the ING tumor suppressors and signaling related pathways.

Key Words: ING1, ING family, Tumor suppressor gene, Cancer, Head and neck cancer

Introduction

Cell proliferation in normal cells is tightly controlled by various regulatory mechanisms. However, certain genetic and epigenetic alterations permit the normal cells to avoid these mechanisms allowing indefinite cellular activities. Cancer is basically a genetic disease. The collection of genetic and epigenetic alterations of multiple genes and chromosomes lead the development of cancer. In this regard, two major groups of tumor-associated genes, oncogenes and tumor suppressor genes (TSGs), have been implicated in the carcinogenic process (Figure 1).

Tumor suppressor genes have been defined as genetic elements whose loss or mutational inactivation allows cell to display one or more phenotypes of neoplastic growth¹⁾. Protein products of

these tumor suppressor genes are known to be involved in negative regulation of the cell cycle and induction of apoptosis by different mechanisms and inactivation of TSGs is one of the crucial steps during carcinogenic process. In this process, the deletion of targeted chromosomal regions eliminates one allele, while inactivating events (mutation, deletion, or promoter hypermethylation) affect the other allele of a concerning TSG²⁾. The detection of frequent loss of heterozygosity (LOH) in a chromosomal locus is considered to be critical evidence for the localization of a TSG (Figure 2).

In fact by using LOH analysis and then positional cloning, many TSGs have been recently identified including well-known p53, RB1, p16, p21, and FHIT etc. Inactivation of these classical TSG usually occurs through deletion of one of its allele and mutation in the rest allele (Class I TSG). However, a new class of TSGs with haploid insufficiency, in which one allele is lost and the remaining allele is haploinsufficient, has been described recently,

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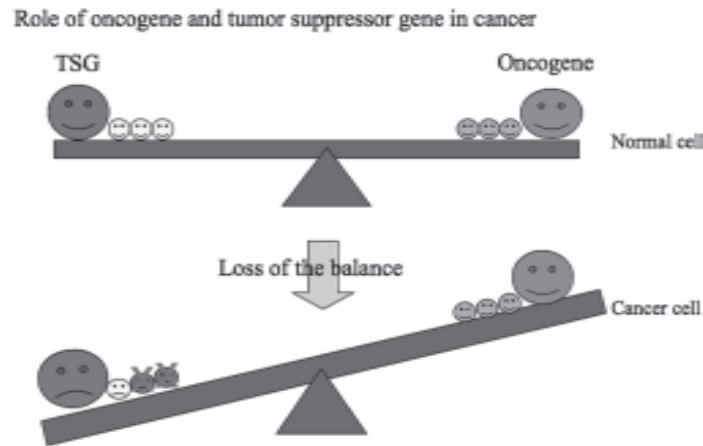


Figure 1. Cell growth and proliferation are regulated by two major groups of genes namely oncogene and tumor suppressor gene. These groups of genes function opposite to each other. While oncogenes promote cell growth and proliferation, tumor suppressor genes inhibit cell growth and proliferation. When the balance between oncogene and tumor suppressor gene is broken, the result is cancer.

and these hemizygous TSGs show a tumor-prone phenotype when challenged with carcinogens (Class II TSG)^{3,4}.

The novel TSG family, inhibitor of growth (ING), belongs to the group of genes that encodes proteins containing structural motifs involved in chromatin remodeling and transcription regulation. The ING family is classified as type II tumor suppressor since its inactivation has been implicated in neoplastic growth of various tumors^{5,6}. Five human ING genes (ING1-5) have been identified and among them, ING1 is the founding member and the most widely studied. ING1 was first isolated using subtractive hybridization between short segments of cDNAs from normal and cancer cells⁷. These randomly fragmented cDNAs interfered with the activity of tumor suppressors by either blocking protein production through anti-sense sequences or abrogating function

in a dominant-negative fashion through truncated sense fragments⁷. Acute expression of transfected constructs encoding this gene inhibited cell growth while chronic expression of antisense ING1 constructs promoted transformation of mouse breast epithelial cells and increased the frequency of focus formation with NIH3T3 cells and protected cells from apoptosis^{7,8}. Using radiation hybrid analysis, ING1 gene was mapped on human chromosome 13q33-34⁹. The other four members have been disclosed through sequence homologies with ING1¹⁰.

Functional Structure of ING Family

All ING family members consist of a highly conserved C-terminal plant homeodomain (PHD) domain that is commonly found in various chromatin remodeling proteins (Figure 3)^{11,12}. Moreover, they contain nuclear localization sequence (NLS) and nuclear conserved region (NCR), which can direct them to the nucleus. However, certain motifs are specifically found in ING members and in their isoforms^{5,13}. These motifs render the ability of the protein to interact with chromatin¹⁴. All the members have distinct N-termini, which dictates the specific role of the ING members and in certain instances their antagonistic regulatory function¹⁵.

After cloning of ING1 cDNA, our group characterized the genomic structure of the human ING1 gene and found that the gene consists of three exons, named 1a, 1b, and 2, and two introns¹⁶. Sizes of the coding region of the exons 1a, 1b, and 2 are 136, 565 and 701 bp, respectively. We also showed that ING1 gene produced at least 3 mRNA variants from 3 different promoters (Figure 3). Two of these variants, p33ING1b consisting of exons 1a and 2, and p24ING1c consisting of a truncated p47ING1a message including the first ATG codon in exon 2, are expressed majorly, while p47ING1a consisting exon 1b and exon 2, was not

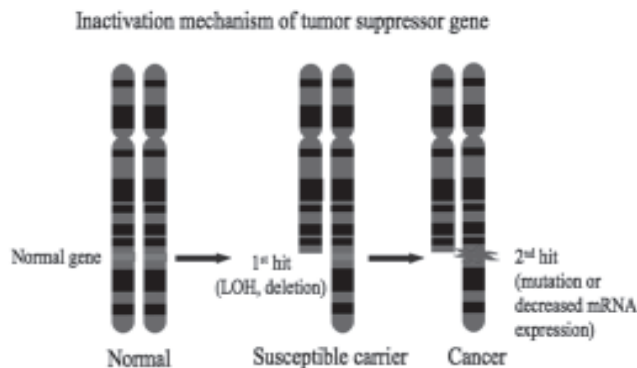


Figure 2. Inactivation mechanism of tumor suppressor gene. It has two copies in normal cell. During carcinogenic process, one copy is deleted through loss of heterozygosity. In this condition, the cell can still control its growth to some extent. However, only one copy is left, these patients are called susceptible carrier for cancer. When the other copy is also inactivated through mutation or decreased mRNA expression, the cell becomes cancerous.

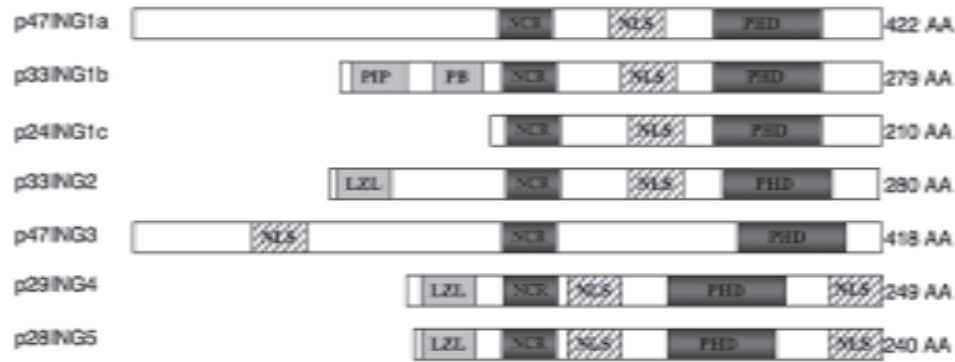


Figure 3. The structure of the ING family proteins. Known domains are shown as follows: Common motifs among all ING members include Plant Homeodomain (PHD), Nuclear Localization Sequence (NLS) and Novel Conserved Region (NCR). The Other like PCNA-interacting protein motif (PIP), partial bromodomain (PB) and leucine zipper-like (LZL) are specifically distinct.

detected in head and neck tissues¹⁶). The N-terminus of p33ING1b contains PCNA-interacting protein motif (PIP) and a partial bromodomain (PB) that seems to regulate protein-protein interactions during chromatin remodeling^{17,18}).

Soon after the cloning of ING1 gene, an ING1-like gene, named ING1L at the time, was isolated from a human fetal brain cDNA library by searching a human EST database for cDNA with sequence similarity to ING1¹⁹). The full-length ING1L cDNA contained an open reading frame of 840 bp, encoding a deduced protein of 280 amino acids with a predicted molecular weight of 32.8 kDa. ING1L was mapped at 4q35 by radiation hybrid mapping and FISH. ING1L showed about 60% homology with ING1 gene and expressed ubiquitously in human tissues. ING1L mRNA was also detected to be significantly higher in colon tumors than in the adjacent normal tissue. After the identification of new members of ING family, ING1L was called as ING2^{20,21}). ING2 was recently shown to negatively regulate cell growth and increase survival in a p53-dependent manner through induction of G1-phase cell-cycle arrest and apoptosis²⁰). ING2 was also induced by the DNA-damaging agents etoposide and neocarzinostatin. Moreover ING2 strongly enhanced the transcriptional-transactivation activity of p53. It has also been shown through a library expression screening using phosphoinositide-affinity resins that ING2 (p33ING2) product is a phosphoinositide (PtdIns)-binding module. p33ING2 has been demonstrated to interact with PtdIns(3)P and PtdIns(5)P through its PHD finger motif, suggesting that PtdIns(5)P recruits or stabilizes p33ING2 in the chromatin and/or interaction with PtdIns(5)P might lead to allosteric activation of p33ING2²²). In addition to the above-mentioned domains, p33ING2 also contains leucine zipper-like (LZL) domain⁵).

Another ING family gene was identified by our group at chromosome 7q31.3 with a protein product of estimated molecular weight of 46.8 kDa (418 amino acids) and was named p47ING3²¹). ING3 is composed of 12 exons spanning over 25 kb genomic

interval. cDNA cloning from human tissues disclosed an alternatively spliced form of the ING3 message but this variant form was devoid of exon 3.

Again by using homology search in genome database, ING4 gene located at chromosome 12p13.31, was identified. It consisted of at least 8 exons spanning over 13 kb genomic interval^{23,24}). ING4 gene produces a mRNA of 1380 nucleotides encoding 249 amino acids. Four different human ING4 mRNAs were generated from juxtaposed splicing sites resulting to the incorporation of 12 nucleotide coding region within a NLS domain^{25,26}). It has also been shown that the small deletion in ING4 protein leads to functional differences between the ING4 variants²⁵). Moreover, alternative splicing could modulate the activity of ING4 tumor suppressor protein²⁶). ING4 also has an LZL domain.

ING5 gene is located at chromosome 2q37.3 and encodes a protein of 240 amino acids corresponding to mRNA with 1068 nucleotides. Like ING4, ING5 has also been identified and characterized having high homology with p33ING1b cDNA²³). At least 4 different splicing isoforms of ING5 have been also observed (Cengiz B, Gunduz M. et al., under submission). p28ING5 likewise contains the domains PHD, NLS, NCR as well as LZL⁵).

Regulatory Activity of ING Family Gene Products

The function of ING is based on its capability to regulate transcription through modifying the chromatin structure by making complexes with histone acetyltransferases (HAT) and histone deacetylases (HDAC). Chromatin remodeling occurs when chemical modification or activation of the ligand-binding system and access to the DNA binding sites take place. With the ING's evolutionary conserved PHD and other domains, different cellular processes brought about by cellular stresses can be regulated either to sites of repair or apoptosis. ING proteins have the ability to physically and functionally interact with integral components of

HAT and HDAC complexes and bind to methylated histones through their PHD domains^{5,10,27}. The association of ING proteins to these complexes leads to acetylation and deacetylation processes and eventually alters gene expression through chromatin remodeling.

ING1 isoforms interact with other proteins in complexes containing HAT activity such as CBP, PCAF, p300, and TRRAP²⁸. However, these isoforms have variations in affinities for the complexes and exerted contradicting effects. Specifically, p33ING1b directly interacts with SAP30 at the N terminus and becomes a part of mammalian Sin3-HDAC complex^{29,30}. These associations have been implicated on ING1's ability to function as a TSG as shown in the consequences of subjecting the cells to various forms of stress. Moreover, ectopic p33ING1b enhanced the G2/M DNA damage checkpoint and reduced cell proliferation emphasizing the essential role of ING1 in cell progression³¹. Although most functions of ING1 have been attributed to this isoform, recently, LN229 cells were shown to differentially activate p47ING1a in response to cisplatin representing a defense to protect the DNA from damage. This effect was specific and independent of the p53 status³². Likewise, p33ING1 was shown to suppress spontaneous lymphoma in mice by negatively regulating cell proliferation and apoptosis independent of the p53 status³³.

ING2 protein has also been found in mammalian Sin3-HDAC complex³⁴. The PHD finger of ING2 was implicated in the negative regulation of cell proliferation through modulation of p53 acetylation in response to DNA damage²². Recently, the ING2 PHD domain has been identified as the first effector domain for methylated H3K4, linking this modification to suppression of transcription^{35,36}. This mechanism may be efficient by switching off proliferation genes as cellular response to acute stress. This emphasized that the biological function depends on the recognition of chromatin modification by the effector proteins rather than by specific modification itself³⁵. Both p33ING1b and p33ING2 gene products also elevated the levels of HSP70 mRNA independent of p53 and down regulated the NF-kappaB pathway using a common mechanism related to the N termini³⁷.

ING3 has been shown to be component of Tip60/NuA4 complex that cooperates with p53 for activation of the p21/waf1 gene. By a member of this complex, ING3 showed strong growth inhibitory activity³⁸. Concurrently, ING3 expression significantly promoted apoptosis by activating Fas/caspase-8 pathway and therefore independent of p53³⁹.

Like all other ING family members, ING4 has been shown to interact with p300 of the HAT complex affecting the transcription of p21/waf1 promoter²³. ING4 and 5 associate with HBO1/HAT complex but specifically ING5 also participates in the MOZ/MORF HAT complex³⁴. Unlike ING2, ING4 PHD doesn't bind to phosphoinositides but binds to H3K4me3 and is possibly involved in transcription activation⁴⁰.

Deregulation of ING Family Genes in Tumors

First clinical studies on ING1 gene demonstrated rearrangement in one neuroblastoma cell line and reduced expression in primary cancers and cell lines^{7,41}. Though rearrangement and deletion of chromosome 13q33-34 region was reported in head and neck cancer, the responsible gene for this deletion ING1 gene was not known at the time. Later in a comprehensive study, we showed tumor specific missense mutations in ING1 gene and frequent deletion at 13q33-34 region for the first time in the world in head and neck squamous cell carcinomas¹⁶. These mutations were found in the PHD zinc finger domain and putative nuclear localization signal, which may abrogate the normal function of ING1 protein. In a separate study, one germline missense alteration and three germline silent alterations of ING1 were detected in 377 primary breast cancers while marked (2-10-fold) decreases in ING1 mRNA expression were seen in 44% of primary breast cancers and in all of ten breast cancer cell lines examined⁴¹. Moreover, the majority of breast cancers showing decreased ING1 expression had metastasized to regional lymph nodes whereas only a small subset of cancers with elevated ING1 expression as compared to adjacent normal tissues were metastatic.

Rare missense mutations in ING1 have been detected in esophageal carcinomas⁴², melanoma⁴³, adenocarcinomas of the esophagogastric junction⁴⁴, non-small cell lung cancer⁴⁵, pancreatic carcinoma⁴⁶, colon cancer cell line⁴⁷, hepatocellular carcinoma⁴⁸, and meningioma⁴⁹, while no mutations were detected in leukemia⁵⁰, Indian oral cancer⁵¹, astrocytoma⁵² and lymphoid malignancies⁵³. On the other hand, decreased expression of ING1 mRNA has been shown in breast cancers^{41,54}, gastric cancers⁴⁷, lymphoid malignancies⁵³, brain tumors⁵², non-small cell lung cancer⁴⁵ and adenocarcinomas of the esophagogastric junction⁴⁴. Most of these studies used primers for mRNA expression analysis in the common exon 2 of ING1 gene since alternative splicing variants of ING1 gene were not intensively investigated in that time. Since the products of different splicing variants of ING1 gene were shown to have different functions and relationship with p53 gene both in mouse and human, recent researches focused on the expression of the each variant in human cancer. Such a study, which examined the p24ING1c and p33ING1b expressions specifically, demonstrated that the p33ING1b is the major variant and exceeds the combined expression of p24ING1c and p47ING1a in hematological malignancies. In our analysis, we could not detect the p47ING1a splicing variant, while p24ING1c and p33ING1b expressions were equally detected in head and neck tissues¹⁶. Specific mRNA expression analysis of p24ING1c and p33ING1b demonstrated various patterns, mostly a decrease of p24ING1c and an increase of p33ING1b in head and neck cancer samples as compared to their normal counterparts (Gunduz M, unpublished data). Since ING1 has 3 different promoters, the difference in expression pattern may be related with carcinogenesis of head and neck cancer through methylation of different promoters or

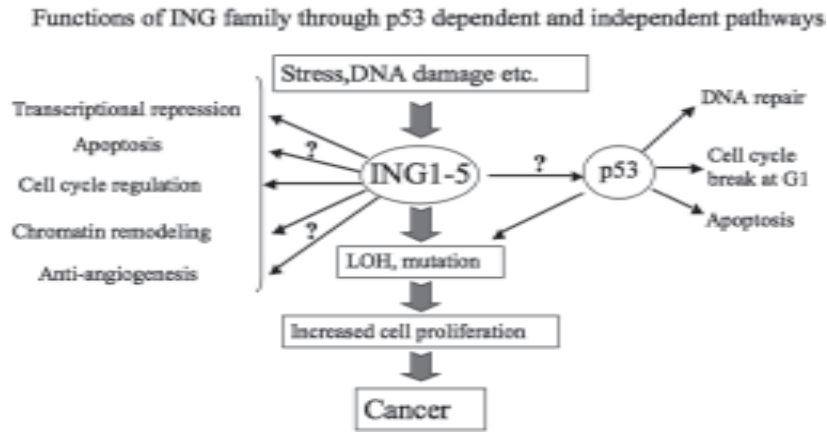


Figure 4. Various roles of ING family genes in apoptosis, angiogenesis, transcriptional and cell cycle regulation, and chromatin remodeling.

abnormalities of upstream or downstream genes.

An interesting finding is that one group showed the down-regulation of nuclear expression of the p33ING1b protein in invasive carcinoma of the breast, acute lymphoblastic leukemia, and melanocytic lesions⁵⁵⁻⁵⁷). Reduced nuclear expression of p33ING1b both in intensity and in the proportion of cells stained was associated with enhanced cytoplasmic p33ING1b expression in a subset of cases. Most tumor suppressors contain nuclear transport signals that facilitate their shuttling between the nucleus and the cytoplasm⁵⁸). This type of dynamic intracellular movement not only regulates protein localization, but also often impacts on function. Therefore, it is possible that nucleo-cytoplasmic modifications of the ING family proteins could occur during carcinogenic process.

Recent studies also showed similar findings of decreased mRNA expression of other ING family members. ING2 was found to be downregulated both at transcriptional and posttranslational level in hepatocellular carcinoma. Moreover the patients with decreased ING2 expression had a poor survival rate in this cancer type⁵⁹). We also recently reported that downregulation of ING3 can be used an independent prognostic factor for poor overall survival⁶⁰). At 5 years follow up, approximately 60% of the patients with normal to high ING3 expression survived, whereas this was 35% in the patients with low ING3 expression in head and neck cancers⁶⁰). On the other hand, a decrease in nuclear ING3 expression was linked to a poor prognosis in cutaneous melanoma⁶¹). Similarly, we and other groups reported chromosomal deletion and/or decrease of ING4 expression in head and neck cancers²¹), glioma⁶²) and breast cancer⁶³). Regarding with ING5, we detected allelic loss⁶⁴), tumor-specific mutation and decreased expression of it (Cengiz B, Gunduz M et al. under submission).

ING family of proteins having NLS can direct themselves into the nucleus and associate with a spectrum of HAT and HDAC

complexes for the maintenance of genomic integrity^{5,10,30,65}). Loss of nuclear ING expression has been observed in various tumors and implicated with tumorigenesis, suggesting that ING is indispensable in controlling cellular regulatory mechanisms. For instance significant reduction of nuclear ING3 was observed in human melanoma indicating the status of ING3 as a prognostic marker and therapeutic target for melanoma⁶¹). The loss of nuclear expression or translocation from nucleus to cytoplasm would probably result to the loss of its tumor suppressor activity⁶). And in events such as p53-dependent pathway, the loss of ING function will compromise the expression of p53. Class II tumor suppressor genes offer great therapeutic possibilities because they are present as non-mutated wild-type alleles in cancer cells. Also endogenous p33ING1b was capable of weakly inducing apoptosis even in the absence of DNA damage, protecting the cell following UV exposure⁶⁶). The sequences responsible for the translocation of p33ING1b into the nucleolus were located in a region common to all splicing isoforms of the ING1 gene indicating that the other two isoforms of the ING1 gene also contain the nucleolar targeting sequences (NTS). This translocation of ING1 protein into the nucleolus upon UV exposure resulted in apoptosis⁶⁶).

Researches have linked ING family to inhibit cell cycle, to promote apoptosis and senescence and to provide aids in reparative process after DNA damage. In addition, ING4 has a specific function in the tumorigenic event that is not directly coupled with regulation of cell cycle⁶⁷). ING4 might be involved in maintaining the contact-inhibited state, fending off a tumorigenic event that leads to loss of contact inhibition. ING4 may achieve this function by affecting the chromatin structure and controlling transcription of genes important in maintenance of contact inhibition⁶⁷). ING4 could also maintain the growth-inhibitory mechanism from extracellular signals or work together with these extracellular signals to implement growth inhibitory function. In both mechanisms, ING4 is indispensable for the extracellular signal

for growth inhibition⁶⁷). Previous studies have likewise implicated ING4 in the inhibition of HIF. Multiple myeloma patients with high IL-8 and microvascular density have considerable reduction in ING4 expression, indicating that ING4 exerted an inhibitory effect on pro-angiogenic molecules (IL-8) and angiogenesis⁶⁸).

Mutations are very rare in ING family genes. Nevertheless, it has now been known that the inactivation of ING family genes at genetic and epigenetic levels plays a major role in tumorigenesis of various tumors. Considering ING genes in the molecular therapy may be of value for the management of various tumors.

Conclusions

Various studies contributed leading to the mutual recognition of ING family as type II TSG with a role in different cellular functions, including apoptosis, angiogenesis, transcriptional and cell cycle regulation, and chromatin remodeling (Figure 4). The structure of the ING gene products having the high-conserved PHD motif is the basis of all its regulatory mechanism through the recruitment of HAT and HDAC complexes. Foreknowingly, HAT and HDAC associations can also recruit other proteins creating more perplexing complexes. Likewise, downstream and upstream molecules involved in the activation or inactivation of ING family are somewhat paradoxical. Notwithstanding, the suppression of ING family in distinct tumors augmented by the formidable existence of ING in pathways related to cell cycle, apoptosis and senescence assert the ING family members in the molecular therapy of distinct tumors. Progress on the knowledge of functions of ING family genes as well as relationship with p53 and other unknown binding molecules will facilitate our understanding of the biochemical mechanisms by which the ING proteins regulate growth, proliferation, senescence and stress response and will help to clarify the functional significance of altered ING family functions in tumorigenesis and cancer progression. This will also enforce efficiency of molecular therapy using each of single ING family genes or combined with other tumor suppressors such as p53. And targeting the complexes in which any of ING family members exists could possibly be used in cancer therapy in near future.

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