Regulation of the MicroRNA Processor DGCR8 by the Tumor Suppressor ING1

Daniel Gómez-Cabello¹, Sergio Callejas², Alberto Benguría², Alberto Moreno¹, Javier Alonso³, and Ignacio Palmero¹

Abstract

The ING family of tumor suppressor proteins controls several cellular functions relevant to antitumor protection, such as cell cycle control, apoptosis, senescence, or migration. ING proteins are functionally linked to the p53 pathway, and they participate in transcriptional control via the recognition of histone marks and recruitment of protein complexes with chromatin-modifying activity to specific promoters. Here, we have investigated the global effect of ING1 in gene regulation through genome-wide analysis of expression profiles in primary embryonic fibroblasts deficient for the Ing1 locus. We find that Ing1 has a predominant role as transcriptional repressor in this setting, affecting the expression of genes involved in a variety of cellular functions. Within the subset of genes showing differential expression, we have identified DGCR8, a protein involved in the early steps of microRNA biogenesis. We show that ING1 binds to the DGCR8 promoter and controls its transcription through chromatin regulation. We also find that ING1 and DGCR8 can cooperate in restraining proliferation. In summary, this study reveals a novel connection between ING1 and a regulator of microRNA biogenesis and identifies new links between tumor suppressor proteins and the microRNA machinery. Cancer Res; 70(5): 1866-74. ©2010 AACR.

Introduction

The ING proteins constitute a family of sequence-related, evolutionary conserved proteins, with tumor suppressor activity in mammals (reviewed in refs. 1, 2). p33ING1 (also known as ING1b) is encoded in the ING1 locus together with two other alternative products: p47ING1a (in humans) and p24ING1c (in humans and mice). Alterations in the ING1 locus have been reported in different types of human tumors, most frequently the reduced expression or mislocalization of ING1 and fewer cases of point missense mutations (reviewed in ref. 3). Furthermore, the inactivation of the Ing1 locus in mice results in an increased incidence of lymphomas (4–6). p33ING1 has been functionally linked to the p53 tumor suppressor pathway and to chromatin regulation. Different activities of p33ING1, such as induction of cell cycle arrest, apoptosis, or DNA repair, require a functional p53 pathway in vitro (7), although p53-independent functions have also been described (8). It has been suggested that ING1 can influence p53 protein stabilization, and/or its posttranslational modification (9), or act as a cofactor in the regulation of target genes (10). On the other hand, p33ING1, in common with other ING proteins, has a general role in transcriptional regulation. ING proteins recognize specific histone methyl marks [preferentially histone H3 trimethylated in Lys4 (H3K4me3)] through their conserved PHD domain (11–15) and allow the recruitment of complexes with histone deacetylase (HDAC) or histone acetyltransferase activity to target genes (16–19). Despite the advances in identifying cellular functions controlled by ING1 or the mechanistic basis for its control of gene expression, the downstream targets of ING1 are largely unknown. Here, we have used primary embryonic fibroblasts derived from mice deficient for the Ing1 locus to investigate the global effect of Ing1 in gene expression and identify genes and processes regulated by Ing1 that may account for its tumor-suppressive action. Among the genes with differential expression, we have identified the microRNA regulator protein Dgcr8, a protein involved in the early steps of microRNA biogenesis. These findings reveal a novel link between tumor suppressor proteins and microRNA biogenesis.

Materials and Methods

Cell culture. Preparation and cultivation of mouse embryonic fibroblasts (MEF) and retroviral infection experiments were carried out as previously described (20). Early-passage MEFs [with less than four population doublings] were used for all the experiments. For lentiviral infections, 293T cells were transiently transfected with the appropriate lentiviral expression vector and the vectors pMD2-G, pMDLg/pRRE, and pRSV-Rev, which encode lentiviral proteins. The medium...
containing lentiviruses was recovered, filtered through a 0.45-μm filter, diluted 1 in 2 with fresh medium, and added to the recipient cells. The same procedure was repeated 12 h later. The following vectors were used for retroviral or lentiviral transduction: pLPC-AU5ING1 (10), pSuper-DGCR8, pSicoR-DGCR8 (21), and the relevant empty vectors. The inducible line EMG was generated by stable transfection with the vectors pWZL-Blast-rtTA and pHRS-AU5-ING1 of the ARE-inducible NARF2 cell line (a kind gift of Gordon Peters, Cancer Research UK; ref. 22). All cells were grown in DMEM containing 10% fetal bovine serum. Bromodeoxyuridine (BrdUrd) incorporation was measured as described (10) using a 6-h pulse. For chromatin immunoprecipitation (ChIP) assays, trichostatin A (TSA) was added at a final concentration of 32 nmol/L 1 h before harvesting the cells. For colony formation assays, cells were seeded at a density of 800 or 8000 per well in six-well plates. After 10 d, cells were fixed with formaldehyde and stained with Giemsa stain solution, and the colonies were counted. For growth curves, cells were seeded in 24-well plates, at 2 × 10⁴ per well, in triplicate. At different time points, cells were trypsinized and counted with a Neubauer chamber.

Analysis of gene expression profiles. CodeLink mouse whole-genome microarrays (Applied Microarrays, Inc.) were used to analyze gene expression profiles. Total RNA was isolated with TriReagent (Sigma), and its integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies). Labeling and hybridization were performed using the CodeLink iExpress assay reagent kit according to the manufacturer’s instructions. After hybridization and washing, slides were scanned in an Axon GenePix scanner and analyzed using CodeLink Expression Analysis software. Data were median normalized, log₂ transformed, and exported to the MeV software (TIGR) for additional statistical analysis and graphical visualization. We analyzed three independent preparations of early-passage MEF for each genotype, each derived from an individual embryo. Genes with ratios between Ing1-deficient and wild-type higher than 2 or lower than 0.5, with statistical significance (P < 0.05, Student’s t test), were selected for further analysis. Functional genomics analysis of differentially expressed genes was carried out with the Fatigo suite.³ Statistical differences between the percentages of Gene Ontology (GO) terms were calculated by Fisher’s exact test.

Chromatin immunoprecipitation. ChIP analysis was performed essentially as described by Weinmann and Farnham (23) with some modifications. For immunoprecipitation, the following antibodies were used: 1 μg of anti-AU5 (Covance Research Products, Inc.), 2 μg of anti-H3K4me3 (Abcam), 1 μg of anti-p53 (DO-1, Santa Cruz Biotechnology), 2 μg of anti-acetylated histone H3 (Upstate Millipore), and 2 μg of anti-acetylated histone H4 (Millipore). The precipitated DNA was analyzed by PCR with the appropriate primers (see Supplementary Data).

Quantitative real-time PCR. Total RNA was isolated with TriReagent, as recommended by the manufacturer. cDNA was generated using reverse transcriptase Moloney murine leukemia virus (Promega), and the real-time PCR was performed in ABI 7900HT or ABI 9700 machines (Applied Biosystems) using SYBR Green for labeling and ribosomal 18S RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as standards.

Western blot. Western blot analysis was carried out as previously described (10) using the following antibodies: anti-DGCR8 (1:300 dilution; PTG-Lab), anti-human p53 (DO-1; 1:500 dilution), anti-AU5 (1:500 dilution), anti-actin (1:10,000 dilution; Sigma), anti-p33ING1 (LG1, 1:1,000 dilution; ref. 10).

Immunofluorescence. Exponentially growing, low-passage MEFs were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated in blocking solution (PBS containing 2% bovine serum albumin) overnight at 4°C. The primary antibody (anti-Dgcr8, 1:100 dilution; PTG-Lab) was added in blocking solution, left for 60 min at room temperature, and washed with PBS containing 0.1% Triton X-100 (three to four washes, 15 min each) followed by incubation with a fluorochrome-conjugated secondary antibody (donkey anti-rabbit Alexa Fluor 488, 1:500 dilution) and washing in the same conditions used with the primary antibodies. Cells were mounted with Vectashield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and analyzed using a Zeiss Axiohot microscope.

Results

Identification of genes regulated by Ing1 in mouse fibroblasts. To investigate the general effect of Ing1 on gene expression, we carried out a genome-wide expression profiling analysis using CodeLink mouse whole-genome expression arrays. We chose for this study primary MEFs of very early passage (see Materials and Methods) to avoid effects of unknown genetic makeup associated with tumor-derived or immortalized cell lines or changes due to the accumulation of divisions in culture. The Ing1-deficient MEFs we have used in this study display a dramatic reduction (>90%) in the expression of all the transcripts of the locus, as a consequence of the insertion of a BetaGeo genetrap cassette. The levels of the p33ING1 protein (the only ING1 peptide detectable in MEFs) are also reduced to the same extent (24). Of the 37,898 probes, representing >30,000 genes, present in the array, we found a significantly differential expression associated to Ing1 status (at least 2-fold change, see Materials and Methods) for 466 of them (~1.2% of the total). This indicates that Ing1 controls the expression of a very specific subset of genes in our system. Of the group of genes showing differential expression, the majority (70%, 328 of 466) were upregulated in Ing1-deficient fibroblasts relative to wild-type counterparts (Fig. 1A). This finding indicates a predominant role for Ing1 as a transcriptional repressor, in agreement with previous biochemical studies (16, 18). As an internal validation of the experiment, we found that the signal for the Ing1 probe was reduced ~10-fold in the Ing1-mutant fibroblasts (Ing1-mutant/wild-type ratio, 0.11; P = 0.0004), consistent with our previous characterization of the expression of the Ing1 locus in this cell type (24). In addition, we found that the expression of the other Ing

http://www.fatigo.org
Ing1 regulates Dgcr8. From the subset of genes with validated differential expression, we chose to analyze in detail Dgcr8, one of the genes with increased expression in Ing1-deficient fibroblasts. Dgcr8 (DiGeorge syndrome critical region 8, also known as Pasha) encodes an RNA-binding protein involved in the early steps of processing of microRNAs (25). Given the growing evidence of the importance of the microRNA machinery in the context of cancer, we wished to know if Dgcr8 could be a direct transcriptional target of Ing1, providing a link between this tumor suppressor protein and the microRNA machinery.

First, we used Western blot and immunofluorescence to investigate the levels and localization of the Dgcr8 protein in normal and Ing1-mutant MEFs (Fig. 2). The amount of Dgcr8 protein was modestly increased in the Ing1-mutant cells (~2-fold), consistent with the data from the array and quantitative RT-PCR (Fig. 2A). Expression of ectopic p33ING1 in Ing1-deficient fibroblasts dramatically reduced Dgcr8 protein levels, further supporting the correlation between Ing1 and Dgcr8 protein levels (Fig. 2B). In accordance with previous reports (26), we found that Dgcr8 shows a predominantly nuclear staining in both wild-type and Ing1-mutant fibroblasts. Dgcr8 showed an even distribution through the nucleoplasm, with some degree of accumulation in speckles in both genotypes (Fig. 2C).

Ing1 is a direct transcriptional regulator of Dgcr8. To test whether Ing1 participates directly in the transcriptional control of the Dgcr8 locus, we performed ChIP experiments. Attempts to detect the endogenous p33ING1 protein on the Dgcr8 promoter in mouse fibroblasts were unsuccessful because of the failure of the anti-ING1 antibody to work in this assay (data not shown). To circumvent this problem, we used a tagged version of p33ING1 in two different experimental settings. First, we used a cell line (EMG) generated in our laboratory from the U2OS-derived, p14ARF-inducible, NARF2 line (22). EMG cells have been engineered to allow doxycycline-inducible expression of AU5-tagged-p33ING1 and independent isopropyl-l-thio-B-D-galactopyranoside (IPTG)-inducible expression of the p53 activator p14ARF (see Materials and Methods). When ING1 expression was induced in addition of doxycycline (Fig. 3A, left), we could easily detect the binding of the ING1 protein to the promoter region of the Dgcr8 locus by ChIP using an antibody against the AU5 tag (Fig. 3A, right, and D). As a control of the specificity of the assay, binding of p33ING1 to the housekeeping GAPDH locus was not detected. These results were confirmed in HCT116 colon carcinoma cells transiently transfected with a vector expressing AU5-tagged p33ING1 (Fig. 3B, left). ChIP analysis with the anti-AU5 antibody again showed binding of ectopic ING1 to the Dgcr8 promoter in ING1-transfected cells and not in vector-transfected controls (Fig. 3B, right, and D).

It has been suggested that ING1 can cooperate with p53 in transcriptional regulation (7). To test whether the presence of ING1 in the Dgcr8 promoter was affected by p53, we increased endogenous p53 levels in the EMG cells by means of the inducible expression of p14ARF (Fig. 3C, left). ChIP...
experiments with the anti-AU5 antibody showed undistinguishable results with or without p53 accumulation (compare Fig. 3C with Fig. 3A), suggesting that the binding of p33ING1 to the DGCR8 promoter region is not affected by p53 levels (Fig. 3C). As a control for the specificity of the detection of ING1 in the DGCR8 promoter, we carried out additional ChIP experiments with an anti-p53 antibody in EMG cells after induction of p14ARF by IPTG (22). We could not detect specific

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>Ratio gg/wt</th>
<th>t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_177346</td>
<td>Gpr149</td>
<td>G protein–coupled receptor 149</td>
<td>11.24</td>
<td>0.0209</td>
</tr>
<tr>
<td>NM_007803</td>
<td>Ctn (EMS1)</td>
<td>Cortactin</td>
<td>10.65</td>
<td>0.002</td>
</tr>
<tr>
<td>NM_023913</td>
<td>Em1</td>
<td>Endoplasmic reticulum to nucleus signaling 1</td>
<td>9.82</td>
<td>0.0009</td>
</tr>
<tr>
<td>NM_025613</td>
<td>Cr1 (Eid1)</td>
<td>EP300 interacting inhibitor of differentiation 1</td>
<td>9.56</td>
<td>0.0157</td>
</tr>
<tr>
<td>NM_020581</td>
<td>Angpt4</td>
<td>Angiopoietin-like 4</td>
<td>9.32</td>
<td>0.019</td>
</tr>
<tr>
<td>NM_175551</td>
<td>Dido1</td>
<td>Death inducer-obliterrator 1</td>
<td>5.17</td>
<td>0.042</td>
</tr>
<tr>
<td>NM_018792</td>
<td>His1</td>
<td>Histone linker H1 domain, spermatid-specific 1</td>
<td>3.80</td>
<td>0.02</td>
</tr>
<tr>
<td>NM_198247</td>
<td>Sertad4</td>
<td>SERTA domain containing 4</td>
<td>3.39</td>
<td>0.02</td>
</tr>
<tr>
<td>NM_019675</td>
<td>Stmn4</td>
<td>Stathmin-like 4</td>
<td>3.04</td>
<td>0.06</td>
</tr>
<tr>
<td>NM_011331</td>
<td>Ccl12 (MCP5)</td>
<td>Chemokine (C-C motif) ligand 12</td>
<td>2.75</td>
<td>0.04</td>
</tr>
<tr>
<td>NM_033324</td>
<td>Dgcr8</td>
<td>DiGeorge syndrome critical region gene 8</td>
<td>2.69</td>
<td>0.04</td>
</tr>
<tr>
<td>NM_008228</td>
<td>Hdac1</td>
<td>Histone deacetylase 1</td>
<td>2.63</td>
<td>0.0159</td>
</tr>
<tr>
<td>NM_028614</td>
<td>Ppp2r1b</td>
<td>Protein phosphatase 2 regulatory subunit A, β isoform</td>
<td>2.58</td>
<td>0.0068</td>
</tr>
<tr>
<td>NM_010545</td>
<td>Rps14</td>
<td>Ribosomal protein S14</td>
<td>0.19</td>
<td>0.003</td>
</tr>
<tr>
<td>NM_013653</td>
<td>Ccl5</td>
<td>Chemokine (C-C motif) ligand 5</td>
<td>0.17</td>
<td>0.026</td>
</tr>
<tr>
<td>NM_009628</td>
<td>Adnp</td>
<td>Activity-dependent neuroprotective protein</td>
<td>0.13</td>
<td>0.03</td>
</tr>
<tr>
<td>NM_011919</td>
<td>Ing1</td>
<td>Inhibitor of growth 1</td>
<td>0.11</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Abbreviations: wt, wild-type; g/g, Ing1-deficient.
binding of p53 to the DGCR8 promoter under conditions where the promoter of the p53 target p21CIP1 could be recovered in chromatin precipitated with the p53 antibody (Supplementary Fig. S1). Note than binding of p53 to the p21 promoter is independent of p53 levels in this cell line (27).

Mechanism of regulation of Dgcr8 by Ing1. To determine the mechanism underlying the regulation of Dgcr8 by ING1, we investigated the status of several histone modifications in the Dgcr8 promoter in wild-type or Ing1-mutant fibroblasts using ChIP. We found that the acetylation of histone H3 and histone H4 in the Dgcr8 promoter was significantly increased in Ing1-mutant cells (Fig. 4A). H3K4me3 is considered a mark of transcriptionally active chromatin and is specifically recognized by ING1 and other ING proteins. The amount of H3K4me3 recovered in chromatin for the Dgcr8 promoter was not grossly altered in Ing1-deficient cells, consistent with the notion that ING1 acts primarily as a reader of this mark but does not influence its methylation (Fig. 4A). Collectively, these results suggest that ING1 contributes to the transcriptional repression of Dgcr8 by the inhibition of histone acetylation through active recruitment of deacetylation complexes. To confirm the involvement of HDACs in the repression of Dgcr8 by Ing1, we treated fibroblasts of both genotypes with TSA, an inhibitor of class I and class II HDACs. Treatment with TSA resulted in an increase of Dgcr8 RNA and protein in wild-type MEFs (Fig. 4B and C), which correlated with increased presence of acetylated histones in ChIP experiments (Supplementary Fig. S2). Ing1-deficient cells retained a limited response to TSA, which could be due to residual Ing1 expression in Ing1-mutant MEFs or to Ing1-independent HDAC activity.

MicroRNA expression in Ing1-deficient fibroblasts. To test whether the altered levels of Dgcr8 observed in

Figure 3. ChIP analysis of the presence of ING1 at the DGCR8 promoter. A, ChIP in the inducible line EMG. Left, cells were treated with doxycycline (Dox) for 24 h to induce AU5-p33ING1 expression; right, immunoprecipitation of chromatin was performed with an antibody against the AU5 tag followed by PCR for the DGCR8 promoter. PCR for GAPDH is shown as a negative control. B, left, ChIP in HCT116 cells transiently transfected with a vector expressing AU5-p33ING1 or an empty vector; right, immunoprecipitation of chromatin was performed as described in A 48 h after transfection. C, ChIP in the inducible line EMG. Left, cells were treated with IPTG for 24 h to induce p14ARF (data not shown) and p53 protein levels; right, immunoprecipitation of chromatin was performed with an antibody against the AU5 tag as described in A. WB, Western blot; −Ab, control without antibody; +Ab, sample with the indicated specific antibody. D, quantitation of ChIP signals from experiments shown in A and B. Error bars indicate SD from two experiments.
all the miRNAs in this subset were upregulated in Ing1.

miRNAs that displayed statistically significant differences in Agilent Technologies. We identified a small subset of mature early-passage fibroblasts using a miRNA microarray from Ing1, suggesting that the group of miRNAs deregulated in share potential target transcripts using the miRanda algorithm, sequence similarity between them, and they were predicted to of the miRNAs with highest scores in this analysis showed se-

miR-192 (the miRNA with the highest upregulation) was vali-
dicated by quantitative PCR (Supplementary Fig. S3C). Some of the miRNAs with highest scores in this analysis showed sequence similarity between them, and they were predicted to share potential target transcripts using the miRanda algorithm, suggesting that the group of miRNAs deregulated in Ing1-deficient fibroblasts might control a common set of targets (Supplementary Fig. S3D).

**Functional link between ING1 and DGCR8.** We explored whether the link between ING1 and DGCR8 expression could have a functional implication. To this end, we stably expressed a short hairpin RNA (shRNA) against DGCR8 in the inducible EMG line, which resulted in a highly efficient reduction of endogenous DGCR8 levels (Fig. 5A, left). As previously described, induction of ING1 expression had a clear antiproliferative effect in these cells, which could be measured in growth curves or in colony formation assays (P = 0.019 in colony formation assays; Fig. 5A, right, and B). Unexpectedly, the independent suppression of DGCR8 by itself also resulted in reduced proliferation (P = 0.01 in colony formation assays). Concomitant induction of ING1 expression and silencing of DGCR8 led to enhanced antiproliferative effect in both assays (compare pSi-DGCR8 + Dox versus pSicoR + Dox; P = 0.12; Fig. 5A and B). The functional connection between both proteins was also investigated in primary MEFs (Fig. 5C). Silencing of endogenous Dgcr8 expression with an RNA interference retroviral vector also reduced the proliferation rate in MEFs, either wild-type or Ing1-mutant, but the reduction in BrdUrd-positive cells was slightly higher in MEFs with functional Ing1 (25.09% versus 18.85%).

**Discussion**

The products of the Ing1 locus participate in several cellular functions with important implications in tumor suppression, such as cell cycle control, apoptosis, DNA repair, or senescence (1, 2). Here, we have taken an unbiased approach to identify Ing1 targets responsible for its cellular functions, namely, the comparison by microarray analysis of the expression profiles of MEFs with the Ing1 locus inactivated with a gene trap cassette, relative to their wild-type littermates. With this experimental setup, we aimed to avoid the possible effects of passage in culture, unknown genetic changes of immortalized or transformed cell lines, or possible nonphysiologic effects due to the use of ectopic gene delivery. The inspection of the global effect of Ing1 status in gene expression revealed that the majority of differentially expressed genes were upregulated in Ing1-mutant cells. These genes are potential targets of Ing1-dependent repression. Our results agree with previous biochemical data showing that the mammalian p33ING1 protein is predominantly associated to repressor complexes with HDAC activity (18, 19). Collectively, these results support the notion that Ing1 has a predominant role as a mediator of transcriptional repression.

GO analysis revealed a very significant enrichment in Ing1-mutant cells of proteins involved in different aspects of chemokine signaling. Interleukin-8, a soluble cytokine, is a well-known target of the related protein ING4 (28, 29). Our results with Ing1 open the possibility that regulation of chemokine or cytokine signaling could be a shared feature of ING proteins. Furthermore, bioinformatic analysis also shows that a large proportion of Ing1-regulated genes contain consensus binding sequences for NF-κB. ING4 can interact with and regulate the activity of NF-κB (28–30). Therefore, our unbiased analysis also supports the existence of cooperation between Ing1 and NF-κB in transcriptional control and indicates that Ing1 and Ing4 might participate in similar signaling pathways. Additional studies aimed to identify Ing1-regulated genes have been reported by other groups using either antisense against
ING1 in a mouse mammary epithelial cell line (31) or over-expression of ING1 or ING2 in human primary fibroblasts (32). Interestingly, there is very limited overlap between our results and those of these studies. The use of different cell types and/or microarray platforms could account for some of these differences. More importantly, unlike the previous studies, we have not used ectopic expression, and instead, we have analyzed genetically defined primary cells differing in their Ing1 status. Therefore, we are confident that our results reflect faithfully the role of endogenous Ing1 in gene regulation. One of the genes with increased expression in Ing1-mutant cells is DGCR8 (also known as Pasha in some species). The DGCR8 protein plays an essential role in the processing of canonical microRNAs in the nucleus (33, 34). DGCR8 and the RNase Drosha form a nuclear complex known as the Microprocessor, which catalyzes the cleavage of the primary miRNA transcripts (pri-miRNA) to yield ∼70-nucleotide-long stem-loop intermediates (pre-miRNAs) that are subsequently exported to the cytoplasm, where they are substrates of Dicer to produce mature miRNAs (35). The increasing evidence of the relevance of alterations in the microRNA machinery in cancer (36) prompted us to investigate further the connection between ING1 and DGCR8 expression. Our data clearly identify Dgcr8 as a novel direct target of transcriptional repression by ING1, which adjusts to the canonical model for ING action, via the direct binding to histone marks and recruitment of

![Figure 5](image-url)

**Figure 5.** Functional link between DGCR8 and ING1. A, left, Western blot of EMG cells infected with a lentiviral vector expressing a shRNA against DGCR8 (pSi-DGCR8) or empty vector (pSicoR) combined with the induction of AU5-ING1 with doxycycline. A, right, growth curves of EMG cells expressing DGCR8 shRNA and/or with induction of AU5-ING1. A representative experiment is shown. Error bars represent SD of triplicates. B, growth of EMG cells expressing DGCR8 shRNA and/or with induction of AU5-ING1. Cells were seeded at 8,000 per well and stained with Giemsa 10 d later. Right, number of colonies from two independent experiments. Error bars indicate SDs. C, left, Western blot analysis of wild-type or Ing1-deficient fibroblasts retrovirally infected with a vector expressing a shRNA against Dgcr8 (pRS-Dgcr8) or empty vector (pRetroSuper). C, right, BrdUrd-positive cells, relative to vector-infected cells, measured by immunofluorescence against BrdUrd (see Materials and Methods). The average and SDs from two experiments are shown.
HDAC complexes to its promoter. To our knowledge, this is the first characterization of the mechanism of transcriptional regulation by ING1 at a specific target gene, and it validates the general model for gene regulation by ING1 and other ING proteins. Alteration in miRNA expression is now recognized as a common feature of human cancers (36). Deregulation of microRNA activity in tumors can occur at different steps of the microRNA biogenesis machinery, with transcriptional regulation of primary miRNA being the best characterized. Our results provide a novel link, acting at the step of miRNA nuclear processing. We show that the tumor suppressor ING1 contributes to the transcriptional regulation of DGCR8 expression, a critical mediator of miRNA biogenesis. The deregulation of Dgcr8 is paralleled by the increased expression of a small subset of mature miRNAs in Ing1-mutant fibroblasts. As ING1 is frequently inactivated in tumors, these observations potentially identify a novel mechanism for altered miRNA function in cancer (i.e., the transcriptional control by tumor suppressors of proteins involved in the processing of miRNAs). The functional effect of altered DGCR8 levels is not unambiguously established. Kumar and colleagues (21) have reported increased proliferation and transformation in tumor cell lines where DGCR8 has been suppressed by RNA interference. On the other hand, mice deficient for the Dgcr8 locus show embryonic lethality (37, 38) and Dgcr8-deficient embryonic stem cells have defects in proliferation and differentiation (38, 39). In line with the latter data, our results indicate that reduced DGCR8 activity is antiproliferative in both transformed and primary cells (Fig. 5; data not shown). The characterization of the mechanism responsible for the antiproliferative action of DGCR8 will be described in detail elsewhere.5 It is feasible that increased expression of DGCR8, like that found in Ing1-mutant cells, might promote proliferation and thus contribute to the transformed phenotype. Impaired processing of pri-miRNAs (40) and altered expression of microRNA processing proteins (41, 42) have been reported in human tumors. Interestingly, microarray data at the Oncomine Cancer Profiling database6 show increased Dgcr8 expression in several tumor types, such as melanoma, glioblastoma, or breast carcinoma. Of note, several of these reports indicate an inverse correlation between ING1 and DGCR8 expression in tumors such as invasive breast carcinoma or glioblastoma. In summary, by studying the global effect of Ing1 depletion in gene expression, we have identified a novel link between the tumor suppressor Ing1 and the microRNA machinery through the transcriptional control of DGCR8. This functional link identifies a novel connection between tumor suppressor proteins and microRNA biogenesis, potentially relevant for the contribution of Ing1 dysfunction to neoplastic transformation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Ana Dopazo and Fátima Sánchez-Cabo for excellent assistance with the microRNA microarray analysis, Gordon Peters for the gift of NARF cells, and Esther Martín-Garrido for the generation of the EMG cell line.

**Grant Support**

Spanish Ministry of Science and Innovation grant BFU/06-10882 (I. Palmero). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 06/10/2009; revised 11/30/2009; accepted 12/08/2009; published OnlineFirst 02/23/2010.

---


6 http://www.oncomine.org

---

**References**


Regulation of the MicroRNA Processor DGCR8 by the Tumor Suppressor ING1

Daniel Gómez-Cabello, Sergio Callejas, Alberto Benguría, et al.

Cancer Res 2010;70:1866-1874. Published OnlineFirst February 23, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2088

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/02/22/0008-5472.CAN-09-2088.DC1

Cited articles
This article cites 42 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/5/1866.full.html#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
/content/70/5/1866.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.