Growth Factor Independence-1 Is Expressed in Primary Human Neuroendocrine Lung Carcinomas and Mediates the Differentiation of Murine Pulmonary Neuroendocrine Cells

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ABSTRACT

Human small cell lung cancers might be derived from pulmonary cells with a neuroendocrine phenotype. They are driven to proliferate by autocrine and paracrine neuropeptide growth factor stimulation. The molecular basis of the neuroendocrine phenotype of lung carcinomas is relatively unknown. The Achaete-Scute Homologue-1 (ASH1) transcription factor is critically required for the formation of pulmonary neuroendocrine cells and is a marker for human small cell lung cancers. The Drosophila orthologues of ASH1 (Achaete and Scute) and the growth factor independence-1 (GFI1) oncoprotein (Senseless) genetically interact to inhibit Notch signaling and specify fly sensory organ development. Here, we show that GFI1, as with ASH1, is expressed in neuroendocrine lung cancer cell lines and that GFI1 in lung cancer cell lines functions as a DNA-binding transcriptional repressor protein. Forced expression of GFI1 potentiates tumor formation of small-cell lung carcinoma cells. In primary human lung cancer specimens, GFI1 expression strongly correlates with expression of ASH1, the neuroendocrine growth factor gastrin-releasing peptide, and neuroendocrine markers synaptophysin and chromogranin A (P < 0.0000001). GFI1 colocalizes with chromogranin A and calcitonin-gene-related peptide in embryonic and adult murine pulmonary neuroendocrine cells. In addition, mice with a mutation in GFI1 display abnormal development of pulmonary neuroendocrine cells, indicating that GFI1 is important for neuroendocrine differentiation.

INTRODUCTION

Lung cancer is the most common cancer in the world and is the leading fatal malignancy in both men and women in the United States (1). It is classified into two major histopathological groups: small cell lung carcinomas (SCLCs) and non–small-cell lung carcinomas (NSCLCs; ref. 2). SCLCs account for 15 to 20% of all human lung cancers and are aggressive lung tumors, which are virtually always fatal (2, 3). Lung tumors with a neuroendocrine phenotype include SCLCs, large cell neuroendocrine carcinomas, and classical and atypical carcinoid tumors. The neuroendocrine phenotype is associated with the production of neuropeptides that may stimulate transformation and tumor growth through paracrine and autocrine signaling (4), and the maintenance of these signaling loops in lung tumors is dependent on the neuroendocrine phenotype (5, 6). Although it is clear that the growth factors secreted by neuroendocrine tumors participate in tumorigenesis and tumor maintenance, the genetic and biochemical pathways underlying neuroendocrine differentiation have not been elucidated. Understanding the molecular basis of neuroendocrine lung carcinomas, especially SCLC, should provide the opportunity for novel therapeutic intervention with significant implications for patients.

Clues to the genetic components of the human neuroendocrine tumor phenotype can be found in the genetic cascade that controls Drosophila peripheral nervous system development. Drosophila peripheral nervous system organ development is regulated by proneural basic helix-loop-helix (bHLH) transcription factors atonal, amos, scute, and achaete (7, 8). These proneural bHLH transcription factors dimerize with daughterless proteins to bind E-box DNA sequences and transactivate target genes, including Delta, which activates Notch signaling in adjacent cells (9). Activation of Notch signaling induces Enhancer-of-split, a bHLH transcriptional repressor, which antagonizes proneural bHLH factor expression and function (10). Proper function of proneural genes is required in most tissues for the expression of senseless, which encodes a zinc-finger transcription factor (sens; ref. 7). Embryonic and adult peripheral nervous system development requires sens: embryos that lack sens specify peripheral nervous system cells, but most of the cells die through apoptosis, whereas adult peripheral nervous system precursors that lack sens fail to become specified. In contrast, forced expression of Sens is sufficient to mediate peripheral nervous system development (7, 11). Thus, sens is a central target of proneural bHLH transcription factors in peripheral nervous system development.

The Achaete-Scute Homologue-1 (ASH1), the mammalian orthologue of Drosophila achaete and scute encoded proteins, is critical for murine pulmonary neuroendocrine cell development (5, 12). Pulmonary neuroendocrine cells are a minor cell population of the airway epithelium, which function as oxygen sensors (13). Newborn mice bearing a disruption of Ash1 completely lack detectable pulmonary neuroendocrine cells and die shortly after birth (5). Although the data are not conclusive, human neuroendocrine carcinomas might be derived from pulmonary neuroendocrine cells (14). Not surprisingly, human SCLC express ASH1 (15). Moreover, expression levels of ASH1 in SCLC tumors correlates with neuroendocrine markers such as gastrin-releasing peptide, 1-dopa-decarboxylase activity and calcitonin (16, 17). However, the role of ASH1 in SCLC is unclear. Transgenic expression of ASH1 in nonneuroendocrine murine lung Clara cells results in hyperplasia but not cancer (6). Dual transgenic expression of ASH1 and the polyoma large T antigen generates neuroendocrine lung tumors, but these are dissimilar to SCLC (6). It is therefore uncertain whether the expression of ASH1 in SCLC is merely a marker of neuroendocrine differentiation or a driving force in neuroendocrine oncogenesis.

The mammalian orthologue of Drosophila Sens is the growth factor independence-1 (GFI1) oncoprotein. Gfi1 was cloned in an insertion mutagenesis screen for targets of the Moloney murine leukemia virus, which could mediate leukemia progression from interleukin 2-
dependent to independent growth (18). Gfi1 is also a common proviral integration target in Moloney virus-induced leukemias (18–22). Indeed, Moloney virus insertions affecting Gfi1 in murine T-cell leukemias are second in frequency only to those affecting Myc (23). Deletion of Gfi1 results in hematopoietic abnormalities (24–26).

We have recently shown that Gfi1 expression is not restricted to the lymphoid lineage (27). Embryonic murine neurons and sensory epithelia also express GFI1. Specifically, Gfi1−/− embryos lose cochlear hair cells and display behavioral defects consistent with inner ear abnormalities (27). Thus, like its Drosophila orthologue Sens in embryos (7, 11), GFI1 appears to play a role in the differentiation of sensory neurons.

Given the critical role of ASH1 in pulmonary neuroendocrine cell development (5) and the clear association between GFI1 and ASH1 orthologues in Drosophila (7, 11), we examined GFI1 expression in developing lungs and lung tumors. Here, we show that in human lung tumors GFI1 is expressed in both neuroendocrine cell lines and in primary human tumor samples. Moreover, the correlation between the expression of GFI1, ASH1, and neuroendocrine markers is highly significant. We previously demonstrated that embryonic mouse lung expresses Gfi1 mRNA in a clustered distribution similar to that of pulmonary neuroendocrine cells (27). Our new data indicate these GFI1-expressing cells in embryonic and adult murine lungs are indeed pulmonary neuroendocrine cells and that Gfi1−/− pulmonary neuroendocrine cells do not express or express aberrant levels of the markers syntophysin (Syn) and calcitonin-gene-related peptide.

These data suggest that GFI1 has an important role in mediating neuroendocrine differentiation and point to a proneural bHLH/GFI1 developmental pathway that is conserved from humans to Drosophila and active in human lung cancer.

MATERIALS AND METHODS

Case Material. Archival paraffin-embedded tissue blocks of primary lung carcinoma were acquired from the pathology archives of Vancouver Hospital and Health Sciences Centre and St. Paul’s Hospital (Vancouver, British Columbia, Canada). Tissue samples were fixed in formalin or Bouin’s fixative. Cases date from 1982 to 2001. A total of 441 cases was selected to build the tissue microarrays (TMAs). Outcome data (1 day to 18.2 years, median = 4.81 years) for all of the cases was available.

Tissue Microarray Construction. TMAs were constructed as described previously (28). Duplicate 0.6-mm tissue cores were used to construct TMAs. Array blocks were sectioned to produce 4-μm sections, and the first section was stained with H&E to assess adequacy. A total of 408 cases was valid for final data analysis and included 123 squamous cell carcinomas, 93 adenocarcinomas, 68 large cell undifferentiated carcinomas, 68 classical carcinoids, 31 atypical carcinoids, 11 large cell neuroendocrine carcinomas, and 14 small cell carcinomas. Fifty cases were omitted from data analysis because of technical problems with immunohistochemistry for one or more markers.

Immunohistochemistry on Human Tissues. Twelve molecular markers were chosen for investigation in this study (Table 1). Immunohistochemical staining was performed with the biotin-streptavidin method. Sections were deparaffinized and antigen retrieval performed. Subsequently, slides were incubated with primary antibody according to a predetermined optimal concentration overnight at 4°C or for 1 hour at 37°C. All slides were scored by the same pathologist (C. Gilks). Staining in nonneural cells or in an inappropriate cellular compartment was not considered to be positive. The scores were entered into blank sector maps of the corresponding array at the time of scoring, and uninterpretable results were eliminated from additional consideration.

Data Analysis and Statistics. Data on the score sheets were converted into a table format using the TMA-Deconvoluter program as described previously (29). Fisher’s exact test using SPSS Graduate Pack 11.0 (SPSS, Inc., Chicago, IL) was used to evaluate the correlation between different immunomarkers. Kaplan-Meier curves and survival estimates were calculated for each outcome, and a log-rank statistic was used to test for differences between groups. A significant difference was declared if the P value from a two-tailed test was <0.05.

Cell Lines and Tissue Culture. SCLC cell lines NCI-H146, DMS53, and NCI-H209, the bronchial carcinoid cell line NCI-H727, and the NSCLC cell lines NCI-H1299, A549 (American Type Culture Collection, Manassas, VA), and CALU1 were used for the experiments in this study. RPMI (Invitrogen, Carlsbad, CA) supplemented with 10% FCS was used to maintain NCI-H146, NCI-H727, and NCI-H1299. Waymouth MB 752/1 was used to maintain DMS53 cells (Sigma, St. Louis, MO). Isov’s modified Dulbecco’s medium for NCI-H209 (Invitrogen), McCoy’s 5A-modified medium for CALU1, and nutrient mixture F12 Ham Kight’s modification (F12K) medium for A549 (Sigma). The human kidney epithelial cell line 293T was maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen). The human acute T-cell leukemia cell line Jurkat was used as a positive control for GFI1 expression and was maintained in RPMI supplemented with 10% FCS (Invitrogen). RNA from the NCI60 panel of tumors was provided by the Developmental Therapeutics Program National Cancer Institute, NIH.

Nuclear Extract Preparation and Western Blotting. Nuclear extracts were prepared using a modified procedure from Dignam et al. (30). Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL), and 25 μg of protein extract were separated on 10% SDS-polyacrylamide gel and electroblotted onto an Immobilon-P membrane (Millipore, Billerica, MA). The membranes were blocked with 5% milk in TBS [50 mmol/L Tris HCl, 150 mmol/L NaCl (pH 7.5)] at 4°C for 16 hours. The blocked membranes were incubated with primary antibodies in 5% milk in TBS [0.05% Tween 20 in TBS] between 1 and 4 hours at room temperature. GFI1 was detected using goat polyclonal antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal antibody was used to detect Hes1 (13). The respective secondary antibodies were added donkey antigoat horseradish peroxidase (HRP)-conjugated IgG (Santa Cruz Biotechnology), Sheep antimonoclonal immunoglobulin-HRP, and donkey antirabbit immunoglobulin-HRP (Amer sham, Piscataway, NJ). The secondary antibodies were prepared in 5% milk TTBs and incubated at room temperature for 1 hour. The membranes were washed five times at room temperature, 5 minutes each wash, and the signals were detected using SuperSignal detection reagents according to the manufacturer’s instructions (Pierce).

Table 1 Immunohistochemical reagents and procedures

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Transient Transfections and Luciferase Assays. DMS53 seeded at 3.2 × 10^5 cells/well or NCI-H146 seeded at 2.4 × 10^5 cells per well in a 96-well format were used for the transient transfection procedure. Lipofectamine 2000 transfection reagent (Invitrogen) was used for the transfection procedure per the manufacturer’s instructions. The click beetle luciferase system (Promega, Madison, WI) was used essentially according to the manufacturer’s instructions. The signals were monitored on a Wallac Victor 1420 Multichannel Reader using a 510/60 filter (red) and 610LP filter (green). A t statistic was calculated on the difference between the values of each measurement of luciferase activity to determine statistical significance for fold repression.

Retroviral Transduction and Xenografts. A FLAG-epitope–tagged GFI1 was cloned into the MIEV retroviral vector (31). Retroviral constructs were transfected into Phoenix cells for virus productions (32). NCI-H146 or A549 were infected (33). Cells were tested for green fluorescent protein (GFP) fluorescence by flow cytometric analysis. GFI1 protein expression was assessed by FLAG-HRP (Sigma) and anti-GFI1 2.5D.17 monoclonal antibodies by Western analysis. Cell cycle analysis was performed by the method of Vindelev (34). For each experiment, 5 × 10^6 MEV- or MEV-GFI1-transfected NCI-H146 cells were injected s.c. into five 4 to 6-week-old NCRNU-M male mice (Taconic farms, Germantown, NY) and measured for tumor diameter and volume. No differences were noted between trends for volume or diameter. The experiment was repeated twice with independently transfected cells.

Mapping the Binding Site of the Anti-Growth Factor Independence-1 mAb 2.5D.17. The TGA stop codons of GFI1, SV40SwapGFI1 (35), GFI1B, and SNAIL were mutated to a BamHI restriction endonuclease site by PCR and cloned into the CMV14 vector (Sigma). The constructs were transiently transfected into the human kidney epithelium 293T cell line using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Nuclear extracts were prepared 48 hours after transfection as described above. The protein extracts were quantified (BCA; Pierce), and 15 μg of GFI1 was used for Western analysis. Cell cycle analysis was performed by the method of Vindelev (34). For each experiment, 5 × 10^6 MEV- or MEV-GFI1-transfected NCI-H146 cells were injected s.c. into five 4 to 6-week-old NCRNU-M male mice (Taconic farms, Germantown, NY) and measured for tumor diameter and volume. No differences were noted between trends for volume or diameter. The experiment was repeated twice with independently transfected cells.

Embryo Staging and Tissue Preparation. Gfi1-mutant mice were generated as described previously (24). Embryos were considered to be E 0.5 days on the morning the vaginal plug was observed. To harvest the embryos, pregnant females were sacrificed by cervical dislocation and the embryos dissected out of the uterus. Portions of the yolk sac or tail were saved for genotyping. Embryos were fixed overnight in 4% paraformaldehyde, dehydrated in an ethanol series, and embedded in paraffin for sectioning according to standard histologic protocols. Ten-micron sections were collected and analyzed by in situ hybridization or immunohistochemistry. Lung tissue for postnatal stages was collected by harvesting the lungs of appropriately aged mice, fixing overnight in 4% paraformaldehyde, dehydrating in an ethanol series, and embedding in paraffin for sectioning. Ten-micron sections were collected and analyzed by immunohistochemistry.

Immunohistochemistry on Mouse Tissues. We used antibodies to PGP9.5 (1:2000 dilution; Affiniti Research Products Limited, Plymouth Meeting, PA), GFI1 (1:1000 dilution; BD Pharmingen), CTRP (1:8000 dilution; Sigma), and GFI1 (1:2000 dilution). Guinea pig antisera to murine GFI1 was generated previously with an immunogen encoding murine GFI1 between the SNAG domain and zinc fingers (27). For single antibody localization, primary antibodies were followed with the ABC Vectastain kit with biotinylated secondary antirabbit antibody (goat anti-rabbit) and streptavidin peroxidase (goat anti-streptavidin) (Vector Laboratories, Burlingame, CA). The slides were rinsed, incubated in Vectastain ABC solution, rinsed again, and the signal was detected with 2 mg/mL 3,3′-diaminobenzidine, 0.02% H2 O2 in PBS. Some slides were counterstained with par-1-hematoxylin. The slides were then dehydrated in a series of alcohols and mounted. For colocalization of GFI1 with neuroendocrine markers CTRP and chromogranin A, both biotin and alkaline phosphate-conjugated secondary antibodies were used. The alkaline phosphatase signal detected with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL each in 70% dimethylformamide) followed by methanol treatment. The slides were then mounted. Numbers of neuroendocrine cells are expressed as number of CTRP-positive cells per microscopic field of view. Low-power images of each mouse lung were taken (n = 3 wild-type and 3 null mice), and all positive cells per image were tallied. For each mouse, the total number of positive cells was divided by the total number of images or fields of view (n = 9 to 15) to determine the cells per field of view.

RESULTS

Growth Factor Independence-1 Is Expressed in Neuroendocrine Human Lung Tumor Cell Lines. ASH1 is expressed in human SCLC (12), and the orthologues of ASH1 and GFI1 interact to inhibit Notch signaling in Drosophila peripheral nervous system development (7). To determine whether an interaction between ASH1 and GFI1 may occur in human SCLC, we first examined a panel of human lung tumor cell lines for the expression of ASH1, the Notch effector human enhancer-of-split-1 (HES1), and GFI1. As expected (39), the neuroendocrine SCLC cell lines DMS53, H209, and NCI-H146 and

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Reverse Transcription-PCR. Total RNA extraction was performed using the ULTASPER RNA isolation system (Biotecx Laboratories, Houston, TX), according to the manufacturer’s recommendations. RNA and genomic DNA were extracted from the human Jurkat cell line was used as a control for the reverse transcription-PCR. Total RNA (5 μg) from each sample was DNase treated (Ambion, Austin, TX) and then used for cDNA synthesis with SuperScript II reverse transcriptase kit (Invitrogen). Random hexamer oligos were used for cDNA synthesis per manufacturer’s instructions. PCR reactions were performed for 20 cycles. The cDNA templates were first analyzed for the levels of the D-glyceroldehyde-3-phosphate-dehydrogenase (GAPDH) housekeeping gene. Products of the reaction were analyzed by Southern with 32 P-labeled oligonucleotides internal to the PCR primer sequences. The resulting signal was quantified on a STORM phosphorimager (PE Biosystems, Foster City, CA) and templates were normalized to give roughly equivalent GAPDH signal. The normalized signals were then analyzed for levels of human enhancer-of-split-1 (HES1), ASH1, and GFI1 expression with the following primers: HES1 forward 5′-AGCGCGGACAT-TCTGAAAATG-3′ and HES1 reverse 5′-CGGACTTCCCCAGCACACTT-3′ (58°C annealing temperature), HES1 internal probe 5′-TGTTGCTCAGCAGCA-GCATCTG-3′ (38); ASH1 forward 5′-GGCTGCCGTGACTTCTC-3′ and ASH1 reverse 5′-CTCCCTCTCCCAAAGGCCACT-3′ (59°C annealing temperature), ASH1 internal probe 5′-CCCTGCTTCAAAAGTCCACT-3′ (39); GAPDH forward 5′-CGAGTCAACGGTGTTGAGT-3′ and GAPDH reverse 5′-AGCCCTTCTTACGGTGTTGAGA-3′ (55°C annealing temperature), GAPDH internal probe 5′-GACCCCTTACGGTGTTGAGA-3′ (39); and GFI1 forward 5′-AAGCCTGCTGCAATGATTGAGT-3′ and GFI1 reverse 5′-TTGTTGGCGTGCTGTTGAGT-3′ (60°C annealing temperature), GFI1 internal probe 5′-ACACGGTTACACAGCTT-3′.

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the bronchial carcinoid H727 express ASH1 RNA (Fig. 1A) and protein (Fig. 1B), whereas NSCLC cell lines H1299, A549, and CALU1 lack ASH1 expression (Fig. 1). In contrast, HES1 was expressed in some NSCLC and SCLC cell lines. HES1 is generally absent in cells with a neuroendocrine phenotype (39). We find that HES1 is expressed in DMS53 and at a low level in H209 (Fig. 1). These data confirm previous reports of HES1 expression in neuroendocrine DMS53 cells (40, 41). HES1 in DMS53 cells may not be functional because Notch signaling or HES1 overexpression in these cells induced significant phenotypic changes (41).

We next examined GFI1 expression. The presence of GFI1 RNA (Fig. 1A) and protein (Fig. 1B) was concordant with that of ASH1. NSCLC cell lines Calu1, A549, H1299 (Fig. 1A) and seven other NSCLC cell lines from the NCI60 panel of tumor cell lines (data not shown) lacked GFI1 expression. The pattern of GFI1 expression in SCLC cell lines was determined with both commercial antiserum (N20) and a monoclonal antibody (2.5D.17; Fig. 1), as well as with a guinea pig antiserum (data not shown). The doublet seen in Western analysis of NCI-H209 nuclear extract may represent an alternatively processed form of GFI1 but was not seen with the commercial or guinea pig antiserum. In conclusion, the expression of GFI1 in human lung tumor cell lines was restricted to those with a neuroendocrine phenotype.

**Growth Factor Independence-1 Is a Functional Transcriptional Repressor in Neuroendocrine Human Lung Tumor Cell Lines.**

We next determined the presence of GFI1 DNA-binding activity in human SCLC cell lines. Nuclear extracts from SCLC cell lines DMS53, NCI-H146, and H209 and the NSCLC cell line A549 were evaluated by electrophoretic mobility shift analysis using an oligonucleotide encoding a canonical high-affinity GFI1-binding site (B30; refs. 35, 37). Nuclear extracts from the DMS53 SCLC cell line show multiple complexes that retard the migration of the B30 oligonucleotide, only one of which is unique in comparison to the A549 NSCLC cell line (Fig. 2A, compare Lanes 2 and 12). The presence of GFI1 in

**Fig. 2.** A, electrophoretic mobility shift analysis of nuclear extracts from the A549 and DMS53 cell lines using an oligonucleotide encoding a high-affinity GFI1 binding site B30. Arrows indicate putative GFI1 complexes. B, transient transcription analysis of GFI1 transcriptional repression activity in the indicated SCLC cell lines. Fold repression represents the activity of a control reporter divided by the activity of a GFI1-responsive reporter. The endogenous activity of GFI1 was significantly augmented by cotransfection of GFI1 expression constructs. Results shown are representative of three separate experiments.

C, MIEV and MIEV-GFI1 retroviral-vector transduced NCI-H146 cell line xenograft growth in NCI-nude mice. The average size of each tumor per day for MIEV (\(\ast\)) and MIEV-GFI1 (\(\ast\)) transduced cells was divided by the number of mice with tumors then plotted as a line, whereas a scatter plot illustrates individual measurements used to generate the average. Western analysis (inset) of transduced NCI-H146 SCLC cells. Results shown are the sum of two independent experiments. W = wild type B30 oligonucleotide probe, M = mutant B30 probe, N = N20 anti-GFI1 goat antisera, Cn = Goat polyclonal antiserum, D = 2.5D.17 monoclonal antibody, Cd = Isotype matched control mouse IgG.

**Fig. 1.** Expression of GFI1, ASH1, and HES1 in human lung cancer cell lines. A, Products of the reverse transcription-PCR analysis of the indicated lung cancer cell line RNA were Southern blotted and probed with an internal \(^{32}P\)-radiolabelled oligonucleotide. B, Western analysis of nuclear extracts from the same cell lines using antibodies to ASH1, HES1 and GFI1. GFI1 expression was detected with both commercially available antisera (N20) and a monoclonal antibody (2.5D.17).
these protein complexes was determined by supershift using two different antibodies: a commercially available goat polyclonal anti-serum and a monoclonal antibody (Fig. 2A). Both reagents clearly resulted in retarded migration of the DMS53-unique complex; however, none of the complexes were affected by the addition of respective isotype controls (Fig. 2A). Thus, these complexes appear to contain GFI1 (Fig. 2A, bottom arrow).

GFI1 binds to a consensus 12-bp DNA sequence with an absolute requirement for AATC (37). Mutation of the AATC core of the consensus sequence ablates DNA binding (35, 37). To determine whether the DMS53-unique complex has similar requirements we performed cold competition. Although excess unlabelled B30 oligonucleotides competed with the labeled B30 oligonucleotides for binding to the DMS53-unique complex (Fig. 2A), an excess of unlabelled oligonucleotides mutated to eliminate the AATC core of the GFI1 binding site did not abolish binding to the same complex (Fig. 2A). Moreover, labeled mutant-B30 oligonucleotides displayed a similar binding pattern with NSCLC and SCLC nuclear extracts (Fig. 2A, compare Lanes 10 and 20). Importantly, the DMS53-unique complex was not observed (Fig. 2A, bottom arrow). We note that the mutant B30 oligonucleotide was not bound by a larger complex and that this complex was also decreased but not disrupted by the addition of GFI1-specific antiserum (Fig. 2A, top arrow). Thus, this larger complex depends on an intact AATC sequence and may contain GFI1.

Complexes similar to those found in DMS53 were observed using nuclear extracts from NCI-H146 and H209 SCLC cell lines (data not shown). Thus, human SCLC cell lines contain GFI1-specific DNA-binding activity.

We have previously shown that DNA binding by GFI1 zinc fingers is required to repress transcription of target genes (35). To address the regulatory function of GFI1 in lung tumor cell lines we performed transient transcription assays. The activity of the herpes-simplex-virus minimal-thymidine-kinase promoter was analyzed with and without B30 high-affinity GFI1-binding sites to determine the activity of GFI1. Similar reporters were previously used to monitor the activity of endogenous GFI1 in lymphocytes (35). In SCLC cell lines, the activity of the GFI1 reporter was mildly repressed in comparison to the control reporter activity (Fig. 2B). Much greater differences were noted in T lymphocyte cell lines (35). The discrepancy between lymphoid and SCLC cells in endogenous GFI1 activity could either be due to ~10-fold lower levels of GFI1 in SCLC cell lines (data not shown) or to deficiencies in the transcriptional machinery necessary for GFI1 function. To delineate between these two possibilities, we cotransfected GFI1 expression vectors with both control and GFI1-responsive reporters. Increased transcriptional repression was induced by cotransfection of GFI1 expression vector (Fig. 2B). Thus, the quantity but not the function of GFI1 is lower in SCLC cell lines in comparison to T lymphocytes. Therefore, GFI1 probably functions as a transcriptional repressor in lung cancer cell lines.

**Growth Factor Independence-1 Overexpression Potentiates SCLC Xenografts in NCI Nude Mice.** Given the ability of GFI1 to function as an oncoprotein in T lymphocytes, we next determined the activity of GFI1 in SCLC xenografts. The MIEV retroviral vector transcribes a bicistronic message encoding the gene of interest and an enhanced jellyfish GFP (31). High-titer VSVg-pseudotyped retroviral supernatants were generated and used to transduce the NCI-H146 SCLC cell line with MIEV-GFI1 constructs and empty vector control. Three days later, the cells were analyzed by flow cytometry for GFP fluorescence. In general, ~60% of NCI-H146 cells were transduced (data not shown). Western analysis with either a monoclonal reagent against GFI1 (2.5D.17), or FLAG-specific antiserum illustrate the expression of FLAG-tagged GFI1 in transduced NCI-H146 cells (Fig. 2C, inset). No differences in cell cycle progression were noted (data not shown); however, subtle differences may be occluded by cells that were not transduced. We determined that 10 million NCI-H146 cells generate a 1-cm tumor in NCI nude mice within 45 days. To increase environmental pressures on the xenograft, we restricted the injection of transduced cells to 5 million transduced NCI-H146 cells. Fifteen days after injection, 40% of the mice injected with GFI1-overexpressing NCI-H146 cells began to show signs of an established tumor burden (Fig. 2C). In contrast, empty vector transduced cells did not begin to establish a tumor until day 24 (Fig. 2C). At all points measured, the difference in tumor diameter (Fig. 2C) and volume (data not shown) was greater on average for MIEV-GFI1 tumors. Because retroviral vectors can alter the genome by insertion mutagenesis, we repeated this experiment with independently transduced cells, which generated essentially the same results. In both experiments, the xenografted MIEV-GFI1–transduced NCI-H146 tumors continue to express FLAG-tagged GFI1 (data not shown). Taken together, these data make it unlikely that an insertion mutagenesis event explains the increase in xenograft establishment and growth and indicate that GFI1 has oncogenic activity in nonhematopoietic cells.

** Forced Growth Factor Independence-1 Expression Alters NSCLC Cell Size and Cell Cycle Progression.** To determine whether the oncogenic effect of GFI1 overexpression is singular to SCLC, we examined NSCLC cells. The NSCLC cell line A549 was transduced with MIEV-GFI1 retrovirus and characterized four days later by flow cytometry to determine the extent of infection. An average of 90% showed GFP expression (data not shown) and Western analysis demonstrated the expression of the FLAG-epitope-tagged GFI1 protein (Fig. 3A). However, cell cycle analysis demonstrated a significant G1 arrest (Fig. 3, B and C; P < 0.05). Cell cycle changes were coincident with a reduction in cell size (Fig. 3D) and a failure of the cultures to propagate (data not shown). We repeated this experiment in Calu1 and in A549 by both retroviral transduction and transient transfection of GFI1 expression vectors with similar results (data not shown).

**Growth Factor Independence-1 Expression Correlates with Expression of Neuroendocrine Markers in Primary Human Lung Tumor Specimens.** We have generated a murine monoclonal antibody against GFI1 (2.5D.17). The specificity of this monoclonal reagent was determined using Western and immunohistochemical analyses with peptide competition (Supplementary Fig. 1). These data indicate that the epitope of the 2.5D.17 monoclonal is within the 20-amino acid GFI1 SNAG transcriptional-repression domain. Immunoreactivity with the 2.5D.17 monoclonal may be associated with the neuroendocrine phenotype of these lung tumors. We analyzed GFI1 expression on a tissue array containing 358 primary human lung tumor specimens. Examples of positive Neuroendocrine tumors are shown (Fig. 4A and Supplementary Fig. 2). We noted that the intensity of the stain is greatest in SCLC tumor specimens (Fig. 4A and Supplementary Fig. 2) and that all SCLC examined stained intensely with the 2.5D.17 monoclonal is within the 20-amino acid GFI1 SNAG transcriptional-repression domain.

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detected by immunohistochemistry, showed strong correlations with expression of ASH1 and the neuroendocrine markers chromogranin A, synaptophysin, and gastrin-releasing peptide. Significantly, the majority of tumors positive for ASH1, synaptophysin, chromogranin A, and gastrin-releasing peptide were also positive for GFI1 (Fig. 4C; P < 0.0000001). None of the other markers studied showed a statistically significant relationship to GFI1 staining. Thus, GFI1 is profoundly associated with ASH1 and the neuroendocrine phenotype in primary human lung cancers.

Growth Factor Independence-1 Is Expressed in Murine Pulmonary Neuroendocrine Cells. We previously determined that Gfi1 mRNA is expressed in embryonic mouse lung tissue (27). At E12.5, Gfi1 is expressed in regional patches of cells, but by E15.5, Gfi1 expression is confined to a punctate pattern resembling pulmonary neuroendocrine cells (27). To determine whether Gfi1-expressing cells are indeed pulmonary neuroendocrine cells, we performed immunohistochemical analysis for pulmonary neuroendocrine cell-specific markers. At E18.5, GFI1 can be visualized in specific clusters of pulmonary neuroendocrine cells. Like for ASH1, we detected no change in level or abundance of wild-type and Gfi1−/− mice positive for PGP9.5 (data not shown). However, CGRP expression is a specific marker in lungs for pulmonary neuroendocrine cells. The number of single pulmonary neuroendocrine cells, clusters of pulmonary neuroendocrine cells (NEB), as well as the number of pulmonary neuroendocrine cells per NEB, was significantly reduced in Gfi1−/− mice (Fig. 5, D and E; P < 0.05). As shown in Fig. 5G, immunohistochemical analysis revealed a 3.4-fold reduction in the number of CGRP-positive cells in Gfi1−/− mice as compared with wild type (Fig. 5, F and G; P < 0.0038). Moreover, the cells which stain positively for CGRP were strongly reduced in intensity in E17.5 Gfi1−/− mice compared with littermate controls (Fig. 5F). In addition, the number of cells positive for the pulmonary neuroendocrine cell-specific marker synaptophysin in Gfi1−/− mice was roughly half of that of wild type (data not shown). Other lung epithelial cell types were not affected (data not shown). We therefore propose that GFI1 is not necessary for the specification of pulmonary neuroendocrine cells but is required for their differentiation and proper maturation.

DISCUSSION

Our analysis of GFI1 expression in human lung cancer reveals a previously unknown link between GFI1 and the neuroendocrine phenotype. Nearly all of the neuroendocrine tumors analyzed expressed GFI1, indicating that GFI1 may have an important function in the generation and/or maintenance of the neuroendocrine phenotype. GFI1 expression is not specific to neuroendocrine tumors, as a significant minority of nonneuroendocrine large cell carcinomas express low levels of GFI1. On the other hand, high levels of GFI1 activity may be required for the expression of neuroendocrine-specific genes by other transcription factors. The defects found in Gfi1−/− lungs support this hypothesis. Specifically, the expression of the neuroendocrine-specific genes synaptophysin and CGRP are reduced in Gfi1−/− lungs. GFI1 is therefore required to mediate the normal differentiation of lung cells with a neuroendocrine phenotype.

ASH1 is a proneural bHLH transcription factor that is critically required for the formation of pulmonary neuroendocrine cells (5). ASH1 functions through dimerization with E2A gene products, and these dimers bind to E-box DNA sequences to transactivate target genes.
genes (42). Inhibitors of ASH1 function sequester binding partners or compete for binding to E-box sequences (42). HES1, the downstream effector of Notch signaling, sequesters binding partners of proneural bHLH genes and represses the transcription of both proneural bHLH genes and their transcriptional targets (40). Hence, HES1 is an ASH1 antagonist. Although Ash1<sup>−/−</sup> mice lack pulmonary neuroendocrine cells, in Hes1<sup>−/−</sup> mice the number of ASH1-expressing cells and pulmonary neuroendocrine cells are increased (13). Thus, the level of functional ASH1 is determined by the corresponding levels of antagonists such as HES1.

The very strong statistical correlation between ASH1 and GFI1 expression in lung cancers may indicate a functional relationship. The <i>Drosophila</i> orthologue of GFI1, Sens, functions to antagonize the <i>Drosophila</i> orthologues of HES1 (7, 11). Specifically, Sens directly binds to orthologues of HES1, resulting in the transactivation of genes encoding proneural bHLH factors (11). Although GFI1 does not contain the protein sequences that mediate Sens protein binding to HES1 orthologues, these data indicate that the functional role of Sens in <i>Drosophila</i> development is to reinforce the developmental program induced by proneural bHLH factors. GFI1 may have a similar function in lung epithelium, which could explain the close association between GFI1 and ASH1 expression. Indeed, our tissue array analyses indicate that GFI1 expression not only correlated with that of ASH1 but also with putative transcriptional targets of proneural bHLH factors and thus a neuroendocrine phenotype. Conversely, mice without GFI1 demonstrate reduced expression of neuroendocrine markers but not a reduction in the number of cells expressing ASH1. In a manner similar to the requirement for Sens in <i>Drosophila</i> proneural bHLH protein activity (7), it is thus possible that the optimal expression of neuroendocrine-specific bHLH target genes depends on GFI1 function.

Recently, the GFI1-related protein GFI1B was suggested to have transcriptional activating properties in erythroid lineage cells (43). Moreover, the <i>Drosophila</i> orthologue of GFI1 can directly repress genes or induce transactivation of genes (11). Here, we show that GFI1 in SCLC cell lines functions as a DNA-binding transcriptional repressor. Therefore, lack of GFI1 would be expected to result in the activation of GFI1 target genes. Thus, GFI1 loss may indirectly result in lower expression of synaptophysin and CGRP, perhaps because the bHLH factors are impaired. In fact, the levels of the ASH1 antagonists, Inhibitor of DNA-binding-1 (ID1) and ID2, were recently demonstrated to be dramatically up-regulated in Gfi1<sup>−/−</sup> thymocytes (26). ID1 and ID2 sequester the E2A gene...
products that are required for ASH1 to bind DNA. It is possible that repression of ID1 and ID2 increases the cellular pool of E2A gene products and thus transcriptionally active ASH1. This possibility requires additional investigation.

GFI1 functions as an oncoprotein in human SCLC. In Moloney murine leukemia virus-induced tumors, activation of the Gfi1 locus by proviral insertion is potently selected (23); however, transgenic expression of GFI1 alone is not overtly oncogenic (44). Conversely, transgenic GFI1 rapidly accelerates leukemogenesis induced by other oncoproteins (44). It is therefore likely that the context of GFI1 expression determines GFI1 oncogenic activity.

Here, for the first time, we demonstrate GFI1 oncogenic activity in nonhematopoietic malignancies. Specifically, SCLC tumor xenograft establishment and growth was accelerated by the forced expression of GFI1. GFI1 activity may therefore be limiting for SCLC growth. Interestingly, although GFI1 overexpression is well tolerated in SCLC cell lines, it impaired growth of NSCLC cell lines. Indeed, the effect of GFI1 overexpression in NSCLC cell lines is similar to the effect of activated Notch expression in SCLC cell lines (45). In human SCLC cell lines, antisense oligonucleotides targeting ASH1 or activated Notch signaling leads to lower ASH1 levels, loss of neuroendocrine target gene expression, and subsequent cell cycle arrest (5, 41). Thus, SCLC may rely on proneural bHLH factors such as ASH1 for autocrine and paracrine stimulation by neuroendocrine growth factors. If GFI1 functions in a manner similar to Sens, it would reinforce the action of bHLH factors and thus provide growth factor support for SCLC tumors. Indeed, the level of GFI1 immunohistochonanical stain was intense in all SCLC tumor samples examined. This was true in three separate laboratories and a total of 18 SCLC specimens (data not shown). Thus, unlike NSCLC tumors, neuroendocrine tumors may provide an epithelial cellular context in which GFI1 functions as an oncoprotein. By analogy to Sens, we would predict that GFI1 action reinforces the activity of proneural bHLH transcription factors that are essential for the expression of neuroendocrine growth factors and therefore autocrine and paracrine stimulation of the tumor. As such, GFI1 may provide a new molecular target for the detection, diagnosis, and treatment of neuroendocrine tumors of the lung.
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