Nuclear Factor I Represses the Notch Effector HEY1 in Glioblastoma

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Abstract
Glioblastomas (GBMs) are highly aggressive brain tumors with a dismal prognosis. Nuclear factor I (NFI) is a family of transcription factors that controls glial cell differentiation in the developing central nervous system. NFIs have previously been shown to regulate the expression of astrocyte markers such as glial fibrillary acidic protein (GFAP) in both normal brain and GBM cells. We used chromatin immunoprecipitation (ChIP)—on-chip to identify additional NFI targets in GBM cells. Analysis of our ChIP data revealed ~400 putative NFI target genes including an effector of the Notch signaling pathway, HEY1, implicated in the maintenance of neural stem cells. All four NFIs (NFIA, NFIB, NFIC, and NFIX) bind to NFI recognition sites located within 1 kb upstream of the HEY1 transcription site. We further showed that NFI negatively regulates HEY1 expression, with knockdown of all four NFIs in GBM cells resulting in increased HEY1 RNA levels. HEY1 knockdown in GBM cells decreased cell proliferation, increased cell migration, and decreased neurosphere formation. Finally, we found a general correlation between elevated levels of HEY1 and expression of the brain neural stem/progenitor cell marker B-FABP in GBM cell lines. Knockdown of HEY1 resulted in an increase in the RNA levels of the GFAP astrocyte differentiation marker. Overall, our data indicate that HEY1 is negatively regulated by NFI family members and is associated with increased proliferation, decreased migration, and increased stem cell properties in GBM cells.

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Introduction
Glioblastomas (GBMs) (or grade IV astrocytomas) are the most common brain tumors in adults [1,2]. Despite aggressive treatment involving surgical resection, radiotherapy, and adjuvant chemotherapy with temozolomide, the median survival for GBM patients is approximately 15 months [3–5]. These tumors are highly infiltrative, resulting in high rates of recurrence and treatment failure [6].

The Nuclear Factor I (NFI) family of transcription factors regulates the expression of the brain fatty acid–binding protein (B-FABP or FABP7) and glial fibrillary acidic protein (GFAP) genes in GBM [7]. The four members of the NFI family (NFIA, B, C, and X) bind to the consensus NFI recognition element 5’-TTGGCA(N2)GCCAA-3’ as homodimers or heterodimers [8–10]. The N-terminal DNA binding and dimerization domain of all four NFI family members is highly conserved; however, the C-terminal domain is more divergent, resulting in variation in transactivation potential [11]. NFIs can both activate or repress transcription, with regulation of transcription being dependent on both promoter context and type of cell or tissue in which the NFIs are expressed [12].

NFI recognition sites are enriched in many brain-specific promoters [13], and NFIs are important regulators of gliogenesis and astrocyte differentiation in the developing central nervous system [14–16]. In particular, NFIA and NFIB are necessary for the onset of gliogenesis downstream of Notch signaling [15,17]. Following glial fate specification, these two NFIs along with NFIX further promote

Abbreviations: APCDD1, adenomatosis polyposis coli downregulated 1; AP2, activating protein 2; bHLH, basic helix-loop-helix; B-FABP, brain fatty acid-binding protein; CoRE, composite response element; EZH2, enhancer of zeste homolog 2; GBM, glioblastoma; GO, gene ontology; GFAP, glial fibrillary acidic protein; HES1, hairy and enhancer of split-1; HEY1, Hes related family BHLH transcription factor with YRPW motif 1; MMD2, monocyte to macrophage differentiation associated 2; MMTV, mouse mammary tumor virus; NEFL, neurofilament light; NFI, nuclear factor I; PAX-6, paired box protein-6; PET, polyethylene terephthalate; SPARC, SPARC like protein-1; WAP, whey acidic protein gene.

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astrocyte differentiation [14, 16, 18–20]. Nfia−/−, Nfib−−, and Nfix−/− mice all display delayed neuronal and glial cell differentiation in the brain [21–27]. Reduced NFIA mRNA levels are associated with high-grade astrocyomas, with 91%, 77%, 48%, and 37% of cells expressing NFIA in grades I, II, III, and IV astrocyomas, respectively [28, 29]. NFIA is enriched in astrocyomas compared to other tumors, with fewer than 5% of cells expressing NFIA in oligodendrogliomas [28]. Furthermore, ectopic expression of NFIA in an oligodendroglioma model promotes conversion to an astrocytoma-like phenotype [19]. Low NFI B mRNA levels are also associated with high-grade astrocyomas, with elevated levels of NFI B RNA correlating with better overall and recurrence-free survival in GBM [30]. NFI B overexpression induces cell differentiation and inhibits GBM tumor growth [30].

To gain insight into the role of NFI in GBM, we carried out chromatin immunoprecipitation (ChIP)—on-chip using a pan-specific NFI antibody to immunoprecipitate NFIs bound to their target genes in U251 GBM cells. A total of 403 NFI target genes were identified, including HEY1, a Notch effector gene. Notch signaling has previously been implicated in regulation of tumor progression in GBM [31–33]. HEY1 is a member of the Hairy/Enhancer of split (E/ spl) family of basic helix-loop-helix transcription factors and is important for maintenance of neural precursor cells downstream of Notch [34]. HEY1 expression increases with increasing astrocytoma tumor grade and correlates with decreased overall survival and disease-free survival [35]. Here, we show that NFI binds to three NFI recognition elements in the HEY1 promoter and negatively regulates HEY1 in GBM cells. Depletion of HEY1 in adherent and neurosphere GBM cultures results in decreased cell proliferation, increased migration, and decreased neurosphere formation. These results suggest a fine balance between levels of NFI transcription factors and the Notch effector HEY1 in GBM, thereby allowing these tumors to express some astrocytic properties while retaining neural stem cell characteristics.

Materials and Methods

Cell Lines, Constructs, siRNAs, and Transfections

The established human GBM cell lines used in this study have been previously described [36, 37]. Cells were cultured in Dulbecco’s modification of Eagle’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum, penicillin (50 U/ml), and streptomycin (50 μg/ml). The primary GBM cultures (A4-004, A4-007, ED512) were prepared by enzymatic dissociation of GBM biopsies obtained with patient consent prior to surgery. A4-004 and A4-007 adherent lines were generated by culturing cells directly in DMEM supplemented with 10% fetal calf serum. GBM tumor neurosphere cultures were generated by plating cells directly in DMEM/F12, supplemented with B27, epidermal growth factor, and fibroblast growth factor. All procedures involving tumor biopsies were approved by the Health Research Ethics Board of Alberta Cancer Committee Protocol #HIREBA.CC-14-0070.

The pCH-NFI expression vectors pCH, pCH-NFIA, pCH-NFIB, pCH-NFIC, and pCH-NFIX were obtained from Dr. R. Gronostajski (State University of New York at Buffalo). The luciferase reporter gene construct was prepared by inserting the 5′ HEY1 flanking DNA from −913 bp to +15 bp into the pGL3-Basic vector (Promega). Stealth siRNAs (Life Technologies) were used to knock down NFIA, NFIB, NFIC, NFIX, and HEY1: NM_005595_6 stealth_919 targeting 5′-GAAAGUUCUCAUA-CUA-CAG-CGUA-3′(NFIA); NM_005596_6 stealth_1020 targeting 5′-AAGCCCAAGUA-UCGUGGCAAAU-3′(NFIB); NM_005597_ stealth_1045 targeting 5′-CAGAGA-GGACAA-GUCA-CUCA-3′(NFIC); NM_002501_ stealth_752 targeting 5′-UGAGGACGA-CACUUGUGGCA-3′(NFI); NM_012258_3 stealth_284 targeting 5′-UUGAGAUCCGGAAC-CAUGUCACUCC-3′(HEY siRNA 2). Scrambled siRNAs (cat. nos. 12935-200 and 12935-300) were used as negative controls. The Stealth siRNAs selected for NFI knockdown have been previously characterized [36].

U251 GBM cells were transfected with plasmid DNA constructs using polyethylenimine (Polysciences Inc.). For knockdown experiments, cells were transfected with 10 nM siRNAs using RNAiMAX-Lipofectamine (Life Technologies). For co-transfection experiments, cells were transfected first with siRNA followed by plasmid transfection 24 hours later. Cells were harvested 60 hours after the last transfection. For 2x transfections with siRNAs, cells were transfected, grown to confluency, replated at 1/7 dilution, and transfected again.

ChIP-on-chip

ChIP to isolate NFI-bound DNA was carried out following Agilent’s mammalian ChIP-on-chip protocol version 10.0. Briefly, −8 × 10^8 U251 GBM cells were cross-linked with 1% formaldehyde for 12 minutes at room temperature, followed by addition of glycerine to 0.125 M to terminate the cross-linking reaction. After cell lysis, nuclei were sonicated 30 × 30 seconds at 30% output (model 300VT, Ultrasonic Homogenizer, BioLogics, Inc.), and Triton X-100 was added to a final concentration of 1%. Cellular debris was removed by centrifugation, and 50 μl of the lysate was frozen at −20°C for input DNA (nonenriched control). The remaining lysate was precleared with Protein-A Sepharose beads (GE Healthcare). The precleared lysate was incubated with 3 μg anti-NFI antibody (N-20 Santa Cruz Biotechnology) and incubated at 4°C for 16 hours. Protein-A Sepharose beads were added and incubated for 2 hours at 4°C. Beads were washed 7x in wash buffer (50 mM Heps–KOH, 500 mM LiCl, 1 mM EDTA, 1% Nonidet-P40, 0.7% sodium deoxycholate) and 1x in TE with 50 mM NaCl at 4°C. Protein-DNA complexes were eluted in elution buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% SDS) at 65°C for 15 minutes.

Linkers (5′-GGGTTGACCGCGAGATCTGAACT-3′, and 5′-GAATTCAGATC-3′) were prepared by annealing at 70°C for 1 minute and cooling slowly to 4°C. Input and ChIP DNAs were amplified by LM-PCR. PCRs containing input or ChIP DNAs, 1x Thermopol buffer (NEB), 250 μM dNTPs, 1 μM LM-PCR primer 5′-GGGTTGACCGCGAGATCTGAACT-3′, and 0.25 U Taq polymerase were carried out as follows: 55°C/4 min, 72°C/3 min, 95°C/2 min, (95°C/30 s, 60°C/30 s, 72°C/1 min) × 15, 72°C/5 min. One hundredth of the resulting PCR products was used in a second round of PCR amplification as described above for 25 cycles. The PCR products were precipitated with ethanol, resuspended in sterile H2O, and diluted to 100 ng/μl.

Input and ChIP DNAs were fluorescently labeled with Agilent Genomic DNA Labeling Kit PLUS (Agilent Technologies). For each reaction, 2 μg input or ChIP DNA was incubated with 5 μl random
primers, 1x buffer, 1x dNTPs, 3 μl 1.0 mM Cyanine 3-dUTP (Cy3) (input DNA) or 3 μl 1.0 mM Cyanine 5-dUTP (Cy5) (ChIP DNA), and 1 μl Exo-Klenow DNA polymerase fragment in a final volume of 50 μl and incubated at 37°C for 2 hours followed by 10-minute incubation at 65°C to inactivate the enzyme. For hybridization, 5 μg Cy3-labeled DNA, 5 μg Cy5-labeled DNA, 50 μg Human Cot1, 1x Agilent blocking agent, and 1x Agilent hybridization buffer per slide were heated for 3 minutes at 95°C followed by incubation at 37°C for 30 minutes and then applied to the Agilent Human Promoter 1 ChIP-on-chip 244K 014706 and 014797 microarray sets (Agilent Technologies) (two independent experiments). Slides were hybridized with shaking (20 RPM) in a hybridization oven at 65°C for 40 hours. The slides were then washed 1x with Oligo aCGH/ChIP-on-chip wash buffer (Agilent Technologies) at room temperature and 1x with Oligo aCGH/ChIP-on-chip wash buffer at 31°C. Slides were scanned on a GenePix 4000B scanner, and data were extracted using Agilent Feature Extraction Software (Agilent Technologies). Data were analyzed using Agilent Genomic Workbench (Agilent Technologies).

**ChIP-PCR**

ChIP-PCR analysis was carried out as previously described [38]. Briefly, U251 cells cross-linked with 1% formaldehyde were resuspended in lysis buffer and sonicated to shear the DNA. Precleared lysates were incubated with either 2 μg IgG or 2 μg anti-NFI antibody (N-20 Santa Cruz Biotechnology), followed by incubation with Protein A-Sepharose beads. Protein-DNA complexes were eluted, and the DNA was amplified using primers flanking putative NFI binding sites located upstream of the HEY1 transcription start site (+1). Primer sequences flanking the −488 to −216 bp region contained two putative NFI binding sites, at −332 to −317 bp and −411 to −396 bp, and primers flanking the −822 to −628 bp region contained one putative NFI binding site, at −794 to −779 bp. The GAPDH promoter was used as the negative control. Input DNA was obtained from cells lysed after the sonication step.

**Electrophoretic Mobility Shift Assay (EMSA)**

EMSAs were carried out as previously described [39]. Putative NFI binding sequences in the HEY1 promoter are listed in Figure 1A. Complementary oligonucleotides (Figure 2B) were annealed and radiolabeled by Klenow polymerase in the presence of α32P-deoxyctydine triphosphate. Oligonucleotides containing mutated NFI binding sites were generated by substituting AA for the conserved GG at positions 3 and 4 of the NFI consensus binding site (Figure 2A). Nuclear extracts were prepared from untransfected U251 GBM cells as described previously [40], and nuclear extracts from U251 GBM cells transfected with pCH, pCH-NFIA, pCH-NFIB, pCH-NFIC, and pCH-NFIX were prepared using the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies). Nuclear extracts (5 μg for untransfected U251 GBM cells, 2 μg for pCH-transfected cells, 3 μg for pCH-NFIA-transfected cells, 4 μg for pCH-NFIB-transfected cells, 1 μg for pCH-NFIC-transfected cells, and 2 μg for pCH-NFIX-transfected cells) were preincubated in binding buffer (20 mM Hapes pH 7.9, 20 mM KCl, 1 mM spermidine, 10 mM dithiothreitol, 10% glycerol, 0.1% Nonident P-40) in the presence of 1.25 μg poly(dl-dC) for 10 minutes at room temperature. Where indicated, a 100x molar excess of competitor oligonucleotide was included during preincubation. Radiolabeled oligonucleotides were added to the reaction mixture and incubated for 20 minutes at room temperature.

![Figure 1](image-url)

**Figure 1.** NFI binds to the HEY1 promoter in vivo. (A) Location of consensus NFI binding sites and putative NFI binding sequences identified upstream of the HEY1 transcription start site (+1). (B) Chromatin immunoprecipitation analysis showing NFI binding to the HEY1 promoter. DNA cross-linked to protein in U251 cells was immunoprecipitated with a pan-specific NFI antibody followed by PCR amplification. Rabbit IgG antibody and GAPDH primers were used as negative controls.
antibody (1:200). rabbit Pax6 complexes (Biotech) and (Sigma) extracts was
Western
For
Membranes
Nuclear
Competitors
-32* 5’ CTG GAG Taa CCG CCG CCG CTC TC 3’
3’ C AIA GCC GGG GGG GGG AAC C 5’
-332* 5’ CCG CCG Taa CCG CCG CCG CCG AGG C 3’
3’ GGG ACC GGG CCG CCG TGC G GTT A 5’
-411* 5’ CCG GAT Taa CCG GCG GCG GCG 3’
3’ CTA AIA GAC CCG CCG CCG CCG G 5’
-794* 5’ GCC CCT aag CTG GTG GCG A 3’
3’ GGA TAC GAC CAG CCG TGA CAC 5’

Figure 2. Binding of NFI to putative NFI binding sequences in the HEY1 promoter. (A) Primers used to generate oligonucleotides for the electrophoretic mobility shift assay, with putative NFI binding sequences in bold. The third and fourth residues in the NFI binding sequences were mutated from GG → AA. These residues are critical for NFI binding. (B) Electrophoretic mobility shift assays were carried out by incubating radiolabeled probes –32 bp, –332 bp, –411 bp, and –794 bp with 3 μg U251 GBM nuclear extracts. DNA-protein complexes were electrophoresed through a 6% polyacrylamide gel buffered in 0.5× TBE. Where indicated, a 100× molar excess of competitors (* denotes mutated NFI binding site) was added to the binding reaction. Where indicated, antibodies (1 μl) to NFI (α-NFI), Pax6 (α-Pax6), or AP2 (α-AP2) were added immediately before the radiolabeled probes.

(a)

(b)

Probes

Competitors

-32 5’ CTG GAG TTG CCG CCG CTC TC 3’
3’ C AAC GCC GGG GGG GGG AAC C 5’
-332 5’ CCG CCG TTG CCG CCG CCG AGG C 3’
3’ GGG ACC GGG CCG CCG TCC G GTT A 5’
-411 5’ CCG GAT TTG CCG GCG GCG GCG 3’
3’ CTA AIA GAC CCG CCG CCG CCG G 5’
-794 5’ GCC CCT GGG CTG GTG GCG A 3’
3’ GGA TAC GAC CAG CCG TGA CAC 5’

Probe

Nuclear Extract

Competitor / Antibody

-32

-332

-411

-794

-32

-332

-411

-794

-32

-332

-411

-794

-32

-332

-411

-794

For supershift experiments, 1 μl anti-NFI antibody (a gift from Dr. N. Tanese, New York University Medical Center), 1 μl anti-AP2 antibody (negative control) (Santa Cruz Biotechnology), or 1 μl anti-Pax6 (negative control) (Developmental Studies Hybridoma Bank) was added with the radiolabeled oligonucleotides. DNA-protein complexes were electrophoresed in 6% native polyacrylamide gels in 0.5× TBE buffer and exposed to film.

Western Blot Analysis

Nuclear extracts were prepared using Thermo-Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Life Technologies). Nuclear extracts were electrophoresed through 8% polyacrylamide-SDS gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were immunostained with mouse anti-HA antibody (Sigma) (1:10,000), rabbit anti-DDX1 antibody (1:5000 [41], or rabbit anti-HEY1 antibody (ARP32512, Aviva Systems Biology) (1:200). Primary antibodies were detected with horseradish peroxidase–conjugated secondary antibodies (Jackson ImmunoResearch Biotech) using Immobilon (EMD Millipore).

Quantitative Real Time-PCR (qPCR)

Total RNA was isolated from GBM cells using the RNAeasy Plus Kit (Qiagen), and cDNA was synthesized with Superscript II reverse transcriptase (Life Technologies). qPCR was carried out using an ABI 7900HT Fast Real-Time PCR System, with gene-specific oligonucleotides labeled at the 5’ end with the fluorescent reporter dye FAM (NFIA, Hs00325656_m1; NFIB, Hs00232149_m1; NFIC, Hs00907819_m1; NFIX, Hs00958849_m1; GFAP, Hs00157674_m1; B-FABP, Hs00361426_m1; NES, Hs04187831_g1: HEY1, Hs01114113_m1; GAPDH, Hs99999905_m1) and Taqman Fast Master Mix (Life Technologies). All samples were assayed in triplicate, and gene expression was normalized to GAPDH. Experiments were repeated three times.

Reporter Gene Assay

U251 GBM cells were cultured in 12-well cell culture plates. Following transfection, cells were harvested in 250 μl of 1× Luciferase Cell Culture Lysis Buffer (Promega) and stored at –80°C. Luciferase activity was measured in 20-μl aliquots of lysate following addition of 100 μl of Luciferase Assay Reagent (Promega) using a FLUOstar Optima microplate reader (BMG Labtech).
**Cells**

**Cell Proliferation Assay**

U251 GBM cells cultured under standard conditions (DMEM supplemented with 10% FCS) and A4-004 GBM cells cultured under neurosphere conditions were transfected with scrambled or HEY1 siRNAs. Forty-eight hours later, transfected cells were seeded in triplicate (30,000 cells per well) in a 12-well plate. Cell growth was measured by counting the cells in triplicate wells every 24 hours for a period of 96 hours using a Coulter Particle and Size Analyzer (Coulter Corporation). Data from three independent experiments were averaged and plotted on a graph.

**Scratch assay**

U251 and A4-004 cells were cultured and transfected with either scrambled or HEY1 siRNAs as described for the cell proliferation assay. Cells were seeded in triplicate in 12-well plates 48 hours posttransfection. Cells were allowed to form a monolayer, at which time a scratch was made in the center of the wells using a P20 pipette tip. Cells were cultured for an additional 24 hours (A4-004) or 30 hours (U251). Digital imaging microscopy (Axiovert 200M, Zeiss) was used to image the cells at two separate positions in each well using a phase contrast lens at 10× magnification (six positions in total for triplicate wells). Metamorph imaging software (Version 7.8.8.0, Molecular Devices) was used to capture a total of 97 images at each position at 15-minute intervals over a period of 24 or 30 hours. TScratch software was used to analyze the images. The percentage open area of the scratch at different time points was measured. The open area of each scratch at 0 hour was normalized to 100% to nullify the effects of minor differences in the initial scratch size in different wells. The open area at subsequent time points is represented relative to their respective 0-hour time point. Three independent experiments were carried out for each cell line.

**Transwell Migration Assay**

U251 and A4-004 cells were cultured and transfected with either scrambled or HEY1 siRNAs as described for the cell proliferation assay. Directional cell migration was measured using the Transwell cell migration assay. Twenty-five thousand cells in DMEM containing 1% fetal calf serum were seeded in the top chambers of 24-well cell culture Transwell inserts (Falcon Cell Culture Inserts). Cells were allowed to migrate through an 8-μm polyethylene terephthalate (PET) membrane towards a chemoattractant (DMEM +10% fetal calf serum) in the bottom chamber for 20 hours. Cells were then fixed with 100% cold methanol for 20 minutes and stained with 1% crystal violet in 20% methanol for 30 minutes at room temperature. Migrated cells were imaged using a Zeiss Axioskop2 plus microscope by capturing different fields of view. Cell counting was carried out using Meta express imaging software. Three independent experiments were carried out for each cell line tested.

**Neurosphere Formation Assay**

Either 200 or 1000 cells were seeded in triplicate in a 24-well low attachment plate (Corning). Cells were allowed to form spheres for a period of 10 days. Digital imaging microscopy (Axiovert 200M, Zeiss) was used to image the spheres using a phase contrast lens at 10× magnification. Total area of all the spheres in each well was calculated for each treatment using Meta express imaging software. Experiments were repeated three times.

**Statistical Analysis**

ChIP-on-chip results from two microarray sets were analyzed using ChIP Analytics software (Agilent Technologies). Identification of putative NFI targets was based on the following parameters: enriched binding to NFI (compared to IgG control) based on a cutoff of Log (2) ratio >0.85 (enrichment of 1.8x) (P < .01). All other experiments were done in triplicate (technical replicated) and were repeated three times (biological replicates). The data shown in the graphs represent an average of all three independent experiments. The statistical significance between two treatments was calculated using an unpaired t test.

**Results**

**ChIP-on-chip of NFI Binding Regions in GBM Cells**

To identify NFI target genes in GBM cells, U251 cells were treated with 1% formaldehyde to cross-link DNA to proteins. Cell lysates were prepared and sonicated to shear the DNA into fragments of ~500 bp. A pan-specific NFI antibody was used to pull down NFIls bound to DNA. This NFI-bound DNA was hybridized to two Agilent Human Promoter 1 arrays (Agilent Technologies) containing probes from ~5.5 kb upstream to +2.5 kb downstream from the transcription start site of ~17,000 RefSeq genes. The data were analyzed with ChIP Analytics software (Agilent Technologies), resulting in the identification of 403 genes with enriched NFI binding based on a cutoff of log (2) ratio >0.85 (enrichment of 1.8-fold) (P < .01) (Supplementary Table S1). The list includes previously identified NFI target genes including *GFAP*, *CDKN1A*, and *NEFL* (neurofilament light) [13].

Gene ontology (GO) enrichment analysis (GO biological process complete annotation data set, 27,378 terms) of NFI putative target genes revealed enrichment in several developmental processes, including system development, organ morphogenesis, differentiation, and specifically cardiovascular, skeletal, and neuronal development (Supplementary Table S2) [45,46]. NFI target genes were also enriched in the category of genes involved in regulation of gene expression, both positive and negative, and transcription from RNA pol II promoters (Supplementary Table S2), suggesting that NFI itself may regulate other transcription factors. In addition, GO enrichment

<table>
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<td>1.94</td>
<td>4.70E-02</td>
</tr>
</tbody>
</table>

GO analysis of putative NFI target genes was carried out. The GO terms represent the biological processes involved.
analysis using the PANTHER GO-slim Biological Process annotation data set, which contains 257 biological process terms, clearly highlights enrichment in development, specifically nervous system development (Table 1) [47].

**Binding of NFI to the HEY1 Promoter**

Of the 403 putative NFI binding regions identified by ChIP-on-chip, 221 were in the promoter regions of genes. One of the putative NFI target genes, **HEY1**, was of particular interest because of its role as a Notch effector gene [48]. HEY1 has previously been shown to be important for maintenance of neural precursor cells [34] and is highly expressed in GBM tumors compared to normal brain [35].

ChIP analysis showed enriched binding of NFI to a microchip probe corresponding to the region upstream of the **HEY1** transcription start site. Sequence analysis of the **HEY1** promoter region from −1100 bp to +1 revealed four putative NFI binding sites located at −32 to −17 bp, −332 to −317 bp, −411 to −396 bp, and −794 to −779 bp (Figure 1A). Of note, the region spanning −30 to −247 bp upstream of the mouse **Hey1** transcription start site has previously been reported to be essential for basal **Hey1** transcription, with additional regulatory sequences located between −247 and −647 bp in mouse (with −647 bp corresponding to −680 bp in human) [49].

To confirm the ChIP-on-chip results, we carried out ChIP analysis in U251 GBM cells using primers corresponding to two regions of the **HEY1** promoter: −216 to −488 bp containing two putative NFI binding sites and −628 to −822 bp containing one putative NFI binding site. DNA cross-linked to NFI in U251 cells was immunoprecipitated with a pan-specific NFI antibody and amplified by PCR. Rabbit IgG and primers to the **GAPDH** promoter were used as negative controls for the ChIP experiments. Bands corresponding to the **HEY1** promoter between −488 to −216 bp and −822 to −628 bp were clearly detected and enriched following immunoprecipitation with an NFI antibody compared to rabbit IgG (Figure 1B). No bands were detected in either the IgG or NFI IP lanes when primers to the **GAPDH** promoter were used.

**Binding of NFI to NFI Recognition Sequences in the HEY1 Promoter**

We used the EMSA to examine NFI binding to the four putative NFI recognition sites (at −32 bp, −332 bp, −411 bp, and −794 bp) located upstream of the **HEY** gene. Double-stranded oligonucleotides

![Figure 3](image-url)

**Figure 3.** Binding of NFIA, NFIB, NFIC, and NFIX to NFI binding sites in the **HEY1** promoter. Nuclear extracts were prepared from U251 GBM cells transfected with control (pCH), NFIA (pCH-NFIA), NFIB (pCH-NFIB), NFIC (pCH-NFIC), or NFIX (pCH-NFIX) expression constructs. (A) Western blot analysis of transfected cells. Nuclear extracts (20 μg) were electrophoresed through an 8% polyacrylamide-SDS gel, electroblotted onto PVDF membranes, and immunostained with α-HA antibody or α-DDX1 antibody. (B) Electrophoretic mobility shift assays were performed with the indicated radiolabeled probes: −32 bp, −332 bp, −411 bp, and −794 bp. Probes were incubated with the indicated nuclear extracts (2 μg pCH, 3 μg NFIA, 4 μg NFIB, 1 μg NFIC, and 2 μg NFIX). Amounts of protein were adjusted to compensate for differences in expression of transfected HA-NFIs. DNA-protein complexes were electrophoresed through a 6% polyacrylamide gel buffered in 0.5× TBE.
Figure 4. Regulation of HEY1 promoter activity by NFI. U251 GBM cells were transfected with 10 nM siRNAs, including control (scrambled), NFIA, NFIB, NFIC, NFIX, or combinations of NFI siRNAs. Where indicated (2×), cells underwent two rounds of siRNA transfection. (A) NFIA, NFIB, NFIC, NFIX, and (B) HEY1 mRNA expression was analyzed by qPCR. GAPDH was used as an endogenous control. Similar data were obtained in two separate experiments. (C) U251 GBM cells were transfected with 10 nM siRNAs, including control (scrambled), NFIA, NFIB, NFIC, NFIX, or combinations of NFI siRNAs, followed 24 hours later by transfection with pGL3/HEY1. Cells were harvested 60 hours later, and luciferase activity was quantified. Changes in relative light units (RLU) are relative to RLU obtained in U251 GBM cells transfected with control (scrambled) siRNA and pGL3/HEY1. The data are from three experiments. SEM is indicated by error bars. Statistical significance, determined using the unpaired t test, is indicated by * (P < .05) and ** (P < .01).
(Figure 2A) corresponding to each putative recognition site were radiolabeled and incubated with nuclear extracts prepared from U251 GBM cells. To address specificity of binding, a 100×-fold molar excess of unlabeled oligonucleotides was used as a competitor. Competitor oligonucleotides included wild-type −32 bp, −332 bp, −411 bp, −794 bp, and mutated −32*, −332*, −411*, −794* bp NFI recognition sites, and the NFI consensus recognition site (Figure 2A).

Two strong and one weak DNA-protein complexes were observed when the −32 bp probe was incubated with nuclear extracts from U251 GBM cells, and one major DNA-protein complex was observed upon incubation of these nuclear extracts with the −332 bp, −411 bp, and −794 bp probes (Figure 2B). Incubation with excess mutated −32* bp oligonucleotide (two key NFI binding residues mutated) resulted in complete loss of shifted bands, indicating that the DNA-protein
complexes observed with the −32 bp probe do not involve NFI binding. These data are further supported by the inability of excess NFI consensus binding site oligonucleotide to serve as competitor for the three DNA-protein complexes observed with the −32 bp probe.

In contrast to the −32 bp probe, addition of excess wild-type competitor oligonucleotides abolished binding to the −332 bp, −411 bp, and −794 bp probes, while addition of excess NFI consensus oligonucleotide significantly reduced the signal intensity of the DNA-protein complexes (Figure 2B). Addition of excess −332 bp oligonucleotide did not significantly affect binding to the radiolabeled −332 bp probe, whereas addition of excess −411 bp and −794 bp oligonucleotides resulted in significant and slight reductions in binding, respectively.

To determine if the observed DNA-protein complexes contain NFI, we incubated the radiolabeled probes with nuclear extracts from U251 GBM cells and an anti-NFI antibody that has previously been shown to supershift NFI-DNA complexes [7,36]. Addition of the anti-NFI antibody resulted in a supershifted band for the −332 bp, −411 bp, and −794 bp probes but not the −32 bp probe (Figure 2B). The relatively weak intensity of the supershifted bands observed with the anti-NFI antibody, combined with the significant decrease in intensity of the DNA-protein complexes, suggests that the anti-NFI antibody impedes binding of NFI to these probes. Alternatively, the weak supershift could be due to the relatively low levels of NFI in U251 cells [7], with the shifted band consisting primarily of non-NFI proteins. Anti-Pax6 and anti-AP2 antibodies had no effect on the protein-DNA complexes regardless of the probe used.

As there are four NFIs, we next asked whether specific members of the NFI family can preferentially bind to the NFI recognition motifs upstream of the HEY1 transcription start site. To do this experiment, U251 GBM cells were transfected with pCH (empty vector), HAtagged NFIA, HA-NFIB, HA-NFIC, or HA-NFIX expression constructs. Nuclear extracts were prepared, and expression of NFIs was analyzed by Western blot. NFIC levels were the highest in the transfected cells, followed by NFIX, NFIA, and NFIB (Figure 3A). To correct for differences in expression levels, we incubated 1 μg of NFIC nuclear extract, 2 μg NFIX nuclear extract, 3 μg NFIA nuclear extract, and 4 μg of NFIB nuclear extract with radiolabeled −32 bp, −332 bp, −411 bp, and −794 bp oligonucleotides. As expected, no DNA-protein complexes were observed with the −32 bp oligonucleotide, indicating that NFIs do not bind to this region.

NFIA, NFIB, NFIC, and NFIX all formed complexes with the −332 bp, −411 bp, and −794 bp oligonucleotides (Figure 3B). Bands of similar intensities were observed when nuclear extracts prepared from each of the four HA-NFI transfected cells were incubated with the −332 bp probe. Similar results were obtained with the −794 bp probe except that band intensities were reduced in the NFIA and NFIB lanes compared to NFIC and NFIX (Figure 3B). In contrast, the only nuclear extract that generated a strong signal when incubated with the −411 bp probe was from HA-NFIX-transfected cells, with only weak bands observed with HA-NFIA and HA-NFIB-transfected cells. Taken together, these results indicate that all four NFIs can bind, albeit with different affinities, to the −332 bp, −411 bp, and −794 bp probes, with NFIA and NFIB showing a relative preference for the −332 bp probe, NFIX showing no preference for any of the three probes, and NFIC showing preference for the −332 bp and −794 bp probes.

Repression of HEY1 Expression and Promoter Activity by NFI

Our combined ChIP and gel shift experiments indicate that NFIs bind to three distinct regions in the HEY1 promoter, suggesting a role for NFIs in the regulation of HEY1 expression. We therefore examined whether changes in NFI levels can affect endogenous HEY1 mRNA levels. U251 GBM cells were transfected with control (scrambled) siRNAs, or siRNAs targeting specific NFIs, alone or in combination. Previously validated NFI siRNAs [36] were used for these analyses, resulting in 75%-93% decreases in NFIA, NFIB, NFIC, and NFIX mRNA levels after one round of transfection (Figure 4A). Endogenous levels of HEY1 mRNA were not significantly altered upon knockdown of single NFIs; however, when all four NFIs were depleted, we observed a 2.4-fold increase in HEY1 mRNA levels (Figure 4B, top panel). Two rounds of NFI siRNA transfections resulted in an even greater increase (4.6-fold) in HEY1 mRNA levels (Figure 4B, bottom panel). These data suggest that multiple members of the NFI family are involved in HEY1 regulation, with NFIs repressing HEY1 promoter activity.

Next, we used the luciferase reporter gene under the control of the HEY1 promoter to investigate the effect of NFI on transcriptional activity. U251 GBM cells were transfected with siRNAs to knock down single NFIs or a combination of all four NFIs, followed by transfection with the pGL3/HEY1 construct containing −915 to +15 bp of the HEY1 promoter upstream of the firefly luciferase reporter gene. Knockdown of NFIA did not affect HEY1 transcriptional activity based on the luciferase assay (Figure 4C). However, transcriptional activity was significantly increased following knockdown of NFIB (3.1-fold), NFIC (6.1-fold), and NFIX (1.6-fold), suggesting that these three NFIs repress transcription from the HEY1 promoter. Knockdown of all four NFIs increased transcriptional activity 5.6-fold compared to control (scrambled) siRNA. As the biggest increase in HEY1 transcriptional activity was observed upon NFIC knockdown, with a similar effect seen upon knockdown
of all four NFIs, these results suggest that NFIC is a key player in the repression of HEY1 promoter activity, at least in the context of an extrachromosomal plasmid reporter gene assay. The combinatorial effect of NFIs on endogenous HEY1 mRNA levels (Figure 4B) clearly indicates that multiple members of the NFI family are involved in endogenous HEY1 regulation.

**HEY1 Expression in GBM Cells**

HEY1 expression has previously been reported in the developing central nervous system and in GBM tumors [34,35]. We carried out quantitative PCR analysis to measure relative HEY1 mRNA levels in a panel of standard GBM cell lines (adherent; cultured in medium containing fetal calf serum), as well as GBM patient-derived adherent
cell lines (cultured in medium containing fetal calf serum) and tumor
neurosphere cultures (serum-free; medium supplemented with
growth factors) (Figure 5, A and B). Overall, there was a trend
towards lower HEY1 RNA levels in cell lines that expressed low levels
of the neural stem cell marker B-FABP [50–53] (Figure 5A). High HEY1
RNA levels were observed in all three GBM tumor neurosphere cell
lines tested (A4-004, A4-007, and EDS12) (Figure 5A). When we compared
adherent cultures and tumor neurosphere cultures derived from the same
patient, we observed considerably higher levels of HEY1 RNA in the
neurosphere cultures, in keeping with HEY1 being more highly expressed
in tumor cells with neural stem cell properties (Figure 5B).

In the developing brain, HEY1 is required for the maintenance of
neural precursor cells [34], whereas NFIA is required for initiation of
gliogenesis and astrocyte differentiation [14,18]. To address a possible
role for HEY1 in the prevention of astrocyte differentiation, we
transfected HEY1 siRNAs into three GBM cell lines: U87 (very low
levels of HEY1; does not express astrocyte differentiation marker
GFAP), U251 (low levels of HEY1; expresses GFAP), and M049 (high levels of HEY1; expresses GFAP). HEY1 RNA levels were
decreased by 85% to 94% in cells transfected with HEY1 siRNA
compared to control (scrambled) siRNA (Figure 5C). HEY1
knockdown had no effect on GFAP RNA levels in U87 cells,
indicating that HEY1 depletion is not sufficient to induce GFAP
expression in cells that do not express endogenous GFAP. However,
there was a ~2× increase in GFAP RNA levels in U251 (1.8-fold) and
M049 (2-fold) GBM cells upon HEY1 depletion (Figure 5D). While
these results do not address the biological relevance of a 2× increase in
GFAP RNA levels, they are in keeping with a role for HEY1 in the
maintenance of neural stem cell properties.

**Effects of HEY1 Depletion on Cell Proliferation and Migration
in GBM**

We transfected U251 GBM cells and A4-004 neurosphere cultures with
HEY1 siRNAs to examine the effect of HEY1 knockdown on
cell proliferation and migration. Both HEY1 siRNAs used for these
experiments decreased HEY1 RNA levels by >90% (U251) and
~80% (A4-004) (Figure 5, E and F). HEY1 protein levels were also
reduced by >70% upon HEY1 depletion (Figure 5A). HEY1
knockdown in both these cell lines resulted in decreased cell
proliferation compared to cells transfected with control siRNAs
(Figure 5, E and F).

Next, we measured the cell motility of U251 and A4-004 cells
transfected with either control or HEY1 siRNAs using the scratch assay.
HEY1-depleted U251 and A4-004 cells both showed increased motility
compared to control cells, closing the wound (scratch) significantly faster
than cells transfected with control siRNAs (Figure 6A; see Supplementary
Figure S1 for 95% confidence intervals). In U251 cells, depletion of
HEY1 by two different siRNAs (siHEY1a and siHEY1b) resulted in
~4.3-fold and ~2.2-fold increases in cell motility, respectively. In A4-004,
HEY1 depletion resulted in 7- to 8-fold increases in cell motility. We also
used the Transwell migration assay to measure the migration of HEY1-
depleted cells compared to control cells. In keeping with the results
obtained with the scratch assay, HEY1-depleted U251 and A4-004 GBM
cells showed significantly higher migration rates compared to cells
transfected with control siRNAs. Specifically, U251 cells transfected with
two different siRNAs showed 3.70- and 5.37-fold increases in migration compared to cells transfected with scrambled (control) siRNAs.

**HEY1 Depletion and Neurosphere Formation**

We transfected A4-004 cells with HEY1 siRNAs to examine the
effect of HEY1 knockdown on their ability to form neurospheres.
Either 1000 or 200 cells were seeded in triplicate in low-attachment
24-well plates and were allowed to form spheres over a period of 10
days. HEY1 depletion resulted in decreased numbers of neurospheres
as well as smaller neurospheres. We therefore measured the
total area of all the neurospheres in each well. When 1000 cells were
seeded, there was a decrease of 32% and 37% in total neurosphere
area in siHEY1a and siHEY1b transfected cells, respectively. When
200 cells were seeded, the decrease in total area was 50% and 59%
for the two HEY1 siRNAs compared to control siRNAs (Figure 7; see
Supplementary Figure S1 for 95% confidence intervals).

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**Figure 5.** HEY1 knockdown reduces neurosphere formation. Either
200 or 1000 A4-004 cells were seeded in triplicate in a 24-well
low-attachment plate. Cells were allowed to form spheres for a period
of 10 days. Sphere formation was analyzed by measuring the total
area of all the spheres in each well. The results are from three
independent experiments. The unpaired t test was used to measure
statistical significance. ** represents P < .01.

**Figure 6.** HEY1 knockdown results in reduced cell migration. (A) U251 GBM and A4-004 (neurosphere) cells were transfected with either
scrambled siRNAs or siRNAs against HEY1 (siHEY1a or siHEY1b) and allowed to reach confluency. A scratch was made in the center of
each well, and cells were allowed to migrate over a period of 30 hours (U251) or 24 hours (A4-004) with live cell monitoring. Graphs
represent percentage open area of the wound (scratch). Each experiment was carried out in triplicate with data obtained from six
different positions for each time point. Experiments were repeated three times, and the unpaired t test was used to measure statistical
significance. Images shown represent 0 hour and 30 hours (U251) or 24 hours (A4-004) time points. (B) Transwell cell migration assay
showing reduced cell migration upon HEY1 knockdown. Twenty-five thousand cells were seeded in the upper chamber and allowed
to migrate across a PET membrane towards medium containing 10% FCS over a period of 24 hours. Migrated cells were fixed, stained,
and counted using Metamorph imaging software. The data shown in the graphs represent an average of three independent experiments. The
unpaired t test was used to measure statistical significance. ** represents P < .01; *** represents P < .001.
Discussion

The NFI family is an important regulator of glial cell differentiation during development [14], with a well-characterized role in the regulation of glial differentiation genes, including GFAP, in both normal brain and GBM cells [36]. We used a ChIP-on-chip approach to identify additional NFI target genes in GBM. DNA sequences from a total of 403 genes were found to be preferentially bound by NFI using a pan-specific anti-NFI antibody. GO analysis of putative NFI target genes identified enrichment of genes involved in multiple biological processes including gene expression, development, and differentiation and, of particular interest, genes involved in nervous system development.

One of the 403 genes identified by ChIP-on-chip was the Notch effector gene HEY1. The HEY family consists of three basic helix-loop-helix (bHLH) proteins (HEY1, HEY2, and HEYL) closely related to the HES family of transcriptional repressors [54]. HEY1 is normally expressed in undifferentiated cells of the developing mouse brain [34]. Ectopic expression of HEY1 in the developing mouse brain inhibits neurogenesis and promotes maintenance of undifferentiated cells [34]. Promoter assays indicate that HEY1 acts by inhibiting the neuronal bHLH genes AεIIl (also known as Math1) and Neuronδ (also known as Math3) [34].

We identified four putative NFI binding sites within a 1000-bp region immediately upstream of the HEY1 transcription start site. Gel shift assays revealed NFI binding to three of these four putative sites: at −794 bp, −411 bp, and −332 bp. Although multiple protein-DNA complexes were observed with the putative NFI binding site at −32 bp, these complexes were competed out with excess cold oligonucleotide mutated at critical NFI binding residues and were not supershifted using anti-NFI antibody, indicating that proteins other than NFI bind to the −32 bp region. Combined data from gel shift and supershift experiments indicate that NFIs bind to the other three NFI recognition sites, at −332 bp, −411 bp, and −794 bp. Gel shift experiments using nuclear extracts prepared from cells that ectopically express individual NFIs indicate differential NFI binding to these sites, with the −411 bp site being the most discriminatory, as only NFIX binds effectively to this region.

Differential binding by different NFI family members in vitro has been previously reported [55,56]. For example, the differential DNA binding specificities of NFI-A4, NFI-B2 and NFI-X1 for the CoRE response element located upstream of the WAP gene were shown to be dependent on other transcription factors binding to this region [56]. As all four NFIs have highly similar DNA binding domains and bind DNA as either homodimers or heterodimers, binding site specificity may be due to NFI interacting partners, structural changes within NFI transcription factors caused by alternative splicing or posttranslational modifications, as well as the relative levels of the different members of the NFI family [55,57]. Thus, differences in the sequences of the three NFI binding sites upstream of the HEY1 gene may allow preferred binding to subsets of NFI recognition sites. In this regard, it is interesting to note that the main differences between the −411 bp NFI recognition sites and that of −332 bp and −794 bp are the last two nucleotides (GC in the case of −411 bp and AG and AC in the case of the −332 and −794 bp regions, respectively) (Figure 4A).

A requirement for knockdown of all four NFIs to detect an effect on endogenous HEY1 RNA levels suggests complex regulation and cross talk between NFI family members. There is considerable variability in the transactivation domain of NFI family members [10,12], and the transactivation potential of heterodimers has previously been reported to be intermediate to that of NFI homodimers [11]. Thus, knockdown of single NFIs, with accompanying changes in NFI interactions, may alter the dynamics of NFI dimerization in the cell but may still result in little to no effect on endogenous HEY1 mRNA levels in the context of an intact cell. It is only when all four NFIs are depleted that their repressive effect on the HEY1 promoter can be overcome. In contrast to the endogenous promoter, single knockdown of NFIB, NFIC, or NFIX, but not NFIA, was sufficient to induce exogenous HEY1 promoter activity. Differences in regulation of NFI-dependent promoter activity in an endogenous (or chromosomal) context compared to an ectopic (or extrachromosomal) context have previously been reported for a number of promoters including B-FABP, GFAP, and MMTV [36,58]. This difference has been explained by a looser organization of the nucleosome structure in episomal DNA compared to chromosomal DNA, allowing easier access to transcription factors [59].

HEY1 expression in GBM correlates with increased tumor grade and decreased survival [60]. Similar to the results reported here, others have shown that HEY1 knockdown decreases proliferation in U87, T98, and U373 GBM cell lines as well as GBM cell lines established from mouse xenografts [35,61]. We extend these studies by demonstrating that HEY1 is associated with higher levels of the neural stem cell marker B-FABP in GBM cells and increased neurosphere formation, in keeping with its proposed role in the brain [34]. Furthermore, HEY1 depletion in GBM cells that already express the astrocyte differentiation marker GFAP results in increased GFAP mRNA levels. In contrast to a previous report indicating that HEY1 knockdown resulted in decreased migration in GBM cell lines [61], our results indicate a significant increase in migration upon HEY1 depletion in GBM cells. This discrepancy may stem from the fact that the pooled siRNAs used for HEY1 depletion by Tsung et al. resulted in increased apoptosis in GBM cell lines established from mouse xenografts [61]. Thus, our results support roles for NFIs and HEY1 in controlling fundamental pro-growth versus anti-growth properties of GBM, as well as support the “go or grow” hypothesis whereby cells with reduced proliferation show increased migration and vice versa [33].

In contrast to HEY1, high NFIA and NFIB mRNA levels correlate with improved patient survival in astrocytomas, with reduced expression of NFIA and NFIB associated with higher-grade astrocytomas [38,39]. In the developing CNS, NFIA and NFIB drive the onset of gliogenesis (gliogenic switch) [14,15,18,19], with NFIX playing a role in the later stages of astrocyte differentiation [20,62]. Nfia−/−, Nfib−/−, and Nfix−/− null mice all show delays in the differentiation of glial cells in developing brain [21–27]. Although NFIC is widely expressed in the CNS, Nfic knockout in mice causes tooth pathologies rather than brain defects, suggesting that its roles in brain are redundant with other NFIs [22,63]. Several studies have shown that NFIs, especially NFIA and NFIB, positively regulate the expression of genes associated with glial cell differentiation (e.g., GFAP, SPARCL1, APCDD1, MMD2) [18,42,43,62] while repressing genes associated with stem cell maintenance (EZH2, HES1) [17,46]. As previously reported, the association between reduced levels of NFIA/NFIB and increased malignancy in astrocytoma is in agreement with NFIs playing similar roles in gliogenesis and gliomagenesis; i.e., promotion of glial cell differentiation properties [65,66]. Our results indicating that NFI knockdown upregulates HEY1 expression add to the repertoire of genes controlled by NFIs that determine stemness versus differentiation properties. It is a well-
known fact that there is considerable heterogeneity in GBM tumors and the cell lines derived from these tumors. Thus, within a single tumor or cell line, there may be NFI-high cells associated with expression of astrocytic markers and less aggressive growth properties, and NFI-low cells associated with increased stemness and more aggressive growth properties. In support of this idea, examination of the astrocytic marker GFAP and neural stem/progenitor cell marker B-FABP in GBM neurosphere cultures reveals little overlap in the expression of these two markers (Figure 8).

Conclusions
In summary, we show that NFI transcription factors expressed in GBM cells bind to the promoters of multiple genes involved in many biological processes. We identify three NFI binding sites in the HEY1 promoter and show that NFI represses HEY1 promoter activity and expression in GBM cells. We demonstrate differential binding of the four members of the NFI family to the different NFI binding sites in the HEY1 promoter. Our results indicate complex interactions between the different members of the NFI family and suggest that NFI dimerization, along with additional transcription factors, is involved in the regulation of the HEY1 gene in GBM. The decrease in cell proliferation and neurosphere formation, along with the increase in cell migration observed upon HEY1 knockdown, supports the “go or grow” hypothesis previously validated for a number of tumor models. We propose that mutually exclusive cell migration and proliferation in GBM cells can be explained at least in part by relative levels of NFIs and HEY1.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2018.08.007.

References


