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Nuclear Factor I Represses the Notch Effector *HEY1* in Glioblastoma[1]

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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(N_5)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 zest 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; SPARCL1, 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 astrocytomas, with 91%, 77%, 48%, and 37% of cells expressing *NFIA* in grades I, II, III, and IV astrocytomas, respectively [28,29]. NFIA is enriched in astrocytomas 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 *NFIB* mRNA levels are also associated with high-grade astrocytomas, with elevated levels of *NFIB* RNA correlating with better overall and recurrence-free survival in GBM [30]. NFIB 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 #HREBA.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 knockdown NFIA, NFIB, NFIC, NFIX, and HEY1: NM_005595_stealth_919 targeting 5′-GAAAGUUCUUCAUACUACAG-CAUGA-3′(NFIA); NM_005596_stealth_1020 targeting 5′-AAGCCACAAUGA-UCCUGCCAAGAAU-3′ (NFIB); NM_005597_stealth_1045 targeting 5′-CAGAGAU-GGACAAGUCACCAUUCAA-3′ (NFIC); NM_002501_stealth_752 targeting 5′-GAGAGUAUCACAGACUCCUGUUGCA-3′ (NFIX); NM_ 012258.3_stealth_284 targeting 5′-UAGAGCCGAACUCAAGUUUCCAUUC-3′ (HEY siRNA 1); and NM_012258.3_stealth_652 targeting 5′-UUGAGAUGCGAAACCAGUCGAACUC-3′ (HEY1 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 2× 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 glycine 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 7× in wash buffer (50 mM Hepes-KOH, 500 mM LiCl, 1 mM EDTA, 1% Nonidet-P40, 0.7% sodium deoxycholate) and 1× 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′-GCGGTGACCCGGGAGATCTGAATTC-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, 1× Thermopol buffer (NEB), 250 μM dNTPs, 1 μM LM-PCR primer 5′-GCGGTGACCCGGGAGATCTGAATTC-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 H_2O, 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