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LABORATORY INVESTIGATION

Transcription factors NFIA and NFIB induce cellular differentiation in high-grade astrocytoma

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Abstract

Introduction Malignant astrocytomas are composed of heterogeneous cell populations. Compared to grade IV glioblastoma, low-grade astrocytomas have more differentiated cells and are associated with a better prognosis. Therefore, inducing cellular differentiation to alter the behaviour of high-grade astrocytomas may serve as a therapeutic strategy. The nuclear factor one (NFI) transcription factors are essential for normal astrocytic differentiation. Here, we investigate whether family members NFIA and NFIB act as effectors of cellular differentiation in glioblastoma.

Methods We analysed expression of NFIA and NFIB in mRNA expression data of high-grade astrocytoma and with immunofluorescence co-staining. Furthermore, we induced NFI expression in patient-derived subcutaneous glioblastoma xenografts via in vivo electroporation.

Results The expression of *NFIA* and *NFIB* is reduced in glioblastoma as compared to lower grade astrocytomas. At a cellular level, their expression is associated with differentiated and mature astrocyte-like tumour cells. In vivo analyses consistently demonstrate that expression of either NFIA or NFIB is sufficient to promote tumour cell differentiation in glioblastoma xenografts.

Conclusion Our findings indicate that both NFIA and NFIB may have an endogenous pro-differentiative function in astrocytomas, similar to their role in normal astrocyte differentiation. Overall, our study establishes a basis for further investigation of targeting NFI-mediated differentiation as a potential differentiation therapy.

Keywords Nuclear factor I · NFIA · NFIB · Glioblastoma · Astrocytoma · Differentiation

Linda J. Richards and Jens Bunt have contributed equally to this manuscript.

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Introduction

Astrocytomas are characterised by high cellular heterogeneity, resulting in complex combinations of proliferating and differentiated cells [1–3]. Compared with grade IV glioblastoma (GBM), lower grade astrocytomas consist predominantly of well-differentiated cells and are associated with reduced aggressiveness and better prognosis [2]. Hence, increasing cellular differentiation could render a tumour less aggressive and improve survival. This argument supports the notion that differentiation therapy could serve as a feasible therapeutic strategy. An example where this strategy has been remarkably successful is the treatment of acute promyelocytic leukaemia (APML) using retinoic acid (RA) [4]. This success is the result of a multitude of studies investigating the role of RA in driving haematopoietic cell differentiation and targeting the fusion protein PML-RARα [5].

Investigation of the response of GBM cell lines to inducers of differentiation did not yield promising results, as the responses appear transient and vary between cell lines [6–8]. This is unsurprising given the endogenous function of these differentiation agents in normal brain cells. For example, RA has different functions depending on cell state, and is able to promote or inhibit differentiation at different stages of astrogliogenesis [9]. It is also ineffective in driving neuronal progenitor cell differentiation [10]. One approach to overcoming such challenges is to target the endogenous mechanisms that are directly involved in producing mature, differentiated glial cells and re-deploy them in astrocytomas.

A promising candidate that may regulate differentiation in astrocytomas is the nuclear factor one (NFI) family of transcription factors. These proteins are essential for normal glial differentiation [11–15]. During development, NFI are expressed in progenitor cells that give rise to various differentiated cell populations in the dorsal telencephalon [16, 17]. Their expression drives the differentiation of these cells [13–15], with NFIA and NFIB expression persisting in mature astrocytes [18]. In *Nfia* and *Nfib* knockout embryos, the generation of mature astrocytes is reduced, and progenitor cells remain in a proliferative state for a prolonged period [11, 12]. Overexpression of these genes is also sufficient to rapidly convert induced pluripotent stem cells into functional astrocytes in vitro [19–21]. These findings reflect the essential role of NFIA and NFIB as regulators of astrocytic differentiation.

In astrocytomas, higher expression levels of *NFI* correlate with lower grade tumours that are composed of more differentiated cells [22, 23]. Loss of heterozygosity (LOH) of *NFIB* as a consequence of chromosome 9p loss is a common occurrence in the tumour progression and is present in 40% of all GBM [24, 25]. The down-regulation of NFI expression may be a mechanism by which tumour cells evade differentiation and thereby remain proliferative. This is in line with studies which report that the *Nfi* loci are common insertion sites in insertional mutagenesis mouse models of gliomas, suggesting that reduced expression promotes tumorigenicity [26, 27].

The induction of NFIA or NFIB in GBM cell lines in vitro induces the expression of astrocyte differentiation markers such as GFAP and FABP7 [13, 23, 28, 29], indicating that overexpression of NFI might be sufficient to drive astrocytic differentiation. NFIA and NFIB function very similarly to drive glial differentiation in the developing brain [13–15]. We demonstrated recently that NFIB overexpression in vitro reduced GBM cell proliferation and inhibited growth when xenografted into mice [23], although whether NFIA plays a similar role is unknown. Here, we investigate whether NFIA and NFIB drive astrocytic differentiation in astrocytomas in vivo. Using expression datasets and immunofluorescence co-staining, we demonstrate that the endogenous expression of NFIA and NFIB positively correlate with each other, and that their expression in astrocytomas is associated with the expression of differentiated cell markers. Furthermore, by manipulating their expression in patient-derived GBM xenografts in vivo, we reveal that induced expression of either NFI is sufficient to promote tumour cell differentiation. These findings suggest that the NFI-pathway is a promising therapeutic target to induce differentiation in astrocytomas.

Material and methods

Collection of GBM tissue and xenografts generation

De-identified fresh and fixed GBM tissues for research from consented patients were obtained from the University of Malaya Biobank and the Wesley Medical Research BioBank with approval from the University of Queensland Human Ethics Committee. The establishment of patient-derived GBM xenografts (QBI-01, QBI-02, and QBI-03) was performed as previously described with minor modifications [30]. Cell line-derived xenografts were generated from U251 [31] and from patient-derived GBM cell lines BAH1, SJH1, RKI1, and WK1 [32].

In silico analyses

To assess gene expression in human glioma samples, public expression datasets GSE50161, GSE43378, GSE4290 (Fig. 1a, b; Supplementary Table S2), GSE108474, GSE16011 (Figs. 1a, b, 2a, b; Supplementary Table S2,

6 and 7), GSE49822, GSE7696, GSE53733, GSE118793 (Fig. 1b), TCGA (Figs. 1a–c, 2a, b; Supplementary Tables 2, 3, 6 and 7), GSE57872 (Figs. 1d, 2c, d; Supplementary Tables 4 and 8), GSE89567 (Figs. 1e, 2c, d; Supplementary Tables 4 and 8) and GSE4271 (Supplementary Table 10) were analysed and visualised using the R2: microarray analysis and visualization platform (http://r2.amc.nl) as previously described [33]. Gene set enrichment analyses were performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.8 online tool [34].

Animals

All breeding and experiments were performed at The University of Queensland in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and with the approval of the University of Queensland Animal Ethics Committee. Animals were housed on a 12 h dark/light cycle with water and food provided ad libitum. The NOD.CB17-Prkdc^{scid}/Arc (NOD-SCID) mice used for patient-derived GBM xenograft experiments were obtained from the Australian Animal Resources Centre.

In vivo electroporation of GBM xenografts

NFI expression in established GBM xenografts was driven using the piggyBac system as described previously [35]. pPBCAGIG, pPBCAGIG-NFIB or pPBCAGIG-NFIA donor plasmids were co-electroporated with pCAG-PBase as a helper plasmid into established xenografts of 0.1–0.2 cm^3. The pCAG-PBase and pPBCAG-eGFP plasmids were kind gifts from Joseph LoTurco, University of Connecticut. Additional details are provided in the Supplementary Methods.

Immunohistochemistry, image acquisition and analysis

Fluorescence immunohistochemistry, image acquisition and analysis were performed as previously described with minor modifications [17, 18]. The primary antibodies used are listed in Supplementary Table 1b. Additional details are provided in the Supplementary Methods.

Statistical analysis

Linear regression was used to determine the correlation of two genes in expression datasets, using the R2 platform as previously described [33]. The differential expression of *NFI* between tumour types was compared with a ratio paired t test on normalised data. To compare the co-staining distribution of NFIA or NFIB with other cell markers between GBM tissues or to determine gene enrichment, a paired one-way ANOVA with Bonferroni's multiple comparison correction was performed. To compare the co-staining of GFP-positive electroporated cells with markers in GBM xenografts between different plasmids, a Welch's t test was performed. These tests were computed with GraphPad Prism 7 (GraphPad Software).

Results

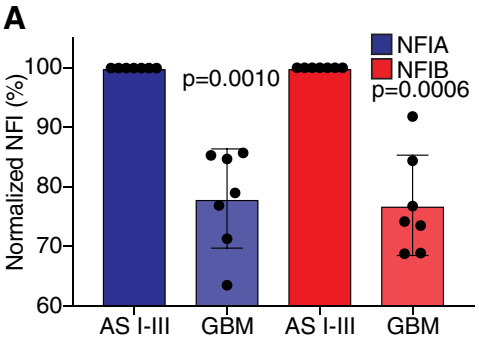
NFIA and NFIB expression is decreased in grade IV GBM

To compare the expression of *NFIA* and *NFIB*, and their relationship in different grades of astrocytomas, we analysed seven large mRNA expression datasets of human astrocytoma samples. Both *NFIA* and *NFIB* expression were reduced in GBM as compared to grade I–III astrocytomas (Fig. 1a), demonstrating that the expression of both *NFIA* and *NFIB* is higher in tumours with more differentiated cells and lower in poorly differentiated tumours. We also observed a positive correlation between *NFIA* and *NFIB* expression in GBM samples across ten datasets (Fig. 1; Supplementary Table 2). Although the strength of the correlation coefficient varied among datasets, this variability may be explained by loss of heterozygosity (LOH) of *NFIB* observed in a subset of GBM samples [24, 25]. Separating tumour samples based on *NFIB* copy number significantly improved the correlation coefficient between *NFIA* and *NFIB* expression in samples where these data were available (Fig. 1c). The trendlines representing the *NFIB*-diploid and haploinsufficient tumour samples had a similar correlation coefficient and were separated by approximately one ^{2}log unit. This meant that when comparing between samples with similar *NFIA* expression, *NFIB* expression was halved in *NFIB*-haploinsufficient samples, as would be expected when one *NFIB* allele is lost. Similar analyses for *NFIA* haploinsufficiency, which is rarely observed in GBM, resulted in only a small improvement in the correlation coefficient (Supplementary Table 3).

NFIA and NFIB are co-expressed at the cellular level in GBM tumours

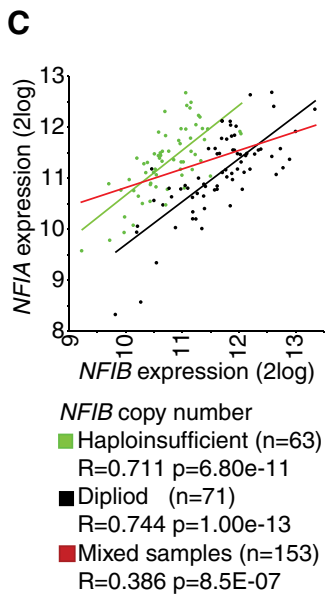
The correlation between *NFIA* and *NFIB* expression in tumour samples does not necessarily mean that these genes are co-expressed within the same cells. We investigated this by first examining *NFIA* and *NFIB* expression in GBM tumours analysed by single cell RNA-seq [3, 36].

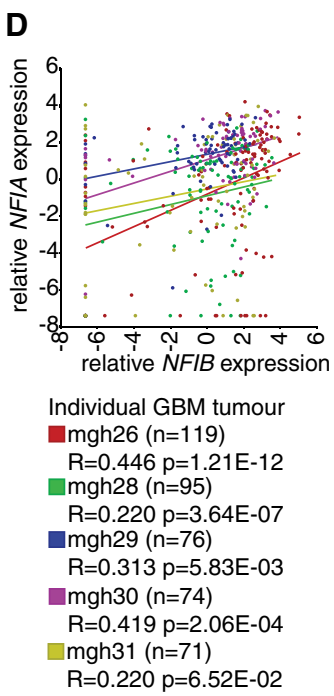
Similar to our observations with whole tumour samples, we observed that the expression of *NFIA* and *NFIB* positively correlated with each other at the single-cell level (Fig. 1d, e; Supplementary Table 4). To validate this at the protein level, we then performed immunofluorescence

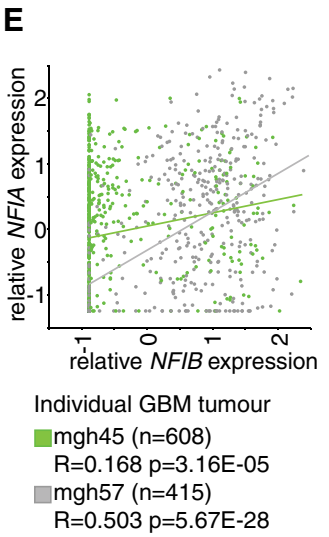


B Correlation of *NFIA* and *NFIB* expression in GBM datasets

Dataset	R-value	P-value	Samples
PFISTER	0.648	1.1E-06	46
DONSON	0.652	2.9E-05	34
HEGI	0.406	4.9E-04	70
FRENCH	0.401	1.6E-07	159
TCGA	0.386	8.5E-07	153
KAWAGUCHI	0.359	4.0E-02	32
LOEFFLER	0.294	1.0E-02	70
STRINGER	0.293	3.5E-01	12
REMBRANDT	0.276	2.5E-05	228
SUN	0.216	6.0E-02	77







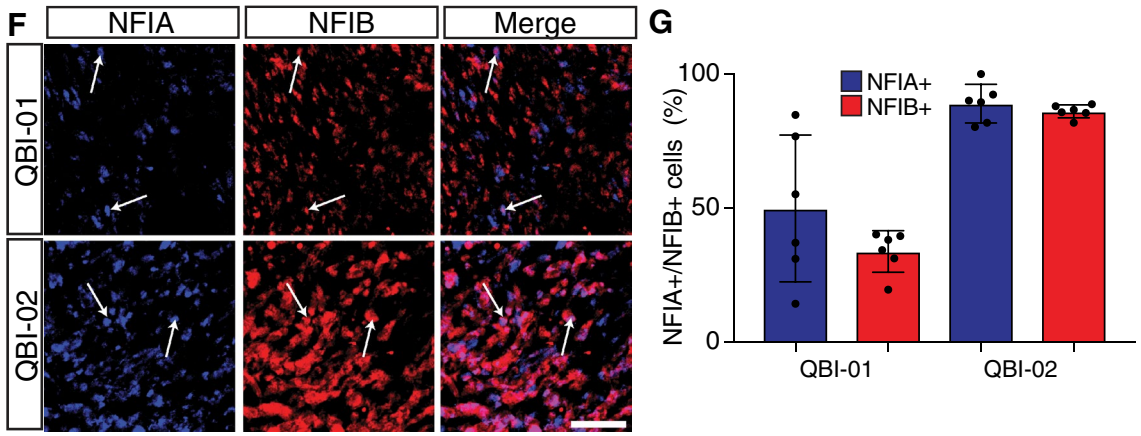


Fig. 1 *NFIA* and *NFIB* share a similar expression pattern in astrocytoma. **a** Based on seven mRNA expression datasets, the expression of both *NFIA* and *NFIB* decreases in grade IV GBM as compared to grade I–III astrocytoma (ratio paired t test). **b** *NFIA* and *NFIB* expression positively correlate with each other within all analysed mRNA expression datasets of GBM (GSE49822, GSE50161, GSE7696, GSE16011, TCGA, GSE43378, GSE53733, GSE118793, GSE108474, GSE4290) **c** By separating GBM samples in the TCGA mRNA dataset based on *NFIB* copy number, the correlation between *NFIA* and *NFIB* expression improves, resulting in higher correlation coefficients for both groups. **d** and **e** *NFIA* and *NFIB* expression positively correlate in single-cell expression data of seven GBM tumours (GSE57872 and GSE89567). **f** Immunofluorescence co-staining of NFIA and NFIB proteins (arrows) in two patient-derived GBM xenograft tissues. **g** Quantification of NFIA+/NFIB+cells as a percentage of all NFIA+or NFIB+cells. Scale bar in f=50 µm. Error bars represent the standard deviation of the mean.

co-staining of NFIA and NFIB in patient-derived GBM xenografts (Supplementary Tables 1a and 5). Quantification was performed with two biological replicates in two non-necrotic 500 × 500 µm regions of interest per tumour. In the QBI-01 xenograft, which represents a tumour with fewer NFI-positive cells in general, half of all NFIA-positive cells co-stained for NFIB, whereas over a third of the NFIB-positive cells were also NFIA-positive (Fig. 1f, g). The co-expression of NFIA and NFIB was observed in more cells for the QBI-02 xenograft, in which at least 80% of cells expressed both NFIA and NFIB. We therefore conclude that

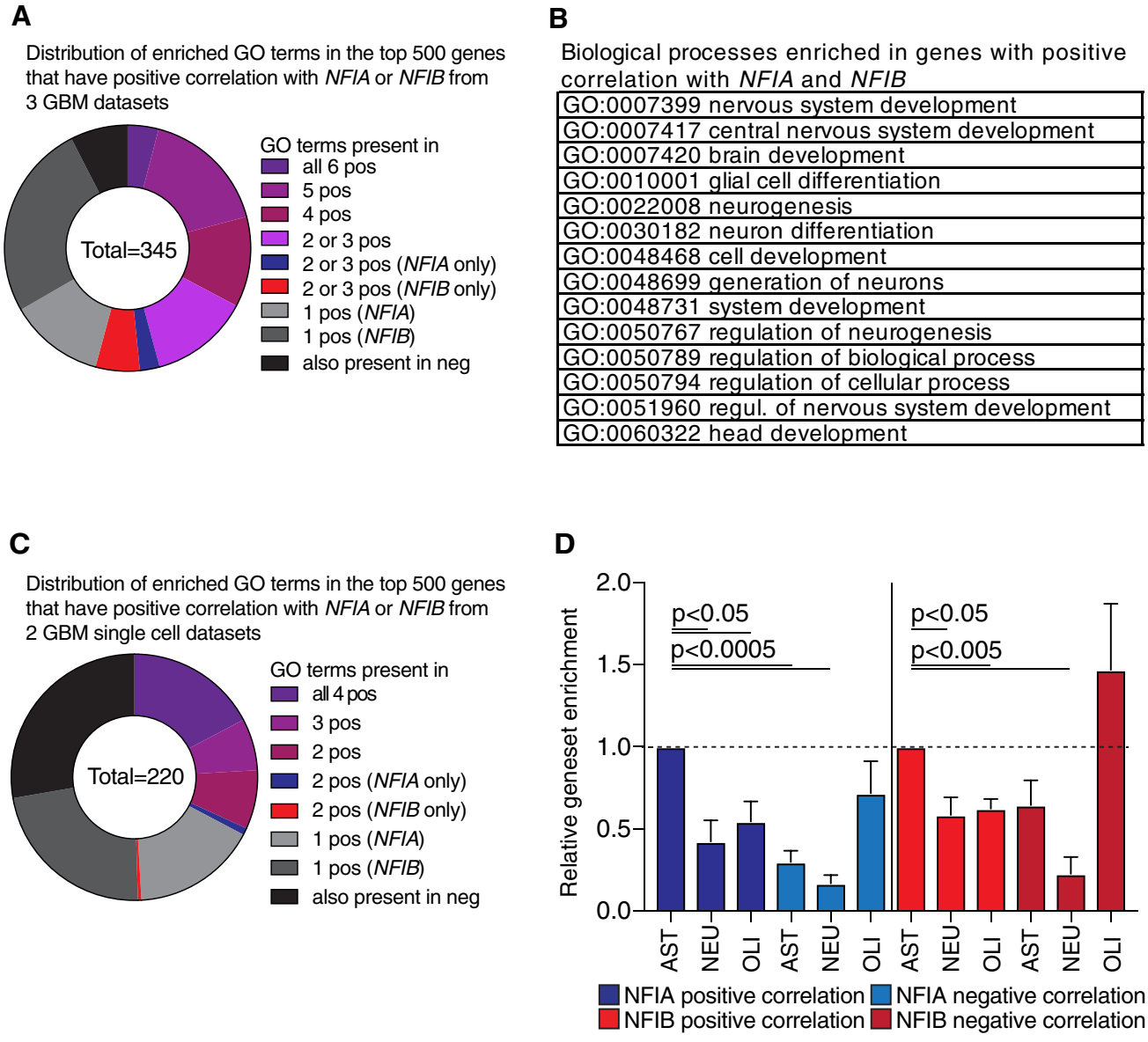


Fig. 2 *NFI* expression in GBM is associated with neurodevelopment and mature astrocytic genes. **a** Gene ontology (GO) analyses were performed on gene sets generated from three large GBM expression datasets (GSE108474, GSE16011 and TCGA). Four gene sets were generated for each large dataset, representing the top 500 genes that positively correlate or negatively correlate with *NFIA* or *NFIB*. Of the 345 GO Biological Process terms obtained from the six positive gene sets (pos), 45% were shared by both *NFIA*- or *NFIB*-correlated gene sets, but were absent from all of the negatively correlated gene sets (neg). **b** The 14 GO terms present in all six gene sets that positively correlated with *NFI* expression. **c** Similar GO analyses were performed on two large GBM single-cell mRNA-seq datasets (mgh45 and mgh57 from GSE89567). Of the 220 GO Biological Process terms obtained from the four gene sets representing the *NFIA* or *NFIB* positively correlated genes, 32% of the GO terms were shared between *NFIA*- and *NFIB*-correlated genes in both datasets. **d** Relative enrichment of genes associated with mature astrocytic (AST), oligodendrocytic (OLI) and neuronal (NEU) signatures derived from the positive and negative gene sets that were generated for all eight datasets. *NFI* positively correlated genes were associated with mature astrocytic genes. Statistical significance was determined using a paired one-way ANOVA with Bonferroni's multiple comparison correction.

NFIA and NFIB show a high degree of co-expression within the same cells in GBM tumours.

NFIA and NFIB expression correlates with astrocytic differentiation genes in GBM cells

GBM tumours consist of a heterogenous mix of cell types [1, 3]. Highly aggressive tumours that consist predominantly of proliferating cells still harbour differentiated tumour cells that are not proliferating [2]. Given their role as regulators of cell differentiation during development, we hypothesised that *NFIA* and *NFIB* are associated with genes that correspond with a differentiated cell state. To investigate this, we performed gene ontology (GO) analyses on gene sets that were independently derived from each of the three largest GBM mRNA expression datasets (GSE108474, GSE16011 and TCGA; Supplementary Tables 6 and 7). We generated four gene sets for each mRNA expression dataset, with each set consisting of 500 genes. The first two sets consisted of the top genes that positively correlated with *NFIA* or *NFIB*, respectively, while the remaining two sets consisted of the top genes that showed negative correlation with *NFIA* or *NFIB*. Each set was then subjected to GO analyses to identify which GO Biological Process terms were enriched.

The six gene sets that were positively correlated with *NFIA* or *NFIB* (two sets for each of the mRNA expression datasets) returned a combined total of 345 unique GO terms ($p < 0.001$) (Fig. 2a; Supplementary Table 7). 114 of these terms (33%) occurred in at least four of the six positively correlated gene sets, but were completely absent from the six gene sets that are negatively correlated with *NFIA* or *NFIB*. These included GO terms associated with transcription, metabolic processes and development. 14 of these terms were shared between all six positively correlated gene sets. These are likely to represent genes that are co-expressed in NFIA+ and NFIB+ cells, and are enriched for terms representative of neurodevelopment and cell differentiation processes (Fig. 2b). Overall, these findings are in line with the strong co-expression of NFIA and NFIB observed in these datasets (Fig. 1b–g), and indicative of their overlapping function in regulating differentiation. Notably, no GO terms associated with proliferation were enriched in any of the positively or negatively correlated gene sets, suggesting that *NFI* expression is indicative of tumour differentiation state and not proliferative potential.

We next performed similar analyses using the two largest single-cell mRNA-seq datasets derived from individual GBM tumours (mgh45 and mgh57 from GSE89567). Other single-cell datasets were excluded, as these did not contain sufficient cells for reliable correlation analyses. The four gene sets representing *NFI* positively correlated genes were enriched for a combined total of 220 GO terms ($p < 0.00005$). 70 of these terms (32%) were shared at least once between the *NFIA* and *NFIB* gene sets, but were completely absent from the gene sets representing negatively correlated genes (Fig. 2c; Supplementary Tables 8 and 9). Similar to our analyses of whole GBM tumours, the 38 GO terms shared between all four positively correlated gene sets represented neurodevelopmental processes, such as neurogenesis, gliogenesis, and cell differentiation (Supplementary Table 9). These findings also held true for gene expression datasets of two GBM cell lines collected from different passages and culture conditions (nob0308 and nob1228 from GSE4271; Supplementary Tables 10 and 11) [37], further supporting the premise that *NFI* expression is a marker of differentiated cells.

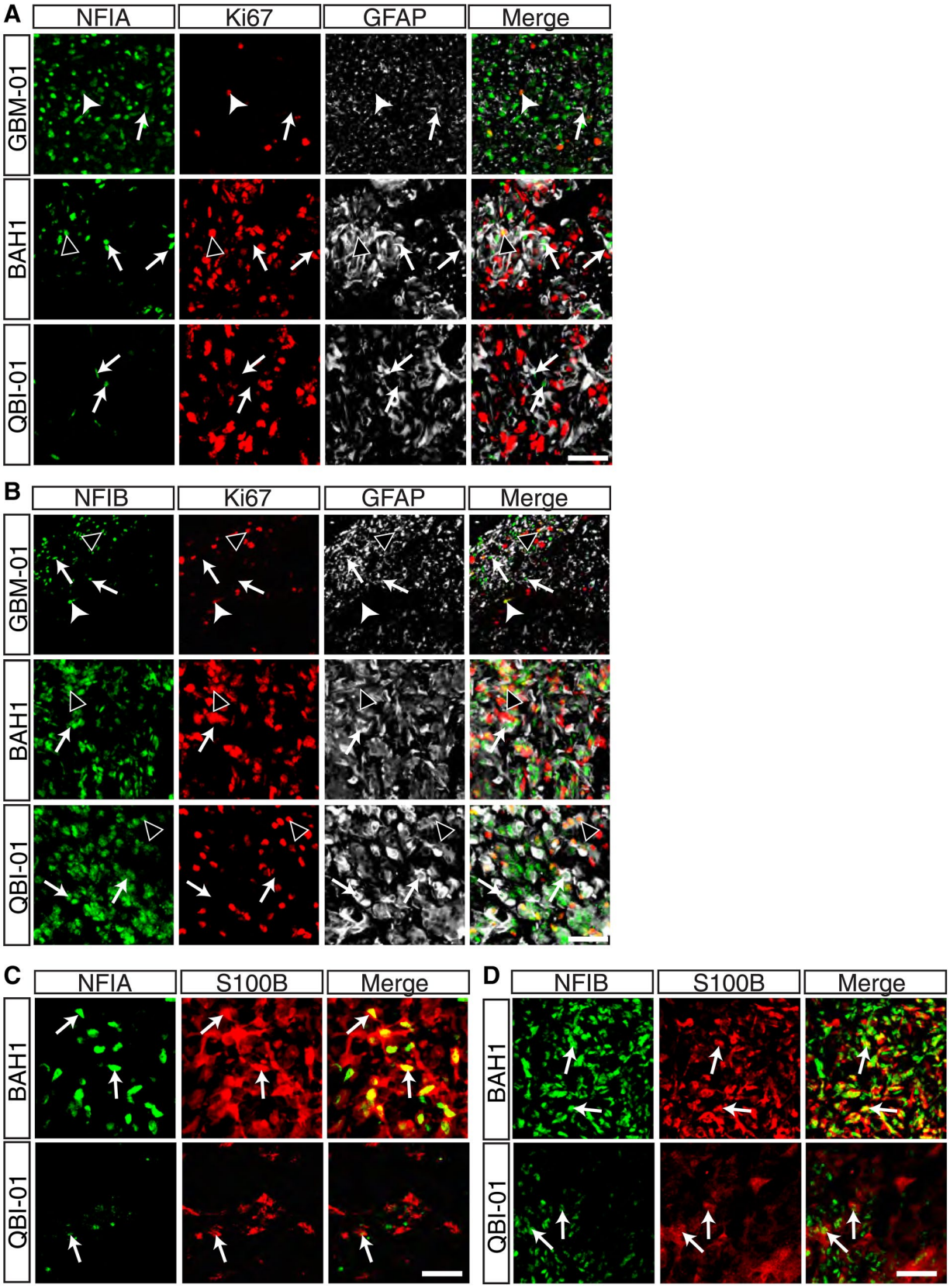
To determine whether the correlated gene sets were associated with specific cell types, we compared these sets with gene expression signatures that represent mature astrocyte, oligodendrocyte or neuron-specific genes as reported by three independent groups (Supplementary Table 12) [38–40]. The *NFIA* and *NFIB* positively correlated gene sets were associated with astrocytic genes (Fig. 2d). In contrast, genes that negatively correlated with *NFIA* or *NFIB* were enriched for oligodendrocytic markers. This suggests that *NFI*-expressing tumour cells are likely to resemble mature astrocytes rather than oligodendrocytes or neurons.

Fig. 3 NFI-expressing cells in GBM are predominantly associated with the astrocytic differentiation marker GFAP. **a** and **b** Representative images of co-staining of NFIA (**a**) or NFIB (**b**) with GFAP and the proliferation marker Ki67. **c** and **d** Co-staining of NFIA (**c**) or NFIB (**d**) with the astrocytic marker S100B in GBM tissues. Closed arrowhead: NFI, GFAP and Ki67 co-localisation; open arrowhead: NFI and Ki67 co-localisation; arrow: NFI and GFAP co-localization. Scale bar (**a**–**d**) 50 µm.

NFIA and NFIB proteins mark non-proliferating, differentiated tumour cells

To determine whether NFI expression is associated with astrocytic markers in GBM tumours in vivo, we performed immunofluorescence co-staining on sections derived from five primary GBM samples (GBM-01 to -05), five xenografts derived from GBM cell lines (BAH1, SJH1, RKI1, WK1, U251) and three patient-derived xenograft tumours (QBI-01 to -03) (Fig. 3; Supplementary Figs. 1 and 2) [32]. All samples expressed both NFIA and NFIB, and displayed co-staining of markers of astrocytic differentiation. Cell counts demonstrated that the majority of NFIA- or NFIB-positive cells (~80%) were also positive for GFAP or S100B (Fig. 4; Supplementary Tables 13 and 14). In contrast, only one third of the NFIA- or NFIB-positive cells co-stained with the proliferation marker Ki67. Hence, NFI expression is associated with the expression of mature astrocytic markers.

To further delineate the differentiation state of NFI-expressing cells, we analysed the combined co-staining of


A
NFIA
Ki67
GFAP
Merge
GBM-01
BAH1
QBI-01
B
NFIB
Ki67
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GBM-01
BAH1
QBI-01
C
NFIA
S100B
Merge
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QBI-01
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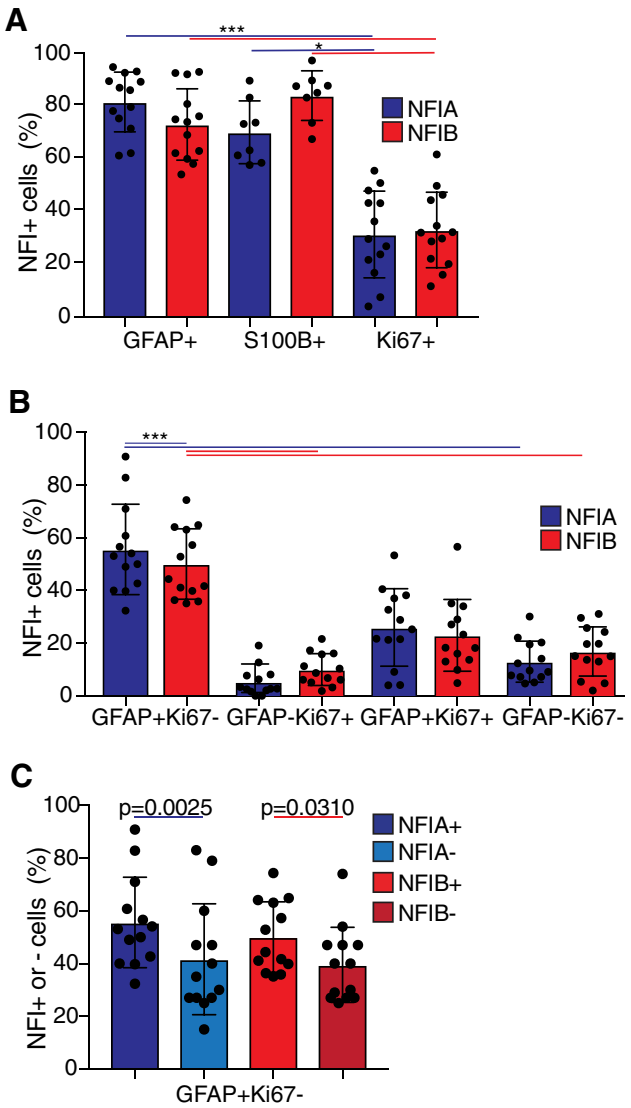


Fig. 4 NFI-expressing cells in GBM are predominantly associated with astrocytic differentiation genes. **a** Quantification of NFIA or NFIB co-staining with astrocytic and proliferation markers, demonstrating the percentage of NFIA+ and NFIB+ cells co-stained for GFAP, S100B or Ki67. **b** The percentage of NFIA+ and NFIB+ cells co-stained with GFAP and Ki67, respectively. **c** GFAP-positive, Ki67-negative cells as a percentage of NFI positive or negative cells. Error bars represent the standard deviation of the mean. Statistical significance was determined using a paired one-way ANOVA with Bonferroni's multiple comparison correction; $*p<0.05$, $***p<0.0005$.

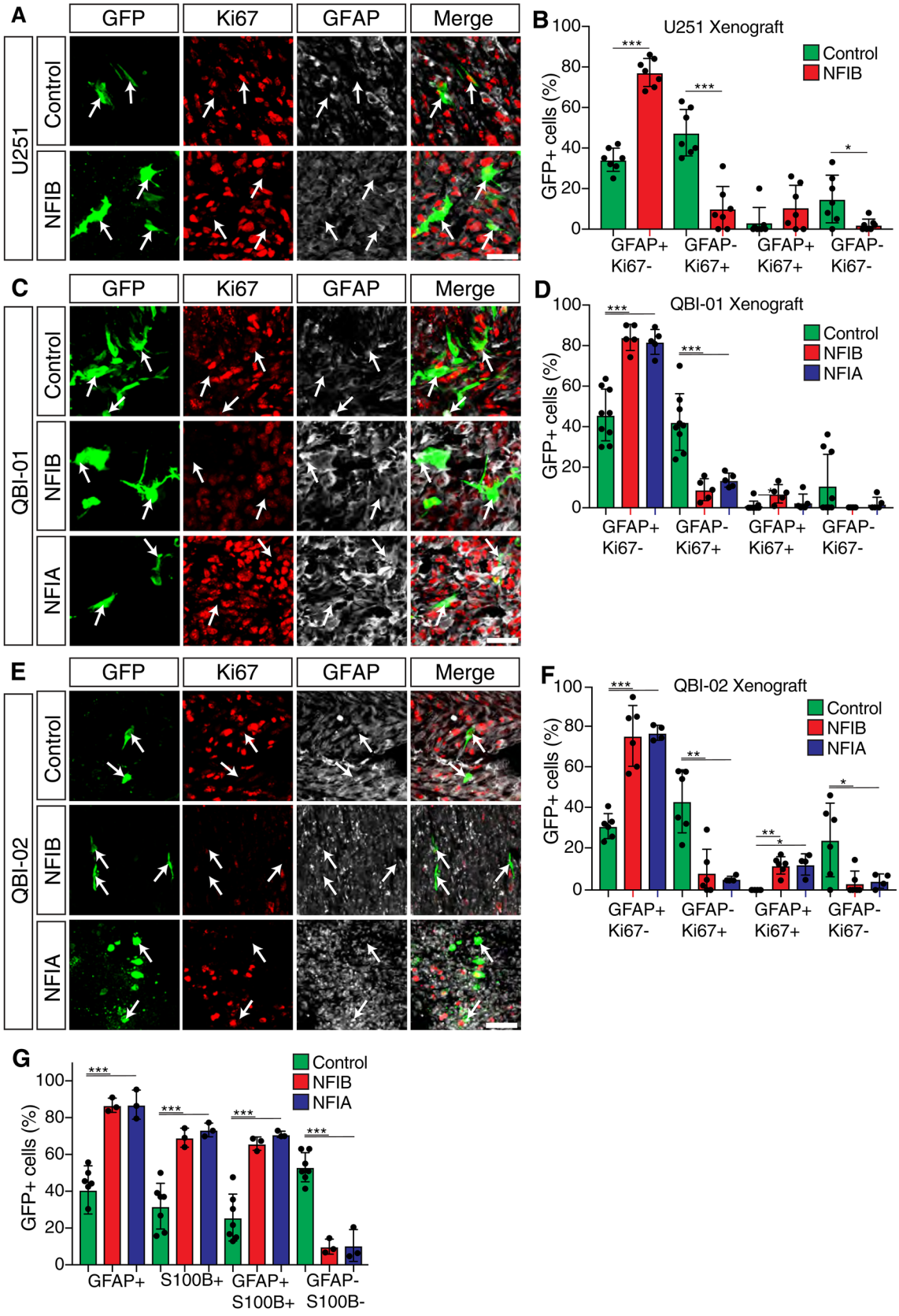
GFAP and Ki67 (Figs. 3, 4; Supplementary Figs. 1 and 2; Supplementary Tables 13 and 14). No difference in the co-staining pattern was observed between NFIA-positive or NFIB-positive cells. Over half of the NFI-positive cells co-expressed only GFAP (Fig. 4c), indicative of more differentiated cells, whereas less than a third of these cells were both GFAP- and Ki67-positive. The identity of the latter cells is questionable, but could perhaps represent cells that have just begun differentiating. We also observed that a subset of GFAP-positive cells was devoid of NFIA or NFIB expression. However, we were unable to determine whether both NFIA and NFIB were concurrently absent in these cells, as our NFIA and NFIB antibodies were derived from the same host species.

Fig. 5 In vivo electroporation of GBM xenografts with NFI overexpression constructs. **a**, **c** and **e** Examples of GFP+ cells (arrows) as a marker of cells electroporated with control, NFIA or NFIB overexpression plasmids in U251 (**a**), QBI-01 (**c**) and QBI-02 (**e**) xenografts. Sections were also stained for the proliferation marker Ki67 (red) and the astrocytic differentiation marker GFAP. **b**, **d** and **f** Quantification of co-localization of the xenografts represented as a percentage of the GFP+ cells in U251 (**b**), QBI-01 (**d**) and QBI-02 (**f**) xenografts electroporated with control, NFIA or NFIB overexpression plasmids. **g** Quantification of co-localization of GFP+ cells in QBI-02 with S100B and GFAP. Error bars represent the standard deviation of the mean. Statistical significance was determined using a Welch's t test; $*p<0.05$, $**p<0.005$, $***p<0.0005$. Scale bars (**a**, **c**, **e**) 50 µm. ▶

Expression of NFIA or NFIB induces tumour cell differentiation

NFI expression is indicative of differentiated cells that express mature astrocytic markers in GBM, but whether these transcription factors can actually drive the differentiation of proliferating cancer cells remains unclear. Overexpression of either NFIA or NFIB is sufficient to induce the expression of astrocytic markers in vitro [20, 23, 28], but this remains to be demonstrated for tumours in vivo. In addition, no study has yet to compare both NFIA and NFIB under similar conditions. To investigate whether NFI expression can drive astrocytic differentiation in vivo, we introduced NFIA or NFIB overexpression plasmids via in vivo electroporation into subcutaneous xenografts. As a proof of concept, we electroporated xenografts of the U251 GBM cell line, which is responsive to NFIB induction in vitro [28], Electroporated xenografts were sectioned and co-labelled for GFAP, Ki67 and green fluorescent protein (GFP), which is indicative of cells that were successfully electroporated (Fig. 5a). The overall number of GFAP-positive cells increased in NFIB-electroporated xenografts as compared to xenografts electroporated with the control plasmid (Fig. 5b; Supplementary Table 15). Specifically, we observed a concurrent decrease in GFAP-negative, Ki67-positive cells and a subsequent increase in GFAP-positive, Ki67-negative cells, indicating that proliferative cells transitioned into a differentiated state upon NFIB overexpression. A small but insignificant increase in the number of GFAP-positive, Ki67-positive cells was observed, suggesting that these could represent proliferative cells that are transitioning to a differentiated state.

To determine whether overexpression of NFIA or NFIB exerts the same effect, we electroporated NFIA, NFIB or


A
GFP
Ki67
GFAP
Merge
U251
Control
NFIB
B
U251 Xenograft
Control
NFIB
GFP+ cells (%)
GFAP+ Ki67-
GFAP- Ki67+
GFAP+ Ki67+
GFAP- Ki67-
C
QBI-01
NFIA
D
QBI-01 Xenograft
E
QBI-02
F
QBI-02 Xenograft
G
S100B+
GFAP+ S100B+
GFAP- S100B-

control plasmids into xenografts representing two patient-derived xenograft lines (Fig. 5c, e; Supplementary Table 15). These lines responded similarly to either NFIA or NFIB overexpression, with the number of GFAP-positive, Ki67-negative cells increasing to approximately 80% in NFIA- or NFIB-electroporated xenografts (Fig. 5d, f). We observed a concurrent reduction in the number of cells that were GFAP-negative, Ki67-positive, and GFAP-negative, Ki67-negative, while GFAP-positive, Ki67-positive cells increased. Immuno-labelling of the QBI-02 xenografts with S100B, another marker expressed in mature astrocytes, returned similar findings (Fig. 5g; Supplementary Fig. 4a and b). Thus, we conclude that either NFIA or NFIB is sufficient to induce astrocytic differentiation in GBM.

Discussion

In this study, we have demonstrated that both NFIA and NFIB function as regulators of astrocytic differentiation in astrocytoma. NFI expression is higher in lower grade astrocytomas, and the expression of NFIA and NFIB in GBM is typically associated with genes representing the mature astrocytic state. Furthermore, both these transcription factors are co-expressed in GBM cells that also express mature astrocytic markers. Most importantly, in vivo overexpression of either NFIA or NFIB in xenografts is sufficient to drive proliferative cells towards astrocytic differentiation. Hence, NFIA and NFIB retain their developmental role as inducers of astrocytic differentiation in astrocytoma, and could therefore act as tumour suppressors within a therapeutic context.

Although strong evidence exists to suggest that NFIB acts as a tumour suppressor in astrocytoma [3, 23, 26, 27], the role of NFIA remains disputable [3, 22, 26, 27, 41, 42]. In vitro experiments suggest that NFIA promotes rather than suppresses cancer cell proliferation [42]. However, our findings demonstrate that both NFIA and NFIB function as tumour suppressors as they drive proliferating cells to differentiate towards an astrocytic fate in vivo. Whether they share similar functions in other tumours requires further study, but this is unlikely to be the case. These transcription factors are widely expressed in many tissues during development, and both oncogenic and tumour suppressive roles have been reported which may be tissue-specific [43]. For instance, NFIA plays a vital role in the development of oligodendrocytes [44, 45], with the expression of NFIA, but not NFIB, being retained in adult oligodendrocytes [18]. In line with this, oligodendrogliomas tend to demonstrate lower *NFIA* expression than astrocytomas, in contrast to *NFIB* [22]. This lower expression may be associated with the partial loss of chromosome 1p31, which is more often observed in oligodendrogliomas than in astrocytomas [46]. Interestingly, overexpression of NFIA in a mouse model of oligodendroglioma resulted in tumours resembling astrocytomas [29]. In spite of this, as this previous study did not investigate whether these cells remained proliferative, another possible interpretation of the findings is that NFIA overexpression caused the cells to differentiate towards the astrocytic lineage, similar to our observations.

NFI proteins have also been implicated as tumour suppressors in other brain cancers, such as the SHH-subtype of medulloblastomas [47, 48]. This role was strongly corroborated using a mouse model of SHH-subtype medulloblastoma with heterozygous deletion of *Nfia* [47]. Compared to normal *Nfia* expression, loss of one allele increased tumour incidence and decreased tumour latency. Hence, NFI may broadly function as inducers of differentiation in different brain tumour cell types.

The identification of the *Nfi* loci as common insertion sites in insertional mutagenesis mouse models demonstrates that reduced *Nfi* expression contributes to glioma tumorigenesis [26, 27]. However, how tumour cells suppress *NFI* expression in astrocytomas in vivo to evade differentiation remains unclear. As mutations of the *NFI* genes are rare, haploinsufficiency of *NFIA* or *NFIB* appears to be the most common pathway through which their expression is reduced. It is also likely that *NFI* expression is regulated on a transcriptional level in proliferating tumour cells that have evaded differentiation commitment. Unfortunately, little is known about the upstream regulators of *NFI* during normal astrogliogenesis or in astrocytoma, so this requires further investigation. However, due to the strong positive correlation between *NFIA* and *NFIB* expression, it is likely that both have similar upstream regulators. A recent study proposed that *NFIA* expression could be mediated by TGFβ in normal gliogenesis [21]. Whether reduced *NFIA* expression in GBM is due to ectopic TGFβ signalling remains to be elucidated. Aside from this, post-transcriptional regulation may also contribute to *NFI* down-regulation. For example, the expression of the microRNAs miR-124 and miR-129 positively correlates with increased glioma grade, but inversely correlates with *NFIB* expression [49, 50]. Indeed in vitro experiments have demonstrated that microRNAs regulate *NFI* expression in astrocytoma cell lines, but their significance in vivo remains to be determined. Greater emphasis on understanding how *NFI* is suppressed in tumour cells is required for effective therapeutic manipulation of the NFI-mediated differentiation pathway.

In addition to direct manipulation of the NFI pathway as a potential differentiation therapy, increased expression of NFI may also act as a biomarker indicative of differentiation when testing novel therapeutic agents or for diagnostic purposes. It is not known whether NFI expression was induced and sustained for the differentiation agents tested [6–8]. The abundance of NFI proteins could even indicate whether a tumour would be more prone to induction of differentiation

for differentiation therapies instead of, or in combination with, the current standard treatment regime. Nevertheless, any diagnostic or prognostic value of NFIA or NFIB to clinical management will require further investigation.

In conclusion, our study demonstrates that both NFIA and NFIB play a direct role in inducing tumour differentiation in astrocytoma. Given that tumours with fewer differentiated cells are associated with a poorer clinical outcome, a deeper understanding of the NFI-mediated differentiation mechanisms may reveal a potential therapeutic strategy to reduce the proliferative potential of GBM cells via differentiation, and thereby improve patient survival.

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Author contributions Study concept and design: KSC, LJR, JB. Acquisition of data: KSC, CRB, ZL, JB. Analysis and interpretation of data: KSC, CRB, ZL, JWCL, LJR, JB. GBM cell lines: BWS, BWD. Pathology: RR, KTW, DG, HA. Drafting of the manuscript: KSC, JB, LJR. Revision of the manuscript: KSC, JWCL, ZL, BWS, RR, KTW, DG, HA, BWD, LJR, JB. Administrative and technical support: CRB. Obtained funding: LJR, JB. Study supervision: LJR, JB.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human material were in accordance with the ethical standards of the institutional research committee (University of Queensland Human Ethics Committee) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All procedures performed in studies involving animals were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and with the approval of the University of Queensland Animal Ethics Committee.

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