A leak pathway for luminal protons in endosomes drives oncogenic signalling in glioblastoma

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Epidermal growth factor receptor (EGFR) signalling is a potent driver of glioblastoma, a malignant and lethal form of brain cancer. Disappointingly, inhibitors targeting receptor tyrosine kinase activity are not clinically effective and EGFR persists on the plasma membrane to maintain tumour growth and invasiveness. Here we show that endolysosomal pH is critical for receptor sorting and turnover. By functioning as a leak pathway for protons, the Na⁺/H⁺ exchanger NHE9 limits luminal acidification to circumvent EGFR turnover and prolong downstream signalling pathways that drive tumour growth and migration. In glioblastoma, NHE9 expression is associated with stem/progenitor characteristics, radiochemoresistance, poor prognosis and invasive growth in vitro and in vivo. Silencing or inhibition of NHE9 in brain tumour-initiating cells attenuates tumoursphere formation and improves efficacy of EGFR inhibitor. Thus, NHE9 mediates inside-out control of oncogenic signalling and is a highly druggable target for pan-specific receptor clearance in cancer therapy.
glioblastoma multiforme (GBM) is the most common and aggressive form of adult primary brain tumour with extremely poor prognosis. Diffuse invasion of tumour cells into the surrounding brain parenchyma makes surgical resection largely ineffective and the pluripotent behaviour of a small group of brain tumour-initiating cells (BTICs) in GBM confers drug resistance and recurrence even after aggressive treatment. As a result, clinical outcomes of patients have failed to show substantive improvement over median survival times of 12 months and innovative approaches to therapy are urgently needed. The key step in moving towards personalized medicine involves identifying specific genes and pathways to be targeted in individual patients. This has been the driving force for compiling databases of genome and proteome changes in GBM patients that are now being successfully mined to uncover novel risk factors and potential therapeutic targets. Using this approach, we made the unexpected discovery that in a subset of glioblastomas, the autism-associated gene SLC9A9 (refs 6–8) was a potent driver of tumour growth and migration, associated with decreased patient survival and increased aggressiveness in vivo. Previously, we showed that loss-of-function mutations in SLC9A9 encoding the endosomal Na+/H+ exchanger NHE9 result in hyperacidification of sorting endosomes and cellular trafficking defects associated with autism. In contrast, we now report that gain of function in NHE9 results in excessive luminal alkalization in BTICs from patient glioblastomas. Although studies of pH, ionic and volume modulation in tumour cells have thus far been restricted to plasma membrane ion transporters, there have been intriguing hints of a link between organelar pH and oncogenic signalling by growth factor receptors. The human papillomavirus type 16 E5 oncoprotein activates growth factor receptors Epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor with concomitant alkalization of Golgi and endosomes, following binding to H+-pumping V-ATPase 16 kDa subunit. Furthermore, pharmacological disruption of V-ATPase activity by bafilomycin A1 was found to augment the extent and duration of EGFR activation. However, a causal link between luminal pH and oncogenic signalling remains to be established, and the prevailing model of the luminal pH set point overwhelmingly focuses on the proton pumping V-ATPase, with the role of proton leak pathways, mediated by endosomal Na+/H+ exchange, being largely understudied.

In this study we demonstrate that NHE9 levels in patient-derived BTICs drive proliferation rates, cell adhesion, migration directionality and speed in vitro and in vivo mouse xenograft models. Mechanistically, these events are mediated by the luminal pH of sorting endosomes with distinct effects on endocytosis and exocytosis of cargo. EGFR is mutated, amplified or transcriptionally upregulated in 50% of human GBM and its downstream signalling network includes highly deregulated and oncogenic components responsible for uncontrolled cell proliferation in most cancers. By drastically attenuating receptor turnover, NHE9 exerts posttranslational control on membrane persistence of EGFR, confers increased resistance to receptor tyrosine kinase inhibitors and drives oncogenic signalling pathways that regulate cell division and migration. Importantly, posttranslational control by NHE9 overrides differences in EGFR transcript levels in GBM tumour cells. Finally, we demonstrate that a pharmacological inhibitor of NHE transporters works together with a known EGFR inhibitor to impair GBM tumour growth. Given the dismal prognosis of glioblastoma, these observations highlight the potential of developing selective anti-NHE9 therapeutics. Taken together, our findings reveal a crucial, hitherto unrecognized role for luminal pH in GBM progression and allow us to propose NHE9 as a promising chemotherapeutic target.

Results

NHE9 affects patient survival and GBM proliferation. Gene expression profiles for NHE9 (SLC9A9) were extracted from human glioblastoma arrays on Oncomine and The Cancer Genome Atlas (TCGA) databases. NHE9 expression varied widely among human glioblastoma subtypes, with 36% of mesenchymal tumours showing significant NHE9 upregulation (Supplementary Fig. 1A). Notably, SLC9A9 was in the top 12% of overexpressed genes in a human gene chip array of 101 samples, with messenger RNA levels averaging 5.7-fold higher (P = 0.012; Student’s t-test) in glioblastomas relative to control neural stem cells (Fig. 1a). Interrogation of the TCGA database revealed that patients with unaltered expression of NHE9 (71.4% of 91 GBM cases) survived longer with the difference in maximal survival between these patients of 65 months (Fig. 1b; P = 0.032; log-rank test). In addition, patients with unaltered expression of NHE9 remained disease free for a median period of 8.4 months after tumour resection. In contrast, NHE9 upregulation (28.6% of 91 GBM cases) correlated with significantly shorter disease-free periods with a median of only 4.8 months (Supplementary Fig. 1B; P = 0.012; log-rank test). A more thorough examination of the TCGA data set including a subset of patients who received neoadjuvant chemotherapy, which included temozolomide (>50% cases), PCV (procarbazine, CCNU and vincristine) and others, revealed that patients with unaltered expression of NHE9 showed strikingly better response and survived longer with a median survival of 58.68 months, in contrast to 15.98 months in patients with NHE9 upregulation (Fig. 1c; P = 0.00002; log-rank test). Furthermore, patients with unaltered expression of NHE9 with neoadjuvant chemotherapy remained disease free after resection for a median period of 33.67 months, in comparison with 11.28 months in patients with NHE9 upregulation (Supplementary Fig. 1C; P = 0.00295; log-rank test). The finding that NHE9 confers resistance to treatment is strengthened by a recent study demonstrating that patients with esophageal squamous cell carcinomas (ESCCs) with NHE9 upregulation showed poor response to neoadjuvant chemoradiotherapy. Taken together, the observations suggest that NHE9 expression levels may have a role in GBM prognosis.

Based on these initial observations, we evaluated NHE9 transcript levels in three clinical GBM specimens (GBM 612, GBM 253 and GBM 276; Supplementary Table 1), cultured in serum-free conditions to enrich for BTIC that maintain phenotype and genotype characteristics of the original tumour. NHE9 expression, similar to that of CD133 (ref. 15), was associated with stem/progenitor characteristics, as seen by a significant decrease in transcript on culturing GBM 612 in serum (Fig. 1d). No transcriptional change was observed for the closely related isoform NHE6 after transfer to serum. Comparative analysis of three early-passage BTIC lines derived from patient tumours showed a 19.4-fold variation in NHE9 transcript levels (GBM 276 < GBM 253 < GBM 612; Fig. 1e), consistent with the variation observed in database surveys. The correlation of NHE9 levels with higher growth rates in GBM 612 relative to GBM 276 was suggestive of a role in tumour cell proliferation (Fig. 1f). To test this hypothesis, we engineered lentiviral-mediated elevation of NHE9 in GBM 276 and, conversely, attenuated NHE9 transcripts in GBM 612 as shown Fig. 1g. No compensatory changes in mRNA levels were observed in the related endosomal isoform NHE6 or V-ATPase subunit V O A1 in these cells (Fig. 1g). Consistent with a role for NHE9 in tumour cell proliferation, growth rates in GBM 276 cells overexpressing NHE9 were significantly higher than vector-transfected control cells (Fig. 1h). Conversely, knocking down NHE9 expression decreased cell proliferation in GBM 612 cells (Fig. 1i). These findings were replicated under both normoxia and hypoxia conditions.
We note that cell viability remained unaltered by introduction or knockdown of NHE9 (Supplementary Fig. 2E). Therefore, we conclude that NHE9 levels regulate proliferation of BTIC in vitro.

NHE9 promotes GBM tumorigenesis and invasion. Next, we evaluated in vivo effects of widely differing NHE9 expression in two human glioblastomas, GBM 276 (Fig. 2a,b) and GBM 612 (Fig. 2c,d), using intracranial xenograft transplants in a nude mouse model (Fig. 2e). Following silencing of NHE9 in GBM 612 (GBM 612 NHE9-), tumour area in implants was visibly reduced relative to vector-transfected control (Fig. 2f,g). These differences were quantitatively significant by an average of ~2.2-fold (Fig. 2h; P = 5.3 × 10^-6; two-tailed Welch’s t-test). Over-expression of NHE9 in GBM 276 (GBM 276 NHE9+) resulted in a significant increase in tumour size (~2-fold on average; P = 6.1 × 10^-5; two-tailed Welch’s t-test) relative to the vector-transfected control (Fig. 2i and Supplementary Fig. 3). Invasive capacity was assessed from the number of cells migrating into the contralateral hemisphere, as described previously. Similar to the observations with tumour size, there was a significant reduction of tumour cell invasion following NHE9 silencing in GBM 612 (P = 0.0025; two-tailed Welch’s t-test) and increased invasion with NHE9 overexpression in GBM 276 (P = 0.0003; two-tailed Welch’s t-test; Fig. 2j,k) when we analysed cell migration in vivo.

Figure 1 | NHE9 expression in glioblastoma affects patient survival, chemoradiation resistance and tumour stem cell proliferation. (a) NHE9 transcript is elevated in glioblastoma tumour stem cells (n = 22) relative to normal neural stem cells (n = 3) (*P = 0.012, Student’s t-test). (b, c) NHE9 transcript is upregulated in 28.6% of cases found in TCGA database when a Z-score of 0.5 was selected. (b) Patients with GBM upregulation of NHE9 had decreased overall survival. (c) Of 21 patients who received neoadjuvant chemoradiotherapy, those with unaltered expression of NHE9 showed better response to treatment and survived longer (P = 0.000002; log-rank test) relative to those with upregulation of NHE9. (d) Quantitative PCR (qPCR) analysis of NHE9, NHE6 and CD133 mRNA from GBM 612 tumour stem cells cultured in medium without or with serum. Transcript levels of NHE9 and CD133 decrease in serum (*P = 0.012, **P = 0.0001, two-tailed Student’s t-test with Welch’s correction, n = 3), whereas NHE6 shows no change. (e) qPCR analysis of NHE9 mRNA in cells from three primary GBM tumours 276, 253 and 612 (**P = 0.0003, ***P = 1.01 × 10^-5, two-tailed Welch’s t-test, n = 3). Inset shows ethidium bromide-stained agarose gel of reverse transcriptase–PCR products of mRNA from the GBM cells. (f) Normalized proliferation of GBM 276 and 612 (***P = 1.87 × 10^-5, **P = 0.0001, two-tailed Welch’s t-test, n = 3) as determined by MTS assay. (g) qPCR analysis showing the efficacy of overexpression and shRNA knockdown of NHE9 in GBM 276 and 612 cells, respectively (**P = 0.0008, ***P = 3.44 × 10^-5, two-tailed Welch’s t-test, n = 3). (h) Normalized proliferation of GBM 276 control cells and 276 cells overexpressing NHE9 (**P < 0.01, two-tailed Welch’s t-test, n = 3). (i) Normalized proliferation of GBM 612 control cells and 612 cells with shRNA knockdown of NHE9 (*P < 0.05, **P = 0.0018, two-tailed Welch’s t-test, n = 3). Bars represent mean ± s.d. All cell cultures were counted, seeded at similar levels and cell densities determined by measuring absorbance at 490 nm (Abs490 nm); initial values were as follows (average of triplicates, s.d.) GBM 276 (0.148, 0.010); GBM 276 + NHE9 (0.155, 0.011); GBM 612 (0.174, 0.022); GBM 612-NHE9 (0.167, 0.041). See related Supplementary Figs 1 and 2.
Patient MRI (GBM 276)

1.5 × 10^5 cells per mouse

GBM BTIC culture

200

GBM 612

GBM 276

GBM 276 NHE9+

Tumour area (% control)

Human nuclei DAPI

Migrating cells (% control)

8 Weeks

Tumour area

Migrating cells

GBM 612

GBM 612 NHE9–

GBM 276

GBM 276 NHE9+

**

***

200

250

300

350

150

100

50

0

GBM 612

GBM 612 NHE9–

GBM 276

GBM 276 NHE9+

**

350

300

250

200

150

100

50

0

GBM 276

GBM 276 NHE9+

**

3,500

3,000

2,500

2,000

1,500

1,000

700

600

500

400

300

200

100

0
Together, both in vitro and in vivo findings confirm a critical role for NHE9 in tumour progression.

NHE9 drives GBM BTIC migration. The diffuse and aggressive infiltration of tumour cells along vascular and white matter tracts is directly responsible for the inefficacy of tumour resection, resulting in mean survival times of only 12–15 months. Central to the invasiveness associated with glioblastomas, as observed in Fig. 2, are tumour cell–substratum interactions and subsequent modification of matrix barriers. In vivo, tumour cells interact with the extracellular matrix in the nanometre scale and respond to topographical cues that affect cell adhesion and gene expression. To study BTIC migration in vitro under conditions of GBM dissemination, we conducted directional migration assays on nano-ridges constructed of transparent polyurethane acrylate coated with laminin (Fig. 3a) as previously reported by our group. These longitudinal tracts closely mimic mechanical cues found in extracellular matrix such as capillaries and nerve fibre tracts along which tumour cells migrate. Biased migration along the grooves was quantified using time-lapse microscopy for a period of 15 h at regular intervals of 15 min in GBM pairs 276 and 612, with NHE9 overexpressed or silenced, respectively. Phase-contrast images of a representative 2-h period for GBM 276 and GBM 276 NHE9 + is shown in Fig. 3b. Quantification of cell speed over time for each population (Fig. 3c,d) and the total average cell speed (Fig. 3e–g) revealed that elevation of NHE9 expression in GBM 276 increased migration speed and consequently distance travelled, whereas attenuation of the high expression of NHE9 in GBM 612 had the converse effect, consistent with the invasion trends observed in mouse xenografts. Next, we evaluated specific parameters of migratory behaviour likely to contribute to invasive ability. Migration alignment, which is the ratio of cell movement parallel to the patterned ridges to that perpendicular to the pattern (Fig. 3h), correlated with migration speed and a significant change in alignment was observed on attenuation of NHE9 in GBM 612 cells (Fig. 3i,j). Migration persistence, ratio of the actual distance travelled by the cell to the shortest distance between the initial and final points (Fig. 3k), was also significantly affected by changes in NHE9 expression in both the GBM lines (Fig. 3l,m). Thus, multiple parameters confirm that NHE9 regulates tumour cell migration in brain cancer.

NHE9 modulates endosomal pH and trafficking. As a starting point to decipher the mechanistic basis for tumour promotion, we focused on GBM 253, in which NHE9 expression was intermediate between GBM 276 and GBM 612 BTIC (Fig. 1e). Ectopic expression of NHE9 in GBM 253 significantly increased cell proliferation (Supplementary Fig. 4A), consistent with results previously reported for the closely related endosomal isoform NHE6. Furthermore, NHE9-GFP localization in tumour cells was similar to that observed in GBM (Supplementary Fig. 5E,F). Therefore, we concluded that NHE9-GFP co-localized with endocytosed transferrin tagged to Alexa Fluor 633, relative to green fluorescent protein (GFP)-transfected controls, as measured by flow cytometry (Fig. 4f,g) and confocal microscopy (Fig. 4h,i). To determine whether increased transferrin accumulation (Fig. 4j) was dependent on Na⁺/H⁺ exchange activity and not a nonspecific effect of gene overexpression, we evaluated the effect of previously described autism-associated loss-of-function mutations in NHE9 (refs 6,8). Single amino-acid substitutions in conserved regions of mammalian NHE9, L236S and S438P (Fig. 4i) were generated and introduced into GBM 253 cells. Mutant NHE9 (S438P) failed to increase cell proliferation, in contrast to wild-type control (Supplementary Fig. 4B). Similar to the wild-type control, loss-of-function NHE9 mutants also co-localized with Tfn-Alexa Fluor 633 (Supplementary Fig. 5G,H), but failed to elevate Tfn-Alexa Fluor 633 accumulation (Fig. 4k). Therefore, we concluded that ion transport activity by NHE9 alters transferrin trafficking in GBM cells.

NHE9 is proposed to function as a leak pathway for protons, limiting the acidification of the endosomal lumen by the proton pumping V-ATPase. Therefore, high levels of NHE9 should make the endosomal lumen more alkaline, via cation/proton exchange activity. To measure endosomal pH (pHe), we took advantage of co-localization of NHE9 with transferrin, as shown in Fig. 4c,d. Luminal pH was quantified by a ratiometric method using pH-sensitive fluorescence of fluorescein isothiocyanate (FITC)-tagged transferrin, normalized for differences in pH-sensitive fluorescence of Alexa Fluor-633 linked to transferrin (Fig. 5a) and calibrated using buffers of known pH (Fig. 5b). Expression of NHE9 in GBM 253 cells resulted in >1 pH unit elevation of pHe from 5.71 ± 0.23 to 6.90 ± 0.47 (Fig. 5c). In contrast, there was no significant change in cytoplasmic pH (Supplementary Fig. 4C,D), as has been previously reported for the closely related endosomal isoform NHE6 (ref. 21). To establish a causal link between compartmental pH and trafficking of transferrin, we evaluated a known alkalinizing agent, monensin, which also mediates Na⁺/H⁺ exchange activity. Luminal pH in GBM 253 cells treated with monensin (500 μM) was also elevated (to 7.02 ± 0.20), similar to cells expressing NHE9 (Fig. 5c). We observed dose-dependent increase in steady state Tfn-Alexa Fluor 633 accumulation with monensin (Fig. 5d). Next, we compared the effect of monensin and NHE9 overexpression on the kinetics of transferrin uptake.
Figure 3 | GBM BTIC migration on a nanopatterned substrate is affected by NHE9. (a) Laminin-coated, longitudinal nanopatterned tracks with the designated dimensions were used for analysis of BTIC migration. (b) Representative time-lapse microscopic images collected every 15 min compare the migration of GBM 276 cells transduced with vector control and NHE9 over a period of 2 h. Population speed, over a period of 5 h, of GBM 276 (c) and GBM 612 (d) cells relative to NHE9 overexpression or knockdown as indicated. Schematic representations of migration speed (e), alignment (h) and persistence (k) of GBM BTICs are shown, along with the formulae used for quantification. GBM 276 BTICs transduced with lentiviral vectors overexpressing NHE9 migrate faster (f) and are more persistent (l) than control cells (***P = 0.0004, ***P = 3.69 x 10^-11, two-tailed Welch’s t-test, n = 75). GBM 612 BTICs treated with NHE9 shRNA show a decreased migration speed (g), alignment (j) and persistence (m) when compared with control virus-transduced cells (***P < 0.0001, *P = 0.036, two-tailed Welch’s t-test, n = 75). There was no significant difference in the alignment between control and NHE9-overexpressing GBM 276 BTICs (i). Bars represent mean ± s.d. Scale bar, 50 μm (c).
**Figure 4 | NHE9 localizes to recycling endosomes and alters transferrin uptake.** (a-d) Representative confocal microscopy images of GBM 253 cells expressing NHE9-GFP. Cells were fixed with 4% PFA and Rab11, and observed by immunofluorescence. Subcellular co-localization of NHE9-GFP (green) with Rab11 (red) is seen in the merge (a) and orthogonal slice (b). Cells were incubated with transferrin tagged to Alexa Fluor-633 (Tfn-633) for 55 min and imaged (c) to show overlap with NHE9 in the perinuclear region and cell periphery, as seen in the orthogonal slice (d). (e) GBM 253 cells were transfected with GFP vector or NHE9-GFP and incubated with Tfn-633 for 55 min, to compare steady-state transferrin uptake. (f–g) GBM 253 cells were subjected to fluorescence-activated cell sorting (FACS) analysis to separate untransfected cells (blue) from GFP-expressing cells (green). Black dashed line indicates average fluorescence intensity (y axis) of GFP positive (x axis) population. There was an increase in average fluorescence of Tfn-633 in cells expressing NHE9-GFP relative to the GFP control, as seen by shift in the population over the black line. (h–i) Fluorescence micrograph of GBM 253 cells treated with Tfn-633 as in (f) and (g), respectively. Fluorescence intensity and exposure settings were the same in the two images. Following background subtraction, fluorescence signal was increased by 2.8-fold in (i) relative to (h). (j) Quantification of Tfn-633 fluorescence from FACS analysis in GBM 253 control cells and 253 cells overexpressing NHE9 (**P = 0.0005, two-tailed Welch’s t-test, n = 3 with > 5,000 cells). Note that the population in P2 may underestimate low expressors of NHE9-GFP in panel 5G. (k) Previously described loss-of-function mutations in NHE9 (L236S and S438P) failed to elevate Tfn-633 accumulation (**P < 0.01, ***P = 2.56 × 10^{-7}, two-tailed Welch’s t-test, n = 3). (l) NHE9 and gene orthologue alignments showing residue conservation of loss-of-function mutations in various species. Bars represent mean ± s.d. Scale bars, 20 μm. See related Supplementary Figs 4 and 5.
and efflux. NHE9 overexpression increased the rate and steady-state levels of transferrin accumulation, whereas it had no effect on the rate of efflux (Fig. 5e–h). In contrast, the increase in transferrin accumulation observed with monensin treatment was largely due to a decrease in the rate of efflux (Fig. 5e–h). Thus, although both NHE9 and monensin alkalinized endosomal compartments to similar extent, they had distinct effects on transferrin uptake and efflux. It was possible that enhanced
transferrin uptake resulting from ectopic expression of NHE9 was the result of elevated surface expression of the transferrin receptor on GBM 253 tumour cells. To test this hypothesis, we observed transferrin-Alexa Fluor 633 bound to GBM 253 cells on ice. Under these conditions, endocytosis was greatly reduced, allowing transferrin receptors present on the cell surface to be visualized. More surface-bound fluorescence was observed in GBM 253 cells transfected with NHE9 (Fig. 5i), consistent with elevated levels of transferrin receptor, which was confirmed by surface biotinylation (Fig. 5j). These findings suggest that NHE9 elevates surface levels of membrane receptors on GBM cells. In summary, we used a variety of experimental approaches, including flow cytometry, confocal microscopy, surface labelling and biotinylation in NHE9-overexpressing or monensin-treated cells, to demonstrate that endosomal Na⁺/H⁺ exchange activity can strongly affect binding and uptake of transferrin at the cell membrane of GBM BTIC. These observations, if extended to other cell surface proteins and growth factor receptors, have the potential to impact oncogenic signalling.

**NHE9 promotes EGFR signalling.** A striking and signature feature of GBM is dysregulated EGFR activity, with gene amplification, mutation and alteration in receptor tyrosine kinase pathways driving tumour growth in over 80% of gliomas. Although EGFR signalling pathways are compelling targets for therapeutic intervention, clinical outcomes for the first generation of anti-EGFR therapeutics have been disappointing in patients with GBM. This lack of efficacy of tyrosine kinase domain inhibitors has been attributed to redundancy in signalling pathways, tumour heterogeneity and mutation-specific differences in inhibitor sensitivity. An independent and alternative strategy to inhibiting receptor tyrosine kinase phosphorylation is to downregulate cell surface availability of the receptor by exploiting negative feedback mechanisms known to control the duration and intensity of EGFR signalling. Based on our finding that NHE9 modulated transferrin receptor levels to regulate ligand uptake, we next investigated the effect of NHE9 on EGFR signalling in GBM BTIC. Growth rates of both GBM 276 and GBM 612 were responsive to EGF and were further increased or decreased, respectively, by NHE9 expression or knockdown (Supplementary Fig. 6A,B). Significantly, proliferation was lowest in the absence of EGF and NHE9 expression, suggesting that NHE9 may synergize with EGFR signalling to promote tumour cell growth (Supplementary Fig. 6A,B). EGFR transcript levels were the same in GBM 216 and GBM 276 (Supplementary Fig. 6C). Strikingly, however, plasma membrane expression of EGFR, as determined by surface biotinylation, was nearly threefold higher in the faster growing and more aggressive GBM 612 BTIC, relative to GBM 276 (Supplementary Fig. 6D). These findings suggested that differences in NHE9 expression between the two GBM cell lines were critical in determining surface expression of EGFR. Confirming this hypothesis, pair-wise experiments indicated that elevation of NHE9 expression in GBM 276 significantly increased plasma membrane persistence of EGFR relative to vector-transfected control (Fig. 6a,c). Conversely, transcriptional attenuation of NHE9 in GBM 612 cells significantly decreased surface expression of EGFR (Fig. 6b,d). Consistent with our findings, there was no correlation in gene expression between NHE9 and EGFR in the TCGA data set (Pearson correlation: −0.157, P-value: 0.141, n = 91), and the subset of patients with mutations in EGFR (15 of 90 patients) was not associated with changes in survival or in NHE9 expression. Thus, we concluded that NHE9 functions posttranscriptionally to regulate EGFR signalling.

Stimulation with EGF triggers receptor endocytosis and lysosomal degradation, resulting in disappearance of ~70% EGFR in GBM 276 cells and ~36.4% in GBM 612 cells within 3h, in the absence of new protein synthesis (Fig. 6e,g, respectively). These differences in EGFR turnover could be directly linked to NHE9 levels. Thus, elevated expression of NHE9 in GBM 276 limited EGFR degradation to ~15%, whereas attenuation of NHE9 expression in GBM 612 tumour cells greatly enhanced EGFR turnover by ~96.4% of initial levels, within 3 h (Fig. 6i and h,i, respectively). Next, we investigated the impact of altered NHE9 expression on EGFR signalling in GBM BTIC. NHE9 knockdown in GBM 612 decreases phosphorylation of EGFR (Fig. 6k,l) and key downstream signalling components that mediate tumour proliferation and invasion, including ERK, Cyclin D and AKT (Fig. 6m). Consistent with these findings, increased NHE9 expression in GBM 276 cells resulted in increased activation of the EGFR signalling pathway (Supplementary Fig. 6E).

Receptor sorting and degradation are dependent on acidic luminal pH in the endo-lysosomal system. We measured endosomal pH, as described for GBM 253 in Fig. 5, in GBM 276 and GBM 612. As predicted, pHc correlated with NHE9 expression in the three GBM cell lines. GBM 276, showing lowest NHE9 levels, also had lowest endosomal pH (5.57 ± 0.13), whereas GBM 612, which had the highest NHE9 levels, had the highest pHc (6.88 ± 0.13). Furthermore, ectopic expression of NHE9 in GBM 276 significantly alkalized pHc (6.61 ± 0.21; P = 0.023) and knockdown of NHE9 in GBM 612 resulted in corresponding acidification (to 6.63 ± 0.095; P = 0.049). Together, these data indicate that dysregulation of EGFR signalling by
NHE9-mediated endosomal pH regulation could be one of the critical mechanisms leading to tumour progression.

Therapeutic potential of NHE9 inhibition. To explore the potential of NHE9 as a therapeutic target in a subset of GBMs, we first tested the effect of NHE inhibitors on GBM BTIC proliferation. The amiloride analogue 5-((N-ethyl-N-isopropyl)amiloride (EIPA) is a general NHE inhibitor24. The IC_{50} for EIPA was 100 μg ml^{-1} for GBM 276 tumour cells and increased by 20% following overexpression of NHE9 (Fig. 7a), consistent with NHE9 being a target for EIPA. We also observed dose-dependent reversal of the NHE9-mediated increase in transferrin uptake in GBM 253 cells by EIPA (Supplementary Fig. 7A). Lower doses of EIPA, sufficient to inhibit NHE1, failed to inhibit growth of GBM 276 or reverse NHE9-mediated increase in cell proliferation (Supplementary Fig. 7B). Overexpression of NHE9 greatly decreased the efficacy of Erlotinib, a known tyrosine kinase inhibitor of EGFR. Addition of EIPA (100 μg ml^{-1}) effectively reversed the drug resistance conferred by NHE9, decreasing IC_{50} for erlotinib from 52 to 31 μg ml^{-1} relative to that of the control GBM 276 cells, 17 μg ml^{-1} (Fig. 7b).

Similarly, treatment with EIPA improved inhibition of GBM 612 cells by Erlotinib (Supplementary Fig. 7C). We show that EIPA reduces EGFR expression in GBM 612 BTIC on addition of EGF (Fig. 7c,d), consistent with the increased efficacy of Erlotinib following EIPA treatment. U0126, a known mitogen-activated protein kinase (ERK1/2) inhibitor, also decreased the cell proliferation in GBM 276 cells overexpressing NHE9 (Supplementary Fig. 7D), suggesting that the proliferation effects of NHE9 in these GBM cells were partly mediated by mitogen-activated protein kinase signalling, a major effector pathway in the EGFR network.

Renewable tumoursphere formation (Fig. 7e) is associated with tumorigenic capacity and rapid tumour progression, and is a significant predictor of clinical outcome25. Knockdown of NHE9 in GBM 612 cells reduced the number of tumourspheres by ~1.6-fold compared with control cells (Fig. 7f,h,i). The diameter of the tumourspheres was also reduced by ~3.3-fold (Fig. 7g–i). Treating GBM 612 cells with 100 μg ml^{-1} of EIPA drastically reduced both the number and diameter of tumourspheres by ~7-fold (Fig. 7f–h,i). Although the EIPA effects could include non-selective inhibition of other NHE isoforms besides NHE9, our results support the therapeutic potential of NHE9 as a drug target for a subset of GBM expressing elevated NHE9 levels. In such cases, bimodal therapy with known EGFR inhibitors could be an effective therapy.

Discussion
A common pathophysiological hallmark of tumours is a perturbation in pH dynamics26. Oncogene-mediated elevation of cytoplasmic pH by constitutive activation of NHE1 and ensuing extracellular acidification have long been recognized as an early step in neoplastic transformation driven by ras and c-mos27,28. In contrast, the role of endosomal pH modulation in oncogenesis has not been studied, although there is emerging evidence that endosomal pH regulation is involved in tumour progression. The current study demonstrates that NHE9, a critical downstream player in both endosomal pH regulation and EGFR signalling, is an effective therapeutic target for a subset of GBMs expressing elevated NHE9 levels.
evidence implicating organellar Na⁺/H⁺ exchangers in various cancers. In hypermutated colorectal tumours, SLC9A9, the gene encoding NHE9, was found to be among the most frequently mutated targets along with ACVR2A, APC, TGFB2, MSH3, MSH6 and TCF7L2 (ref. 29). Consistent with this finding, a genome-wide scan for colorectal cancer susceptibility identified linkage to chromosome 3q, with SLC9A9 being one of the candidate genes (ref. 29). Furthermore, a genome-wide case–control study of ESCC including 300 ESCC cases and 300 matched controls discovered significant associations of single-nucleotide polymorphisms in SLC9A9 with ESCC risk (ref. 31). The link between NHE9 and ESCC is strengthened by a recent study demonstrating

Figure 7 | NHE inhibitor EIPA mitigates resistance to Erlotinib, EGFR persistence and tumoursphere formation in GBM BTICs. (a) GBM 276 cells were treated for 24 h in the presence of different concentrations of EIPA and cell viability was measured with a colorimetric MTS assay. The graph shows IC₅₀ for EIPA is elevated in cells overexpressing NHE9 relative to vector control (*P < 0.05, two-tailed Welch’s t-test, n = 3). (b) GBM 276 control cells, NHE9-overexpressing cells and NHE9-overexpressing cells treated for 24 h with 85.83 µg ml⁻¹ (EIPA IC₅₀ of 276 NHE9+ cells) were treated with different concentrations of EGFR inhibitor Erlotinib and cell viability was measured by MTS assay (n = 3). (c–d) GBM 612 cells were growth factor starved, pre-incubated with cycloheximide (CHX) for 2 h and treated with EGF (20 ng ml⁻¹) for the times indicated before cell lysates were collected and analysed by western blotting. Six hundred and twelve cells treated with EIPA (85.83 µg ml⁻¹) showed an increase in EGF-stimulated EGFR degradation compared with vector control (c) and representative blots are shown (n = 3). EGFR bands in c were normalized to their GAPDH control and graphed as a percentage of controls (d) without the addition of EGF. (e) Adherent GBM BTICs not cultured in the presence of EGF were dissociated and transferred to non-adherent conditions (without laminin or poly-L-ornithine coating). Cells were cultured in StemPro NSC SFM media with EGF (20 ng ml⁻¹) for 21 days before tumoursphere formation was analysed. (f–g) Image J software was used to determine the number (f) and diameter (g) of tumourspheres from GBM 612, GBM 612 cells transfected with NHE9 shRNA and GBM 612 cells treated with EIPA (85.83 µg ml⁻¹; *P < 0.05, **P < 0.01, ***P < 0.0001, two-tailed Welch’s t-test, n = 3). (h–j) Representative phase-contrast images of the tumourspheres are shown. Scale bar, 50 µm. Bars represent mean ± s.d. See related Supplementary Fig. 7.
that patients with ESCC with NHE9 downregulation (>2-fold) showed complete response to neoadjuvant chemo-radiotherapy. Despite these clinical and genetic links, the molecular role of NHE9 in carcinogenesis was previously unknown. There has been one report showing that ectopic expression of NHE7, a closely related isoform of NHE9 that localizes to the trans-Golgi network, increased tumorigenicity in a breast cancer cell line. Although the underlying mechanisms were not investigated, the study showed that whereas tumour invasion was enhanced by both NHE1 and NHE7, only NHE7 could promote anchorage-independent growth and cell adhesion.

There is new evidence from model organisms as well as mammalian cells that luminal pH in the secretory-endosomal pathway is set by a balance of proton pump and leak, and is critical for normal vesicle trafficking. Although the importance of the H⁺ pumping V-ATPase is firmly established, we are only beginning to appreciate the complementary role of eNHE as a H⁺ leak pathway for the precise tuning of luminal pH. Pathophysiological elevation or loss of NHE9 activity is associated with glioblastoma (this study) or autism, as a consequence of excessive luminal alkalization and acidification, respectively (Fig. 8a). Early endosomes transit through a series of increasingly acidic compartments, culminating with delivery and degradation of cargo at the lysosome (Fig. 8b, left panel). Luminal pH affects the fate of cargo, both by modulating acid-activated hydrolases and by directing vesicle traffic through one of the several routes. Thus, premature activation of vacuolar acid hydrolases in the yeast mutant lacking the eNHE orthologue, NHX1, results in degradation of chaperone protein VPS10 in the prevacuolar compartment and missorting of cargo to the extracellular medium. Similarly, enhanced turnover of EGFR resulting from silencing or inhibition of NHE9 may be due to acidification of the endolysosomal system and activation of acid hydrolases. On the other hand, receptors may escape degradation by recycling back to the plasma membrane instead of being sorted to the late endosome/lysosome. We show
that elevated levels of plasma membrane receptors seen in NHE9-overexpressing GBM cells greatly enhance Alexa Fluor-labeled transferrin uptake by TfnR-mediated endocytosis and downstream signalling from EGFR.

The EGFR signalling network is a powerful driver of glioblastoma47. Ligand-bound EGFR not only activates intracellular tyrosine kinases directly but synergistically transactivates other cell surface receptors to ignite signalling cascades that control cell proliferation, invasion and apoptosis. Yet, despite the importance of EGFR signalling, multiple clinical trials using EGFR kinase inhibitors have had disappointing clinical outcomes due to their lack of tumour cytotoxicity. Although kinase inhibition of EGFR results in decreased cell proliferation, cell death appears to require the loss of EGFR protein, consistent with vastly different in vivo phenotypes of EGFR knockout and mutants with greatly attenuated kinase activity8,39,39. The resistance of GBM to EGFR kinase inhibitors has been attributed to a host of confounding factors, including activation of compensatory pathways, constitutively active mutations and kinase-independent prosurvival roles of EGFR. For example, EGFR protects cancer cells from autophagic death by stabilizing the active glucose transporter SGLT1, independent of its kinase function40. There is growing realization that targeting EGFR for degradation could be a more effective strategy than inhibiting kinase activity. Recently, it was shown that depletion or inhibition of a dual specificity tyrosine phosphorylation-regulated inhibiting kinase activity, DYRK1A, targets EGFR for lysosomal degradation, severely compromising self-renewal ability of GBM BTIC41.

Similarly, the tumour suppressor Mig-6 protein recruits internalized EGFR to the late endosome for eventual lysosomal degradation, by association with the SNARE protein STX8 (ref. 42). Another example is that of LRIG, a transmembrane protein that controls CBL recruitment and EGFR downregulation, found to be attenuated in many glioblastomas43,44. Taken together, several independent studies have borne out that EGFR membrane persistence is key to oncogenic activity.

Resveratrol, a potent SIRT1 activator, has anti-tumour property45. Treatment of GBM BTIC with resveratrol showed profound effect on brain tumour stem cell renewal, proliferation and downstream signalling from EGFR.

Methods

GBM cell cultures. All glioblastoma tumour samples were obtained with written informed consent from patients through the Department of Neurosurgery, Johns Hopkins Hospital (Baltimore, MD), following Institutional Review Board guidelines and as previously described51,52. Patient tumour grade and type was determined by a neuroradiologist (Supplementary Table 1). Culture conditions were in 5% CO2 incubator at 37°C as follows: GBM 253 primary glioblastoma cells were subcultured in DMEM/F12 (1:1) Nutrient Mixture, 10% fetal bovine serum (Invitrogen) and 1% HyClone Antibiotic Antimycotic Solution (Thermo Scientific). GBM 276 and GBM 612 glioblastoma stem cells were cultured in one of two ways: (1) as serum-free cultures on laminin and poly-l-ornithine-coated plates35 using StemPro NSC SFM (Invitrogen) without EGF, or (2) as tumour spheres with EGF (20 ng ml−1). EGF was only added to adherent cell cultures during experimental procedures.

Plasmids. Full-length mNHE9-EGFP was cloned into FuGw lentiviral vector as previously described6. Quik-Change-Site-Directed Mutagenesis Kit was used to make all point mutations (Strategene). HnNHE9 short hairpin RNA (shRNA) sequence (5′-CCGGCCCTCCATTAAGGAGAGTTTTTCAAGAGAAAACTCTCTGAGAGTTTTTCAAGAGAAAACTCTCTGAGAGTTTTTCG-3′) targeted shRNA sequence (Sigma-Aldrich) (5′-CACAAGATGAAACGACGCAAA-3′) were cloned into pLKO.1 lentiviral vector with a puromycin-selectable marker.

Construction of lentiviruses and cell transduction. pCMV-dR8.9 and pMD2.G packaging plasmids were co-transfected with pLKO.1-9NE9 shRNA in HEK293 cells for 48 h. FuGW mNHE9-EGFP was co-transfected 1:1 with 2nd Generation Packaging Mix (ABM) into HEK293 cells according to manufacturer’s instructions. Virus was collected 48 and 72 h after transfection and Lenti-X Concentrator (Clontech) was added to concentrate lentiviral stocks. Glioblastoma cells infected with shRNA constructs were incubated with virus for 48 h and selected with puromycin (0.75 μg ml−1). GBM cells infected with mNHE9-EGFP virus were assayed 72 h after infection.

Quantitative PCR analysis. mRNA was isolated from cell cultures using the RNeasy Mini Kit (Qiagen), following the manufacturer’s instructions, and optional RNA treatment with DNase I (Roche) was done. Complementary DNA was synthesized using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, 4387406), following the manufacturer’s instructions. Quantitative real-time PCR analysis was performed using the 7500 Real-Time PCR System (Applied Biosystems) using Taqman Fast universal PCR Master Mix (Applied Biosystems, 4352042). Taqman gene expression assay probes were used: Hs00234732_m1 (SLC3A6/NHE6), Hs010545318_m1 (SLCA9/NHE9), Hs00184593_m1 (ATP6AP1/V-ATPase subunit), Hs01076078_m1 (EGFR), Hs01009250_m1 (ATP6AP2/V-ATPase subunit), Hs01067607_m1 (EGFR), Hs01009250_m1 (ATP6AP1/V-ATPase subunit), Hs01076078_m1 (EGFR), Hs01009250_m1 (ATP6AP2/V-ATPase subunit). Each experiment was performed with following quantitative PCR cycling conditions: 2 min at 50°C, 10 min at 95°C, then 40 cycles of 15 s at 95°C and 1 min at 60°C. Ct values were used for all manipulations and were first normalized to endogenous control levels by calculating the ΔCt for each sample. Values were then calculated relative to control, to generate a ΔΔCt value. Fold change was calculated using the equation: expression fold change = 2ΔΔCt. The fold change of three independent (biological) replicates were run to account for assay variance.

Cell proliferation assay. Cell growth was monitored using the Celltiter 96 Aqueous One Solution Cell Proliferation Assay (MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), Promega), which is based on metabolic activity as a proxy for cell number45. Briefly, 0.5 × 104 cells were seeded in 96-well plates, and treated with control medium or 0.1 μM AVE-0497, a potent, specific, non-peptide, ATP-competitive inhibitor of Cyclin D-dependent kinase 1. Cells were incubated for 72 h at 37°C. At each time point, 20 μl of Celltiter 96 Aqueous One Solution reagent was added to each well and incubated for 2 h at 37°C. The absorbance was measured at 490 nM using a 96-well plate reader and normalized to D1.

Intracranial GBM injections and immunohistochemistry. All animal protocols were approved by the Johns Hopkins Animal Care and Use Committee. In vivo tumorigenesis and invasion of cells expressing either NHE9-GFP or NHE9 shRNA were assessed in 4- to 6-week-old male athymic nude mice (NCr-nu/nu, NCI) using a previously described brain tumour model33. Briefly, a total of 150,000 cells were injected into the right hemisphere of the mouse brains (coordinates X1.5,
Y1.34, Z3.5 relative to brevica). Each experimental group had eight animals each. Animals dying before the termination of the experiment were excluded from the analysis. Mice were killed 8 weeks after injection, and fixed and stained as previously described. Briefly, mice were subjected to a transcardiac perfusion using 4% paraformaldehyde as fixative. Dissected brain was postfixed overnight at 4°C in the same fixative, embedded in OCT compound (Tissue-Tek), frozen at −80°C and sectioned. Then, cryosections were stained with an antibody against human nuclei (mouse monoclonal IgG1, 1:250, Millipore) and were imaged to calculate tumour size and invasiveness by morphometric analysis using Image J (NIH). The slides were scanned, coded in a blinded manner and counted to prevent observer bias.

**Immunofluorescence.** Cultured GBM cells on coverslips were pre-extracted with PHEM buffer (60 mM Pipes, 25 mM HEPES, 10 mM EGTA and 2 mM MgCl₂, pH 6.8) containing 0.025% saponin for 2 min, then washed twice for 2 min with PHEM buffer containing 0.025% saponin and 8% sucrose. The cells were fixed with a solution of 100 mM sucrose and 8% PBS for 30 min at room temperature, and blocked with a solution of 1% BSA and 0.025% saponin in PBS for 1 h. Primary antibodies were diluted 1:100 in 1% BSA and incubated with cells 1 h or overnight. Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG (both Invitrogen) were used at 1:1,000 dilution for 30 min. Cells were mounted onto slides using Dako Fluorescent Mounting Medium and were imaged using a Zeiss LSM510-Meta confocal microscope.

**Protein turnover and western blotting.** Protein was assessed and analysed using a previously described directional migration assay. Briefly, nanogrooved substrates made of transparent polyurethane acrylate were coated with ultraviolet-assisted capillary lithography. The nanogrooved surface was coated with laminin and cell migration was quantified using time-lapse microscopy. Observation was done using a Cascade 512B II CCD camera equipped to an Olympus IX81 inverted microscope in a chamber controlled at 37°C. Images were acquired with a Photometrics CoolSNAP FX digital camera and processed for analysis. Mice were killed 8 weeks after injection, and fixed and stained as previously described. Briefly, cells were rinsed and incubated in serum-free medium for 30 min and then exposed to 100 μg ml⁻¹ transferrin conjugated to Alexa Fluor 633 in 1% BSA solution for varying amounts of time. Washes were done accordingly (as described) and cells were mounted onto slides using Dako Fluorescent Mounting Medium. Fluorescence microscopy images were acquired with a Photometrics CoolSNAP FX digital camera and processed using Scion software and Adobe Photoshop CS2 software. Internal fluorescence was quantified using ImageJ software, n = 100, for each time point for each experiment. Exocytosis was measured as a loss of fluorescence at various time points similarly.

**Cell surface biotinylation.** For surface biotinylation, cells were incubated with 1 μg ml⁻¹ biotin as previously described. Briefly, cells were washed three times with ice-cold PBS and incubated with 1 μg ml⁻¹ Sulfo-NHS-LC biotin in PBS at 4°C for 20 min. Excess NHS groups were quenched using 100 mM glycine followed by three washes with PBS. Protein superantigens were mixed with 120 μl of immobilized Neutradvin beads and incubated at 4°C overnight, with gentle rotation. Beads collected by centrifugation were washed three times with lysis buffer and surface proteins labelled with biotin were separated by SDS–PAGE and analysed by immunoblotting. All blots were cropped to show relevant bands.

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