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Alterations in cellular metabolome after pharmacological inhibition of Notch in glioblastoma cells

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Notch signaling can promote tumorigenesis in the nervous system and plays important roles in stem-like cancer cells. However, little is known about how Notch inhibition might alter tumor metabolism, particularly in lesions arising in the brain. The gamma-secretase inhibitor MRK003 was used to treat glioblastoma neurospheres, and they were subdivided into sensitive and insensitive groups in terms of canonical Notch target response. Global metabolomes were then examined using proton magnetic resonance spectroscopy, and changes in intracellular concentration of various metabolites identified which correlate with Notch inhibition. Reductions in glutamate were verified by oxidation-based colorimetric assays. Interestingly, the alkylating chemotherapeutic agent temozolomide, the mTOR-inhibitor MLN0128, and the WNT inhibitor LGK974 did not reduce glutamate levels, suggesting that changes to this metabolite might reflect specific downstream effects of Notch blockade in gliomas rather than general sequelae of tumor growth inhibition. Global and targeted expression analyses revealed that multiple genes important in glutamate homeostasis, including glutaminase, are dysregulated after Notch inhibition. Treatment with an allosteric inhibitor of glutaminase, compound 968, could slow glioblastoma growth, and Notch inhibition may act at least in part by regulating glutaminase and glutamate.

Glioblastoma (GBM) are one of the most lethal cancers,[1] and a subpopulation of cells generally known as glioma stem cells (GSCs) are thought to be major drivers of tumor progression and therapeutic resistance.[2] Molecular regulators of GSCs therefore represent potential therapeutic or diagnostic targets. Suppression of developmental signaling cascades important in non-neoplastic stem cells has emerged as a promising strategy to target stem-like cells in cancers,[3] and one such pathway is Notch.

Notch regulates numerous processes during embryonic and adult development,[4] including neural stem cell biology,[5] and its oncogenic role has been demonstrated in many tumors outside the brain[6] as well as in GBM.[7] The importance of understanding the effect of key oncogenes on the cellular metabolome in cancer is becoming increasingly appreciated.[8] Recent studies suggest that Notch can regulate cellular metabolism in normal tissues,[9–11] and in some tumors outside the brain such as leukemia[12] or breast cancer.[13] However, little is known about the effects of Notch on the metabolism of brain tumors including GBM.

We therefore examined the effects of Notch inhibition on the global metabolome in GBM neurospheres. Prior studies have shown that pharmacological inhibition of the enzyme gamma-secretase, responsible for terminal activating cleavage of the Notch receptor, effectively targets the stem-like cell population in GBM.[14] The gamma-secretase inhibitor MRK003 was therefore used to treat GBM neurospheres sensitive to Notch blockade in terms of pathway suppression, as well as insensitive control lines. Inhibition of Notch activity caused a range of metabolic changes in the GBM cells as

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What's new?
Glutamate metabolism may play a key role in brain tumor growth. These authors investigated the effects of blocking Notch, an important developmental pathway for some stem cells. They tested the change in levels of various metabolites in brain tumor cells when Notch was blocked. They found a drop in glutamate levels, a change that did not occur when they blocked Notch in human neural stem cells nor did they observe this loss of glutamate when they suppressed other signaling pathways. Perhaps, the authors suggest, Notch regulates tumor growth via glutamate metabolism, and hindering glutaminase could be a useful therapeutic avenue.

measured by high-resolution proton nuclear magnetic resonance spectroscopy (^{1}H-NMR), including phosphocholine as well as metabolites associated to glycolysis and glutaminolysis. Given the previously reported important role of glutamate in gliomagenesis,[15] we decided to focus our studies on this metabolite and found decreased levels of intracellular glutamate in glioma cells after Notch blockade. This was not observed after treatment with pharmacological compounds suppressing WNT or mTOR signaling, or by the alkylating agent temozolomide, suggesting that these changes are not the generic result of tumor growth inhibition or modulation of signaling cascades important in GSCs. Moreover, treating MRK003-sensitive cells with the glutaminase inhibitor compound 968 reduced their overall growth, but not that of MRK003-insensitive cells.

Material and Methods

Cell lines and cell culture

The GBM1 and 040922 lines were derived as previously described, and generously provided by Dr. Vescovi.[14] GBM10[16] and GBM14[17] were established in our laboratory from intraoperative tumor specimens obtained from the Department of Neurosurgery, Johns Hopkins Hospital. U87 and LN229 were purchased from American Tissue Culture Collection (www.atcc.com). All cells were cultured as neurospheres (NS) in serum-free and glutamine-rich conditions as described before,[16] and passaged at least twice per week, except for LN229, which was propagated in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, NY) supplemented with 10% fetal calf serum (Life Technologies).

Human fetal neural stem cells (fNCS) were obtained from first trimester human fetal autopsy specimens as described[18] in concordance with German law and Ethics Board evaluation (Division of Stereotactic Neurosurgery, University Medical Center Freiburg, Germany) and cultured as neurospheres. This study was also approved by the Johns Hopkins Institutional Review Board. Cell line identity was confirmed for all cultures by analysis of nine short tandem repeats (STR) plus a gender determining marker, Amelogenin using the StemElite kit (Promega, Madison, WI) in the John Hopkins Core Facility for DNA fragment analyses (Supporting Information File S1).

Suppression of the NOTCH pathway

Twelve hours before the start of the experiments, cultures were dissociated to single-cell suspension and plated at a concentration of 1.5×10^6 cells per 1 ml of media to ensure exponential growth status. The next day, media were replaced with media containing the gamma-secretase inhibitor MRK003 (Merck Research Laboratories, Kenilworth, NJ) or dimethyl sulfoxide (DMSO, #D9170, Sigma-Aldrich, Saint Louis, MO) as vehicle control. Based on our previous experience with MRK003,[14] we applied MRK003 in a final concentration of 1 μM for 48 hr to achieve significant pathway inhibition as confirmed by reduction of canonical target genes (*HES1* and *HEY1*) expression but only low to moderate cell death. With this treatment regime, we aimed to minimize secondary effects on the metabolome introduced through cell degradation.

Extraction and quantification of metabolites from *in vitro* cultures using high-resolution ^{1}H-NMR

Cells were harvested, washed twice in 1× PBS (Life Technologies) and quantified using Trypan blue (#T8154, Sigma-Aldrich) exclusion assay (three independent counts). A minimum of 7×10^6 cells per sample were used for each extraction and total cell numbers were used to normalize between MRK003 and DMSO counterparts. The methanol–chloroform–water (1/1/1, v/v/v, all Sigma-Aldrich) dual-phase cell extraction protocol was applied to obtain water- and lipid-soluble metabolites as previously described.[19] The lyophilized water-soluble extracts were resolved in 495 μl deuterium oxide (D_2O, #151882, Sigma-Aldrich) supplemented with 5 μl D_2O containing 0.05% 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid (TSP, #450510, Sigma-Aldrich) as internal concentration standard. The extracts were analyzed in the Department of Radiology, Johns Hopkins Hospital on a Bruker Avance 500 spectrometer operating at 11.7 T using a 5-mm HX inverse probe run at 25°C as previously described.[19] The fully relaxed ^{1}H-NMR data were postprocessed and metabolites were quantified through peak integration using Mestrenova v10.0 (Mestrelab Research, Santiago de Compostela, Spain, CA). For each sample, Notch activation status was assessed by quantification of canonical target gene expression.

ELISA-based quantification of glutamate

Colorimetric quantification of glutamate was performed through glutamate dehydrogenase-mediated oxidation using

EnzyChrom™ Glutamate Assay Kit (EGLT-100, BioAssay Systems, Hayward, CA) according to the manufacturer's protocol. Each step of sample preparation was performed on ice if not mentioned otherwise. In brief, cells were harvested, washed two times in 1× PBS (Life Technologies) and lysed in 1× PBS containing 1× proteinase inhibitor (#P2714-1BLT, Sigma-Aldrich). Cell membranes were broken through a three-time-performed freeze–thaw cycle using a dry-ice/ethanol bath and a water bath warmed to 37°C. Total protein concentration determined by Protein Assay Dye Reagent (#500–0006, Bio-Rad, Hercules, CA) was used to normalize the individual samples of each group. Absorptions reflecting concentrations of total protein (595 nm) as well as glutamate (565 nm) were measured on an Epoch plate reader (BioTek Instruments, Winooski, VT).

Whole genome expression profiling

Whole human genome gene expression was measured as described before[20] using 44k microarray technology (Agilent Technologies, Santa Clara, CA)-based assessment performed at the Johns Hopkins Oncology Microarray Core, with labeling, hybridization and detection performed according to the manufacturer's instructions (Agilent Technologies). All analyses were performed using software packages available from the R/Bioconductor platform for statistical computing. Briefly, we used a generalized linear model approach for differential gene expression detection, and performed gene set enrichment analysis using Analysis of Functional Annotation, as previously described.[21] Similarly, all processes for gene annotation, raw expression data and MIAME (Minimal Information about a Microarray Experiment) have been described by our lab before.[20] We considered genes with a false discovery rate of $<5\%$ as differentially regulated. The data can be accessed through the NCBI GEO data portal under http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE71769.

Targeted gene expression analyses and Western blot

The relative abundance of gene transcripts was determined using SYBR-green-based ddCt-method on an IQ™5-system (Bio-Rad) normalized to beta-actin housekeeping gene expression. The sequences for the primers used in this study are listed in Supporting Information File S2. For Western blotting we used anti-glutaminase antibody #12855-1-AP (Proteintech, Chicago, IL, 1:1,000) and anti-beta-actin antibody #sc-47778 (Santa Cruz Biotechnology, Dallas, TX, 1:1,000).

Analyses of cellular growth and apoptosis

For all assays, cells were dissociated to single-cell suspension and viable cells quantified using the MUSE Count & Viability Assay Kit (#MCH100102, Merck KGaA) on a Muse Cell Analyzer (#0500–3115, Merck KGaA). For growth studies, 5,000, 10,000 or 20,000 cells (dependent on cell line and assay) were plated in a 96-well plate in 100 µl triplicates. Biologically active cell mass was then measured using the TiterBlue assay (#G8081, Promega, Madison, WI) according to the manufacturer's description on an Infinite M1000Pro plate reader (Tecan, Morrisville, NC). Cell Titer Blue reagent (20 µl per well) was added directly to the cells, incubated for 2 hr at 37°C followed by fluorescence intensity measurement at 560ex/590em nm.

Apoptotic cells were quantified using the AnnexinV & Dead Cell Kit (#MCH100105, Merck KGaA, Darmstadt, Germany) on the Muse Cell Analyzer according to the manufacturer's protocol. A minimum of 2,000 gated events were acquired.

Additional pharmacological treatment

All pharmacological treatment experiments required the dissociation to single cells before the start of the experiment (1.5×10^5 NS cells per 1 ml of media, 3.5×10^5 cells LN229 per cm^2 culture area). We treated the cells in the indicated concentrations with the alkalyting agent temozolomid[1] (TMZ, #T2577, Sigma-Aldrich), dual TORC1/2 inhibitor INK-128/Milenum0128[22] (MLN, #S2811, www.selleckchem.com), porcupine inhibitor LGK974 to interrupt WNT ligand/receptor interaction[23] (Xcess Biosciences, San Diego, CA, #M60106-2S) and glutaminase inhibitor compound 968[24] (Millipore, #352010). The indicated drug concentrations diluted in growth media were compared to the individual vehicle control (DMSO).

Determination of WNT/beta-catenin pathway activity

WNT/beta-catenin pathway inhibition through LGK974 was confirmed through bioluminescence-based quantification of occupied putative beta-catenin T-cell factor/lymphoid enhancer factor (TCF/LEF)-binding sites using the stable integration of 7TFP *firefly* luciferase reporter as described before.[25] Infectious lentiviral particles were generated using the third-generation lentiviral packaging system and stable integration was selected through puromycin (Sigma- Aldrich) resistance at a concentration of 2 µg/ml.

For each measurement, cells were harvested, washed in 1× PBS and lysed according to the manufacturer's descriptions using the Dual-light combined Luciferase & beta-Galactosidase Reporter Gene Assay System (#T1003, Life Technologies). Luminescence was read at an emission wavelength of 490 nm on the Infinite M1000Pro plate reader (Tecan) and normalized to beta-galactosidase activity. A robust IC_{50} was achieved at 5 µM LGK974 at 48 hr as defined by previous experiments.[26]

Figure generation

Sigma Blot 8.0 (Systat Software, San Jose, CA), Prism v5 (GraphPad Software La Jolla, CA) and Illustrator C4 (Adobe System, San Jose, CA) were used to generate the figures.

Statistical evaluation

Statistical analyses were performed using a two-tailed Student's *t*-test and presented as mean values plus standard deviation if not indicated otherwise. The *p*-values ≤ 0.05 were considered statistically significant.

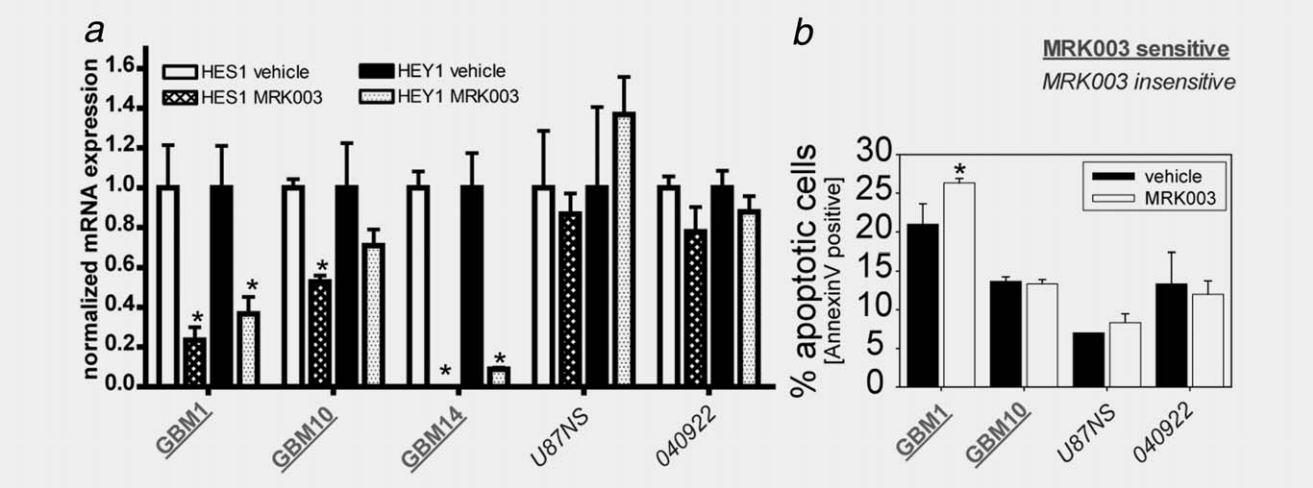

Figure 1. Glioma cell lines with different sensitivity to Notch blockade with MRK003: confirmation of successful suppression of Notch signaling through inhibition of transcription of canonical pathway targets *HES1* and *HEY1* distinguishes between MRK003-sensitive (GBM1, GBM10 and GBM14) and -insensitive (U87NS and 040922) cells (*a*, $p \leq 0.05$). This treatment led to minimal induction of apoptosis in GBM1 (21–27% AnnexinV-positive cells) and had no effect on GBM10, U87NS and 040922 (*b*, $p \leq 0.05$).

Results

Altered metabolic profiles in glioma neurospheres after Notch suppression

It has been shown that while some GBM are sensitive to GSIs, others are resistant to Notch blockade.[27] mRNA levels of the canonical Notch pathway targets *HES1* and *HEY1* were measured to confirm sensitivity or resistance in five glioma lines. Four of these were originally derived as neurospheres, while U87 was continuously passaged as neurospheres in serum-free media for these studies and designated as U87NS. Significant suppression of mRNA levels for several direct transcriptional targets of Notch was achieved in the GBM1, GBM10 and GBM14 lines, but not in U87NS or 040922 cells (Fig. 1a, $p \leq 0.05$). On the basis of this, we classified GBM1, GBM10 and GBM14 as MRK003 sensitive, with U87NS and 040922 designated MRK003 insensitive.

High-resolution ^{1}H-NMR was used to compare metabolic changes in MRK003-sensitive and -insensitive lines. Although this technique allows for precise identification and quantification, it requires extremely large numbers of cells, thus the initial survey was performed on only the two fastest growing lines in each group, GBM1 and U87NS. The levels of GSI used for these studies resulted in a modest induction of apoptosis for GBM1 and had no effect on AnnexinV in the other tested cell lines (Fig. 1b, $p \leq 0.05$).

We identified a broad range of metabolites with characteristic peak formations of singulet (S), duplet (D) or multiplet (M), including valine/isoleucine (Val/Iso) at 0.9–1.0 ppm (M), threonine (Thr) at 1.30 ppm (D), lactate (Lac) at 1.33 ppm (D), alanine (Ala) at 1.47 ppm (D), *N*-acetylaspartylglutamate (NAAG) at 2.05 ppm (S), glutamate (Glu) at 2.33 ppm (M), glutamine (Gln) at 2.41 ppm (M), glutathione (GSH) at 2.50 ppm (M), total creatine (tCre) at 3.03 ppm (D), free choline (fCho) at 3.18 ppm (S), phosphocholine (PC) at 3.22 ppm (S), glycerophosphocholine (GPC) at 3.24 ppm (S), glycine (Gly) at 3.55 ppm (S) and myo-inositol (myo) at 4.05 ppm (M). Representative findings are shown in Figure 2a, and the summary of three independent experiments in each of the two lines in Figure 2b. Interestingly, although a number of metabolite levels decreased after Notch blockade in the sensitive GBM1 cells, no corresponding decreases were seen in MRK003-insensitive U87NS except for myo-inositol (Fig. 2b). Significant decreases were noted in glutamate, glutamine, free choline and phosphocholine after Notch pathway inhibition. Myo-inositol levels also decreased in the GBM1 and significantly reduced in U87NS cells after GSI treatment. Due to technical issues in one GBM1 repetition only two of the three independent samples could be quantified for this metabolite, and therefore no statistical comparison of myo-inositol was performed in these cells. Interestingly, although no other significant decreases in metabolite levels were seen in U87NS cells, threonine and lactate levels were significantly increased.

Reduction of glutamate after Notch inhibition

We focused on changes to glutamate, and Figure 3a shows a more detailed view of a representative spectra of this region, with a 36% mean reduction ($p \leq 0.05$) in GBM1 cells as compared to a nonsignificant 7% increase in U87NS cells after Notch inhibition. To verify this observation and extend it to additional lines, we used an ELISA-based method based on oxidation of glutamate, which was more easily performed on small samples. This confirmed that Notch pathway suppression reduced intracellular glutamate levels by 50% and 26% in the GSI-sensitive lines GBM1 and GBM10 ($p \leq 0.05$). No glutamate reduction was noted in the insensitive U87NS and

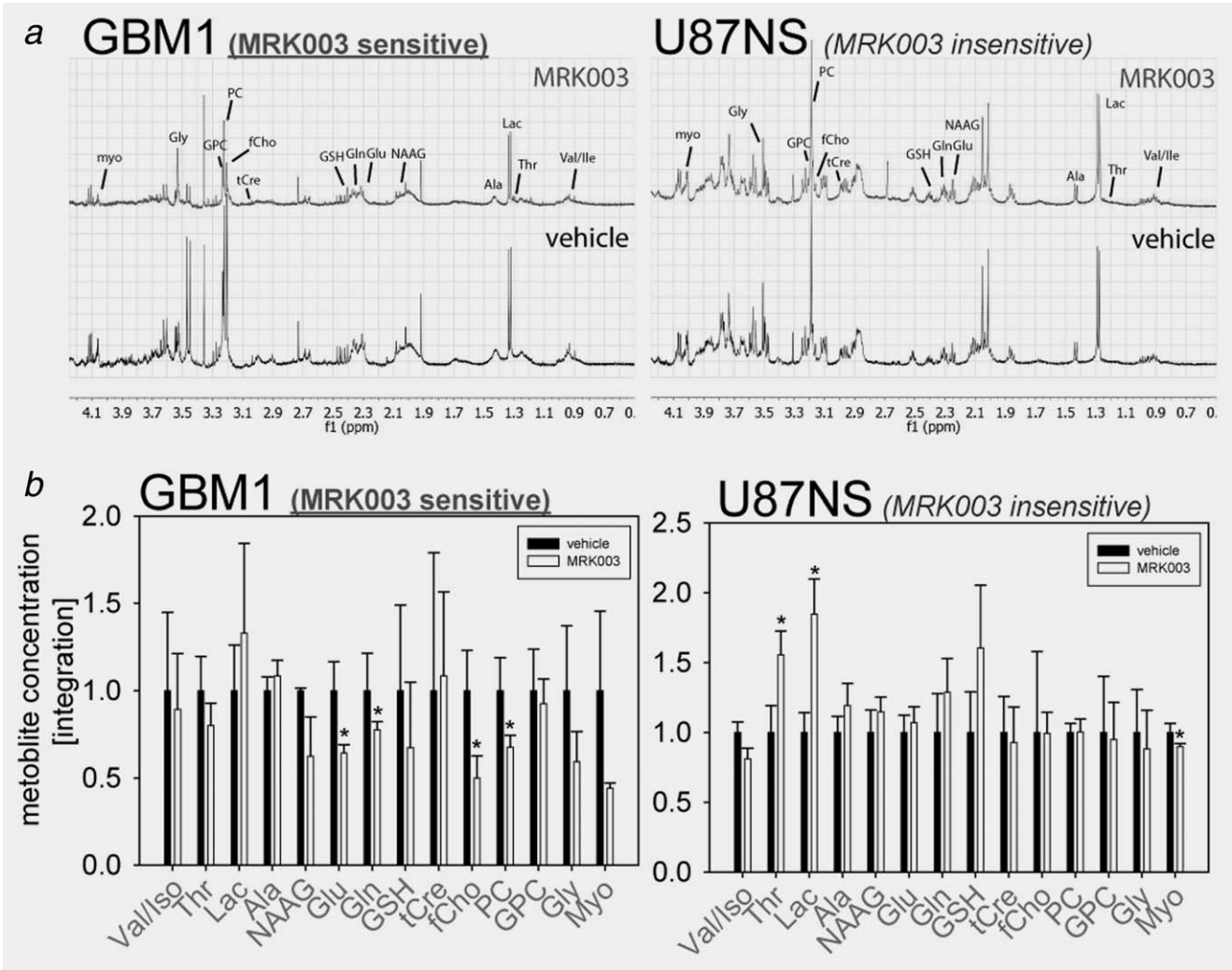

Figure 2. Global metabolic profiling of GBM neurospheres after MRK003 administration with proton magnetic resonance spectroscopy (^{1}H-NMR): assessing the metabolic composition of *in vitro* metabolic extracts of glioma cells sensitive (GBM1) and insensitive (U87NS) to MRK003 *via* ^{1}H-NMR with and without drug exposure identified various metabolites including valine/isoleucine (Val/Iso), threonine (Thr), lactate (Lac), alanine (Ala), *N*-acetylaspartylglutamate (NAAG), glutamate (Glu), glutamine (Gln), glutathione (GSH), total creatine (tCre), free choline (fCho), phosphocholine (PC), glycerophosphocholine (GPC), glycine (Gly) and myo-inositol (myo) (*a*). Quantification of three independent rounds for each represented as mean plus SEM (*b*) ($p \leq 0.05$).

040922 neurospheres (Fig. 3b). In addition, although MRK003 inhibited Notch in human fetal cortical neurospheres, it did not reduce levels of intracellular glutamate (Fig. 3c), suggesting that the observed Notch–glutamate interplay is differentially regulated in non-neoplastic neural stem cells.

Treatment with other chemotherapeutics did not reduce glutamate

To determine if the observed glutamate reduction might be a general consequence of treatment, we examined the effect of other pharmacological agents on glutamate levels. The standard alkylating agent temozolomide (TMZ) and the mTOR inhibitor INK-128/MLN0128 (MLN) were tested on the MRK003-sensitive cell lines GBM1 and GBM10. Both TMZ and MLN caused a profound decrease in cellular growth (Fig. 4a), and after 48 hr apoptosis in GBM1 was induced to a degree similar to that seen with MRK003, but no corresponding glutamate reduction was detected in these samples (Fig. 4b). Thus, chemotherapeutic growth inhibition and increased apoptosis is not always associated with glutamate alterations in glioma neurospheres.

We also examined if other pathways important in non-neoplastic and GSCs might regulate glutamate. A number of groups have shown that WNT signaling blockade depletes stem-like glioma cells.[28] We have recently shown that the porcupine inhibitor LGK974, which targets WNT signaling by modulating ligand processing, can also inhibit the pathway and reduce survival in glioma neurospheres.[26] For these LGK974 studies, LN229 cells were used instead of GBM10, as LN229 is characterized by higher WNT activity similar to

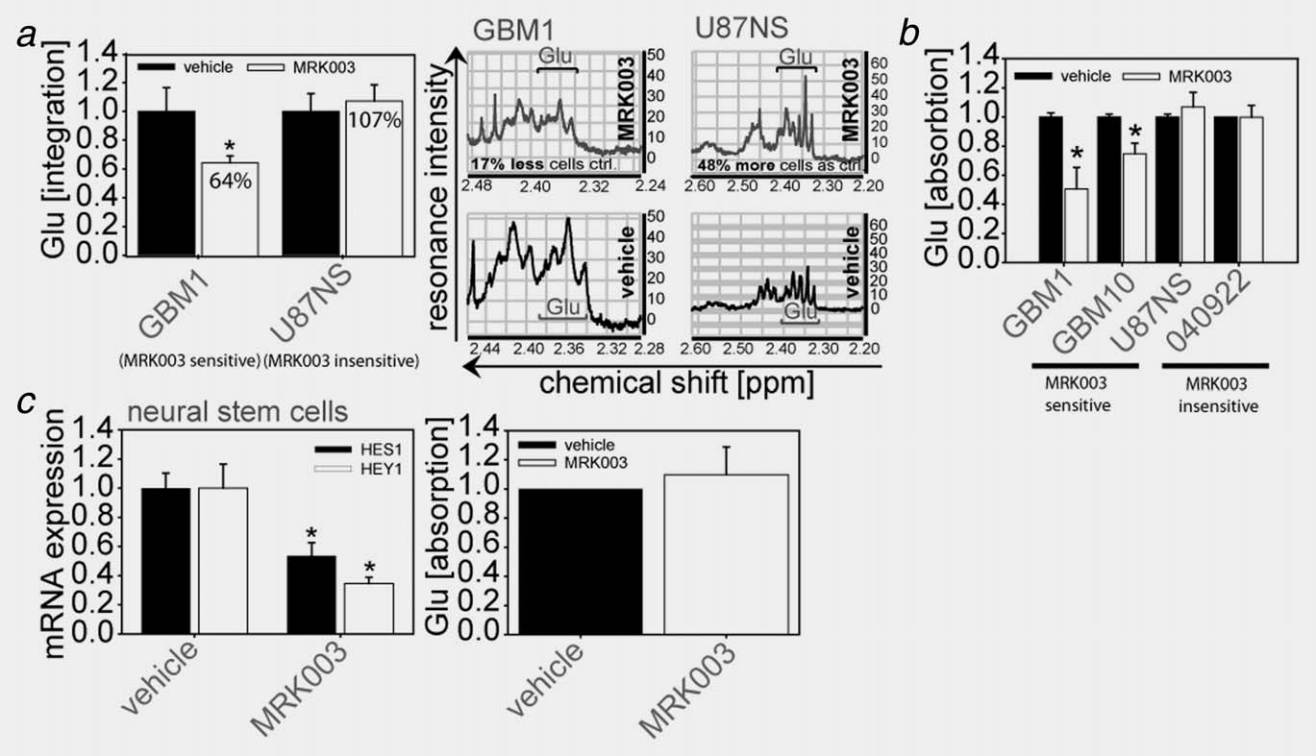

Figure 3. Reduction of intracellular glutamate after Notch blockade: MRK003-sensitive GBM1 reduces levels of intracellular glutamate but not in MRK003-insensitive U87NS cells as assessed with ^{1}H-NMR (*a*). In addition, MRK003-sensitive cells GBM1 and GBM10 exhibit lower intracellular glutamate levels after Notch blockade as confirmed with glutamate dehydrogenase-mediated quantification of glutamate oxidation which was not seen in MRK003-insensitive U87NS and 040922 (*b*). Notch blockade did not reduce glutamate in human fetal neural stem cells as assessed with the ELISA kit (*c*) ($p \leq 0.05$).

that seen in the upper range of snap-frozen GBM specimens.[26] Inhibition of the pathway after LGK974 administration was confirmed by reduction in luciferase WNT reporter signals, with no corresponding effects on glutamate levels (Fig. 4c).

Notch blockade alters expression of multiple regulators of glutamate homeostasis

To determine the mechanism by which Notch was altering glutamate levels, we examined global transcriptomes from MRK003-sensitive GBM1, GBM10 and GBM14 cells after 2 μM MRK003 treatment. This identified a number of dysregulated genes important for glutamate homeostasis, which are listed in Figure 5a. To confirm this, we performed targeted gene expression analysis *via* RT PCR for a panel of core regulators of cellular glutamate metabolism in our cell lines. This includes NAAG-cleaving glutamate carboxypeptidase II (*GCPII*), a member of the soluble carrier (SLC) membrane transporter family responsible for intracellular glutamate uptake (*SLC1A3*) as well as the glutamine- to glutamate-hydrolyzing glutaminase (*GLS1*) (Fig. 5b).

Drug treatment significantly reduced the expression of *GCPII* in the MRK003-sensitive GBM1 (40%) and GBM14 (20%) cultures but not in MRK003-insensitive U87NS or 040922 cells. No *GCPII* transcription was detected in GBM10. *SCL1A3*, which helps import glutamate into the cell, was significantly reduced in most MRK003-sensitive lines after Notch blockade, but not in MRK003-insensitive ones. Interestingly, MRK003-sensitive cells expressed higher baseline levels of glutaminase as compared to insensitive cells (Fig. 5c), they were the only ones to show a decrease in *GLS1* as mRNA after Notch blockade (Fig. 5b).

Treatment with inhibitor of glutaminase reduced cell growth

If reductions in glutamate represent a major mechanism through which Notch blockade slows glioma growth, one would predict that direct inhibition of this metabolic pathway would have a similar effect. We therefore treated our neurosphere cultures with the glutaminase inhibitor compound 968[24] and observed significant growth inhibition in MRK003-sensitive lines, whereas no effect was seen on growth of MRK003-insensitive cultures (Fig. 6a). A similar pattern of response in MRK003-sensitive cells was seen when less selective glutaminase inhibitors such as acivicin or 6-diazo-5-oxo-L-norleucine (DON) were used (data not shown). No additive effect was seen after combination treatment with both MRK003 and compound 968 (Fig. 1b).

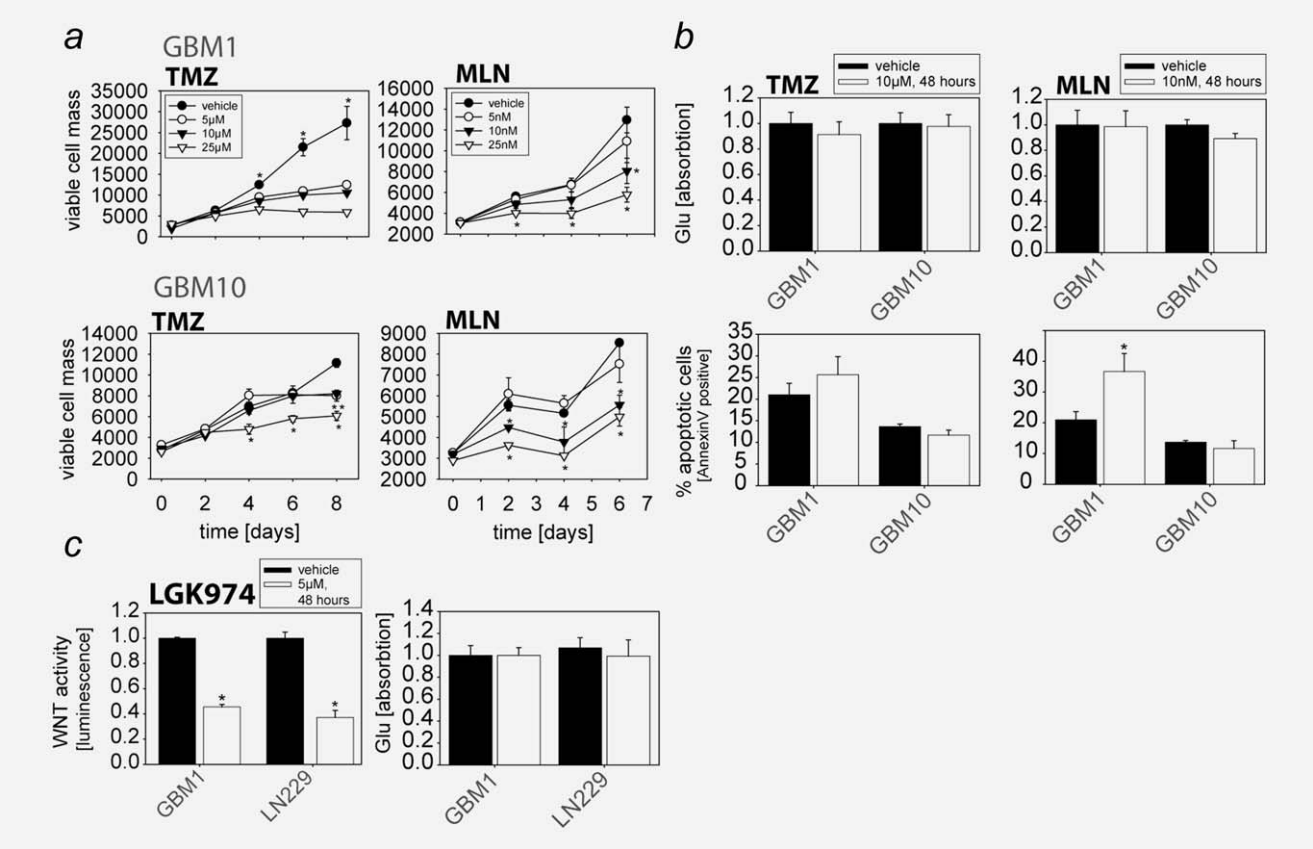

Figure 4. Other chemotherapeutic treatments did not reduce levels of glutamate: administration of alkylating agent temozolomid (TMZ) or mTOR inhibitor MLN0128 (MLN) significantly impaired glioma cell growth as shown for MRK003-sensitive cell lines GBM1 and GBM10 (*a*). Treatment with TMZ and MLN for 48 hr induced apoptosis for GBM1 but not for GBM10 while glutamate levels remained unchanged (*b*). Porcupine inhibitor LGK974 inhibited transcriptional activity of WNT/beta-catenin signaling in GBM1 and LN229, but had no significant effect on glutamate (*c*) ($p \leq 0.05$).

Discussion

Despite the importance of Notch in normal development[29] and oncogenesis,[6,7] little is known about how this signaling cascade can regulate cell metabolism. Our data indicate that pharmacological Notch blockade alters intracellular levels of a range of metabolites in glioma cells. In general, we saw decreased metabolite levels, while treatment of neurospheres insensitive to Notch inhibitor MRK003 show no reductions in these metabolites.

Notch blockade significantly reduced the onco-metabolite phosphocholine (PC). PC has been found to be particularly high in fast-dividing glioma cells[30] and elevated in malignant high-grade brain tumors when compared to low grades.[31,32] Moreover, PC has been shown to be downstream of mTOR in GBMs,[33,34] and our data now add a link to Notch. Additionally, our NMR studies showed that the MRK003 treatment led to reduction of glycine (gly) in both MRK003-insensitive and -sensitive GBMs reaching statistical significance in the latter. Of note, gly has recently been shown to be involved in the regulation of survival of hypoxic glioma cells,[35] a population of cells particular enriched with stem-like characteristics,[36] and is known to be positively associated to rapid cancer cell proliferation.[37] Furthermore, we found lactate, an indicator of active glycolysis, to be increased after treatment with the Notch inhibitor in both glioma subgroups. This is concordant with very recent discoveries that Notch stimulates cellular anaerobic glucose metabolism in breast cancer[13] and during macrophage activation.[38]

Besides these alterations, we found a significantly different response in the two glioma groups after drug treatment in terms of intracellular glutamine (gln) and glutamate (glu), as we detect significantly lower levels of these two metabolites in MRK003-sensitive GBM1 but not in MRK003-insensitive U87NS after treatment. We focused in particular on changes to glutamate, given the many prior studies implicating it in the biology of brain tumors,[15] and confirmed its reduction after Notch blockade with oxidation-based quantifications. These changes may be linked to increased differentiation of the tumor.

It has previously been shown that differentiated glioma cells possess lower levels of glutamate as compared to stem-like GBM cells[39] and high levels of intracellular glutamate in glioma neurospheres are associated with increased clonogenicity as well as with increased expression of GSC marker

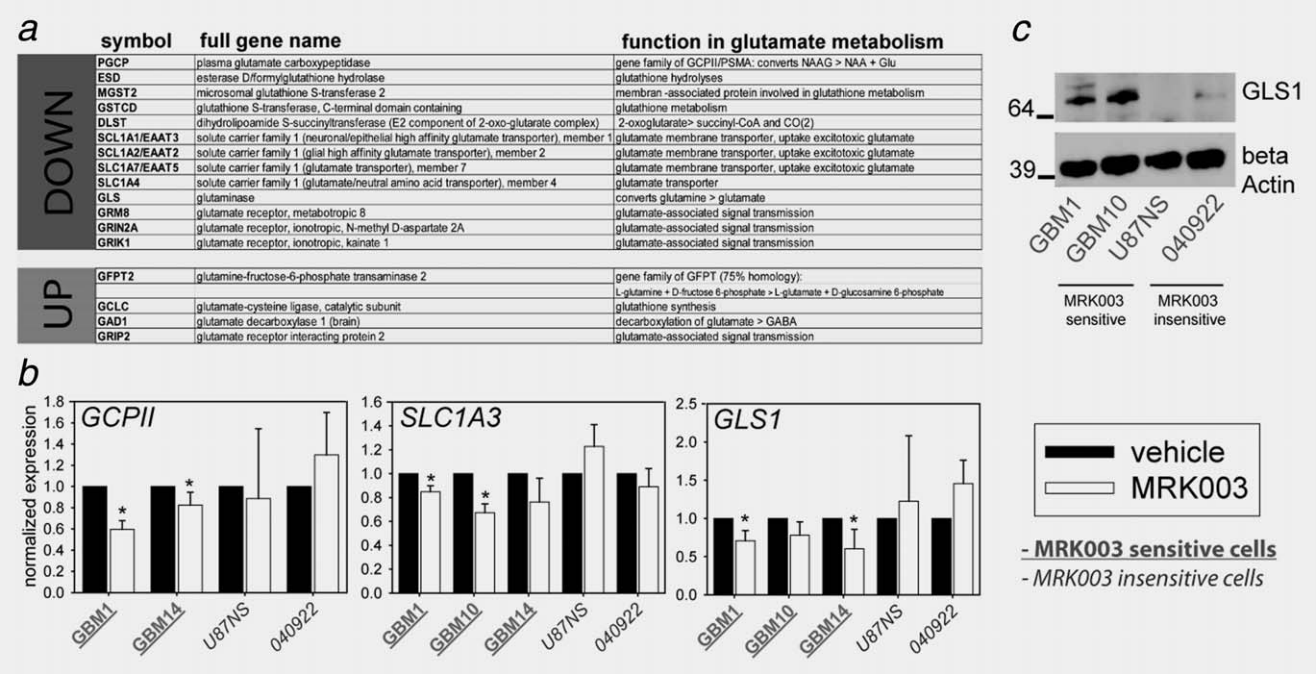

Figure 5. MRK003 modulates expression of multiple genes regulating glutamate homeostasis: microarray analysis of MRK003-sensitive cells GBM1, GBM10 and GBM14 after suppression of Notch revealed the altered expression of multiple genes involved in glutamate homeostasis (*a*). RT PCR-based quantification of mRNA transcription confirmed the suppression of genes involved in glutamate metabolism after Notch blockade including glutamate carboxypeptidase II (*GCPII*), soluble carrier (SLC) membrane transporter *SLC1A3* as well as kidney-type glutaminase (*GLS1*) in MRK003-sensitive glioma neurospheres but not in MRK003-insensitive cultures (*b*). MRK003-sensitive cells have higher baseline expression of glutaminase as compared to MRK003-insensitive cells (*c*) ($p \leq 0.05$).

CD133.[40] Moreover, Angulo-Rojo *et al.* found that Notch induces glutamate consumption during terminal differentiation of astrocytes.[41] Glutamate has also been shown to serve as growth stimulus for murine GSCs and that the neocortex, a brain region rich in glutamate, generates a metabolic niche advantageous for the growth of brain tumors.[42]

Our findings are consistent with prior studies implicating Notch in glutamate regulation. For example, glutamate levels are particularly high in glioma cells clustering with the PDGFRA+ signature of proneural GBMs[43]—the subset of gliomas with high Notch activation [27] . This glutamate accumulation is thought to possibly be due to overactive glutamate uptake mediated through the members of the soluble carrier (SLC) membrane transporters family.[43] However, further studies are needed to determine whether the lowered glu level in our cells is a consequence of altered consumption or production.

Regardless of the mechanism by which glu is regulated by Notch, it appears to be a promising therapeutic target. We found that Notch blockade suppressed transcript levels of cardinal glutaminolysis regulator glutaminase (GLS). GLS is known to promote cancer cell proliferation[44,45] and its suppression can impair tumor growth. Previously, genetic GLS suppression slowed GBM growth, and pharmacological suppression with BPTES preferentially acted on glioma cells with mutant isocitrate dehydrogenase 1 (*IDH1*).[46] We treated our cells with the recently discovered GLS inhibitor compound 968,[24] which acts through a different principle of allosteric inhibition as compared to BPTES.[47] This suppressed growth of MRK003-sensitive cells which were not *IDH1* mutant, but failed to slow growth of MRK003-insensitive cells. Therefore, our data indicate that GLS suppression in some GBMs can have antigrowth benefits independent of *IDH1* status, and suggest that the therapeutic benefit of MRK003[14] might be, at least in part, mediated by interference with glutamate/glutamine metabolism. This is supported by the observation that the addition of compound 968 to MRK003 did not result in an increased antigrowth effect, suggesting that they may be acting *via* a common mechanism.

Importantly, the commonly used chemotherapeutic agent TMZ, the inhibitor of mTOR signaling MLN, and the WNT inhibitor LGK974 could reduce cellular growth and induce apoptosis, but did not reduce glutamate in the same fashion as Notch blockade, suggesting that this change was not a general effect of all agents targeting brain tumors. Interestingly, glioma cells resistant to mTOR inhibition have recently been shown to induce glutamine metabolism as compensatory prosurvival strategy resulting in elevated intracellular glutamate,[48] although we do not see signs of this in our lines after 48 hr treatment with MLN.

Taken together, our data suggest that Notch blockade can have widespread effects on brain tumor cellular metabolism, including glycolysis, glycine and choline metabolism as well

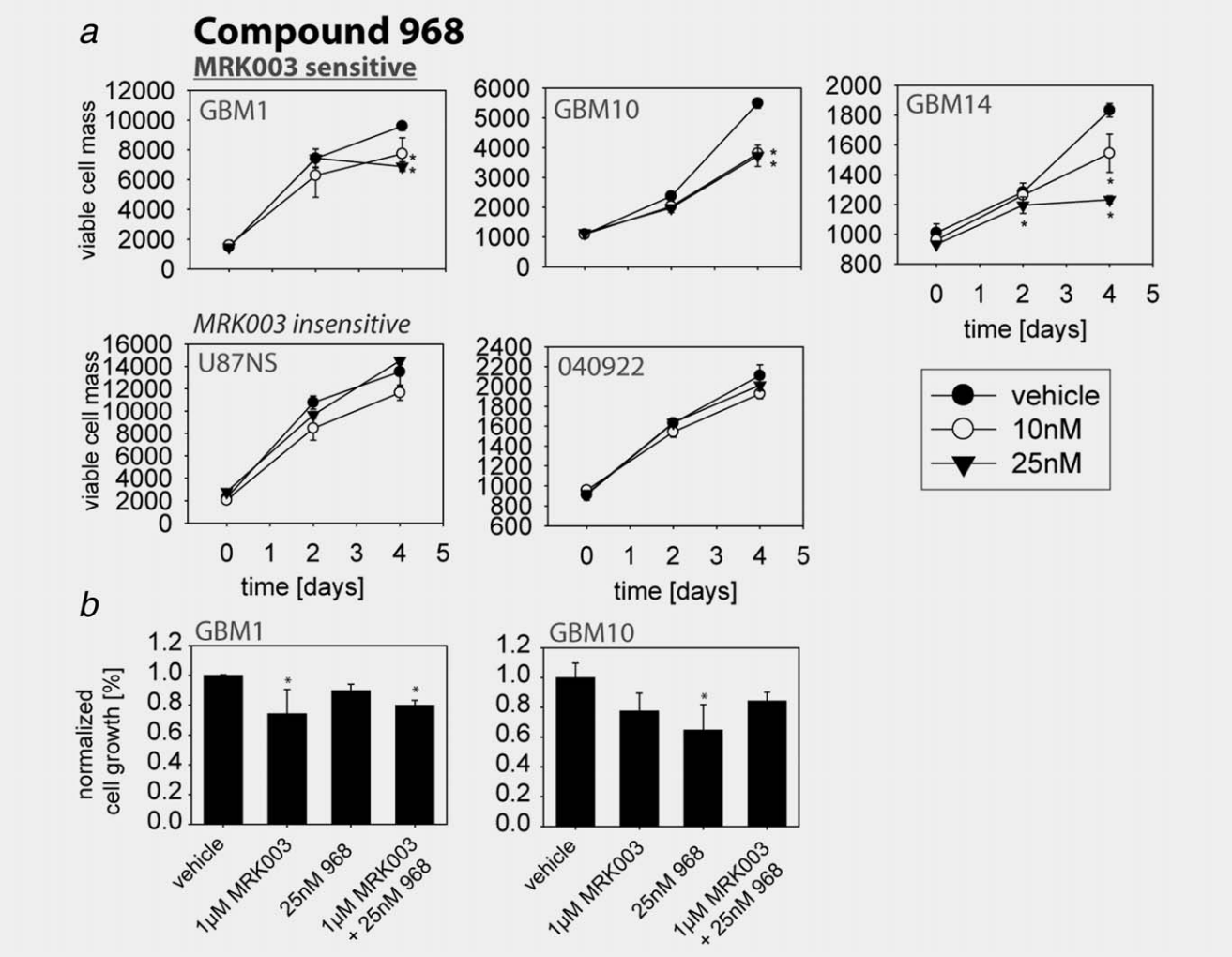

Figure 6. Treatment with pharmacological inhibitor of glutaminase compound 968 can slow glioma cell growth in MRK003-sensitive but not in MRK003-insensitive cells (*a*, $p \leq 0.05$). No additive effect of MRK003 in combination with compound 968 on cell growth was observed (*b*, Day 4 for fast-growing GBM1, Day 6 for GBM10, $p \leq 0.05$).

as glutaminolysis, which extend beyond simple manifestations of growth inhibition. It should be noted that Notch inhibition was achieved pharmacologically, and the possibility of off-target effects of MRK003 on glutamate or other metabolites cannot be excluded. In our studies, the reduction of glutamate was observed in glioma cells but not in neural stem cells following Notch blockade (Fig. 3c), suggesting that the regulation of glutamate metabolism may differ between cancerous and noncancerous brain stem cells. Finally, our results also suggest utility in monitoring glutamate levels in GBM as a measure of Notch treatment response, as this could potentially be performed noninvasively with ^{1}H-NMR.

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