miR-125b controls apoptosis and temozolomide resistance by targeting *TNFAIP3* and *NKIRAS2* in glioblastomas

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Diffusely infiltrating gliomas are among the most prognostically discouraging neoplasia in human. Temozolomide (TMZ) in combination with radiotherapy is currently used for the treatment of glioblastoma (GBM) patients, but less than half of the patients respond to therapy and chemoresistance develops rapidly. Epigenetic silencing of the O⁶-methylguanine-DNA methyltransferase (MGMT) has been associated with longer survival in GBM patients treated with TMZ, but nuclear factor κ B (NF- κ B)-mediated survival signaling and TP53 mutations contribute significantly to TMZ resistance. Enhanced NF- κ B is in part owing to downregulation of negative regulators of NF- κ B activity, including Tumor necrosis factor alpha-induced protein 3 (TNFAIP3) and NF- κ B inhibitor interacting RAS-like 2 (NKIRAS2). Here we provide a novel mechanism independent of TP53 and MGMT by which oncogenic miR-125b confers TMZ resistance by targeting *TNFAIP3* and *NKIRAS2*. GBM cells overexpressing miR-125b showed increased NF- κ B activity and upregulation of anti-apoptotic and cell cycle genes. This was significantly associated with resistance of GBM cells to TNF α - and TNF-related inducing ligand-induced apoptosis as well as resistance to TMZ. Conversely, overexpression of anti-miR-125b resulted in cell cycle arrest, increased apoptosis and increased sensitivity to TMZ, indicating that endogenous miR-125b is sufficient to control these processes. GBM cells overexpressing TNFAIP3 and NKIRAS2 were refractory to miR-125b-induced apoptosis resistance as well as TMZ resistance, indicating that both genes are relevant targets of miR-125b. In GBM tissues, high miR-125b expression was significantly correlated with nuclear NF- κ B confirming that miR-125b is implicated in NF- κ B signaling. Most remarkably, miR-125b overexpression was clearly associated with shorter overall survival of patients treated with TMZ, suggesting that this microRNA is an important predictor of response to therapy.

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Introduction

Gliomas, which account for at least 60% of primary brain tumors in adults, are among the prognostically most discouraging neoplasia in human. Depending on an individual constellation of clinical variables and histological parameters, median survival of patients ranges from some months to 12–15 years.¹ Glioblastoma multiforme (GBM) represents the highest grade (WHO grade IV) within the prevailing astrocytic category of tumors, and present either as a *de novo* neoplasm (primary GBM) or as the endpoint of tumor progression from a lower grade precursor (secondary GBM). With optimal treatment, the mean survival time of patients with GBM is <15 months.

Surgical resection of GBM is essentially palliative as the tumor infallibly recurs because of its pervasively infiltrative growth.² Patients receiving a combination of radio- and chemotherapy using the alkylating agent temozolomide (TMZ) tend to have a significantly longer overall and progression-free survival, but not all patients benefit from therapy and resistance develops rapidly in those patients.^{1,3}

O⁶-methylguanine-DNA methyltransferase (MGMT) promoter methylation^{2,4,5} and mutations in the isocitrate dehydrogenase (IDH) subunits IDH1 and IDH2^{6,7} correlate with a higher rate of objective response to TMZ.

Constitutive activity of nuclear factor κ B (NF- κ B) signaling, which occur in up to 90% of GBMs, is an important regulator of proliferation, invasion and apoptosis.^{8,9} NF- κ B is one of the major factors modulating the ability of cancer cells to resist apoptosis and to contribute to chemoresistance.^{10,11} NF- κ B comprises two subunits, commonly p50 (NFKB1)/p65 (RelA), which in its inactive state is held in the cytoplasm by the inhibitor of NF- κ B (I κ B). I κ B is regulated by I κ B kinase (IKK): upon stimulation by external signals or stress, IKK is activated and phosphorylates I κ B and, thereby, targets I κ B to ubiquitin-mediated protein degradation. As a result, NF- κ B is released and translocates into the nucleus where it transactivates multiple genes involved in proliferation, invasion and apoptosis (reviewed by Nakanishi and Toi¹¹).

Little information is available on the mechanism that may account for constitutive activity of NF- κ B in gliomas. Tumor

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Abbreviations: GBM, glioblastoma multiforme; IDH, isocitrate dehydrogenase; I κ B, inhibitor of κ B; MGMT, O⁶-methylguanine-DNA methyltransferase; miRNA, microRNA; NF- κ B, nuclear factor κ B; NKIRAS2, NF- κ B inhibitor interacting RAS-like 2; TMZ, temozolomide; TNFAIP3, Tumor necrosis factor alpha-induced protein 3; TRAIL, TNF-related inducing ligand

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necrosis factor alpha-induced protein 3 (TNFAIP3, also known as A20), which acts in a negative feedback loop to block NF- κ B activity and is significantly associated with resistance to TMZ, is frequently downregulated in GBM.¹² TNFAIP3 catalyzes the cleavage of K63-linked ubiquitin chains and the conjugation of K48-linked polyubiquitin chains, thereby targeting receptor-interacting serine-threonine kinase 1 for degradation.^{13,14} Other negative regulators including NF- κ B inhibitor interacting RAS-like (NKIRAS) 1 and 2, which interfere with proteosomal degradation of I κ B, have also been implicated in regulating NF- κ B activity,^{15–18} but their role in gliomas is poorly understood. In addition, the molecular mechanism of downregulation of these negative regulatory proteins in gliomas remains largely unknown.

In this study, we tested the hypothesis, whereby microRNAs (miRNAs), short regulatory sequences that control gene expression at the posttranscriptional level, would be implicated in the regulation of NF- κ B activity in gliomas. miRNAs are incorporated into RNA-induced silencing complex where they bind to target mRNAs and, depending on the degree of complementarity to their target sequence, confer mRNA degradation and/or translation inhibition of the target gene (reviewed by Iorio and Croce¹⁹). miRNAs are involved in the regulation of most basic cellular processes and are also implicated in tumorigenesis where they act as oncogenic or tumor suppressing miRNAs (reviewed by Hwang and Mendel²⁰).

Here we show that miR-125b, a brain-enriched miRNA, is implicated in the regulation of NF- κ B activity in GBM by targeting *TNFAIP3* and *NKIRAS2*. We show for the first time that, owing to downregulation of these targets, miR-125b controls proliferation and apoptosis, and confers TMZ resistance of GBM cell lines in a MGMT- and TP53-independent manner. These results can be extrapolated to clinical cases of GBM, as miR-125b expression significantly correlates with nuclear NF- κ B in surgically obtained GBM samples indicated that miR-125b is directly implicated in TMZ resistance.

Results

miR-125a/b directly target *TNFAIP3* and *NKIRAS2* in GBM cell lines. To identify miRNAs that are able to induce NF- κ B signaling, we searched target prediction databases for putative target genes implicated in negative regulation of NF- κ B using miRNAs that are overexpressed in GBM tissues.²¹ Target prediction algorithms using target scan (www.targetscan.org), miRDB (<http://mirdb.org>) or DianaLab (<http://diana.cslab.ece.ntua.gr>) revealed that *TNFAIP3* and *NKIRAS2* are putative targets of oncogenic miR-125a and miR-125b. miR-125a/b binding sites of eight nucleotides in length were detected in the 3' UTR of each gene and in the coding region of *TNFAIP3* (Supplementary Figure S1A). To assess direct regulation by miR-125a/b, luciferase constructs containing predicted miR-125a/b binding sites from the 3' UTR of the respective genes were cloned downstream of the luciferase reporter gene (Supplementary Figure S1B). U87 or LN-18 GBM cell lines, transiently transfected with miR-125a/b precursors, displayed significantly lower luciferase activity for reporter constructs containing the binding site for

TNFAIP3 or *NKIRAS2* relative to the control construct containing no binding site (Figures 1a and b). In contrast, downregulation of luciferase activity was abrogated in constructs in which the miR-125a/b binding site was mutated (Figures 1a and b and Supplementary Figure S1B). Consistent with these findings, the *TNFAIP3* and *NKIRAS2* mRNA levels were significantly reduced in U87 or LN-18 cells that were transiently transfected with miR-125a/b precursors (Figure 2a). In addition, the *TNFAIP3* mRNA level was inversely correlated with the level of miR-125b in 452 GBM samples from the Cancer Genome Atlas (TCGA) database (Supplementary Table S1). Under the same conditions, *TNFAIP3* and *NKIRAS2* proteins were strongly reduced (Figure 2b). These results clearly indicate that miR-125a/b directly target *TNFAIP3* and *NKIRAS2*.

Endogenous miR-125b is sufficient to regulate *TNFAIP3* and *NKIRAS2*. Luciferase activity of the reporter construct containing the target site of *TNFAIP3* was almost maximally reduced by endogenous miR-125a/b (Figure 1a, precursor control). In contrast, transfection of GBM cells with miR-125a/b precursor did not lead to a further reduction in luciferase activity (Figure 1a). To confirm that endogenous miR-125b is sufficient to downregulate *TNFAIP3* and *NKIRAS2*, GBM cell lines were transduced with a lentiviral

Figure 1 miR-125a and miR-125b directly target *TNFAIP3* and *NKIRAS2* in GBM cells. Luciferase constructs containing the wild-type (TS) or mutated (mTS) miR-125a/b target site from *TNFAIP3* or *NKIRAS2* were co-transfected with pre-miR-125a/b or pre-control into GBM cells. Luciferase activity is presented for *TNFAIP3* (a) and *NKIRAS2* (b) relative to the activity obtained with the construct Luc containing no target site ($n=3$)

vector expressing antisense miR-125b giving rise to five-times lower levels of miR-125b (Figure 2c). Indeed, anti-miR-125b elicited a 2–2.5-fold increase in the mRNA level (Figure 2c) and an increase, up to fivefold, in the protein level (Figure 2d) of TNFAIP3 and NKIRAS2. In contrast, anti-miR-125a was barely able to affect the mRNA or protein level of TNFAIP3 or NKIRAS2 (data not shown) suggesting that miR-125b is the relevant miRNA. In agreement with this finding, the level of miR-125b was up to 20 times higher than that of miR-125a in both GBM cell lines and GBM tissues (Supplementary Figure S2). Hence, we focused on miR-125b in subsequent experiments.

miR-125b is implicated in the regulation of NF- κ B activity. To assess whether miR-125b is able to induce NF- κ B activity, reporter assays were performed using a construct containing the luciferase gene under the control of a NF- κ B-inducible promoter. miR-125b precursor elicited a twofold higher luciferase activity in U87, LN-18 and U251 GBM cells compared with scrambled control (pre-control) (Figure 3a and Supplementary Figure S3). Consistent with these findings, blocking endogenous miR-125b by antisense miR-125b resulted in reduced luciferase activity of U87 cells (Figure 3a). Luciferase activity was barely detectable in LN-18 cells overexpressing anti-miR-125b (data not shown). However, results consistent with those for U87 cells were obtained, when LN-18 cells were treated with TNF α , an inducer of NF- κ B activity (compare Figures 3a and b).

To confirm NF- κ B activation using an independent approach, nuclear and cytoplasmic fractions of cells cultured in the presence or absence of TNF α were analyzed by western

blotting. The level of nuclear p65 was higher in cells transfected with pre-miR-125b relative to cells transfected with pre-control irrespective of whether they were cultured in the presence or absence of TNF α (Figure 3c). Complementary results were obtained for cytoplasmic fractions giving rise to reduced p65 levels in cells overexpressing miR-125b (Figure 3d). In addition, miR-125b conferred prolonged I κ B phosphorylation and concomitantly enhanced I κ B degradation (Figure 3d) consistent with a concept that negative feedback loops mediated by TNFAIP3 and NKIRAS2 are abrogated in cells overexpressing miR-125b.

NF- κ B is a transcriptional activator of anti-apoptotic and proliferative genes.²² Transient transfection with miR-125b precursor gave rise to increased levels of anti-apoptotic genes *Bcl-2* (Figure 4a) and *c-IAP2* (Figure 4b) in 5/5 and 3/5 GBM cell lines, respectively, whereas the levels of *XIAP*, *survivin*, *Bcl-xL* and *Bcl-w* mRNAs were not affected (Supplementary Figure S4). In agreement with this finding, miR-125b and *Bcl-2* were directly correlated at the RNA level in the cohort of TCGA ($P < 0.0001$, Supplementary Table S1). The cell cycle gene *CCND1* was induced in 4/5 cell lines upon transfection with miR-125b precursor (Figure 4c).

Phenotypic analysis of miR-125b transfected cells.

Populations of U87 or LN-18 cells overexpressing miR-125b precursor were 1.5–2-times higher in cell number, whereas populations transduced with anti-miR-125b were 25% lower 3 days post transfection relative to scrambled control (Figure 5a). To assess cell cycle progression, GBM cells were transduced with anti-miR-125b and subsequently treated with nocodazole to induce cell cycle arrest in G2/M.

Figure 2 TNFAIP3 and NKIRAS2 are regulated by miR-125a/b. (a) TNFAIP3 or NKIRAS2 mRNA levels by real-time PCR of cells transiently transfected with pre-miR-125a/b relative to cells transfected with pre-control ($n = 3$). (b) Western blot of GBM cells transiently transfected with pre-miR-125b relative to pre-control. (c) mRNA levels ($n = 3$) and (d) protein levels of TNFAIP3 and NKIRAS2 in cells lentivirally transduced with anti-miR-125b relative to cells transduced with anti-control

Figure 3 miR-125b induces NF- κ B activity. (a) NF- κ B reporter activity of GBM cells overexpressing miR-125b or anti-miR-125b relative to control cells ($n=6$). (b) NF- κ B reporter activity of LN-18 cells overexpressing anti-miR-125b. Cells were induced with 10 ng/ml TNF α for 4 h beginning 20 h post transfection ($n=6$). Expression of (c) nuclear p65 and (d) cytoplasmic p65, I κ B α and phospho I κ B α in LN-18 cells transiently transfected with pre-miR-125b or pre-control by Western blotting. Cells were induced with 10 ng/ml TNF α beginning at 48 h post transfection. The ratio of plkB α to I κ B α protein levels is presented below the corresponding Western blot

In the case of U87 cells transduced with anti-control, 41% of the population were in G1, whereas 75% of the cells transduced with anti-miR-125b were in this phase of the cell cycle ($P=0.0033$), indicating that miR-125b promotes G1 progression. In contrast, anti-miR-125b had no effect on cell cycle progression of LN-18 cells (Figure 5b).

To assess apoptosis, a caspase 3 cleavage assay was performed. U87 or LN-18 cells overexpressing miR-125b were significantly protected from TNF α or TNF-related inducing ligand (TRAIL)-induced apoptosis (Figure 5c). Conversely, experiments performed with cells transduced with anti-miR-125b revealed an increased rate of TNF α -induced apoptosis (Figure 5d).

Figure 4 miR-125b induces the expression of NF- κ B responsive genes. mRNA levels of *Bcl-2* (a), *c-IAP2* (b) and *CCND1* (c) by real-time PCR in GBM cell lines overexpressing miR-125b relative to control transfected cells ($n=3$)

miR-125b induces NF- κ B activity by targeting TNFAIP3 and NKIRAS2. miR-125b protects GBM cells from apoptosis, but is this owing to TNFAIP3 and/or NKIRAS2 repression? To address this question, miR-125b-refractory retroviral expression constructs were made containing the coding region of *TNFAIP3* or *NKIRAS2*. Both constructs lack miR-125a/b target sites in the 3' UTR, but the *TNFAIP3* construct contains a predictive miR-125a/b target site in the coding region which, however, did not affect protein expression (Supplementary Figure S5A). U87 or LN-18 cells transduced with *TNFAIP3* or *NKIRAS2* expression vectors gave rise to high level expression of the respective gene (Supplementary Figures S5B and C).

NF- κ B activity was significantly reduced in cells overexpressing TNFAIP3 and NKIRAS2, although this was more

pronounced in U87 cells than in LN-18 cells (Figure 6a). To assess if ectopic TNFAIP3 or NKIRAS2 can rescue the phenotype obtained with miR-125b, NF- κ B activity was

analyzed by luciferase reporter assays. miR-125b elicited a 1.8-fold induction of luciferase activity in U87 cells transduced with control plasmid (pBABE), whereas only a 1.2–1.3-fold induction of luciferase activity was obtained in cells ectopically expressing NKIRAS2 or TNFAIP3 (Figure 6b). Comparable results were also obtained in LN-18 cells giving rise to a 1.5-fold induction in pBABE cells and a 1.2–1.3-fold induction in NKIRAS2 or TNFAIP3 cells, respectively.

In agreement with these results, miR-125b was less efficient in cells ectopically expressing TNFAIP3 or NKIRAS2 than in pBABE control to induce nuclear accumulation of NF- κ B (Figure 6c, upper panel). In contrast, more NF- κ B was retained in the cytoplasm, whereas less phospho I κ B was expressed in cells ectopically expressing TNFAIP3 or NKIRAS2 following transfection with miR-125b precursor (Figure 6c, lower panel). In conclusion, these results indicate that ectopic TNFAIP3 or NKIRAS2 can restore miR-125b-induced activation of NF- κ B.

Resistance to apoptosis by miR-125b depends on the expression of TNFAIP3 and NKIRAS2. TNF α -induced apoptosis was higher in cells ectopically expressing TNFAIP3 or NKIRAS2 compared with pBABE control, consistent with a reduced NF- κ B activity (Figure 7a). However, the ability of miR-125b to protect cells from TNF α -induced apoptosis was significantly compromised in cells ectopically expressing TNFAIP3 or NKIRAS2. This was based on the finding that miR-125b elicited a 1.9 times lower apoptosis rate in pBABE control, but only a 1.3–1.4 times lower rate in cells expressing TNFAIP3 or NKIRAS2 compared with cells transfected with pre-control (Figure 7a).

miR-125b confers resistance to TMZ independent of MGMT. Enhanced NF- κ B activity is clearly associated with resistance to TMZ.²³ Consistent with this finding, U87 or LN-18 cells transfected with miR-125b precursor were significantly more resistant to TMZ than cells transfected with pre-control (Figure 7b). Conversely, cells overexpressing anti-miR-125b revealed a higher sensitivity towards TMZ compared with the control (Figure 7c).

To assess if resistance to TMZ by miR-125b depends on the expression of TNFAIP3 or NKIRAS2, retrovirally transduced LN-18 cells were analyzed. Consistent with the results obtained with untransduced cells, pBABE control cells overexpressing miR-125b were significantly more resistant to TMZ than control transfected cells (compare Figures 7b and d). In contrast, miR-125b was significantly compromised in its ability to confer resistance to TMZ in LN-18 cells ectopically expressing TNFAIP3. NKIRAS2 proved to be

Figure 5 miR-125b induces proliferation and apoptosis resistance in GBM cells. (a) Population growth of U87 and LN-18 cells overexpressing miR-125b or anti-miR-125b relative to the control using the resazurin assay ($n = 3$). (b) Cell cycle analysis of nocodazole-treated cells by flow cytometry ($n = 3$). (c) Apoptosis. Cells were transfected with pre-miR-125b or pre-control and treated with 10 ng/ml TNF α or 250 ng/ml TRAIL for 48 h beginning 24 h post transfection. Apoptosis was assessed using the ApoTox-Glo Triplex assay ($n = 3$) (d) LN-18 cells overexpressing anti-miR-125b or anti-control were induced with 10 ng/ml TNF α for 24 h and subjected to the ApoTox-Glo Triplex assay ($n = 3$)

Figure 6 miR-125b induces NF- κ B activity by targeting *TNFAIP3* and *NKIRAS2*. (a) NF- κ B activity of GBM cells retrovirally transduced with miR-125b-refractory *TNFAIP3*, *NKIRAS2* or pBABE control relative to untransduced cells ($n = 6$). (b) NF- κ B activity of retrovirally transduced cells overexpressing miR-125b relative to pre-control transfected cells ($n = 6$). (c) Western blot analysis of nuclear (upper panel) and cytoplasmic (lower panel) fractions of retrovirally transduced cells overexpressing miR-125b

less efficient than *TNFAIP3* to restore miR-125b-induced resistance to TMZ (Figure 7d).

NF- κ B is a known transactivator of *MGMT*. To assess if miR-125b confers TMZ resistance by inducing the expression of *MGMT*, the steady-state level of *MGMT* mRNA was analyzed in GBM cell lines overexpressing miR-125b. However, *MGMT* mRNA levels were unaffected in 4/5 cell lines (Supplementary Figure S6), indicating that miR-125b induces TMZ resistance independent of *MGMT*.

miR-125b levels correlate with nuclear NF- κ B in the cancer tissue and with the patient's survival. Formalin-fixed paraffin-embedded tumor tissues, obtained from a collective of 60 GBM patients,²⁴ were analyzed for the expression of miR-125a/b (Supplementary Figure S2). Kaplan–Meier survival curves revealed that miR-125b is an unfavorable prognostic marker for patients treated with TMZ in combination with radiotherapy (Figure 8a upper panel, $P = 0.02$, log-rank Mantel–Cox). The median survival for subgroups expressing high levels and low levels of miR-125b was 9 and 18 months, respectively, and the hazard ratio was 0.26. In contrast, miR-125a was not a predictor of response to TMZ (Figure 8a lower panel, $P = 0.14$). To validate our collective, all tissues were also analyzed for the *MGMT* promoter methylation status and IDH1/2 mutation status. Both markers proved to be favorable prognostic markers in this collective (Supplementary Figures S7A and B).

miR-125b expression was not correlated with *MGMT* methylation or IDH mutation suggesting that miR-125b is an independent prognostic marker (Supplementary Figure S8). To assess if miR-125b affects NF- κ B activity in tumor tissues, tissue sections were analyzed by immunohistochemistry

using an antibody against p65 (Supplementary Figure S9). Nuclear localization of NF- κ B was correlated significantly with miR-125b expression (Figures 8b, $P = 0.03$), indicating that the results obtained in cell lines are also relevant for tumor tissues. Again, no significant correlation was obtained between miR-125a levels and nuclear NF- κ B (Figure 8b).

Discussion

miR-125b is an 'oncomiR' which plays a central role in molecular dysfunctions linking inflammation with cancer.²⁵ It is implicated in proliferation, apoptosis and invasion and is considered to be an important modulator of drug resistance in different tumor systems.²⁶ However, controversy exists whether miR-125b is able to induce or inhibit these processes. Different proapoptotic genes including *TP53*, *Bmf*, *SIRT1*, *Bak1* and *Puma*^{27–30} or anti-apoptotic genes including *Bcl-2*, *Mcl-1*, *Bcl-w*^{31–33} were identified as targets of miR-125b. Importantly, these studies often solely relied on data from miR-125b overexpression and did not include rescue experiments to show that the newly identified targets are relevant in miR-125b-mediated cell death or proliferation responses.

In this study, we show that miR-125b induces proliferation and anti-apoptosis by inducing NF- κ B activity in GBM cell lines. This is based on the findings that miR-125b induces: (a) activity of a NF- κ B reporter construct, (b) phosphorylation and degradation of *I κ B* and, as a consequence, nuclear localization of NF- κ B and (c) expression of known mediators of NF- κ B signaling including *Bcl-2*, *c-IAP2* and *CCND1*. The results obtained *in vitro* may be extrapolated to clinical cases of GBM, as miR-125b expression significantly correlates with nuclear NF- κ B in surgically obtained GBM samples.

Furthermore, endogenous levels of miR-125b are sufficient to regulate proliferation and resistance to apoptosis as indicated by anti-miR-125b lentiviral transduction experiments (Figure 5). In agreement with our results, miR-125b was among the top

hits in a functional genomics screen for miRNAs regulating NF- κ B activity in HEK293 cells,³⁴ but this was not further investigated. High level expression of miR-125b³⁵ and enhanced NF- κ B activity^{8,9} are both confined to grade IV (GBM) gliomas suggesting that miR-125b is less important in low grade gliomas.

miR-125b directly targets negative regulators of NF- κ B activity including NKIRAS2 and TNFAIP3. Although both miR-125a and miR-125b were shown recently to control the expression of TNFAIP3 and NF- κ B activity in DLBCL,³⁶ endogenous levels of miR-125b, but not miR-125a, were sufficient to affect the expression of TNFAIP3 in GBM cells. Thus, the findings obtained in one cell system cannot be extrapolated to another cell system. The finding that TNFAIP3 regulation by miR-125b is particularly important for GBM cells is illustrated by the fact that luciferase activity of a construct containing the miR-125a/b target site from TNFAIP3 was almost maximally reduced by endogenous miR-125b (Figure 1a). Further evidence for TNFAIP3 and NKIRAS2 being relevant targets of miR-125b in GBM cells is based on our findings that NF- κ B activity, anti-apoptosis and TMZ resistance induced by miR-125b were abrogated in GBM cells ectopically expressing miR-125b-refractory TNFAIP3 or NKIRAS2. The rescue was not complete as TNFAIP3 and NKIRAS2, both of which contribute to NF- κ B activity, were not ectopically expressed in the same cell. TNFAIP3 forms part of a negative feedback loop to shut off NF- κ B signaling.^{13,14} Both miR-125b³⁷ and TNFAIP3³⁸ are induced by NF- κ B activity. In non neoplastic cells, one role of miR-125b may be to modulate NF- κ B activity by counteracting TNFAIP3 induction. In contrast, this negative feedback loop is abrogated in GBM cells owing to overexpression of miR-125b, resulting in prolonged NF- κ B signaling. Consistent with this notion, miR-125b conferred prolonged I κ B phosphorylation and concomitantly enhanced I κ B degradation (Figure 3d).

Reduced *TNFAIP3* mRNA level in GBM is strongly associated with TMZ resistance,¹² but the underlying molecular mechanism is largely unknown. We are the first to show that this is at least in part owing to miR-125b. Thus we provide a novel mechanism of TMZ resistance in GBM cells. Consistent with this finding, miR-125b expression in the tumor tissue is significantly correlated with the survival of patients treated with TMZ. These findings are important as TMZ is the major chemotherapeutic agent which is used for the treatment of GBM, but <40% of GBM patients initially respond to therapy and resistance develops rapidly.³⁹

In addition, we show that NKIRAS2 is directly implicated in apoptosis and contributes to chemosensitivity of GBM tumors (see Figures 7a and d) and, thus, may constitute a new

Figure 7 miR-125b confers resistance to apoptosis and TMZ by targeting *TNFAIP3* and *NKIRAS2*. (a) Retrovirally transduced LN-18 cells were transfected with pre-miR-125b or pre-control and induced with 10 ng/ml TNF α for 24 h ($n = 3$). Apoptosis was assessed using the ApoTox-Glo Triplex assay. (b) Cell viability of GBM cells overexpressing miR-125b in the presence of 200 μ M TMZ. Cell viability was assessed 3 days after treatment with TMZ using resazurin assay ($n = 3$). (c) Cell viability of GBM cells overexpressing anti-miR-125b or anti-control in the presence of TMZ ($n = 6$). (d) Cell viability of LN-18 cells retrovirally transduced with miR-125b-refractory TNFAIP3, NKIRAS2 or pBABE control following transfection with pre-miR-125b or pre-control in the presence of TMZ ($n = 6$).

Figure 8 miR-125b expression is directly correlated with NF- κ B activity in GBM tissues and with the patient's overall survival. **(a)** Comparison of the overall survival of patients expressing >120% or <120% miR-125a/b in the tumor tissue relative with the level of miR-125a/b in U87 cells using Kaplan-Meier survival plots. The threshold was defined using a density blot, which gave rise to a bimodal distribution with best separation at 120%. All patients were treated with TMZ. Significance of correlation was assessed using log-rank (Mantel-Cox) test. **(b)** Correlation of miR-125a/b expression and nuclear p65 in GBM tissues. A tumor with >25% nuclear p65 was considered as constitutive active for NF- κ B⁴⁸

chemoresistance marker. NKIRAS2 was reported to disturb cytokine-induced NF- κ B activation.^{15,16} Consistent with previous findings,¹⁶ ectopic expression of NKIRAS2 resulted

in significantly less NF- κ B activity (Figure 6a). Surprisingly, miR-125b was reported to induce rather than reduce the expression of NKIRAS2 in primary human macrophages,⁴⁰ but the molecular mechanism was not further investigated. We are the first to show that NKIRAS2 is not only a direct, but also a relevant target of miR-125b in GBM cells.

miR-125b-induced TMZ resistance was independent of TP53. This was indicated by the fact that although LN-18, T98G, HS683 and U251 cells carry mutations or deletions in the *TP53* gene,^{41,42} miR-125b was capable of inducing Bcl-2 expression and protecting these cells from apoptosis to an extent similar to that of TP53 proficient U87 cells (Supplementary Figure S4). Likewise, miR-125b did not affect the level of MGMT expression in most cell lines. Thus we conclude that miR-125b induces resistance of GBM cells to TMZ independent of MGMT or TP53. In addition, high level expression of miR-125b was not inversely correlated with the presence of IDH mutations or promoter methylation of the MGMT gene in GBM tissues. Thus, miR-125b seems to be an independent predictor of response to TMZ. Interestingly, U87 cells transduced with anti-miR-125b were considerably less resistant to TMZ than anti-miR-125b transduced LN-18 cells, which might be because of the fact that anti-miR-125b induces cell cycle arrest in G1/G0 in the former.

In conclusion, our results indicate that miR-125b is an important regulator of NF- κ B activity by targeting *TNFAIP3* and *NKIRAS2* and, thereby, affects proliferation, apoptosis and resistance to TMZ. The finding that miR-125b is a predictor of response to TMZ in GBM patients underlies the importance of miR-125b in NF- κ B mediated processes. Our results suggest that miR-125b represents an interesting target for adjuvant therapy as blocking miR-125b by antagoniRs would allow us to modulate the activity of NF- κ B in GBM tumors.

Materials and Methods

Cell lines and culture conditions. The human GBM cell line U87 MG, LN-18, U251, HS683 and T98G were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Iscove's modified Duplecco's medium (Sigma-Aldrich, Buchs, Switzerland) supplemented with 2 mmol/l L-alanyl-L-glutamine, 1% penicillin/streptomycin and 5–10% fetal bovine serum (Sigma-Aldrich) at 37 °C and 5% CO₂.

Constructs. Luciferase constructs containing wild-type or mutated miR-125a/b target sites from *TNFAIP3* (BC114480) and *NKIRAS2* (NM_001001349), respectively, were obtained by cloning double-stranded oligonucleotides into an *Xba*I site downstream of the luciferase gene of pcDNA3.0_luc.⁴³ The coding region of *NKIRAS2* was amplified from genomic DNA using the primer pair *NKIRAS2_fw* and *NKIRAS2_rv* and cloned between the *Bam*HI and *Xho*I sites of pBABE-puro (Addgene, Cambridge, MA, USA). A fragment encompassing the 5' end of *TNFAIP3* was amplified from genomic DNA using the primer pair *TNFAIP3-5'_fw* and *TNFAIP3-5'_rv* and released by cleavage with *Bgl*II and *Hind*III. A fragment containing the 3' end of *TNFAIP3* was released from pEGFR-C1-A20 (Addgene) by cleavage with *Hind*III and *Xho*I. Both fragments were cloned between the *Bam*HI and *Xho*I sites of pBABE-puro to give rise to pBABE-TNFAIP3. Primers used for cloning are indicated in Supplementary Table S2.

Transfection. Transfection was performed using Effectene, HiPerFect or Attractene reagents (Qiagen, Hombrechtikon, Switzerland) essentially as described⁴³ except that 37.5 nM miRNA precursor *hsa-miR-125a-5p* and *hsa-miR-125b-5p*, pre-miR negative control #1 (Ambion, Carlsbad, CA, USA) was used for transfection. Luciferase constructs and pGL4.32 reporter plasmid (Promega AG, Dübendorf, Switzerland) were used at 1 μ g/ml and pRI-SV40 (Promega) was used at 50 ng/ml transfection mix, respectively.

Lentiviral and retroviral transduction. U87 and LN-18 cells were transduced with a lentiviral expression vector for antisense hsa-miR-125b-1 (MZIP125b-PA-1) or scrambled control (System Bioscience, Mountain View, CA, USA). Lentiviruses were produced as described.⁴⁴ Cells were analyzed 3 days post transduction. For ectopic expression of miR-125b-refractory TNFAIP3 and NKIRAS2, U87 and LN-18 cells were transduced with pBABE-puro retrovirus (Addgene) as described.⁴⁵ Retrovirally transduced U87 and LN-18 cells were selected with 0.8 μ g/ml and 1.5 μ g/ml puromycin, respectively.

Luciferase activity assays, cell cycle analysis, apoptosis assay and cell viability assay. NF- κ B reporter assays and luciferase activity assays for target validation were carried out 30 and 48 h post transfection, respectively, using a dual luciferase reporter assay (Promega) and an Infinite 200 reader (Tecan, Maennedorf, Switzerland).

Cell cycle analysis was performed essentially as described previously.⁴⁶ Lentivirally transduced cells were treated with 80 ng/ml nocodazole for 16 h beginning at 3 days post transduction. Flow cytometry was performed using a LSR flow cytometer (Becton Dickinson, Allschwil, Switzerland) and FlowJo software Version 9.5.3 (Tree Star, Ashland, OR, USA).

Apoptosis and viability were assessed using the ApoTox-Glo Triplex assay (Promega). Apoptosis was induced by treating the cells with 10 ng/ml TNF α (PeproTech, Rocky Hill, NJ, USA) or 250 ng/ml TRAIL (R&D Systems, Oxon, UK). Cells were analyzed 24–48 h following stimulation.

TMZ (Sigma-Aldrich) sensitivity was assessed using the metabolic resazurin assay (Sigma-Aldrich) in cells cultured in the presence of TMZ for 3 days.

RNA isolation and Real-time PCR. RNA extraction and real-time PCR was performed as described in.⁴³ Quantitative PCR of anti-apoptotic markers was carried out using Quantitect primers (Qiagen); all other amplifications were done using TaqMan assay (Applied Biosystems, Rotkreuz, Switzerland). Quantitative PCR was performed using the one-step PCR system (Applied Biosystems). The mean C_T was determined from triplicate experiments. miRNA and mRNA levels were normalized to the level obtained for RNU48 and GAPDH, respectively. Changes in expression were calculated using the $\Delta\Delta C_T$ method.

Cell fractionation and western blot analysis. Cell fractionation was performed using the nuclear extract kit according to the manufacturer's instruction (Active Motif, Carlsbad, CA, USA). Protein concentrations were determined using the Pierce BCA assay (Thermo Scientific, Reinach, Switzerland); 5 μ g were loaded per lane on a 4–20% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories AG, Reinach, Switzerland). Separated proteins were transferred to PVDF membranes using the transfer turbo blot system (Bio-Rad). Monoclonal antibodies used in this study were directed against TNFAIP3 (clone 59A426, diluted 1:100, Abcam, Cambridge, UK), NKIRAS2 (clone ab57303, diluted 1:500, Abcam), I κ B α (clone E130, diluted 1:10 000, Abcam), phospho-I κ B α (clone 5A5, diluted 1:2000, Cell Signaling, Beverly, MA, USA), α -tubulin (clone B512, diluted 1:2000, Sigma-Aldrich), histone 3 H3 (clone D1H2, diluted 1:2000, Cell Signaling) and p65 (polyclonal, diluted 1:2000, Abcam). Secondary goat anti-mouse-HRP and goat anti-rabbit HRP antibodies (Bio-Rad) were used at 1:5000 or 1:7000, respectively. Protein levels from total or cytoplasmic extracts were normalized to α -tubulin and protein levels from nuclear extracts were normalized to histone 3. Quantification of protein bands were performed using a luminescent image analyzer LAS-4000 (Fujifilm, Dielsdorf, Switzerland) and Multi Gauge software (Fujifilm Version 3.0).

Immunohistochemistry. For immunohistochemistry, 3- μ m formalin-fixed paraffin-embedded sections were treated with 25 mM citrate buffer, pH 6, in a pressure cooker essentially as described.⁴⁷ Anti-p65 (Abcam) was used at a 1:50 dilution. Mouse IgG1 (1:20, Dako, Glostrup, Denmark) was used as a negative control. Sections were incubated with EnVision+ system (labeled polymer HRP anti-mouse, Dako) for 30 min at room temperature, visualized with 3,3'-Diaminobenzidine (Sigma-Aldrich) for 8 min and counterstained with haematoxylin.

Laser capture microdissection, IDH1/2 mutation analysis and MGMT promoter methylation analysis. Formalin-fixed paraffin-embedded tissues from 58 surgical GBM samples were used for miRNA expression, NF- κ B, MGMT and IDH1/2 analysis. Clinical characteristics of the patient's collective are described elsewhere.²⁴ Tumor tissue was collected by laser capture microdissection as described.⁴³ All experiments using human specimens

were done in compliance with the ethical guidelines of the Institute of Pathology, University of Bern, and were reviewed by the institutional review board.

Statistics. Statistical analyzes were performed and Kaplan–Meier plots were generated using the GraphPAD prism software (La Jolla, CA, USA). Statistical differences were calculated using unpaired two-tailed Student's *t*-test. A probability of $p \leq 0.05$ was considered statistically significant. Ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Conflict of Interest

The authors declare no conflict of interest.

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