

Mutant EGFR is required for maintenance of glioma growth in vivo, and its ablation leads to escape from receptor dependence

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Epidermal growth factor receptor (EGFR) gene amplification is the most common genetic alteration in high-grade glioma, and \approx 50% of EGFR-amplified tumors also harbor a constitutively active mutant form of the receptor, Δ EGFR. Although Δ EGFR greatly enhances tumor growth and is thus an attractive target for anti-glioma therapies, recent clinical experiences with EGFR kinase inhibitors have been disappointing, because resistance is common and tumors eventually recur. Interestingly, it has not been established whether Δ EGFR is required for maintenance of glioma growth in vivo, and, by extension, if it truly represents a rational therapeutic target. Here, we demonstrate that in vivo silencing of regulatable Δ EGFR with doxycycline attenuates glioma growth and, therefore, that it is crucial for maintenance of enhanced tumorigenicity. Similar to the clinical experience, tumors eventually regained aggressive growth after a period of stasis, but interestingly, without re-expression of Δ EGFR. To determine how tumors acquired this ability, we found that a unique gene, *KLHDC8*, herein referred to as Δ E (Substitute for Δ EGFR Expression)-1, is highly expressed in these tumors, which have escaped dependence on Δ EGFR. Δ E-1 is also expressed in human gliomas and knockdown of its expression in Δ EGFR-independent "escaper" tumors suppressed tumor growth. Taken together, we conclude that Δ EGFR is required for both glioma establishment and maintenance, and that gliomas undergo selective pressure in vivo to employ alternative compensatory pathways to maintain aggressiveness in the event of EGFR silencing. Such alternative pathways function as substitutes for Δ EGFR signaling and should therefore be considered as potential targets for additional therapy.

tumorigenicity | glioblastoma | epidermal growth factor receptor

Malignant gliomas are the most common type of primary brain tumors. Glioblastoma, the most malignant form of glioma, remains mostly incurable despite intensive treatment including surgical resection, irradiation, and chemotherapy (1). Epidermal growth factor receptor (EGFR) gene amplification occurs in \approx 30–50% of all glioblastomas, and the amplified genes are frequently rearranged (2, 3). An in-frame deletion of exons 2–7 of EGFR (referred to here as Δ EGFR), is the most commonly occurring mutant form of the EGFR in these tumors (4), is constitutively activated, and confers enhanced tumorigenicity to glioblastoma cells in vivo by reducing apoptosis and increasing proliferation (5–7). Patients with tumors expressing Δ EGFR have a poorer prognosis than those that do not (8, 9).

Δ EGFR has been used as a tumor-specific drug target in kinase inhibitor, antibody-based immunotherapy, immunotoxin, peptide vaccination, and small interfering RNA (siRNA) approaches (10). For such approaches to be ultimately successful, the target would ideally be required for the maintenance of tumor growth, not only for its initiation. This notion has been demonstrated for other oncogenes such as ras, and wild-type-EGFR in other tumor types (11–14) but has not been shown for Δ EGFR in glioblastoma or any other tumor type. Moreover, although it has been shown that a glioblastoma subgroup coexpressing Δ EGFR and PTEN initially

respond to EGFR targeted therapy (15), recent experience shows that even responsive tumors will eventually grow through the therapy (16, 17). Therefore, an understanding of the mechanisms that tumor cells use to overcome receptor inhibition is of substantial importance in designing useful therapies.

In some cases, resistance to EGFR-targeted therapy arises either due to desensitizing mutations in the kinase itself or through the activation of alternate oncogenic pathways (18). Recently, it has also been shown that other receptor tyrosine kinases, such as PDGFR, IGF1-R, and c-Met can be concurrently up-regulated in glioblastomas, resulting in compensation for decreased signaling by Δ EGFR (19–21). All of these studies demonstrate that tumor cells are adept at overcoming single targeted therapies and suggest that understanding the various mechanisms used to this end could provide very useful information with which to design more effective therapies that are more difficult to overcome. Here, we sought to define such mechanisms by using glioma cells that conditionally express Δ EGFR in vivo to test the requirement of Δ EGFR for maintenance of enhanced tumor growth and to uncover alternate pathways that gliomas may employ to bypass Δ EGFR inhibition.

Results

Glioma Cells that Express Δ EGFR Regulated by Doxycycline. To determine whether ongoing Δ EGFR expression was essential for tumor establishment and maintenance, we developed a tetracycline-regulatable expression system, in which the Δ EGFR level in tumor xenografts is repressed by administering doxycycline-containing drinking water to engrafted mice. We selected the human high-grade glioma cell line U373MG for these studies because it requires Δ EGFR expression for xenograft establishment in mice. As shown in Fig. S1A, U373MG cells constitutively expressing Δ EGFR formed tumors in nude mice, whereas neither control U373MG-Luc cells nor U373MG-wt.EGFR cells were able to do so. To construct tetracycline-regulatable expression in these cells, a U373MG clone that expresses the tetracycline-controlled transactivator (tTA) was first produced and verified. Then, either p Δ CMV/(tetO)- Δ EGFR-IRES2-EGFP or p Δ CMV/(tetO)- Δ EGFR-DK-IRES2-EGFP vectors that express Δ EGFR or its kinase-deficient mutant, DK, together with enhanced green fluorescent protein (EGFP) from a single bicistronic mRNA, were engineered into this tet-regulatable U373MG clone (Fig. S1B). Cells that had higher GFP expression

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were collected by two rounds of fluorescence activated cell sorting to enrich for cells that highly expressed Δ EGFR. In these tet-regulatable Δ EGFR-expressing U373 cells, both Δ EGFR and GFP expression could be silenced within 2–4 days after exposure to the tetracycline analog, doxycycline (Dox) (Fig. 1A and Fig. S1C and D).

Growth of U373MG Cells Expressing Tetracycline-Regulated Δ EGFR In Vitro and in Vivo. To determine whether tetracycline-regulated Δ EGFR imparted enhanced in vitro growth to U373MG cells, cell proliferation was analyzed by WST assays. Consistent with previous results (5), there were no significant differences in the in vitro growth rates between cells expressing Δ EGFR (-Dox) and cells silenced for Δ EGFR expression (+Dox), as well as no adverse effect of doxycycline on U373MG cells that constitutively express Δ EGFR (Fig. S1E).

To investigate the tumorigenicity of tetracycline-regulatable Δ EGFR-expressing U373MG cells in vivo, cells were engrafted s.c. into nude mice. Mice that were given doxycycline in their drinking water (+Dox) failed to develop tumors even after 80 days. However, when dox was omitted from the water (-Dox), Δ EGFR was expressed and this resulted in rapid tumor formation (Fig. 1B). When tumors were allowed to establish for 32 or 48 days, and then switched to +Dox conditions, resulting in the complete silencing of Δ EGFR expression (Fig. 1C), these tumors underwent an extended period of stasis (Fig. 1B). As a control, U373MG cells expressing tetracycline-regulated DK did not form tumors in nude mice. These results indicate the dependency of establishing U373 gliomas and of their continued enhanced growth in vivo on catalytically active mutant EGFR.

Decreased Proliferation in U373MG Tumors Silenced for Δ EGFR Expression. To investigate the effects of Δ EGFR silencing in the tumors, cell proliferation rates, apoptotic indices and signaling downstream of activated EGFR after administration of doxycycline were analyzed. Tetracycline-regulatable U373MG cells (3×10^6) expressing Δ EGFR were injected s.c. into the flanks of nude mice and Δ EGFR-driven tumors were allowed to establish. When tumor volumes reached an analyzable size ($272 \pm 138 \text{ mm}^3$ at day 56), these mice were separated into two groups, and doxycycline-containing water was provided to one group only. Tumors continued to grow in mice that remained on normal drinking water (-dox), whereas tumors regressed in mice receiving doxycycline-containing water (+dox). Tumors were resected at 4 days after doxycycline administration when -dox tumors reached a size of $433 \pm 237 \text{ mm}^3$ and +dox tumors reached a size of $211 \pm 86 \text{ mm}^3$. Tumors were also

harvested at 8 days after doxycycline administration in a separate experiment when -dox tumors reached a size of $357 \pm 300 \text{ mm}^3$ and +dox tumors reached a size of $141 \pm 95 \text{ mm}^3$. The growth rate of tumors during the first 4 days with and without suppression of Δ EGFR were statistically different ($-20 \pm 15\%$ and $+45 \pm 22\%$, respectively; $P < 0.001$; Student's *t* test), and a significant ($P < 0.001$; Student's *t* test) reduction in proliferation (K_i -67) was observed in Δ EGFR-negative tumors at 4 and 8 days (Fig. 2A and Fig. S2A and B). These tumors also showed a slight increase ($P = 0.064$ at 4 days and $P < 0.05$ at 8 days; Student's *t* test) in apoptosis (TUNEL staining) (Fig. 2B and Fig. S2C), whereas there was no significant difference ($P > 0.1$; Student's *t* test) in microvessel area (CD31 staining) (Fig. S2D).

In those tumors that no longer expressed Δ EGFR, diminished expression of the antiapoptotic molecule Bcl- x_L was observed as would be expected for reduced Δ EGFR signaling (Fig. 2C) (6). There was no difference in phosphorylation of serine residue of Akt, which is likely due to the strong possibility that growth factors present in the tumor microenvironment elicit signaling through the PI3-K pathway even in the absence of Δ EGFR. Also as expected for tumors that exhibit growth stasis, the expression of proteins involved in cell cycle progression and proliferation, such as CyclinA, CyclinE, CDK2, CDK4, CDK6, and activation of Stat1/3 showed trends toward decreases in Δ EGFR-negative tumors compared to Δ EGFR-positive control tumors (Fig. 2C). Accordingly, p27, which acts in a negative fashion to prevent cell cycle progression, was expressed at very low levels in Δ EGFR-positive tumors and increased in Δ EGFR-negative tumors (Fig. 2C). Interestingly, we also observed a substantial increase in the phosphorylation of both ERK and p38 MAPK after silencing of Δ EGFR (Fig. 2C), suggesting that Δ EGFR signaling in vivo is mediated primarily by pathways other than MAPK. Furthermore, the tumor regression we observed indicates that the increase in MAPK phosphorylation is not immediately sufficient to support further tumor growth at these early timepoints. Overall, these results indicate that initial tumor regression after silencing of Δ EGFR expression is predominantly marked by a reduction in cell proliferation, which is governed by alterations in STAT1/3 transcriptional activity as well as apoptotic and cell cycle machinery.

Breakthrough U373MG Tumor Growth Independent of Δ EGFR. After an extended period of stasis, some tumors [11 of 19 total individual tumors (58%)] in which Δ EGFR expression had been repressed by exposure to dox eventually regained aggressive growth over the period of the experiment. When they did so, they then grew with

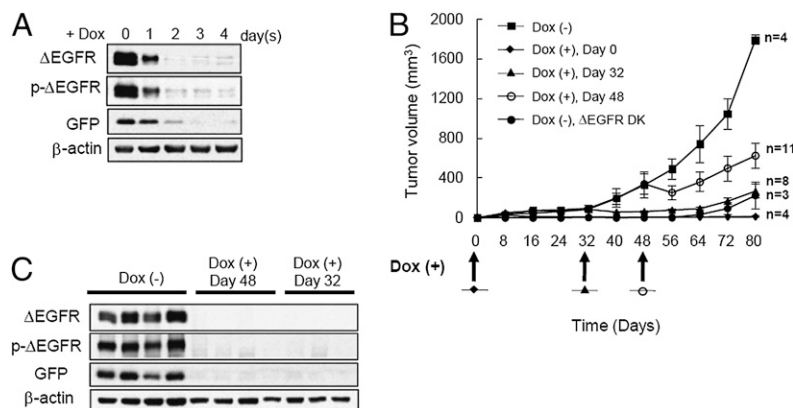


Fig. 1. Δ EGFR-dependent tumor growth of U373MG cells expressing doxycycline-regulatable Δ EGFR. (A) Western blot analysis of proteins from whole-cell lysates with the indicated antibodies of engineered U373MG cells cultured in medium containing 1 μ g/mL dox for 0, 1, 2, 3, and 4 days. (B) Tumor growth of U373MG cells (3×10^6) expressing dox-regulatable Δ EGFR injected s.c. into the flanks of nude mice that received dox-containing drinking water as indicated by arrows at 0, 32, or 48 days. One group of mice received U373MG cells expressing kinase-defective Δ EGFR (DK) and no doxycycline. Similar experiments were done at least twice with consistent results. (mean \pm SEM) (C) Western blot analysis of lysates from mouse tumors in A that received no dox or received dox at day 32 or 48.

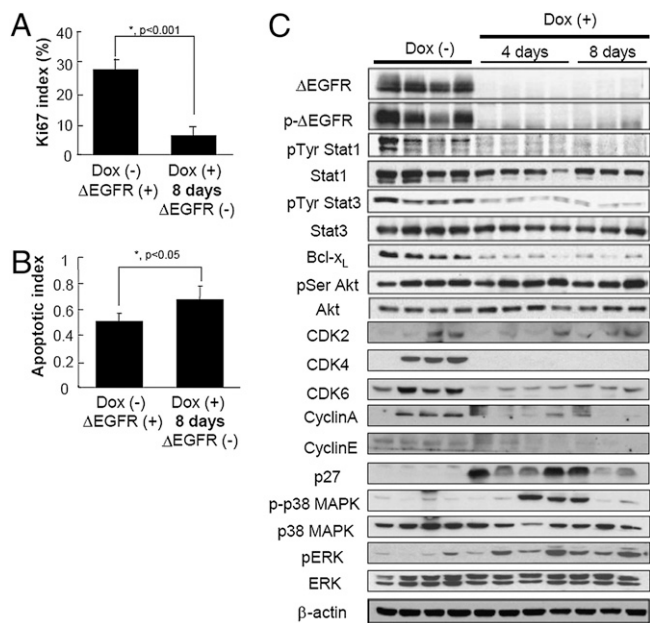


Fig. 2. Growth characteristics and pathway profiling of U373MG tumors upon silencing of Δ EGFR expression. Mice bearing established s.c. U373MG dox-regulatable Δ EGFR tumors remained on normal drinking water [Dox (-)], or were administered dox-containing drinking water for 4 or 8 days [Dox (+)], and tumors were resected and analyzed. (A) Proliferation index as measured by counting proportion of K_i -67-positive cells (mean \pm SD; *, $P < 0.001$, Student's t test). (B) Apoptotic index as measured by percentage of TUNEL-positive cells (mean \pm SD; *, $P < 0.05$, Student's t test). (C) Western blot analysis of proteins from tumor lysates with the indicated antibodies. Each lane represents a tumor from an individual mouse.

kinetics similar to Δ EGFR-expressing tumors (indicated by the horizontal arrows in Fig. 3A). Western blot analysis demonstrated that these breakthrough tumors, hereafter referred to as “escapers,” did not result from the re-expression of Δ EGFR (Fig. 4), implying that this outgrowth might be prompted by compensatory pathways that confer similar enhanced tumorigenicity as does Δ EGFR. These breakthrough tumors showed an increase in cell proliferation (K_i -67 staining) compared with the initial silencing of Δ EGFR, but proliferation in the escapers remained lower than that observed in the original Δ EGFR-dependent tumors (Fig. 3B). In contrast, apoptosis in the escapers as measured by TUNEL staining was substantially decreased compared to the Δ EGFR-dependent tumors (Fig. 3C). There was no significant difference in microvessel area by CD31 staining ($P > 0.1$, ANOVA; Fig. S3A). Together, these results suggest that resistance to apoptosis rather than an increase in proliferation may be a predominant mechanism for escape.

Identification of Pathways that Compensate for Lack of Δ EGFR Expression and Promote Tumor Development. To uncover the mechanisms underlying the acquisition of Δ EGFR-independent growth, we first investigated proteins and signaling pathways that were initially affected by Δ EGFR silencing to determine if compensation led to the emergence of escaper tumors. Western blot analysis revealed that some escaper tumors expressed the growth-promoting proteins CyclinA, CyclinE, CDK2, CDK4, and CDK6 at a similar level as Δ EGFR-dependent tumors (Fig. 4), which is in contrast to the very low levels of these proteins observed during the period of stasis after initial Δ EGFR silencing (Fig. 2C). Furthermore, most breakthrough tumors exhibited a low level of growth-inhibiting p27 protein (Fig. 4), again in contrast to the increased p27 observed immediately after Δ EGFR silencing (Fig. 2C). The majority of breakthrough tumors also exhibited slightly

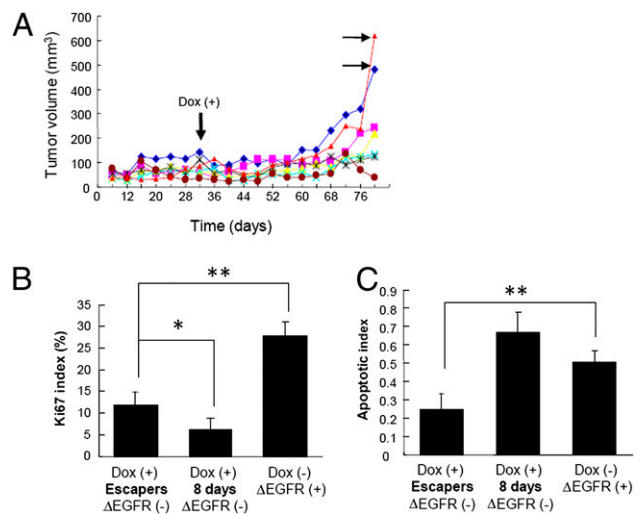


Fig. 3. Relapse of U373MG tumors after silencing of Δ EGFR expression. (A) Tumor growth of U373MG cells (3×10^6) expressing dox-regulatable Δ EGFR injected s.c. into the flanks of nude mice administered dox-containing drinking water 32 days after injection. Each line represents an individual growth curve of a tumor in a mouse from one typical experiment. Horizontal arrows indicate representative cases of Δ EGFR-independent “escaper” tumors. (B) Proliferation index as measured by counting proportion of K_i -67 positive cells in escaper and Δ EGFR-dependent (with Dox for 8 days from day 32 or without Dox) tumors (mean \pm SD; $P < 0.0001$, ANOVA; *, $P < 0.05$; **, $P < 0.01$, Tukey–Kramer HSD test). (C) Apoptotic index in same tumors as part (B), as measured by TUNEL staining (mean \pm SD; $P < 0.0001$, ANOVA; **, $P < 0.01$, Tukey–Kramer HSD test).

elevated Stat1/3 phosphorylation (Fig. 4) in comparison with the very low levels observed during the initial response (Fig. 2C). Bcl- χ_L was expressed at levels similar to Δ EGFR-positive tumors (Fig. 4), in striking contrast to the down-regulation of this antiapoptotic protein after Δ EGFR silencing (Fig. 2C). There was no alteration in the phosphorylation of AKT (Fig. 4). Interestingly, compensation by cell cycle progression proteins and Stat transcription factors, although likely contributory in some tumors, was not a uniform or consistent observation among the escapers (Fig. 4). The increased phosphorylation of ERK and p38 MAPK that was observed initially after Δ EGFR silencing (Fig. 2C), however, was clearly maintained in all but one escaper tumor (Fig. 4). This finding suggests the possibility that suppression of these pathways by Δ EGFR, although not able to promote tumor growth or maintenance initially after Δ EGFR silencing, may impact later breakthrough tumor growth.

To confirm that the breakthrough tumors had acquired a stable Δ EGFR-independent tumorigenic phenotype, secondary cell lines were established from Δ EGFR-independent tumor xenografts, and their tumorigenic behaviors were compared to those of cells established from Δ EGFR-dependent xenografts. Established cell lines, whose Δ EGFR expressions were suppressed by doxycycline, were reinjected into nude mice. As shown in Fig. S3B, most of the cell lines established from breakthrough tumors showed substantial tumorigenic ability in the absence of Δ EGFR signaling (i.e., in the presence of doxycycline), whereas all three cell lines established from Δ EGFR-dependent tumors (size of tumors when resected for culture were 72, 56, and 64 mm³, respectively) could only develop small tumors in the presence of doxycycline at 84 days after inoculation (Fig. S3C). These same Δ EGFR-dependent cell lines were able to develop tumors rapidly in the absence of doxycycline when Δ EGFR was expressed (Fig. S3D). Even though there was an obvious difference in tumor growth in vivo in mice, no obvious differences in cell growth could be detected in vitro between original U373MG cells expressing tetracycline-regulatable Δ EGFR and representative Δ EGFR-independent U373MG cell lines (Fig. S3E). These results indicate

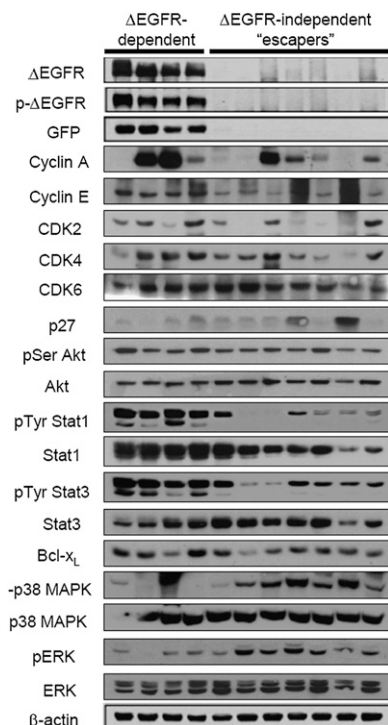


Fig. 4. Analysis of Δ EGFR-dependent vs. escaper tumor protein expression and signaling pathway preferences. Western blot analysis with the indicated antibodies of lysates derived from s.c. tumors from mice without dox in the drinking water (Δ EGFR-dependent) and escaper tumors that showed regrowth after a period of stasis after initial dox administration as in Fig. 3 (Δ EGFR-independent escapers). Analysis was performed at least three times for each antibody with consistent results.

that the breakthrough tumor phenotype of Δ EGFR-independent escaper tumors is stable upon cell line establishment and serial xenografting.

Interestingly, we did not detect any obvious reactivation of expected downstream pathways in established Δ EGFR-independent cell lines without serum stimulation (Fig. S3F). Using RTK arrays to search for other receptor tyrosine kinases that could potentially substitute for Δ EGFR (21), we observed modest increases in the expression of insulin-like growth factor 1 receptor (IGF1R) in these Δ EGFR-independent cell lines (Fig. S3F), which has been previously reported, to compensate for inhibited Δ EGFR signaling through PI3K-Akt activation (19). However, we also observed up-regulation of IGF1R in cell lines established from large s.c. tumors that depended on Δ EGFR expression for their growth, suggesting that IGF1R and Δ EGFR expression may not be mutually exclusive and that IGF-1R is unlikely to play the sort of compensatory role that develops when Δ EGFR expression is silenced.

To more closely examine candidates that may be responsible for this Δ EGFR-independent *in vivo* growth, we next used microarrays to compare gene expression profiles of Δ EGFR-independent escaper cell lines to those of control cell lines (DK and Δ EGFR cells grown in the presence and absence of dox and Δ EGFR-dependent tumor-derived cell lines). Informatic analysis of the data revealed 25 up-regulated and 10 down-regulated genes (34 and 14 probe sets, respectively) with expression patterns that suggested their potential for being responsible for Δ EGFR-independent growth (Fig. S4I and Tables S1 and S2). To determine whether the breakthrough tumors were the trivial consequence of selection of preexisting aberrant cells within the original populations, DNA from Δ EGFR-independent cells was analyzed by using high-resolution long oligonucleotide-based CGH arrays. Although this approach would

allow the detection of copy number alterations, such as genomic amplifications or deletions, that may occur either in the original population or during resumption of tumor growth (22), genomic differences between cell lines were extremely rare (Fig. S4B), indicating that most of the changes in gene expression in U373 Δ EGFR-independent tumors likely resulted from epigenetic modifications or transcriptional alterations in cells of the same genotypes.

***SΔE-1/KLHDC8A* Is Expressed in Human Glioblastoma and in Tumors Growing Independent of Δ EGFR.** Among the list of the 35 candidate genes determined by RNA expression microarray analysis, we focused on a unique gene, Kelch domain containing 8A (*KLHDC8A*), because this gene showed the second highest fold increase (4.6-fold) in Δ EGFR-independent cell lines and public microarray databases indicated its striking overexpression in glioblastoma tissues compared to normal brain tissue (Figs. S4A and S5A). To verify the microarray data, we performed quantitative real-time PCR (qPCR) (Fig. S5B) and Northern blot analysis (Fig. S5C) and found remarkably increased expression of *KLHDC8A* in cells established from tumors attaining growth independent of Δ EGFR. Because this gene was representative of several genes overexpressed in Δ EGFR-independent cell lines, we refer to it as Substitute for Δ EGFR Expression 1 (*SΔE-1*). It was highly expressed in xenografts that showed aggressive tumor growth in mice after they escaped from suppressed Δ EGFR expression, whereas only very low expression was detected in tumors of various sizes whose growth was supported by Δ EGFR expression (Fig. S5B). *SΔE-1* expression was also significantly higher in human glioblastoma specimens than in normal brains (Fig. S5D). To further explore expression of this gene, we raised a rabbit anti-human *SΔE-1/KLHDC8A* polyclonal antibody, which also recognized the mouse *Klhdc8a* homolog (Fig. S5E). Using this antibody, we observed marked overexpression of *SΔE-1/KLHDC8A* exclusively in tumors that were able to grow rapidly after escaping suppressed Δ EGFR expression (Fig. 5A), and in a series of human glioblastoma tissues (Fig. S5F), although proteins detected in both sets of samples were a few kilodaltons smaller in molecular mass than that of overexpressed full length *SΔE-1* protein. This size difference suggests that additional modification or processing of predicted full-length protein may be necessary for this gene to become functional. Interestingly, the established escaper cell line with the least tumorigenic potential, Esc-3, showed the lowest *SΔE-1* protein expression (Fig. 5A).

Immunohistochemical (IHC) analysis using the unique antibody demonstrated that *SΔE-1/KLHDC8A* protein was expressed in >85% of GBM patient tumors ($n = 33$) (Table S3). In most tumors, 10–40% of tumor cells expressed the protein and the intensity of staining in individual tumor cells was heterogeneous with low to moderate staining detected in most patient samples (Fig. S5G and Table S3), while low in normal brain (23, 24).

Suppression of *SΔE-1/KLHDC8A* Expression Decreases Tumorigenicity of U373 Δ EGFR-Independent Cells. To investigate the significance of increased expression of *SΔE-1/KLHDC8A*, shRNAs were used to knock down the expression of *SΔE-1/KLHDC8A* in two representative Δ EGFR-independent U373 cell lines (Fig. 5B Inset). This resulted in a significant suppression in tumor formation when compared to control cells producing a negative control shRNA (Fig. 5B). Taken together, these results suggest that *SΔE-1/KLHDC8A* is required for the tumorigenic potential of derived Δ EGFR-independent U373 cells.

Discussion

Here, we tested the dependence of glioblastoma on Δ EGFR expression for growth. The results demonstrated that catalytically active Δ EGFR is required for tumor establishment and, upon silencing its expression, is required for the maintenance of the enhanced tumor growth conferred by the receptor. After a period of tumor regression or stasis, resumption of growth (“escape”)

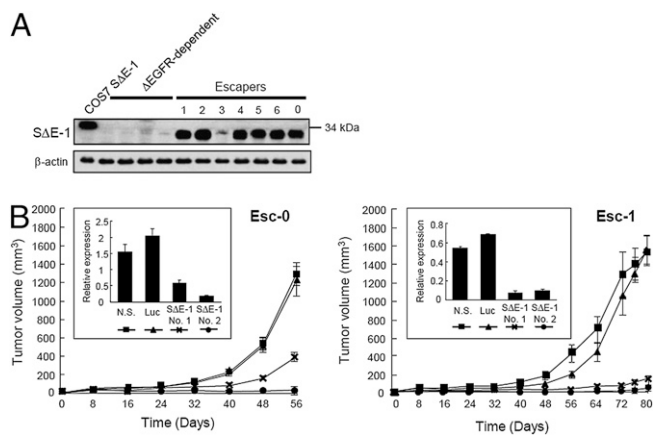


Fig. 5. Increased expression of $S\Delta E-1/KLHDC8A$ in $\Delta EGFR$ -independent U373MG cells and GBMs and effect of knock-down on tumorigenicity. (A) Western blot analysis of lysates from $\Delta EGFR$ -dependent or -independent escaper tumors as in Fig. 4 with a polyclonal rabbit anti- $S\Delta E-1$ antibody. Cos7 cells transfected with $S\Delta E-1$ were used as a positive control for the antibody. (B Inset) Two tet-regulatable U373MG cell lines established from escaper tumors, Esc-0 and Esc-1, were infected with shRNA-expressing retroviruses targeting $S\Delta E-1/KLHDC8A$ (No. 1 or No. 2), a nonspecific sequence (N.S.), or Luciferase (Luc), and stable cell lines were established. qPCR analysis of $S\Delta E-1/KLHDC8A$ gene expression was performed in the cell lines expressing the indicated retroviral shRNA constructs. Each bar represents the mean \pm SD of three replicates. y axis represents the relative gene expression level. These cells (3×10^6) were injected s.c. into the flanks of nude mice fed with dox-containing drinking water. Tumor volumes were measured at the indicated times to generate the growth curve of tumors with knockdown of $S\Delta E-1/KLHDC8A$ expression. $n = 8$ mice for all groups, mean \pm SEM.

often occurred, reminiscent of clinical trials showing eventual resistance to receptor-targeted therapy with drugs such as tyrosine kinase inhibitors or antibodies. As potential mechanisms of $\Delta EGFR$ -independent growth, we initially suspected that $\Delta EGFR$ acquired unregulated expression or that compensatory pathways were activated that might phenocopy $\Delta EGFR$ activity (18, 25). However, neither unregulated $\Delta EGFR$ overexpression nor apparent reactivation of expected downstream signaling (either as compensation for $\Delta EGFR$ or through activation of other receptor tyrosine kinases) could be observed. These results suggested that other unsuspected pathways might have been activated that became critical in these $\Delta EGFR$ -independent cell lines. To uncover such pathways, we analyzed cell cycle, signal transduction, and apoptosis pathway associated proteins as well as assessed global gene expression changes by expression microarray analyses. In summary, these approaches illustrated that the majority of escaper tumors restore their tumorigenic growth chiefly through a switch in signaling pathways, from a $\Delta EGFR$ -driven signature of activated Stat1/3, low p38, and Erk activation to one of low Stat1/3 activity, high p38 and Erk activation, reminiscent of a signaling fingerprint associated with EGFR activation (20), despite the absence of this receptor. Additionally, down-regulation of Bcl-xL seen upon initial $\Delta EGFR$ silencing was restored when tumors resumed growth, in agreement with suppression of apoptosis as a major mechanism leading to emergence of the escaper phenotype.

Analysis of the escaper transcriptome identified a group of up-regulated genes among which was a unique gene, termed $S\Delta E-1/KLHDC8A$. This gene appears to be responsible for tumor relapse after $\Delta EGFR$ silencing in several of the breakthrough tumors. This conclusion was supported by demonstrating that shRNA-based knock-down of $S\Delta E-1/KLHDC8A$ led to decreased tumorigenicity and implies an important link with pathways using this gene and $\Delta EGFR$ -targeted therapies. $S\Delta E-1/KLHDC8A$ is a 350-amino acid protein that belongs to the kelch repeat superfamily and shares

>95% amino acid sequence identity with the predicted mouse or rat *klhdc8a* homolog. Each kelch motif is a segment of 44–56 amino acids in length and a repeat of five to seven kelch motifs forms a β -propeller tertiary structure (26). Many members of this protein family, including $S\Delta E-1/KLHDC8A$ before our study, have not been cloned or functionally characterized. In addition to the kelch domain, many superfamily members contain BTB (broad-complex, tramtrack, and bric à brac) or other conserved domains, whereas $S\Delta E-1/KLHDC8A$ consists of seven kelch repeats and falls into a propeller structure-only subgroup. Several kelch proteins have been shown to associate with the actin cytoskeleton through their β -propeller structure or to colocalize with actin-rich organelles. *Klhdc2*, another propeller only kelch family member, has been reported to be highly expressed in muscle and is essential for myogenic differentiation and migration (27).

The gene encoding $S\Delta E-1/KLHDC8A$ is located at human chromosome 1q32.1, a region where genomic amplification has been reported in gliomas (28). One cDNA microarray study, which analyzed nearly 20,000 genes, showed that $S\Delta E-1/KLHDC8A/FLJ10748$ is 1 of 50 genes whose increased expression was significantly associated with poor prognosis in glioblastomas (29). Consistent with qPCR results in our study, an examination of the cancer profiling databases, Oncomine (www.oncomine.org; ref. 23) (<http://cgap.nci.nih.gov/SAGE>; ref. 24), show significantly higher expression of $S\Delta E-1/KLHDC8A$ in high-grade glioma ($P < 0.01$; Student's t test), as well as in medulloblastoma, prostate carcinoma, and invasive breast carcinoma, compared with normal tissues and other solid tumors (30–33).

On the other hand, our survey of several routinely used glioblastoma cell lines has not shown appreciable baseline levels of $S\Delta E-1/KLHDC8A$ expression, suggesting that expression of this gene is difficult to maintain in the conventional culture conditions of glioblastoma cell lines or that it is not needed for the growth of such cells in vitro. We also experienced decreasing $S\Delta E-1/KLHDC8A$ expression during establishment of $\Delta EGFR$ -independent cell lines from xenograft tumors by using conventional growth conditions. This loss of $S\Delta E-1/KLHDC8A$ expression during cell line establishment is reminiscent of the observation that expression of $\Delta EGFR$ itself is difficult to maintain during in vitro establishment of glioblastoma cell lines, while it is maintained in serially passaged xenografted tumors (34, 35). Analysis of the public database, Laboratory for Systems Biology and Medicine (www.lsbm.org), indicates that the expression of $S\Delta E-1/KLHDC8A$ is high in fetal brain, cerebellum, thalamus, pituitary gland, ovary, and neuronal stem cells, whereas expression is low in other tissues such as normal adult brain and differentiated astrocytes. Taken together, the spatial expression of $S\Delta E-1/KLHDC8A$ leads us to speculate that in addition to its role in tumor formation and maintenance, this molecule may play a pivotal role in the maintenance of the neural stem cell population.

Glioblastomas may use several mechanisms to evade therapies targeted at $\Delta EGFR$ or other receptors. The presence of redundant active tyrosine kinase receptors such as PDGFR, IGF1-R, and c-Met contributes in part to this resistance (19–21), whereas increased expression of $S\Delta E-1/KLHDC8A$, or similarly acting molecules and their pathway interactions, might also play an important role. The heterogenous staining within tumors and across patients raises the possibility that such heterogeneity and potential resistance of $S\Delta E-1/KLHDC8A$ cell subpopulations may contribute to eventual resistance of GBM patients to EGFR inhibition. Further studies using biopsy evaluation of $S\Delta E-1/KLHDC8A$ expression pre- and posttreatment will be needed to evaluate this possibility. In summary, these studies point to the idea that targeting both the primary target and potential escape mechanisms might have better therapeutic results. Indeed, the utility of combination therapies targeting multiple pathways simultaneously has been reported (36) and suggests that this area warrants considerable further attention.

Materials and Methods

Cell Culture and Biological Reagents. The human high-grade glioma cell line, U373MG, was cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin in a 95% air/5% CO₂ atmosphere at 37 °C. U373MG cells, which overexpress Δ EGFR, wild-type (wt) EGFR and control luciferase genes (*Lux*) were engineered as described (5, 6).

Engineering Tet-Regulatable Δ EGFR-Expressing Glioma Cells. U373MG cells were first infected with the pRevTet-Off (Clontech) retrovirus and were selected in media containing neomycin (G418) to create a stable Tet-regulatable cell line. Approximately 20 independent clones were then isolated by fluorescence activated cell sorting (FACS) and subsequently screened for high inducibility and low background by using the luciferase expressing pRevTRE-Luc virus. Four clones with high luciferase induction in medium without doxycycline and with effective suppression in medium containing doxycycline were selected. These clones were further tested, and a clone that was not tumorigenic in the absence of Δ EGFR expression in nude mice and possessed the same ploidy as parental U373MG, as assessed by FACS analysis, was chosen to establish doubly stable, Δ EGFR- and DK-inducible cell lines. To achieve this, the p Δ CMV/(tetO)- Δ EGFR-IRES2-EGFP or p Δ CMV/(tetO)- Δ EGFR DK-IRES2-EGFP vectors were cotransfected with a plasmid harboring a puromycin resistance gene into the tet-regulatable U373MG clone by calcium phosphate precipitation and cells were selected in medium containing puromycin. The pool of clones was then harvested and FACS sorted twice to enrich cells that had higher expression of green fluorescent protein (GFP) when cultured in medium without doxycycline.

Xenografts. For s.c. tumors, 2 or 3 \times 10⁶ U373MG cells in 0.1 mL of PBS were injected into the flanks of nude mice. To suppress the expression of Δ EGFR, mice were fed with water containing 1.25% sucrose and 0.5 mg/mL of doxycycline (Dox). Tumor volumes were defined as (longest diameter) \times (shortest diameter)² \times 0.5. All of the procedures were approved by the Animal Care and Use Committee of the University of California. Tumors were resected immediately after perfusion with 20 mL of cold PBS, snap frozen in liquid nitrogen and stored at -80 °C until use. Total RNA was isolated from frozen tumor samples using the RNeasy kit (Qiagen) following manufacturer's instructions.

To establish secondary tumor cell lines from xenografts, resected tumor pieces were immediately stored in sterile, ice-cold PBS containing 100 units/mL penicillin, and 100 μ g/mL streptomycin. Tumor pieces were washed thoroughly in cold PBS and then minced. These tissue pieces were suspended in RPMI 1640 (Invitrogen) medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin and seeded into Petri dishes. After 12 h of incubation at 37 °C, regular growth medium was added and contaminated mouse tissues were eliminated by culturing in medium containing puromycin.

For additional details, please see *SI Materials and Methods*.

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