Internalized CD44s splice isoform attenuates EGFR degradation by targeting Rab7A

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CD44 has been postulated as a cell surface coreceptor for augmenting receptor tyrosine kinase (RTK) signaling. However, how exactly CD44 triggers RTK-dependent signaling remained largely unclear. Here we report an unexpected mechanism by which the CD44s splice isoform is internalized into endosomes to attenuate EGFR degradation. We identify a CD44s-interacting small GTPase, Rab7A, and show that CD44s inhibits Rab7A-mediated EGFR trafficking to lysosomes and subsequent degradation. Importantly, CD44s levels correlate with EGFR signature and predict poor prognosis in glioblastomas. Because Rab7A facilitates trafficking of many RTKs to lysosomes, our findings identify CD44s as a Rab7A regulator to attenuate RTK degradation.

CD44s | splice isoform | EGFR | Rab7A | GBM

Abnormal activation of receptor tyrosine kinase (RTK) and its downstream PI3K/Akt signaling is a common mechanism that drives cancer progression. The cell surface molecule CD44 has been regarded as a cancer stem cell marker in many types of cancers (1–5), and increasing evidence has suggested a role for CD44 in promoting cancer progression (6–12). CD44 was reported to act as a coreceptor for RTKs, including EGFR, and stimulates signaling downstream of RTKs (13–15). However, the mechanism by which CD44 triggers RTK-dependent signaling has remained unclear. Adding another layer of complexity, CD44 undergoes extensive alternative splicing, producing two families of isoforms, termed CD44v and CD44s. The CD44v splice isoforms contain at least one of its nine variable exons, whereas the CD44s isoform is devoid of all variable exons. The contribution of specific isoforms of CD44 in RTK signaling has been elusive.

The majority of glioblastoma (GBM) displays hyperactivation of RTKs with greater than 45% of specimens harboring EGFR amplification or mutation (16). A challenge for treating GBM patients with RTK inhibitors (RTKIs) is the acquired drug resistance, due in part to the cross-activation of RTKs converging on PI3K-Akt (17, 18). Thus, proteins that promote the signaling of not only EGFR but also many other RTKs could be ideal targets for GBM.

Here, we report an unexpected finding that the CD44s splice isoform attenuates endocytosis-mediated EGFR degradation, thus sustaining downstream Akt signaling. We identify the small GTPase, Rab7A, as a major downstream target for CD44s in mediating EGFR degradation. Because Rab7A promotes the trafficking of many RTKs to the lysosomal pathway, CD44s may serve as a potential therapeutic target not only by impairing the EGFR-signaling cascade but also possibly through elimination of cross-activated RTK signaling in response to therapeutic resistance in GBM.

Results

CD44s Inhibits Lysosome-Mediated EGFR Degradation and Sustains AKT Signaling. The CD44 gene produces CD44s and CD44v family of splice isoforms. To examine the effect of CD44 on EGFR signaling, we used the HT1080 and U87MG cancer cells that predominately express CD44s and the experimentally immortalized mammary epithelial HMLE cells, which express CD44v as the major isoform (*SI Appendix*, Fig. S1*A*). Silencing CD44 in the CD44s-expressing HT1080 and U87MG cells caused a strikingly faster rate of EGFR degradation in response to EGF stimulation, accompanied by a pronounced inhibition of Akt signaling (Fig. 1 *A* and *B*). By contrast, silencing CD44 in the CD44v predominantly expressed HMLE cells did not show any detectable differences on EGFR degradation (*SI Appendix*, Fig. S1*B*). We also used 293FT cells that show undetectable levels of CD44 and found that ectopically expressing CD44s but not CD44v3-v10 cDNA inhibited EGFR degradation, resulting in sustained levels of EGFR and Akt activation (*SI Appendix*, Fig. S1 *C* and *D*). These results show that CD44s, but not CD44v, attenuates EGFR degradation, resulting in enhanced Akt signaling.

We then analyzed the *EGFR* mRNA levels and found no significant differences in control and CD44 knockdown cells (*SI Appendix*, Fig. S1*E*). Treatment of cells with the protein synthesis inhibitor cycloheximide (CHX) did not alter the kinetics of EGFR degradation in CD44-silenced cells (*SI Appendix*, Fig. S1*F*). FACS analysis of cell surface EGFR as well as EGF tracing showed no differences in EGFR internalization between control and CD44 knockdown cells (*SI Appendix*, Fig. S1 *G* and *H*). These results excluded the effects of CD44s on EGFR transcription, protein synthesis, or internalization.

Significance

Abnormal EGFR signaling is frequently observed in glioblastoma multiforme (GBM). However, the clinical benefit of EGFR inhibitors has been limited. Here we show that the cell surface molecule CD44s splice isoform acts as a signaling modulator that attenuates EGFR degradation and sustains EGFR's downstream AKT signaling. CD44s internalizes in cells and inhibits Rab7A-mediated EGFR trafficking for degradation, resulting in sustained EGFR protein levels. CD44 depletion combined with EGFR inhibitor results in a robust and synergistic GBM cancer cell killing. Because CD44s-mediated inhibition on Rab7A also affects other receptor tyrosine kinases (RTKs), inhibiting CD44s may be an exciting approach for perturbation of multiple RTKs in GBMs.

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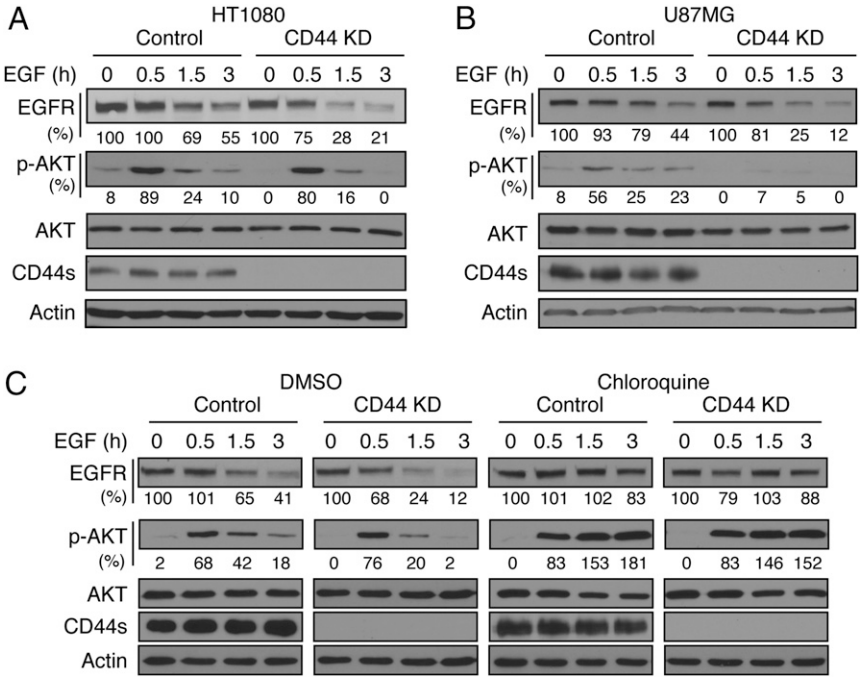

Fig. 1. CD44s inhibits lysosome-mediated EGFR degradation and sustains Akt signaling. (*A* and *B*) Depletion of CD44s by CD44 shRNA-mediated knockdown (KD) promoted EGF-induced EGFR degradation and attenuated its downstream AKT signaling in HT1080 cells. Control or CD44 KD HT1080 (*A*) or U87MG (*B*) cells were serum-starved and stimulated with EGF (100 ng/mL) for the indicated times. Cell lysates were collected for Western blot analysis with indicated antibodies. (*C*) Chloroquine abolished the effect of EGFR degradation caused by CD44 KD. HT1080 control or CD44 KD cells were serum-starved and stimulated with EGF (100 ng/mL) in the presence or absence of Chloroquine (100 μM) for the indicated times. In all panels with Western blot analysis, the quantification value of EGFR and p-AKT at each time point was normalized to the loading control Actin and total Akt, respectively, and shown below the panel.

Because EGFR degradation is mediated by EGF-induced endocytosis, we treated cells with chloroquine, a compound that prevents endocytosis-mediated degradation in the lysosome. Chloroquine treatment almost completely restored levels of EGFR and p-Akt reduced by CD44s loss (Fig. 1*C*). Taken together, CD44s may act on lysosome-mediated EGFR degradation, warranting us to investigate its underlying mechanism. In the following studies, we used cells that predominantly express CD44s with no detectable CD44v. Thus, shRNA knockdown of CD44 eliminates CD44s in these cells.

Loss of CD44s Promotes EGFR Accumulation in Late-Endosome/Lysosome Compartments. To determine the effect of CD44s on EGFR trafficking, we analyzed the distribution of EGFR in early and late endosomes. We found no differences in the colocalization of EGFR and the early endosomal marker EEA1 in control and CD44-silenced cells at 15 min after EGF stimulation (*SI Appendix*, Fig. S2*A*). However, loss of CD44s resulted in a preferential enrichment of EGFR in the LAMP1$^+$ late-endosome/lysosome compartments (Fig. 2*A*) in cells 30 min after EGF stimulation, at which time EGFR was trafficked to the late endosome, but its degradation was yet to take place (see Fig. 1*A* for time course of EGFR protein degradation). These results suggest that CD44s inhibits EGFR trafficking from the early endosome to the late endosome/lysosome and are in line with our observations that CD44s depletion increases EGFR degradation.

We also examined the localization of CD44s. CD44s colocalized with both EEA1 and LAMP1, but showed preferential localization to LAMP1$^+$ late-endosome/lysosome compartments (*SI Appendix*, Fig. S2*B*). CD44s and EGFR also colocalized on the cell surface and inside the cells (*SI Appendix*, Fig. S2*C*). Because coimmunoprecipitation assays showed that CD44s and EGFR were detected to interact only in roughly half of the experimental repeats, we reasoned that the effect of CD44s on EGFR endocytosis might be mediated by a CD44s-interacting protein.

CD44s Interacts with Rab7A and Colocalizes with Rab7A in LAMP$^+$-Endosomal Compartments. To identify factors that mediate CD44s-dependent EGFR trafficking, we stably expressed CD44s with a C-terminal FLAG-tag in HT-1080 cells and found that CD44s was enriched in lipid raft fractions, cosedimenting with the lipid raft marker flotillin-1 (*SI Appendix*, Fig. S2*D*). These fractions were pooled and immunoprecipitated with a FLAG antibody. Mass spectrometry identified Rab7A as the top hit of CD44s-interacting proteins (Fig. 2*B*).

Rab7A belongs to the Ras-like small-GTPase family and regulates endocytosis-mediated protein trafficking (19). In particular, Rab7A facilitates trafficking of RTKs, including EGFR, from early endosome to late endosome and lysosome for their ultimate degradation (20, 21). Reciprocal immunoprecipitation assays showed that CD44s coimmunoprecipitated with Rab7A and vice versa (Fig. 2*C*). Yeast two-hybrid assays showed that CD44s and Rab7A physically interact (Fig. 2*D* and *SI Appendix*, Fig. S2*E*). In addition, we found that CD44s and Rab7A colocalized as punctate structures inside cells (Fig. 2*E*) and that they colocalized with LAMP1 and EGFR (Fig. 2*F* and *SI Appendix*, Fig. S2 *F* and *G*). These observations prompted us to investigate whether CD44s-mediated EGFR endosomal trafficking is through its interaction with Rab7A.

CD44s Prohibits Rab7A-Mediated EGFR Degradation. Rab7A is cycled between its inactive (i.e., GDP-bound) and active (i.e., GTP-bound) conformations, and the GTP-bound Rab7A promotes EGFR trafficking to the late endosome/lysosome for degradation (20). When ectopically expressing a GDP-bound Rab7A mimic, Rab7A/T22N dominant negative (DN) mutant, we found that EGF-induced EGFR degradation was prevented (*SI Appendix*, Fig. S3*A*), in agreement with previous findings that the GTP-bound Rab7A is required for EGFR degradation (20).

We next investigated whether CD44s renders reduction of the levels of GTP-bound Rab7A, hence inhibiting EGFR trafficking to lysosomes for degradation. Immunoprecipitation using a Rab7A antibody that specifically binds its GTP-bound form showed that loss of CD44s led to a pronounced increase of the GTP-bound Rab7A (Fig. 3*A*). Because RILP is a Rab7A effector and interacts with the GTP-bound form of Rab7A, we measured the interaction of Rab7A/RILP as a readout of Rab7A-GTP level (*SI Appendix*, Fig. S3*B*) (22–25). We found that loss of CD44s caused a drastic increase of the Rab7A-GTP signal that was pulled down by the GST-fused Rab7A-binding domain of

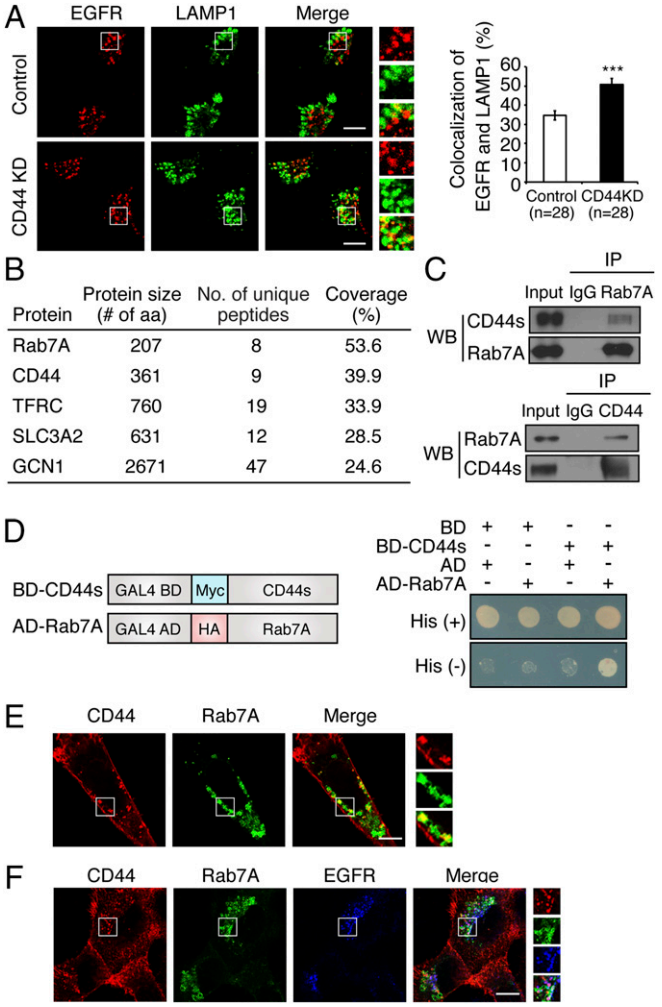

Fig. 2. Loss of CD44s promotes EGFR traffic from early endosomes to lysosomes and CD44s interacts and colocalizes with Rab7A. (*A*) CD44 KD resulted in increased accumulation of EGFR in late endosomes/lysosomes. HT1080 control or CD44 KD cells were stimulated with EGF (100 ng/mL) for 30 min, fixed, and costained with EGFR (red) and the late-endosome/lysosome marker LAMP1 (green). The colocalization percentage (%) of indicated numbers of cells (*n*) are presented as mean ± SEM; ***$P < 0.001$ (Student's *t* test). (Scale bars, 10 μm.) (*B*) Results of mass spectrometry analysis. Percent coverage was calculated by dividing the number of amino acids in unique peptides by the total number of amino acids in the protein. (*C*) Western blot images of reciprocal immunoprecipitation showing that CD44s and Rab7A interact. pcDNA-mCD44s and GFP-HA-Rab7A were cotransfected in 293FT cells. (*D*) CD44s and Rab7A interacts in a yeast two-hybrid assay. CD44s and Rab7A were fused to GAL4-binding domain (BD) and activation domain (AD), respectively (*Left*). Images of colonies with indicated transformants grown on His (+) and His (–) plates (*Right*). (*E* and *F*) Confocal imaging analysis of colocalization of CD44, Rab7A, EGFR, and LAMP1. GFP-HA-Rab7A plasmid were transfected in HT1080 cells, starved, and stimulated with EGF (100 ng/mL) for 30 min. The cells were stained with indicated antibodies. (Scale bars, 10 μm).

RILP (GST-RILP-RBD) (Fig. 3*B*). These results indicate that loss of CD44s promotes the accumulation of the GTP-bound form of Rab7A, and, as a consequence, it caused an increased colocalization of EGFR and Rab7A (*SI Appendix*, Fig. S3*C*).

Using GFP-labeled Rab7A wild type (WT) and its mutants, GTP-bound mimic Rab7A/Q67L and GDP-bound mimic Rab7A/T22N, we sought to determine their colocalization with CD44s. The Rab7A/Q67L mutant showed similar punctate structures as the WT Rab7A, whereas Rab7A/T22N showed mostly diffuse distribution (Fig. 3*C*) (26). Interestingly, CD44s predominantly colocalized with Rab7A/Q67L, but not Rab7A/T22N (Fig. 3*C*). Once again, these results suggest that CD44s interacts with the Rab7A GTP-bound form.

Given that loss of CD44s causes an increase in Rab7A-GTP and that CD44s colocalizes preferentially with the Rab7A-GTP mimic, the Rab7A/Q67L mutant, we speculated that CD44s promotes the conversion from Rab7A-GTP to Rab7A-GDP. To this end, we analyzed the GTP hydrolysis efficiency using the purified His-tagged Rab7A protein and a CD44s-immunoprecipitated fraction of lysates. The GTP hydrolysis of Rab7A was ∼27% in control reactions (Fig. 3*D* and *SI Appendix*, Fig. S3*D*). When CD44s-containing immunoprecipitants were added to the reaction, Rab7A GTP hydrolysis was increased to 51% (Fig. 3*D* and *SI Appendix*, Fig. S3*D*). Thus, results from these experiments indicate that CD44s accelerates the conversion from Rab7A-GTP to Rab7A-GDP, thereby inhibiting Rab7A activity.

The above results prompted us to determine whether CD44s acts through Rab7A to attenuate EGFR degradation. We ectopically expressed the Rab7A/T22N DN mutant in CD44-silenced cells. As expected, expressing the Rab7A/T22N mutant in the CD44-silenced cells restored the levels of EGFR (Fig. 3*E*) and Akt activation (*SI Appendix*, Fig. S3*E*) to levels comparable to control cells. Expressing Rab7A/T22N also redistributed EGFR away from $LAMP^+$-late endosomes/lysosomes in CD44 knockdown cells to a distribution similar to the control cells (Fig. 3*F* and *SI Appendix*, Fig. S3*F*). Together, these results indicate that CD44s attenuates EGFR degradation by eliminating the Rab7A GTP-bound form, thus perturbing EGFR trafficking to the late endosome/lysosome.

CD44s Reduction Sensitizes GBM Cells to EGFR Inhibitor Erlotinib. EGFR amplification is associated with poor prognosis in several types of cancers; among them, EGFR amplification is prevalent in GBM (27). Several RTK inhibitors (RTKIs), including the EGFR inhibitor erlotinib, have been used in GBM treatment, but the clinical benefit of these RTKIs has been limited due to drug resistance (28, 29). A mechanism underlying this RTKI resistance is that coactivation of RTKs converges on PI3K-Akt in GBM, conferring insensitivity to any single RTKI agent (17, 18). Interestingly, previous studies using two different animal models showed that CD44 depletion inhibits GBM tumor growth in vivo (7, 12). As loss of CD44 promoted Rab7A-mediated EGFR degradation and because Rab7A is a cargo transport protein translocating RTKs to the late endosome/lysosome for degradation (30), we examined whether silencing CD44s sensitizes cells to the EGFR inhibitor erlotinib by promoting the degradation of EGFR and, potentially, other RTKs.

Indeed, knockdown of CD44 in U87MG cells drastically decreased Akt signaling to an undetectable level compared with erlotinib treatment alone (compare lanes 5 and 6 of Fig. 4*A*). Moreover, loss of CD44 exhibited an additive killing effect on GBM cells, including patient-derived glioblastoma-initiating cells (GICs), when combined with erlotinib (Fig. 4*B*). These GBM cells expressed predominantly the CD44s isoform (*SI Appendix*, Fig. S4*A*), indicating that CD44s reduction sensitizes GBM cells to erlotinib treatment.

Further characterization of the GICs showed that loss of CD44s impaired Akt activation and that Akt activation was restored when Rab7A was silenced (Fig. 4*C*). The CD44s-depleted GICs also showed decreased cell viability and neurosphere-forming ability, defects that can be suppressed by silencing Rab7A (Fig. 4*D* and *SI Appendix*, S4*B*). These results indicate that CD44s activates Akt signaling and potentiates the survival of GBM cells through Rab7A.

To examine whether other RTKs, in addition to EGFR, were affected by CD44s depletion, we analyzed the levels of PDGFR and c-MET, RTKs that were associated with GBM (16, 31–34). Whereas PDGFR levels were not detectable in our collection of

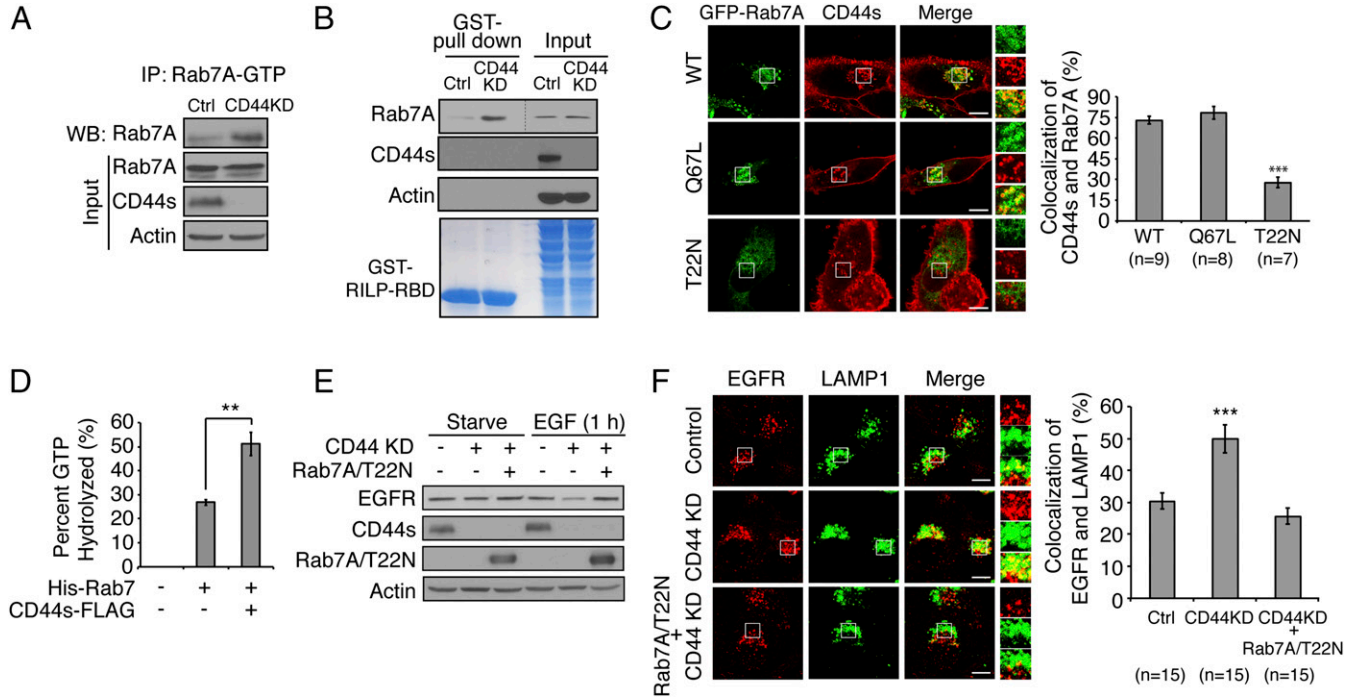

Fig. 3. CD44s inhibits Rab7A activity and prohibits Rab7A-mediated EGFR degradation. (*A*) CD44 KD increased the GTP-bound active form of Rab7A. A Rab7A-GTP antibody was used for immunoprecipitation in Rab7A-overexpressing HT1080 control and CD44 KD cells, and a Rab7A antibody was used for Western blot. (*B*) CD44 KD increased the binding of Rab7A to RILP-RBD. Cell lysates from HT1080 control and CD44 KD cells were used for GST-RILP-RBD pull down. Western blot images are shown. Recombinant GST-RILP-RBD protein was visualized by Coomassie Blue staining. (*C*) CD44s preferentially colocalizes with Rab7A/Q67L. Images were taken 30 min after EGF stimulation in HT1080cells expressing GFP-Rab7A WT, Q67L, or T22N plasmids. Colocalization of internalized CD44 and Rab7A were plotted. (*D*) CD44s promoted Rab7A GTP hydrolysis. Purified His-Rab7A protein was incubated with FLAG-beads immunoprecipitated lysates from control or CD44s-FLAG–expressing cells. Percentages of hydrolyzed GTP were shown. $^{**}P < 0.01$ (Student's *t* test). (*E*) Rab7A/T22N restored EGFR protein level in CD44 KD cells. Western blot analysis of HT1080 control, CD44 KD, or CD44 KD overexpressing Rab7A/T22N cells were shown after 1 h of EGF stimulation. (*F*) Rab7A/T22N expression in CD44 KD cells redistributed EGFR away from LAMP1$^+$ loci to levels comparable to control cells. Cells were costained with EGFR (red) and LAMP1 (green) after 30 min of EGF stimulation. The colocalization percentage (%) of the indicated numbers (*n*) of cells are presented as mean ± SEM; $^{***}P < 0.001$ (Student's *t* test). (Scale bars, 10 μm.)

GBM cells, depletion of CD44s caused a significant reduction in the protein expression of c-MET, even at basal levels (*SI Appendix*, Fig. S4*C*), without affecting *c-MET* mRNA levels (*SI Appendix*, Fig. S4*D*). Notably, expressing the Rab7A/T22N DN mutant partially restored c-MET levels in CD44s-depleted cells (*SI Appendix*, Fig. S4*E*), suggesting the notion that CD44s attenuates c-MET protein degradation at least in part by inhibiting the Rab7A activity. Consistently, silencing CD44 in conjunction with a c-MET inhibitor SU11274 showed robust inhibition of HGF-induced Akt activation (*SI Appendix*, Fig. S4*F*) and an increase in cell killing (*SI Appendix*, Fig. S4*G*), supporting our hypothesis that loss of CD44s sensitizes GBM cells to RTKIs.

CD44s Positively Correlates with EGFR Signature and Predicts Poor Survival. To examine the clinical relevance of CD44s, we analyzed the expression of CD44 splice isoforms in the TCGA database of GBM patients. CD44s was preferentially expressed in GBM tumors showing a greater than two orders of magnitude expression compared with CD44v (Fig. 5*A*). CD44s expression was higher in tumors from patients who had shorter survival or recurrence (Fig. 5*B* and *SI Appendix*, Fig. S5*A*). Kaplan–Meier survival curve also revealed that higher CD44s expression correlated with poor survival (Fig. 5*C*).

Gene Ontology (GO) enrichment analysis revealed that CD44s-associated gene signatures were involved in biological processes such as intracellular-signaling cascade, small-GTPase–mediated signal transduction, regulation of protein kinase cascade, and vesicle-mediated transport (*SI Appendix*, Fig. S5*B*), supporting a role for CD44s in regulating RTK signaling via endocytosis.

Consistent with our findings that CD44s attenuates EGFR protein degradation, genome-wide gene set enrichment analysis (GSEA) of TCGA datasets revealed that the CD44s gene-set exhibited significant positive association with the EGFR pathway in GBM (Fig. 5*D*) and other cancer types including colon cancer, liver cancer, lung cancer, and pancreatic cancer (*SI Appendix*, Fig. S5*C*), suggesting a universal role for CD44s in EGFR signaling.

We also analyzed protein levels of EGFR and CD44 in a cohort of clinical GBM samples by immunohistochemistry (IHC). High levels of CD44 expression correlated with poor survival (Fig. 5*E*). Moreover, coexpression of CD44 and EGFR correlated with shorter survivals in GBM patients (Fig. 5*F*). Whereas we were not able to quantitate CD44 isoforms at the protein levels due to the lack of isoform-specific antibodies, we suspect that the detected CD44 protein expression was largely contributed by CD44s because our TCGA analysis showed two orders of magnitude enrichment in CD44s expression (Fig. 5*A*). Taken together, these results support the role of CD44-EGFR axis in the clinical aggressiveness of patient GBMs.

Discussion

Our findings presented in this study reveal that the splice isoform CD44s attenuates EGFR protein degradation, resulting in prolonged activity of Akt signaling. Endocytosis is the major regulator for EGFR signaling. Upon internalization, EGFR is trafficked to the lysosome for degradation, damping its downstream signaling. Alternatively, EGFR is recycled back to the cell

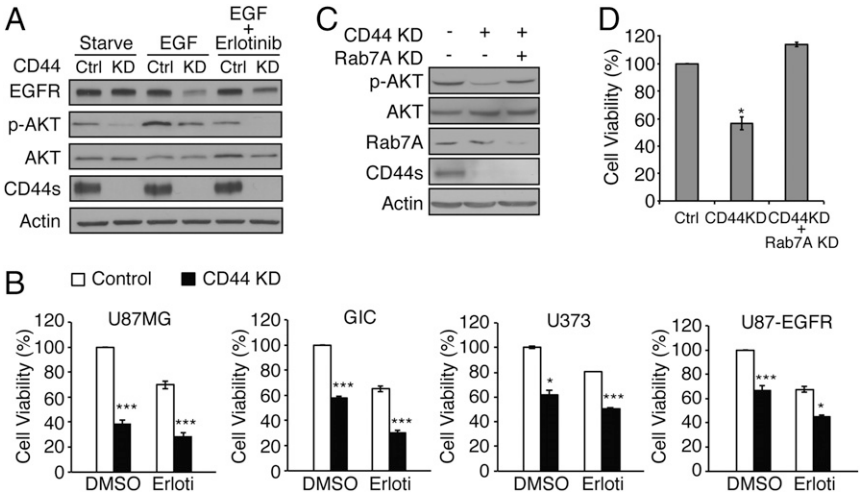

Fig. 4. CD44s reduction sensitizes GBM cells to cell death in response to EGFR inhibitor erlotinib. (*A*) CD44 KD diminished EGF-induced p-AKT signaling and showed additive inhibitory effect with erlotinib. U87MG control and CD44 KD cells were starved and stimulated with EGF (100 ng/mL) in the presence or absence of erlotinib (10 μM) for 1 h. Cell lysates were collected for the indicated antibody detection. (*B*) CD44 KD in combination with erlotinib (10 μM) showed additive effect on inhibiting cell viability in GBM cell lines, U87MG, GIC, U373, and EGFR-expressing U87MG (U87-EGFR). Cell viability was measured 72 h after plating. (*C* and *D*) Rab7A KD restored Akt signaling (*C*) and cell viability (*D*) in CD44s-depleted GIC cells. Data are mean ± SEM; $n = 3$. $^{*}P < 0.05$; $^{***}P < 0.001$ (Student's *t* test).

surface to sustain EGFR signaling. Thus, the net effect of EGFR signaling depends on the balance between signal attenuation through degradation and signal continuation through recycling (30, 35–37). The cargo transport protein Rab7A in its GTP-bound form plays an essential role in EGFR degradation by regulating EGFR endocytic trafficking to the late endosome/lysosome. In this study, we have identified that CD44s is a negative regulator of Rab7A. We demonstrate that CD44s preferentially interacts with the Rab7A-GTP form and accelerates the conversion from Rab7A-GTP to Rab7A-GDP, thus inactivating the Rab7A activity and inhibiting EGFR degradation. These data define a previously unrecognized function of the splice isoform CD44s, but not CD44v, for EGFR signaling. Notably, because Rab7A promotes endocytosis-mediated degradation of RTKs, it is conceivable that blocking the CD44's function on inactivating Rab7A may lead to perturbation of multiple RTK-signaling cascades in tumor cells. Supporting this notion, we found a similar activity of CD44s on c-Met. Thus, it would be interesting to investigate whether CD44s attenuates the degradation of other RTKs through the same mechanism. Also, further mechanistic study on CD44s-mediated Rab7A inactivation would provide profound insights into the role of CD44s-mediated RTK signaling in tumor progression.

CD44 belongs to the class I transmembrane glycoprotein family. Previous work implicated a role for CD44 as a coreceptor for RTKs, resulting in activation of intracellular signaling in response to extracellular cues (13). With the complexity of CD44 alternative splicing that produces multiple splice isoforms, the underlying mechanisms mediated by CD44 and its isoforms have remained largely elusive. Unlike the previous thought that CD44 acts on the cell surface for RTK activation, this study provides evidence that CD44, in particular, the CD44s splice isoform, internalizes from cell surface to the endosomal compartments and affects protein stability by blocking Rab7A-mediated EGFR translocation and degradation. Interestingly, besides our findings, other mechanisms downstream of CD44 were reported to contribute to GBM progression. CD44 was shown to attenuate the activation of the hippo-signaling pathway (7). It was also demonstrated that the intracellular domain (ICD) of CD44 promotes GBM growth via CBP/p300-dependent enhancement of HIF-2a activity (12). These results together with our findings indicate that CD44 acts through diverse and perhaps parallel mechanisms to achieve the aggressive phenotype of GBM. In line with this view, TCGA analysis of GBM patient data showed significant association between high levels of CD44s and poor survival. These findings suggest the promise of targeting CD44s for the treatment of GBM. Unlike conventional antibody depletion of pan-CD44, isoform-specific antibodies against CD44s or nanoparticle-conjugated CD44s shRNA strategy may be developed to inhibit the CD44s-mediated signaling.

Aberrant PI3K-AKT–signaling activation is observed in the majority of GBMs (16, 38). This study describes a previously unprecedented mechanism by which CD44s internalization and binding to Rab7A impedes the degradation of RTKs, resulting in prolonged Akt signaling. Notably, results from our recent work demonstrated that Akt signaling is sustained by CD44s-mediated positive feedback loop through the production of hyaluronic acid

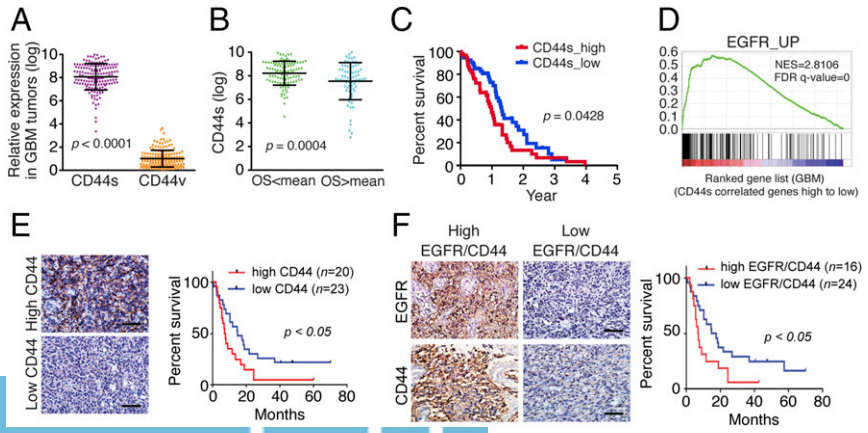

Fig. 5. CD44s levels are increased in GBM patient specimens, predict poor prognosis, and correlate with an EGFR signature. (*A*–*C*) TCGA analysis of 172 GBM patient specimens showed that CD44s was the predominant splice isoform in GBM (*A*), was enriched in tumors that showed lower overall survival (*B*), and predicted poor survival (*C*). (*D*) GSEA analysis indicated positive correlation between the EGFR signature and the CD44s signature. (*E* and *F*) Immunohistology analysis of patient GBM specimens indicated that CD44 (*E*) and CD44 in conjunction with EGFR (*F*) predicted patient poor survival.

(HA) that is required for and produced by CD44s-stimulated Akt activation (39). Interestingly, HA is the major ligand of CD44s, and its binding to CD44s triggers the HA/CD44 complex internalization (40–43). It is conceivable that this trigger of CD44s internalization allows CD44s to interact with Rab7A and to inhibit Rab7A's activity for RTK degradation. The resulting activation of Akt signaling downstream of RTKs then fuels into the CD44/HA/Akt positive feedback circuit, producing more HA and sustaining Akt activity. As tumor cells nearly invariably evolve sustained PI3K/Akt signaling as an effective means to circumvent apoptosis and maintain survival, the study described here may underlay the mechanism for those tumor cells that do not acquire PI3K/Akt mutations to achieve this end and thus support the notion of therapeutically targeting CD44s. In contrast to CD44s-mediated Akt activation, CD44v was shown to augment EGF-stimulated MAPK activation (44, 45). The divergent downstream signaling activation mediated by different splice isoforms of CD44 thus emphasizes the importance of alternative splicing in controlling signaling cascades. Given the estimated occurrence of alternative splicing as 92–94% in human genes, our results strongly argue that isoform specificity should be considered when investigating the role of a gene, such as CD44, in tumor progression. This information helps develop therapeutic interventions that target specific splice isoforms for the treatment of advanced cancers, including GBM.

Materials and Methods

Detailed information about plasmid constructs, reagents, cell culture, and experimental procedures describing generation of stable cell lines, Western blot and immunoprecipitation, immunofluorescence, fluorescence-activated cell sorting (FACS), Rab7A activity assay, cell viability assay, EGFR degradation assay, yeast two-hybrid, GST-RILP-RBD pull down, EGF internalization, Rab7A GTP hydrolysis, and statistics can be found in *SI Appendix*. The study was approved by the Institutional Review Board of Northwestern University.

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