

Endosomal trafficking of the EGFR mutant, EGFRvIII, results in exosomal secretion that triggers astrocyte reactivity

Kimiya Memarzadeh

University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences

Anthony N Patrizz

University of Texas John P and Katherine G McGovern Medical School

Edward C. Koellhoffer

University of Texas John P and Katherine G McGovern Medical School

Monica Gireud-Goss

University of Texas John P and Katherine G McGovern Medical School

Andrew Bean (✉ andrew_j_bean@rush.edu)

<https://orcid.org/0000-0002-4615-2742>

Research article

Keywords: Proliferation, migration, EGFRvIII, trafficking, exosome

Posted Date: October 11th, 2019

DOI: <https://doi.org/10.21203/rs.2.15913/v1>

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Abstract

Background: The epidermal growth factor receptor (EGFR) variant three (EGFRvIII) mutation is linked with approximately one third of Glioblastoma Multiforme (GBM) tumors and is associated with poor patient prognosis. Persistent signaling due to a lack of the EGFR ectodomain and inefficient degradation have been suggested to underlie the tumorigenic properties of EGFRvIII.

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Methods: Cell viability and trans-well migration assays were used to determine the effects that expression of the oncoprotein, EGFRvIII, had on glioma cells. A cell-free reconstitution assay developed by our laboratory was utilized to determine trafficking of EGFR and EGFRvIII at the late endosome and determine molecular requirements for inward budding of proteins into the MVB. Western Blot Analysis and Nanosight Tracking Analysis were used to characterize exosomes by protein marker presence and vesicle size, respectively. Immunohistochemistry and Western Blot analysis were used to determine astrocyte reactivity marked by GFAP expression.

Results: Like the parental EGFR, we observed that EGFRvIII is internalized into the intraluminal vesicles of late endosomes / multivesicular bodies (MVBs) but does not follow the canonical pathway by which wild-type EGFR is degraded following MVB fusion with lysosomes. These studies suggested that EGFRvIII is secreted on exosomes, the intraluminal vesicles that are secreted upon MVB fusion with the plasma membrane, suggested that EGFRvIII is localized in a subset of MVBs that preferentially fuse with the plasma membrane rather than with lysosomes which may account for its decreased degradation. Astrocytes are a component of the GBM tumor microenvironment with which tumor cells interact in a paracrine manner. EGFRvIII-containing exosomes derived from GBM cells induce changes in astrocytes that mimic reactive astrogliosis including an increase in glial fibrillary acidic protein (GFAP).

Conclusions: These studies reveal novel aspects of the endocytic trafficking of EGFRvIII that underlie its reduced degradation and the mechanism by which it is packaged into exosomes for secretion. Moreover, EGFRvIII secretion on exosomes can facilitate changes in the tumor microenvironment to enable tumor growth.

Background

Glioblastoma (GBM) is a malignant tumor arising in brain tissue of adults¹⁻⁴. Primary GMB tumors are grade IV gliomas that arise from astrocytes, glial cells that possess star-like processes that intercalate into brain tissue making complete surgical resection of the tumor difficult. While surgery, radiotherapy,

and chemotherapy are current therapeutic approaches, they are not adequate as there is currently no GBM cure and the median survival period is less than 24 months^{1,2,5}.

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that regulates of cellular function via downstream signaling⁶⁻⁹. Upon ligand binding at the plasma membrane, EGFR is internalized and transits to the late endosome/multivesicular body (MVB) prior to MVB-lysosome fusion and receptor degradation, a mechanism that regulates receptor signal tuning^{8,10}. Unregulated EGFR signaling results in aberrant cell proliferation, angiogenesis, and tissue invasion¹¹ and underlies several malignancies including GBM¹²⁻¹⁷. EGFR protein overexpression occurs in approximately 35–65% of GBM cases (Brennan 2013) and of these cases, 65–75% also carry a genetic mutation in the EGFR, commonly the EGFR-variant III (EGFRvIII)^{13,14,18-23}. The EGFRvIII mutation results in the deletion of 801 base pairs that span exons 2–7^{24,25} removing 267 amino acids from the ligand-binding domain of the receptor^{15,26}. Inability of EGFRvIII to bind its ligand results in decreased autophosphorylation and internalization from the cell surface²⁷. Despite the absence of ligand binding, EGFRvIII constitutively initiates downstream signaling cascades that promote cell proliferation and migration^{15,26,28-30}. It is possible that the differences in trafficking of EGFR and EGFRvIII contribute to the oncogenic effect of the mutant receptor protein²⁸ and the poor prognosis of those patients whose GBM tumors express EGFRvIII^{1,31}.

GBM tumors are highly invasive and the interaction between tumor cells and their environment is critical to their proliferation and invasion^{5,32-35}. Astrocytes are a prominent component of the tumor microenvironment^{36,37}. Paracrine signaling between GBM and astrocytes has been documented and GBM-induced activation of tumor-associated astrocytes can result in increased proliferation and influence tumor cells in ways that promote GBM invasion into healthy tissue³⁸⁻⁴⁰. Activated astrocytes secrete tumor promoting factors that alter the signaling and cytokine profile of normal astrocytes resulting in a more favorable environment for tumor cell proliferation and migration⁴⁰⁻⁴³. The mechanisms underlying the information transfer between GBM tumor cells and surrounding astrocytes likely involve exchange of various genetic and proteinaceous factors^{41,44,45}.

Extracellular vesicles (EVs) are small membrane-bound vesicles that transfer nucleic acids, proteins, and lipids between cells as a means of intercellular communication^{46,47}. Interestingly, EGFRvIII is secreted by glioma cells on EVs that can be transferred to neighboring glioma cells resulting in incorporation into the plasma membrane and increased downstream signaling⁴⁸. Though EGFRvIII is found on EVs secreted from GBM cells, the origin and biogenesis of EGFRvIII-containing EVs has not been studied. In this regard, understanding the molecular itinerary of EGFRvIII prior to secretion could unveil a mechanism to alter the trafficking of the oncoprotein and provide potential therapeutic benefit. We observed that EGFRvIII was inefficiently degraded although the receptor was internalized into intraluminal MVB vesicles. Since EGFRvIII appears to take a similar itinerary into intraluminal MVB vesicles as the parental EGFR but is not degraded like the parental receptor, we hypothesized that EGFRvIII is secreted from GBM cells on

exosomes, nanovesicles that are released from cells following the fusion of MVBs with the plasma membrane^{1,48,49}. Further, we predicted that exosomes containing EGFRvIII might affect cells within the tumor microenvironment.

Methods

Cell Culture

U87MG cells were a gift from the laboratory of Dr. Frederick Lang and originally purchased from ATCC (ATCC HTB–14). Cells were cultured as a monolayer in 10-cm plastic plates in Modified Eagle Medium with non-essential amino acids (MEM + NEAA, Corning) containing 10% Fetal Bovine Serum (FBS, Sigma) under 5% CO₂ at 37°C. Before each experiment, cells were passaged by removing them from the plate using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) and seeded into 10-cm tissue culture plates.

Western Blotting

Cells were collected by scraping and were centrifuged at 1500 x g for 10 minutes, resuspended in a small volume of RIPA (1% Triton X–100, 6.1% SDS, 150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP–40) buffer with a protease inhibitor cocktail (10 mM leupeptin, 1 µg/µL pepstatin, 0.3 mM aprotinin, and 1.74 µg/µL PMSF) (Thermo Fisher Scientific, 78438), lysed using sonication (5 pulses of 1 second at output control 3, Branson Sonifier 250, VWR Scientific), and centrifuged at 2000 x g to separate membranes from cytosolic protein. Protein concentrations were quantified using BCA protein assay (Thermo Fisher, A53225) according to the manufacturer's instructions. Thirty µg of protein were mixed with 6x loading buffer (50 mM Tris-HCl at pH 6.8, 4% SDS, 60% glycerol, 0.6% bromophenol blue, and 30% β-mercaptoethanol), boiled for 5 minutes at 100°C, and separated using polyacrylamide gel electrophoresis (SDS-PAGE, 5% stacking, 10% separating) and transferred to nitrocellulose membranes under standard conditions. Membranes were blocked using 5% nonfat dry milk in PBS or 5% BSA in PBS based on manufacturer instructions. Membranes were incubated overnight at 4°C with anti-EGFR antibody (Invitrogen), anti-Alix antibody (abcam), anti-Flotillin antibody (abcam), anti-GFAP antibody (abcam) or anti-V5 antibody (Invitrogen). Membranes were subsequently washed three times with PBS-T and then incubated in a goat anti-mouse or anti-rabbit secondary antibody per primary antibody species (1:5000 for EGFR, 1:5000 for V5, 1:2000 for Alix, 1:2000 for Flotillin, and 1:2000 for GFAP) conjugated to horseradish peroxidase (Sigma, A0545) for 1 hour at room temperature. The resulting signal was visualized using chemiluminescence (SuperSignal West Pico, Thermo Fischer Scientific, 34580) and film.

Degradation Assay

U87MG cells were cultured to 80% confluency. Cells were washed with serum-free MEM media and starved in serum-free media for 2 h (37 °C, 5% CO₂). The medium was aspirated, and cold serum-free

media supplemented with EGF (50 ng/ml) and cyclohexamide (5 ug/ml) was added. Plates were placed on ice and shaken at 4 °C for 1 h.

Cells were rinsed and either kept on ice (0 min) or incubated with warm serum-free media supplemented with cyclohexamide (5 ug/ml) (5% CO₂, 37 °C) for 30, 60, or 90 min. Cells were lysed as above, and 30 µg of protein from each sample was subject to SDS-PAGE and Western blotting. Proteins were visualized with ECL and exposed to autoradiography film. Bands were quantified using ImageJ.

Cytosol preparation and Cell-Free Reconstitution

Mammalian and *S. cerevisiae* cytosol as well as cell-free reconstitution assays were performed as previously described in Gireud-Goss et al.⁵³ For cell-free reconstitution experiments testing dependence of EGFR and EGFRvIII on cholesterol, following the final centrifugation step (90,000 x g for 30 minutes) endosomes used in reactions including Methyl-β cyclodextrin (MβCD) (Sigma-Aldrich) were incubated with 20mM MβCD at 37°C for 15 minutes. The endosomes were then centrifuged at 90,000 x g for 30 minutes, and resulting endosomes were incorporated into standard reactions. Reactions either contained U87 cytosol, U87 cytosol with 20mM MβCD, or U87 cytosol with 20mM MβCD and 1mM cholesterol (Sigma-Aldrich). All reactions were adjusted to have a final volume of 50 µL as described in Gireud-Goss et al.⁵³

Criteria for Calculating Reaction Efficiency in Cell-Free Sorting Assay

All experimental reactions were normalized to starting endosomal controls to obtain the reaction efficiency. To obtain reaction efficiency, the amount of EGFR on starting membranes added to each reaction was compared to the amount of EGFR in reactions that contained membranes, cytosol, ATP and had been cleaved by trypsin.

Exosome Isolation

U87 cells were culture in exosome-free Exosome isolation was performed by a series of serial centrifugation steps previously described in Thery et al.⁶⁵ Briefly, exosome-free media was prepared by centrifugation of FBS overnight at 100,000 x g at 4 °C. Supernatant was added to MEM with non-essential amino acids for a final concentration of 10% FBS in media. U87MG cells at approximately 60–70% confluence were culture in exosome-free media for 72 hours (10 plates for western blot, 5 plates per well of astrocytes in 6-well dish). Conditioned media was collected and centrifuged at 300 x g for 10 minutes, supernatant was spun at 2,000 x g for 20 minutes, supernatant was sput at 10,000 x g for 30 minutes. The resulting supernatant was filtered through.45 µm PES filter. Conditioned media was then centrifuged at 100,000 x g for 1 h, supernatant was discarded, pellet was washed with sterile 1X PBS and again centrifuged at 100,000 x g for 1 h. The resulting pellet was either resuspended in loading buffer for western blot analysis, or in sterile 1X PBS for astrocyte treatment.

NanoSight Tracking Analysis (NTA)

NTA measurements were performed on extracellular vesicles isolated from U87MG cells as described in They et al.⁶⁵ using a NanoSight NS300 instrument following the manufacturer's instructions. NTA is performed by measuring the rate of Brownian motion of particles in a low volume light scattering system (NanoSight Ltd., Amesbury, United Kingdom). Results are presented as mean size of vesicles (x-axis) and concentration of particles per mL of solution (y-axis). Samples were examined in triplicate.

Almar Blue Cell Viability Assay

U87MG parental, EGFR wild-type overexpressing, or EGFRvIII overexpressing cells were diluted 1:1 with trypan blue and counted with the Countess automated cell counter (Invitrogen). Cells were then diluted in MEM media to enable plating at a confluence of 1,500 cells and a total volume of 100 μ L per well in a 96-well plate and incubated for 4 hours to allow adherence. Following 4 hours, 10 μ L Alamar Blue was added to cells (in triplicate per cell-line per time point) and incubated at 37°C for 4 h. The plate was then light protected and absorbance was measured at (excitation λ 565, emission λ 605) (PerkinElmer Multimode plate reader, EnSpire). This was repeated at 24, 48, and 72 hours post-plating. Samples assessed daily were compared to the absorbance of cells at Day 0. Statistical significance was calculated using a one-way ANOVA and Dunnett's multiple comparisons test. All statistical analysis was performed with GraphPad Prism software version 7.

Transwell Migration Assay

EGF was added to MEM for a concentration of 100 ng/mL, and 750 μ L was added to wells of a 12 well plate. Falcon Cell Culture inserts (Corning Life Sciences, ECM508) with 8 μ m pores were submerged in the wells. U87MG parental, EGFR wild-type overexpressing, or EGFRvIII overexpressing cells were diluted 1:1 with trypan blue and counted with the Countess automated cell counter (Invitrogen). U87MG cells were diluted in serum-free MEM media at a concentration of 1.5e5 cells per μ L of serum-free MEM media and added to the cell culture insert. The cells were allowed to incubate overnight 37°C with 5% CO₂.

The following day the cells were washed and fixed for microscopy. Non-invading cells were scrubbed from the top surface of the membrane using a cotton-tipped swab moistened with media. Next, the membranes were fixed with 100% methanol for 5 minutes at room temperature. The membranes were then rinsed in diH₂O for 1 minute and stained with a 1 μ g/mL DAPI in PBS with 0.1% Tween for 20 minutes at room temperature protected from light. The membranes were then washed again in diH₂O for 1 minute and inverted to dry. Lastly, a fine-tipped scalpel was used to cut the membranes from the insert. The membranes were placed bottom-side-up on a glass microscope slide. FluroSave reagent (Millipore Sigma, 345789) was added drop wise to the membranes. They were then covered with glass coverslips and then sealed with nail polish.

The glass slides were stored at 4°C protected from light and imaged with 10x magnification on a fluorescent microscope. The instrument was set for DAPI with an excitation wavelength of 358 nm and an emission wavelength of 461 nm. At least three 10x fields on each membrane were counted, and the average number of cells that had migrated through the membrane was counted to compute the number of cells per high-powered field (HPF). The experiment was performed in triplicate and significance was determined with a one-way ANOVA and Dunnett's multiple comparison test using GraphPad Prism.

Primary Astrocyte Cell Culture

Experimental use of animals was conducted in compliance with approved protocols from the Institutional Animal Care and Use Committee of the University of Texas Health Science Center. Animal numbers of each group were calculated by power analysis and animals were randomly assigned to each group where applicable. All murine mouse pups were obtained from breeding in-house from adult C57Bl/6 mice obtained from Charles River.

P0.5-P3 mouse pups were humanely euthanized on ice and brains were removed for dissection. Cortices were isolated and enzymatically digested in 1.5mL sterile tubes using an enzymatic digestion kit (Neural Dissociation Kit (P), Miltenyi Biotec). Cortices were homogenized using sterile 1000uL and then 200uL pipet tips (USA Scientific). Finally, cells were then diluted and plated on Poly-D-Lysine-coated flasks or glass cover slips at a density of $\sim 2 \times 10^5$ live cells/well. After 24hr media was aspirated, adherent cells were gently rinsed in 1mL of media to remove debris, and fresh media was added. Following confluence of the astrocyte monolayer, the remaining microglia were then depleted at 14 DIV by exposure to a 50 mM solution of leucine methyl ester (pH = 7.4) for 2 hours. The following day, the media was changed again to remove any floating (dead) microglia, and the astrocyte monolayer was visually inspected under polarized light to confirm successful microglial depletion (fewer than 5% of visible cells by area).

Statistical Analysis

Statistical significance was determined using the Prism 7 software. Prior to analysis, samples were tested for normality using the Shapiro-Wilk test. If the samples followed a normal distribution, then either a Paired t-test or a one-way ANOVA followed by post-hoc analysis (Tukey or Dunnett's test) were performed. If samples were not normal, then the Kruskal-Wallis analysis was utilized. A p-value of <0.05 was considered statistically significant with an $n = >3$.

Results

Truncation of ligand binding domain yields inefficient EGFR degradation

Wild-type EGFR transits the canonical endosomal pathway for membrane proteins where it is ultimately internalized into the ILVs of MVBs and is degraded upon fusion of the MVB with the lysosome. We compared EGFRvIII degradation with the parental EGFR by examining whole cell EGFRwt and EGFRvIII levels following ligand stimulation in U87 glioblastoma cells. Following 90 min of EGF stimulation, cells stably overexpressing wild-type EGFR had 26.3 +/- 8.5 % cellular EGFR remaining compared with U87 cells stably overexpressing EGFRvIII which had 141.5 +/- 42.1% receptor present after 90 minutes ($p < 0.05$), confirming the inefficient degradation of EGFRvIII ²⁸.

EGFRvIII is internalized into intraluminal vesicles of the multivesicular body

Differences in degradation between the parental EGFR and EGFRvIII may result from divergence in their intracellular trafficking itineraries. While disparities in internalization rates may affect the speed of degradation, we observed that total levels of cellular EGFRvIII were lower than the levels of parental EGFR either after ligand stimulation or at steady state (Fig. 1). To understand whether a divergence in itinerary may underlie the difference in degradation we examined the trafficking of EGFR subsequent to its internalization. We focused on a critical step in the endocytic pathway where a choice between degradation and recycling is made ^{8,50,51}. Using a cell-free reconstitution approach ⁵² we examined whether, like EGFRwt, EGFRvIII is trafficked through the late endosome and subsequently internalized into the intraluminal vesicles (ILVs) of the MVB. The cell-free assay measures cargo movement into the intraluminal vesicles of the MVB using an antibody specific to the V5 epitope fused to the intracellular domain of EGFRwt and EGFRvIII that are expressed in U87 cells. If membrane proteins are internalized into the ILVs of MVBs, the intracellular epitope is no longer accessible to exogenously added trypsin and therefore protected from digestion. The epitope is then detected via immunoblotting following reaction completion. If the epitope remains exposed to the extracellular environment, as would occur if the membrane protein was not internalized from the endosomal membrane into the ILV, it would be digested by exogenous trypsin and would no longer be detectable, indicating that the receptor remained on the limiting membrane of the endosome ⁵²⁻⁵⁴.

We observed that EGFRvIII is internalized into ILVs (Figure 2). A portion (20.9%) of the receptor present on the limiting membrane of the late endosome was protected from trypsin digestion while 56.9% of EGFRwt was protected from trypsin digestion (Figure 2). These results suggest that despite the reported inefficient endocytosis and lack of degradation of EGFRvIII ²⁸, this mutant EGFR can be internalized into the ILVs of MVBs. Since EGFRvIII is inefficiently degraded and yet enters the ILVs of MVBs, it is possible that EGFRvIII-containing MVBs are not destined to fuse with lysosomes resulting in EGFRvIII degradation. An alternative MVB itinerary that would be compatible with these data (ILV internalization and decreased degradation) is that EGFRvIII may be internalized into a discrete population of MVBs that may fuse with the plasma membrane instead of lysosomes, which would result in secretion of EGFRvIII on exosomes.

Internalization of wild-type EGFR but not EGFRvIII into the MVB is a cholesterol-dependent process

If MVBs containing EGFRvIII follow a different itinerary (e.g. fusing with the plasma membrane) than those containing parental EGFR, that fuse with lysosomes^{55–58} these data imply that there are discrete populations of MVBs^{58,59}. Because of the apparent differences in itineraries of EGFRwt and EGFRvIII, we used EGFRwt and EGFRvIII as proxy cargo with distinct destinations with the hope of identifying cellular factors that may differentiate these potential MVB populations.

There are differences in the lipid composition of MVBs that fuse with lysosomes and the plasma membrane⁶⁰. Cholesterol is enriched in lipid rafts and lipid rafts have been implicated in various membrane budding events⁶¹. Additionally, cholesterol is concentrated on ILVs of the MVB⁶² suggesting that cholesterol may play a role in inward budding events at the MVB. To determine whether cholesterol is required for MVB sorting of transmembrane cargo, endosomes were depleted of cholesterol using Methyl- β cyclodextrin (M β CD). Endosomal membranes treated with 30 mM M β CD had significantly decreased sorting of EGFRwt into internal vesicles (Figure 3). In contrast, EGFRvIII internalization was not significantly affected by M β CD. The inhibition of EGFR protease protection in M β CD- treated endosomes was rescued by the addition of soluble cholesterol (Figure 3). These data suggest that cholesterol is required for internalization of the parental EGFR into ILVs, but not for the endosomal internalization of EGFRvIII into ILVs, and imply that sorting of cargo that is destined for lysosomal degradation may have differing lipid requirements than those destined for exosomal secretion.

Glioma cells secrete EGFRvIII on exosomes

EGFRvIII is efficiently internalized into ILVs but is not efficiently degraded, thus we postulated that MVBs containing EGFRvIII might not fuse with lysosomes. To determine whether EGFRvIII-containing MVBs may fuse with the plasma membrane we examined whether exosomes (ILVs secreted into the extracellular space) containing EGFRvIII were secreted from U87 cells. Exosomes were isolated from U87 glioma cells expressing either EGFRwt or EGFRvIII. We isolated extracellular vesicles by differential centrifugation and examined whether these vesicles contained the V5 tagged EGFRwt and/or EGFRvIII as well as exosomal marker proteins. We confirmed that U87 cells secrete both EGFRwt and EGFRvIII on exosomes. Additionally, nanoparticle tracking analysis was used to confirm that EVs isolated from U87 cells overexpressing EGFRwt or EGFRvIII were enriched in exosomes and found that the average size of particles isolated from cells expressing either receptor were not significantly different and within a size range previously reported for exosomes at 151.1 $\pm$ 66.2 nm and 162.8 $\pm$ 77.1 nm respectively with peaks at 104nm and 112nm^{47,63,64}.

Glioma cells overexpressing EGFRvIII possess exacerbated oncogenic phenotypes compared to cells overexpressing wild-type EGFR

Expression of EGFRvIII is associated with poor GBM outcomes^{26,31,66}. We examined whether expression of EGFRvIII may exacerbate tumor cell proliferation and migration compared to expression of EGFRwt. Cell proliferation and migration were measured in U87MG parental cells, U87MG cells stably expressing the wild-type EGFR, and U87MG cells stably expressing EGFRvIII. EGFRvIII proliferated significantly ($p < 0.001$) more than U87MG parental cells or U87MG cells expressing EGFRwt over a 72 hr time period following plating. GBM tumor cells (U87MG) were seeded in a transwell and quantitation of their migration through the filter revealed that EGFRvIII-expressing cells migrated in nearly twice the numbers as parental U87MG and EGFRwt expressing U87MG cells. These data suggest that expressing of EGFRvIII favors tumor growth and results in accentuated oncogenic phenotypes such as cell proliferation (tumor growth) and migration (tumor invasion).

EGFRvIII positive exosomes result in increased astrocyte activation compared to wild-type EGFR positive exosomes

Astrocytes make up a large portion of the tumor microenvironment and secreted factors from reactive astrocytes have been implicated in creating a favorable environment for GBM tumor growth^{33,67}. Since we observed that EGFRvIII is secreted on exosomes from U87MG tumor cells (Figure 4), we examined whether EGFRvIII-containing exosomes secreted from U87 glioma cells affect naïve astrocytes. We hypothesized that EGFRvIII might activate primary astrocytes which might enable a favorable environment for tumor cell proliferation and/or migration. Because the main component of astrocyte intermediate filament, the glial fibrillary acidic protein (GFAP), is upregulated in reactive astrocytes, we used it as a marker of activation in cultured cortical astrocytes⁴².

Primary cortical astrocytes were treated with exosomes isolated from the conditioned media of U87MG cells overexpressing EGFRwt or U87MG cells overexpressing EGFRvIII. Following treatment for 24 hours, astrocytes were either scraped from the 10-cm dish, lysed and probed for GFAP via SDS-PAGE or cultured on glass coverslips, fixed and stained for immunofluorescent imaging. These results showed that treatment with EGFRvIII-containing exosomes resulted in an upregulation of GFAP in astrocytes when compared to EGFRwt. Additionally, immunofluorescent staining revealed that EGFRvIII is transferred from U87 glioma cells into astrocytes via exosome secretion as demonstrated by staining for V5-tagged EGFRvIII.

Discussion

Expression of the mutant EGFR, EGFRvIII, occurs in about 50–60 percent of EGFR-overexpressing GBM tumors and results in poor patient prognosis^{68,69}. While the canonical pathway used to tune EGFR signaling involves its internalization from the plasma membrane followed by lysosomal degradation, previous studies have shown that EGFRvIII is not appreciably degraded and have postulated that it is largely recycled back to the plasma membrane following internalization resulting in continuous downstream signaling²⁸ and tumorigenesis^{31,70}. We examined the itinerary of EGFRvIII and unexpectedly observed that it is internalized into the intraluminal vesicles (ILVs) of the late endosome/MVB. Sorting of membrane proteins from the limiting endosomal membrane into the ILVs is the canonical pathway that targets them for degradation when MVBs fuse with lysosomes^{71–73}. We observed that EGFRvIII is sorted into a pathway that typically results in membrane protein degradation, although the receptor is not efficiently degraded in the same tumor cells.

EGFRvIII is internalized into the ILVs of MVBs but not appreciably degraded, thus we hypothesized that the MVBs containing EGFRvIII may have an alternate itinerary from the canonical pathway taken by non-mutant EGFRs. Our observation that EGFRvIII is present on exosomes secreted by glioblastoma cells that possess markers found on MVBs, suggests that EGFRvIII-containing MVBs are a discrete population of MVBs independent of those that fuse with lysosomes. Subpopulations of MVBs have been suggested^{63,74} although their characterization has been difficult because discrete populations have not been isolated. While our data suggest that cholesterol is required for internalization of cargo into MVBs that will fuse with lysosomes, but not for MVBs that can fuse with the plasma membrane, other molecular details of these populations are lacking. It seems likely that in addition to differing lipid composition, different SNARE proteins present on discrete MVB populations may mediate fusion events with different target membranes. Isolation of these MVB populations and understanding their physiological significance will require examination of their capacity to fuse with different targets.

Secretion of exosomes provides a mechanism for tumor cells to interact with their surrounding microenvironment. The microenvironment may be tumor promoting by enabling angiogenesis, tumor cell invasion, proliferation, and chemoresistance⁶⁷. We observed that exosomes secreted from tumor cells expressing EGFRvIII, but not the parental EGFR, increased signs of astrogliosis in primary cultured astrocytes including swollen cell bodies and increased intermediate filament protein GFAP immunoreactivity. Since astrogliosis, in the context of GBM, has often been reported to be tumor promoting and EGFRvIII is also associated with enhanced tumorigenesis, we hypothesized that exosomal secretion of EGFRvIII may affect astrocytes in a manner that would be tumorigenic. EGFRvIII has previously been shown to be present on extracellular vesicles (EVs) that promote GBM proliferation⁷⁰, however, not all EVs are endocytic in origin and previous studies have not determined whether EGFRvIII is secreted on exosomes. The distinction in EV populations is important in that their cargo depends in part on the cellular compartment from which they originate (e.g. plasma membrane vs. endosomes)^{63,65,75,76}.

Distinct subpopulations of MVBs that allow cargo to be targeted to different destinations suggests that there might be molecular signals that enable the sorting of cargo into those MVBs destined for lysosomal degradation versus those destined for exosomal secretion. Understanding and manipulating these signals might allow for directing specific cargos toward secretion or degradation. An interesting therapeutic application relevant to GBM could take advantage of the potential ability to misdirect EGFRvIII into a degradative pathway rather than the exosomal secretory pathway, which may diminish oncogenic effects on neighboring cells in the surrounding tumor microenvironment.

Conclusions

In conclusion, results of this study indicate that reduced EGFRvIII degradation and packaging into exosomes at the MVB underline a mechanism by which the oncoprotein is secreted into the tumor microenvironment. In addition to characterization of EGFRvIII enriched exosomes secreted by glioma cells, these studies revealed that EGFRvIII secretion on exosomes stimulates reactive astrogliosis ultimately enabling tumor growth.

Abbreviations

EGFR—epidermal growth factor

EGFRvIII—epidermal growth factor variant III

GBM—glioblastoma multiforme

MVB—multivesicular body

ILV—intraluminal vesicle

EVs—extracellular vesicles

NTA - nanosight tracking analysis

EDTA - ethylenediaminetetraacetic acid

GFAP—glial fibrillary acidic protein

M β CD - Methyl- β cyclodextrin

Declarations

Ethics approval and consent to participate

None of the cell lines required ethics approval for their use in this study.

Consent for publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare that they have no competing interests

Funding

The materials in these studies were supported by National Institute of Health (CA166749). KM was supported in full by the Frederick and Marilyn Lummis Fellowship through the MD Anderson UTHealth Graduate School of Biomedical Sciences. The funding bodies did not have any role in the design of the study and collection, analysis and interpretation of data and in writing of the manuscript.

Authors' contributions

KM performed the majority of the experiments. AP, EK, and MGG were blinded to and performed the ICC imaging. KM and AB conceptualized the project, analyzed and interpreted the experiments, and drafted/edited the manuscript. All authors read and approved the final manuscript

Acknowledgements

We thank Drs. Neal Waxham and Joo Eun for providing the cortical astrocytes used in this study. We thank Max Odem for providing useful insight into the statistical analyses of these data.

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Figures

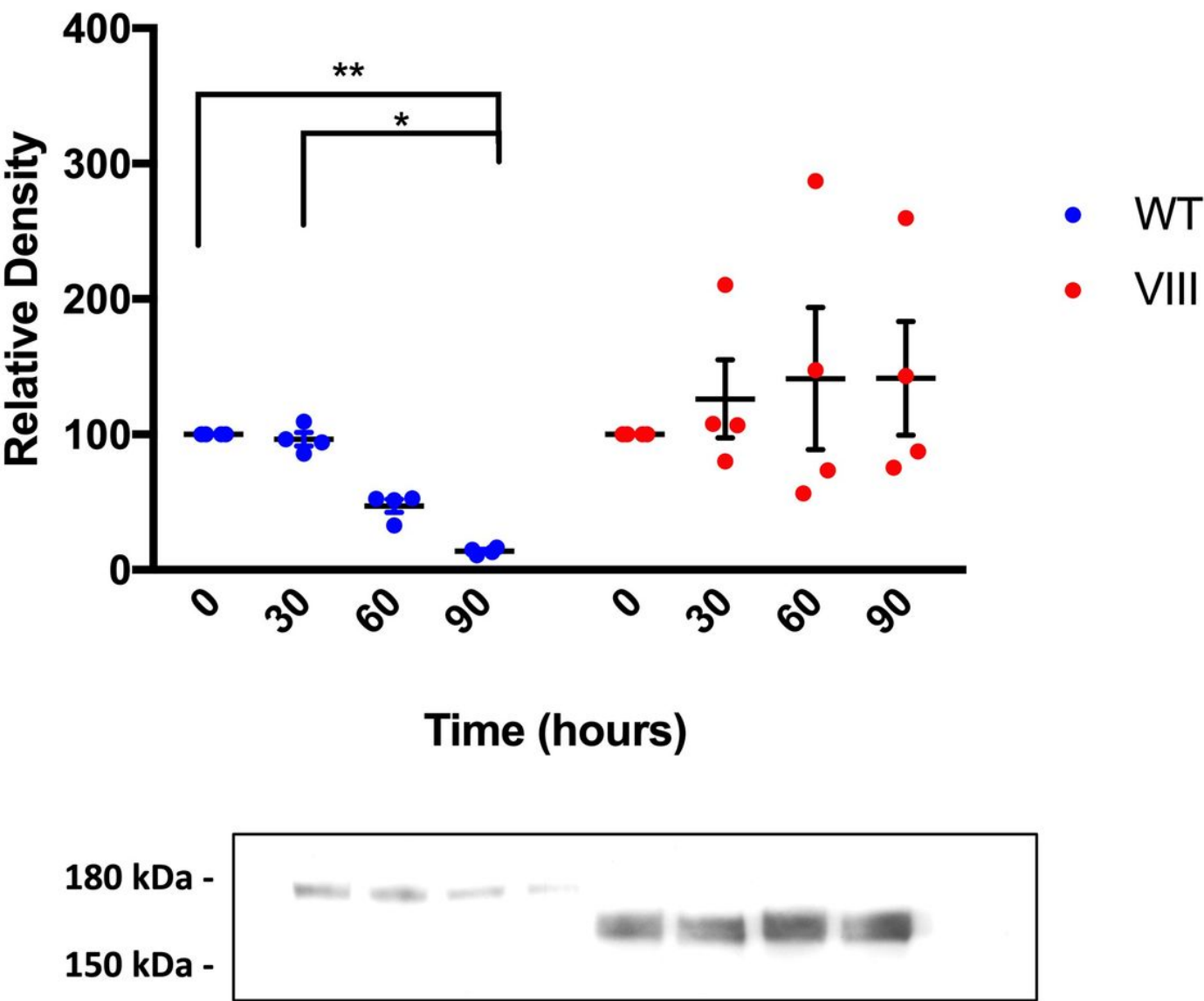


Figure 1

EGFRvIII is inefficiently degraded by U87 cells following ligand stimulation. U87 cells stably overexpressing V5-tagged EGFRwt or EGFRvIII were serum starved for 2 hours. Cells were incubated on ice with EGF ligand for 1 hour. Cells were then incubated at 34 degrees in fresh media for 30, 60, and 90 minutes prior to being lysed, proteins separated using SDS-PAGE, transferred to nitrocellulose, and the resulting blots immunoblotted with an anti-V5 antibody. The levels of EGFRwt after 90 min EGF stimulation are significantly decreased compared to t=0 ($p < 0.01$), while mutant EGFRvIII levels are not significantly different from t=0 after the same stimulation. Data represents mean $\pm$ S.E.M normalized to t=0 (n=4). * denotes $p < 0.05$, ** denotes $p < 0.01$

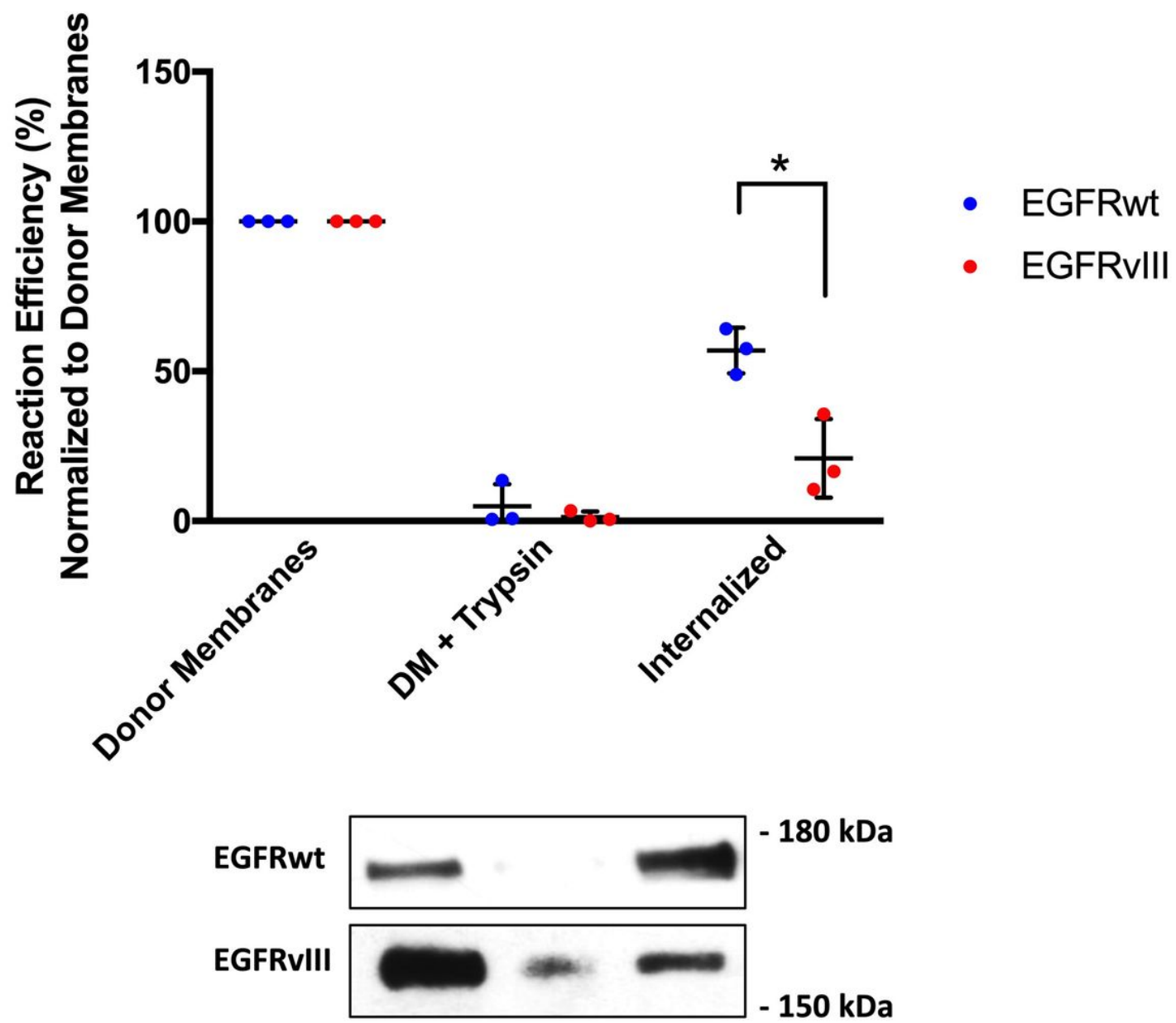


Figure 2

EGFRvIII is internalized into late endosomes / multivesicular bodies. Endosomes from U87 cells stably overexpressing V5-tagged EGFRwt (blue) or EGFRvIII (red) were harvested following serum-starvation and EGF ligand stimulation, and incorporated into cell-free reactions with U87MG cytosol and ATP, conditions

allowing for ILV formation. Immunoblot analysis for V5 enables quantitation of EGFRwt or EGFRvIII internalized into ILVs relative to the amount of receptor on the MVB limiting membrane prior to incubation (starting membranes). Data represent mean +/- S.E.M. (n=3) normalized to control. *p<0.05

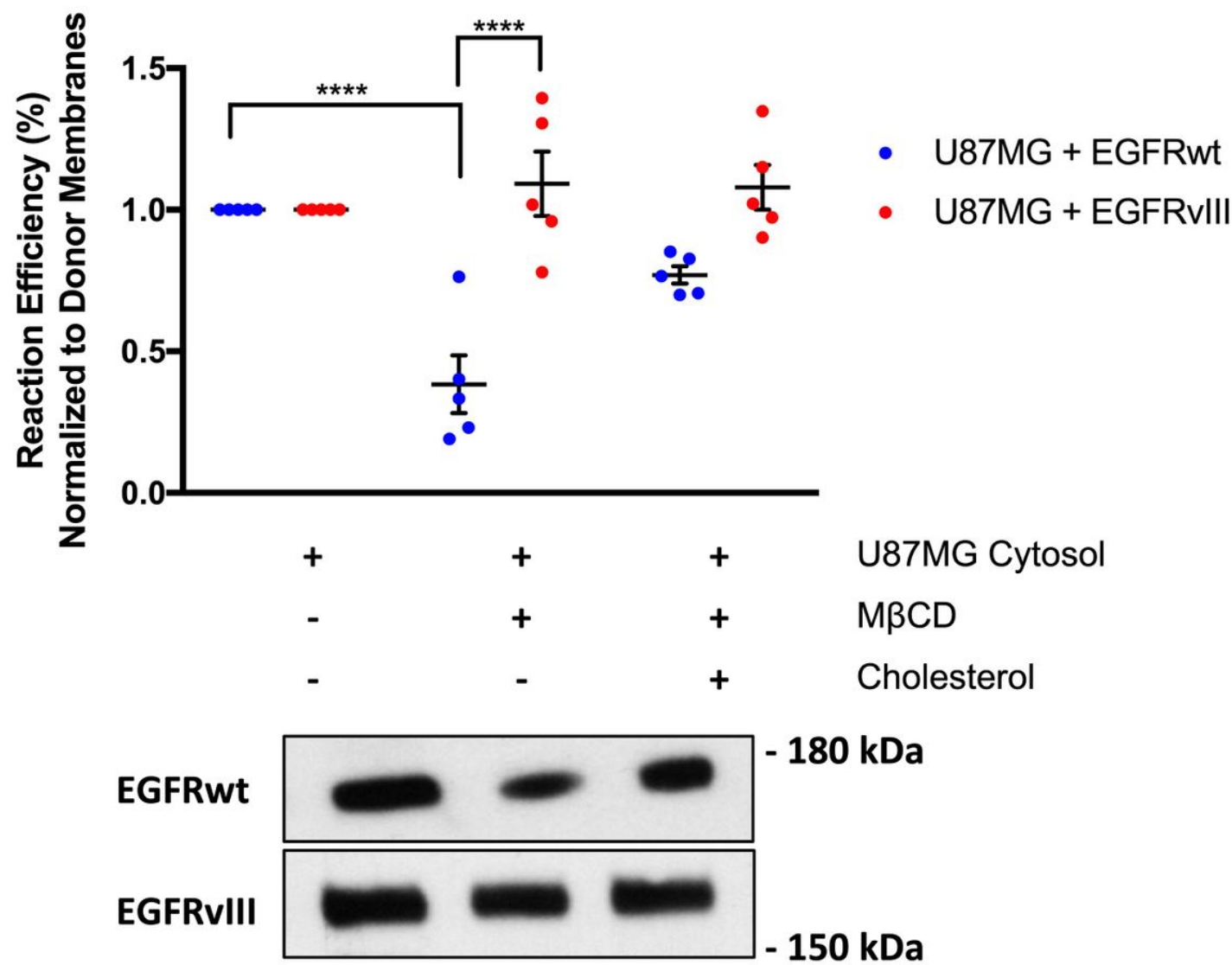


Figure 3

Internalization of EGFRvIII into MVBs is a cholesterol-independent process. Endosomes from U87 cells stably overexpressing V5-tagged EGFRwt (blue) or EGFRvIII (red) were harvested following serum-starvation and EGF ligand stimulation. Endosomes were incubated with MβCD prior to incorporation into reactions containing MβCD. All reactions were incubated with U87MG cytosol and contained either no MβCD, only MβCD, or MβCD with soluble cholesterol. Immunoblot analysis for V5 enables quantitation of EGFRwt or EGFRvIII internalized into ILVs relative to the amount of receptor on the MVB limiting membrane prior to incubation (starting membranes). Data represent mean +/- S.E.M. (n=5) normalized to control. * denotes p<0.05, ** denotes p<0.01, **** denotes p<0.0001

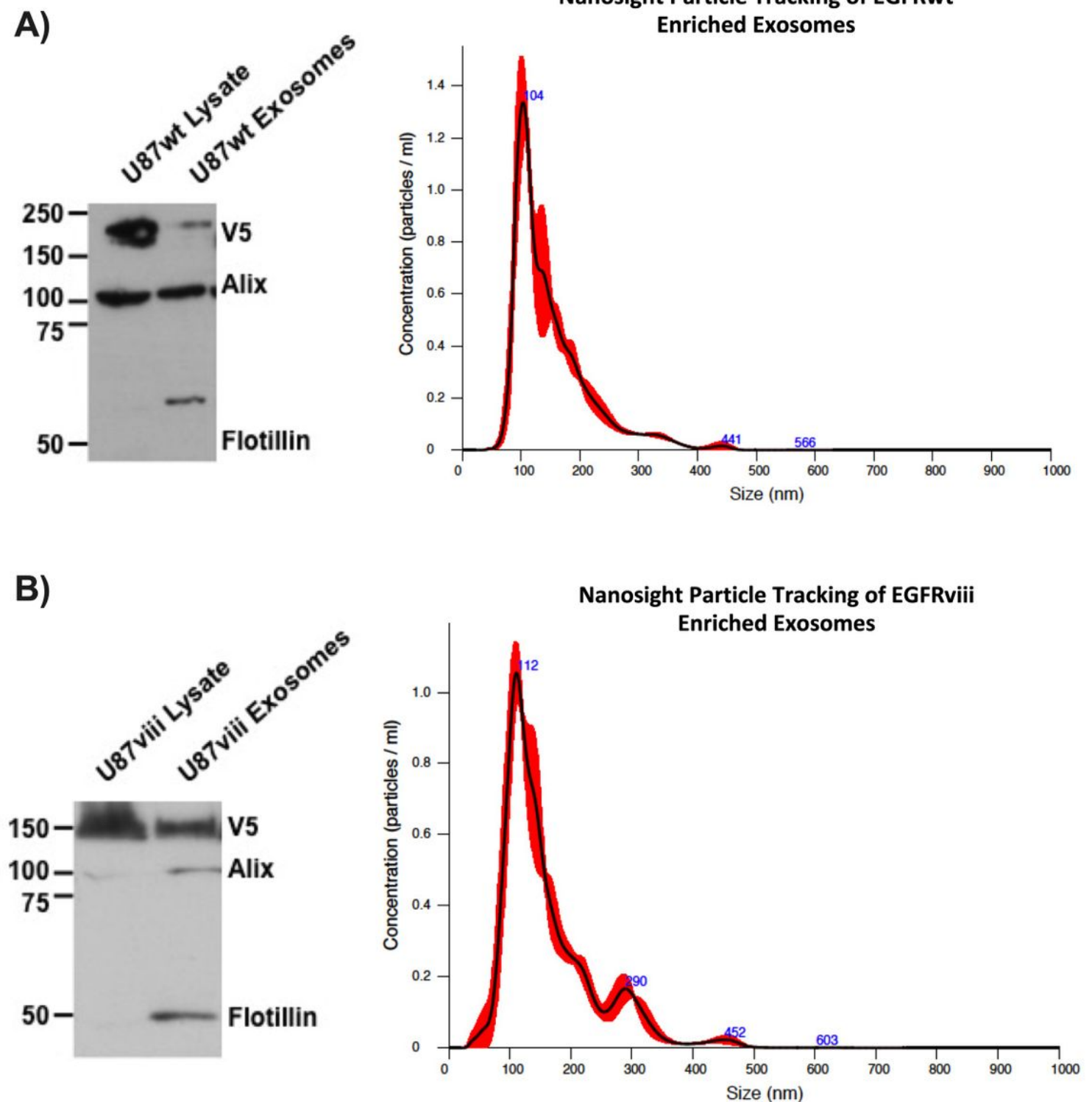


Figure 4

EGFR and EGFRvIII are enriched on exosomes secreted from U87MG cells. U87MG cells stably overexpressing V5-tagged EGFRwt (A) or EGFRvIII (B) were incubated in exosome-free media for 72 hours. Conditioned media was harvested and subjected to a series of centrifugation steps to isolate exosomes as described in Théry et al⁶⁵. Exosomes and cell lysate from secreting cells are probed for V5 (tagged receptor of interest), Alix, and Flotillin-1 (exosome markers). Representative western blots are shown and

show that EGFRvIII expression is stronger in exosomes than wild-type EGFR. Nanosight tracking analysis revealed that exosomes secreted from U87MG cells expressing EGFR or EGFRvIII were 151.1 +/- 66.2 nm and 162.8 +/- 77.1 nm with peaks at 104 nm and 112 nm respectively.

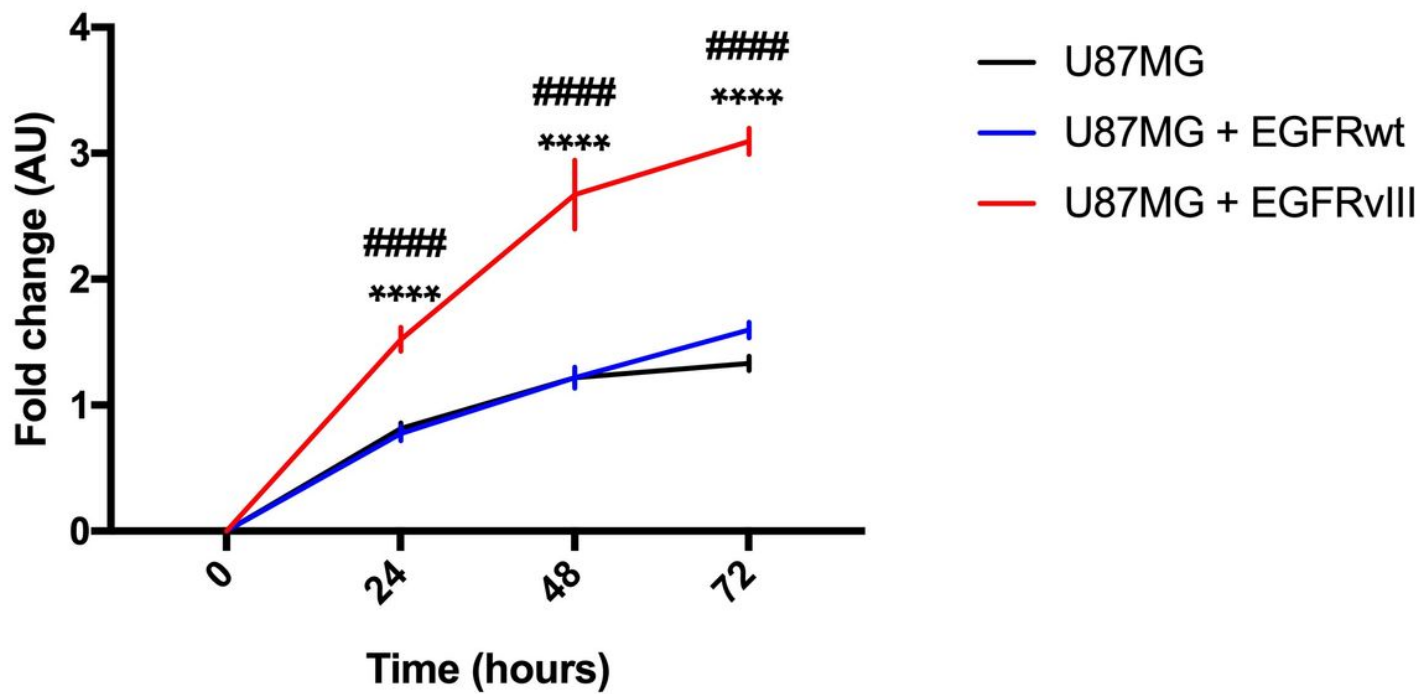


Figure 5

EGFRvIII expression exacerbates U87MG proliferation in vitro. Parental U87 cells, and U87 cells stably overexpressing EGFRwt or EGFRviii were plated at a 1500 cells / well. Following 24, 48, and 72 hours, cells were treated with Alamar Blue Cell viability solution (ThermoFisher Scientific) and cell viability was recorded by measuring fluorescent intensity. Data represents mean +/- S.E.M normalized to t=0 (n=4). **** and #### denotes $p < 0.0001$ U87MG + EGFRvIII compared to U87MG and U87MG + EGFRwt respectively.

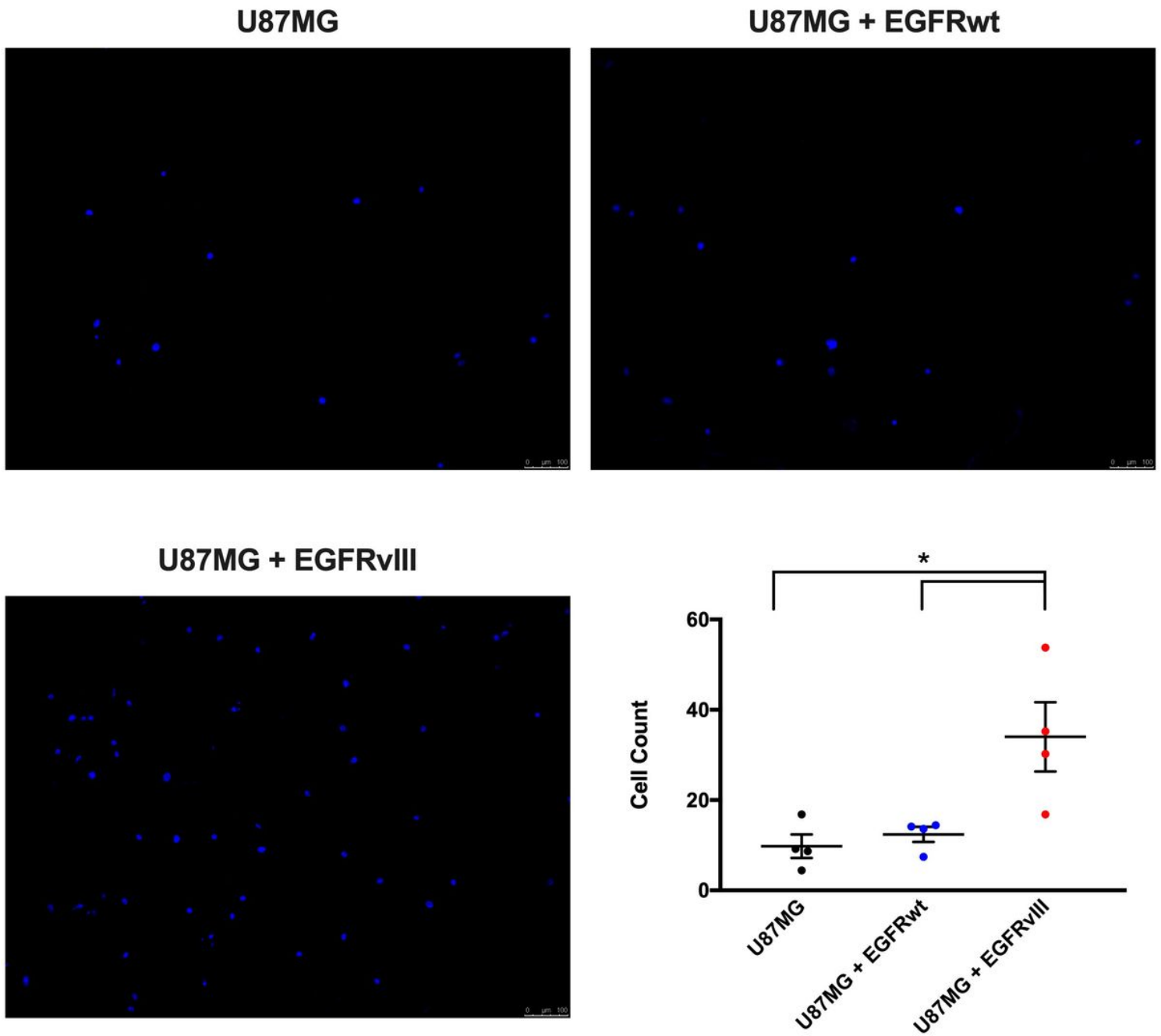


Figure 6

EGFRvIII expression enhances U87 cell migration. Parental U87 cells, and U87 cells stably overexpressing EGFRwt or EGFRviii were plated in trans-well inserts (15,000 cells per insert). Inserts were submerged in media containing 100ng / mL EGF ligand and allowed to migrate for 24 hours. Following 24 hours membranes of the trans-well insert were fixed and stained with DAPI to quantify cells that had migrated through the membrane. Data represents mean +/- S.E.M (n=4). * denotes p < 0.05

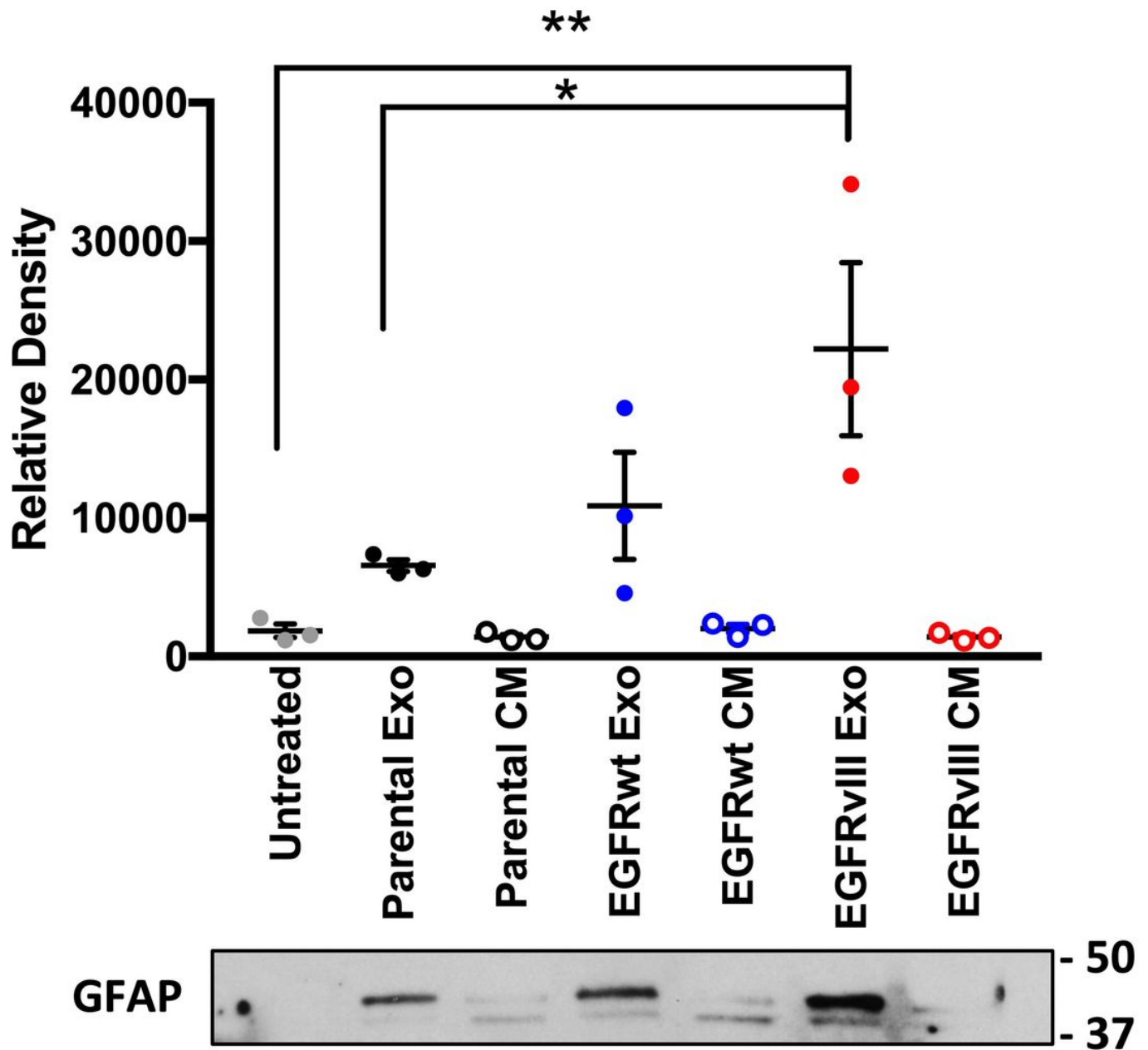


Figure 7

Exosomes enriched in EGFRvIII exacerbate astrocyte activation. Equal numbers of U87MG parental cells (black), cells overexpressing EGFR (blue) or cells expressing EGFRvIII (red) were cultured in exosome-free media for 72 hours. Exosomes were isolated as previously described⁶⁵. Cultured primary cortical astrocytes were treated with exosomes (filled circle) or conditioned media with exosomes removed (open circle). Following treatment for 24 hours, astrocytes were scraped, lysed and probed for GFAP via SDS-PAGE. Data represents mean $\pm$ S.E.M (n=3). * denotes $p < 0.05$, ** denotes $p < 0.01$

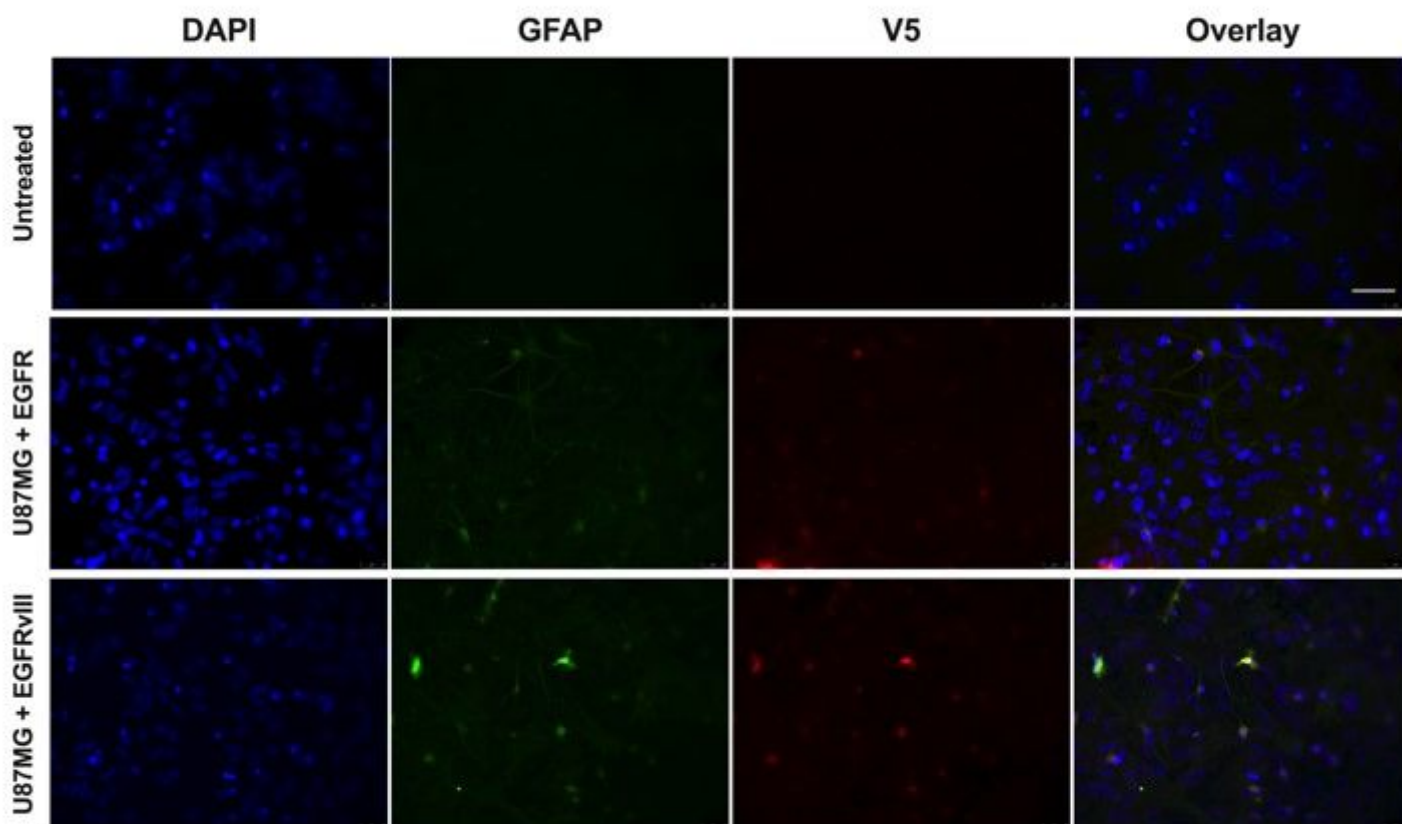


Figure 8

U87MG exosomes transfer EGFR and EGFRvIII to astrocytes and increase GFAP expression. Representative immunofluorescent images of primary cortical astrocytes treated with exosomes isolated from U87MG cells expressing V5-tagged EGFR or EGFRvIII. Images collected at 40x magnification were taken 24 hours after exosome treatment. Astrocytes were triple stained with DAPI, GFAP, and V5 antibodies showing incorporation of EGFR and EGFRvIII into the astrocyte. Scale bar indicates 100µm.