STAT3 Suppresses The AMPKα-ULK1-Dependent Induction of Autophagy In Glioblastoma Cells

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STAT3 suppresses the AMPKα-ULK1-dependent induction of autophagy in glioblastoma cells

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Running title: STAT3 is a novel transcriptional repressor of autophagy in glioblastoma

# Equal contribution

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Abstract

Despite advances in molecular characterization, glioblastoma (GBM) remains the most lethal type of brain tumor with high mortality rates in both pediatric and adult patients. The signal transducer and activator of transcription 3 (STAT3), is an important oncogenic driver of GBM. Although STAT3 reportedly plays a role in autophagy of some cells, its role in cancer cell autophagy remains unclear. In this study, we found STAT3 phosphorylation on Y705 and S727 residues, acetylation on K685, and tri-methylation on K180 was constitutive in GBM cell lines. Tyrosine phosphorylation of STAT3 in MT330 GBM cells suppresses autophagy, whereas knockout (KO) of STAT3 increases ULK1 gene expression, increases TSC2-AMPKα-ULK1 signaling and Prom1/CD133 expression, decreases p62 gene expression and accumulation, and increases lysosomal Cathepsin D processing, leading to the stimulation of autophagic flux. Rescue of STAT3-KO cells by the enforced expression of wild-type (WT) STAT3 reverses these pathways and inhibits autophagic flux. Conversely, expression of Y705F-STAT3 and S727A-STAT3 mutants in STAT3-KO cells did not suppress autophagy. Inhibition of ULK1 activity (by MRT68921) or its expression (by siRNA) in STAT3-KO cells inhibits autophagy flux and sensitizes cells to apoptosis. Taken together, our findings suggest that both STAT3 serine and tyrosine phosphorylation play critical roles in STAT3-dependent autophagy in GBM and the potential of targeting this pathway to treat GBM.
Abbreviations

STAT3: signal transducer and activator of transcription 3; GBM: glioblastoma; SQSTM1: sequestome 1 (also known as p62); Baf: Bafilomycin A1; WB: western blot; BECN1: Beclin1; DAPI: 4’6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; LC3-I/LC3-II: microtubule associated light chain 3-I/-II; mTORC1: mammalian target of rapamycin complex 1; TSC2: tuberous sclerosis complex 2 (also known as Tuberin); TMZ: Temozolomide; Prom1: Prominin-1 (also known as CD133); PTMs: post-translational modification(s)
Introduction

Glioblastoma multiforme (GBM) is a highly aggressive and lethal brain tumor with poor prognosis \(^1\). Despite recent advancements in the molecular characterization of GBM, the median survival of patients is only between 10-15 months \(^2,3\). GBM presents major treatment challenges due to its therapeutic resistance and drug accessibility, which is limited by the blood-brain barrier \(^4\). There is a compelling clinical need for a deeper understanding of GBM to develop new and targeted approaches to treatment.

Although GBM is characterized by marked intra-tumoral heterogeneity at both cellular and molecular levels, the PTEN/PI3K/Akt/mTOR signaling axis is a major factor in GBM biology \(^5-10\). In addition, accumulating evidence has shown that Signal Transducer and Activator of Transcription 3 (STAT3), is an important oncogenic driver in many cancers including GBM \(^11,12\). Under normal physiological conditions, cytoplasmic STAT3 undergoes phosphorylation at Tyrosine (Y)-705 and Serine (S)-727 residues. STAT3 tyrosine phosphorylation induces homodimerization and/or heterodimerization with other STAT family proteins, nuclear translocation, and DNA binding, leading to the induction of cytokine responsive genes \(^13\), and anti-apoptotic genes \(^14\). The role of S727 phosphorylation is less well understood, but studies suggest that it may be required for STAT3’s maximum transcriptional activity \(^15\). STAT3 is constitutively phosphorylated in GBM cancer stem cells (GSCs) and inhibiting STAT3 phosphorylation attenuates GSC-driven tumor growth \(^16,17\), showing that STAT3 plays a critical role in GBM tumorigenesis.

Phosphorylation-independent STAT3 pathways have also recently been identified. Upon cytokine treatment, the histone acetyl-transferase (HAT) CBP/p300 acetylates STAT3 on lysine-
which enhances DNA binding, transactivation activity, and nuclear localization. Conversely, histone deacetylases (HDACs) promote STAT3 deacetylation and inhibit the transcription of STAT3 target genes. Apart from acetylation, STAT3 function can be modulated through other post-translational modifications (PTMs), such as methylation, which reduces STAT3 binding to DNA, and gene transcription. STAT3 can also be methylated in GBM, increasing STAT3 transcriptional activity. Although PTMs are critical for the pleiotropic functions of STAT3, it is unknown whether the crosstalk between STAT3 phosphorylation, acetylation, and methylation modifies GBM tumorigenesis and its therapeutic resistance.

Autophagy is a highly conserved cellular catabolic process that recycles damaged organelles, protein aggregates, and other toxic intracellular debris. Autophagy has a complex and context-dependent role in tumor development and cancer therapy. Although autophagy suppresses primary tumor growth, it is required for advanced tumor growth with elevated metabolic demand and promotes multiple steps in tumorigenesis. Constitutive activation of mTOR signaling impairs basal autophagy in GBM, which enhances proliferation and pluripotency of GSCs. Conversely, restoration of autophagy through mTOR inhibition reduces the invasive potential of GSCs, suggesting that mTOR hyperactivation sustains GSC metabolism through suppressing autophagy. In addition, increased autophagy has been associated with both tumor survival and chemoresistance in GBM, and for the antitumor effects of Temozolomide (TMZ) combined with radiotherapy.

Prominin-1 (Prom1/CD133) is a well-known cancer stem cell biomarker, but little is known about its role in GBM. Recent studies have implicated Prom1-mediated autophagy in cancer cell survival. We have shown that genetic deletion of Prom1 activates both mTORC1
and mTORC2-dependent pathways causing inhibition of autophagy flux in human retinal pigment epithelial (RPE) cells\textsuperscript{40}. Thus, Prom1 may represent a novel molecular target in the regulation of human cancers with defective autophagy and acquired resistance to chemotherapy.

Although these studies underscore the relevance of autophagy in GBM, little is known about the function of STAT3 signaling in regulating autophagy in GBM. Pharmacologic inhibition of either JAK2 (using SAR317461)\textsuperscript{41} or STAT3 (using AG490)\textsuperscript{42} stimulates autophagy in GBM cells. Nuclear STAT3 inhibits autophagy by upregulating anti-autophagy genes and downregulating pro-autophagy genes\textsuperscript{43}. An inverse correlation between phosphorylated STAT3 and a stimulator of autophagy, Beclin1, has also been observed in GBM\textsuperscript{44}. Based on these observations, we sought to define the specific STAT3-dependent signaling mechanisms that modulate autophagy and thereby, impact GBM tumorigenesis and chemosensitivity. Utilizing CRISPR/Cas9 knockout of STAT3 in GBM lines and STAT3 restoration with phosphorylation-defective mutants, we demonstrate that aberrant STAT3 activation suppresses autophagy, which may be exploitable therapeutically.
Results

STAT3 deletion increases autophagy flux

We used CRISPR/Cas9 lentivirus encoding different gRNAs to delete the STAT3 gene in MT330 GBM cells; as a control, cells were transduced with empty vector (EV). Absence of STAT3 protein in MT330 cells was validated in whole cell extracts by immunoblotting with antibodies to STAT3 (Fig. 1A). STAT3 expression was restored in the STAT3 knockout cells by transduction with lentiviral vectors encoding either the WT-STAT3 or the STAT3 mutants (Y705F and S727A). We found that the Y705F mutant protein had higher electromobility compared to S727A mutant and WT-STAT3 in MT330 cells. Our results demonstrate that STAT3 was phosphorylated in EV transduced MT330 cells on both Y705, and Ser727, acetylated on Lys(K)685, and trimethylated on K180 (Figs. 1A-D). While STAT3-KO completely abolished STAT3 phosphorylation, acetylation, and trimethylation, rescue of KO cells with WT-STAT3 significantly increased these post-translational modifications (PTMs) in MT330 cells. Expression of the Y705F mutant in KO cells completely blocked Y705 phosphorylation (Fig. 1B) and significantly decreased S727 phosphorylation (P=0.0363), whereas expression of the S727A mutant significantly blocked S727 phosphorylation (Fig. 1B) but increased Y705 phosphorylation (P=0.0365). Of note, expression of these STAT3 mutants did not significantly alter STAT3 acetylation at K685. However, expression of the Y705F-STAT3 mutant significantly increased STAT3-trimethylation at K180 (Figs. 1C-D). These results demonstrate interdependency of the phosphorylation of Y705 and Ser727 and trimethylation of K180, but independency of the acetylation of K685 in MT330 cells.

To characterize the mechanisms by which STAT3-deficiency induces autophagy in MT330 GBM cells, we examined AMPKα and Unc-51-like kinase 1 (ULK1) phosphorylation
status in STAT3-KO MT330 cells. AMPKα is activated in response to cellular stress \(^{45}\) and activates autophagy either through ULK1 or by impairing mTOR-dependent inhibition of ULK1 \(^{45,46}\). Deletion of STAT3 in MT330 cells significantly increased AMPKα activity in the presence and absence of bafilomycin A1 (Baf), as determined by the ratio of phosphorylated AMPKα (assessed by Thr172 phosphorylation in the catalytic domain) \(^{47}\) to unphosphorylated AMPKα (Figs. 1E-F). Rescue of STAT3-KO cells with WT-STAT3 blocked AMPKα activation. Expression of the Y705F and S727A mutants in STAT3-KO cells had significantly higher levels of AMPKα activation compared to STAT3-KO cells treated with or without Baf (Figs. 1E-F). Since AMPKα activates autophagy through ULK1 phosphorylation at multiple sites, we next assessed ULK1 phosphorylation at S555 and S638 residues (Figs. 1G-H). STAT3 deletion in MT330 cells markedly increased total ULK1 protein expression and phosphorylation at all sites, which were sustained after Baf treatment (Fig. 1G). Our results demonstrate significantly higher levels of phosphorylated ULK1 S555 in STAT3-KO cells compared to EV (Fig. 1H). Given that ULK1 acts as a direct target for both mTORC1 and AMPKα \(^{48}\), these data suggest that ULK1 is a critical mediator of autophagy in GBM cells. Expression of the Y705F mutant construct in KO cells showed a similar effect on phosphorylated ULK1 S555. However, cells expressing the S727A mutant had no significant increase in phosphorylated ULK1 (Fig. 2H) suggesting that S727-STAT3 phosphorylation requires AMPKα but not ULK1 to suppress autophagy. Reconstitution of STAT3 activity in KO cells completely reversed these effects.

Since AMPKα inhibits mTORC1 by phosphorylating and activating TSC2 \(^{49}\), we examined TSC2 phosphorylation in STAT3-KO MT330 cells. We found that KO of STAT3 significantly increased TSC2 phosphorylation on both S1387 and Thr1462 sites, which was reversed with WT-STAT3 expression. STAT3-KO cells treated with or without Baf showed
significantly higher levels of TSC2 phosphorylation (Fig. 1I-J). Expression of Y705F mutant showed a similar effect. By contrast, cells expressing the S727A mutant showed significantly lower levels of phosphorylated TSC2 T1462 but not that of S1387 phosphorylation, demonstrating that phosphorylation on S727 selectively regulates TSC2 T1462 phosphorylation. Thus, AMPKα activation in STAT3-KO cells interacts through multiple pathways to activate autophagy, by directly activating ULK1 and by indirectly impairing mTOR-dependent inhibition of ULK1 through activation of TSC2 S1387 phosphorylation. Since Akt inhibits TSC2 via phosphorylation on T1462, resulting in mTORC1 activation, it is possible that mTORC1 could phosphorylate and inhibit ULK1. This may be counteracted by robust ULK1 phosphorylation at S555 leading to its sustained activity, which is required for maintaining autophagy flux in STAT3-KO cells.

To further assess the impact of STAT3 deletion and rescue of KO cells with STAT3 mutants, MT330 cells were treated with and without an inhibitor of autophagy, Baf. Baf is a specific inhibitor of vacuolar-type ATPase that is known to prevent the fusion of autophagosomes with lysosomes resulting in LC3-II accumulation and blockade of the autophagic flux. Since, autophagy is a dynamic process where microtubule-associated protein light chain-I (LC3-I, precursor) is rapidly converted to lipidated LC3-II, we examined the relative levels of LC3-I and LC3-II in MT330 cells treated with or without Baf. Under basal conditions, LC3-II accumulation was undetectable in control MT330 EV cells and treatment with Baf had no effect on LC3-II accumulation, showing minimal basal autophagy flux (Fig. 2A-B). In contrast, STAT3 deletion in MT330 cells significantly increased basal LC3-II accumulation, which was further potentiated in response to Baf treatment. To demonstrate that induction of autophagy flux in MT330 cells was due to loss of STAT3, KO cells were reconstituted with WT-
STAT3. Rescue with WT STAT3 completely inhibited basal and Baf-induced LC3-II accumulation (Fig. 2A-B). These results demonstrate that KO of STAT3 enhances LC3-II accumulation and autophagy flux, and that the effect is reversed by re-expression of WT-STAT3. Expression of Y705F and S727A phosphorylation-defective mutants also significantly increased basal and Baf-induced LC3-II accumulation as with STAT3-KO cells, providing further evidence for the requirement of functional STAT3 in autophagy suppression. Together, these results show that loss of STAT3 function enhances LC3-II lipidation and autophagic flux through activation of the AMPKα/ULK1/TSC2 signaling axis. These effects are completely reversed by expression of WT-STAT3, but not by its mutants.

The dependence of autophagy on STAT3

The p62 protein is an inverse marker for the induction of autophagic flux and high expression of p62 is found in GBM patient tumors, suggesting constitutive inhibition of autophagy. High p62 levels were also observed in control MT330 (EV) cells, but p62 levels were significantly decreased in STAT3-KO cells consistent with activation of autophagy in these cell lines (Fig. 2A, C). Baf treatment partially increased p62 levels in STAT3-KO cells suggesting impaired autophagosomal degradation. Rescue of KO cells with WT-STAT3 restored p62 expression to that of control EV cells demonstrating the role of STAT3 in down-regulation of autophagy. Expression of Y705F mutant significantly decreased p62 expression, evidence of increased autophagosomal degradation and re-activation of autophagy (Figs. 2A, C). Conversely, expression of the S727 mutant did not significantly decrease p62 expression, suggesting a decline in its autophagosomal degradation. Our results, therefore, strengthen the possibility of differential autophagy regulation by STAT3 mutants, in which the S727A mutant and other key
autophagy molecules function in concert, to induce autophagic activity independent of p62 degradation.

Additional evidence for the activation of autophagy was seen by an increase in the levels of mature heavy chain Cathepsin-D in STAT3-KO cells, which was blocked when KO cells were rescued with WT-STAT3. In cells expressing phosphorylation defective STAT3 mutants, the levels of mature Cathepsin D were higher compared to control EV cells, albeit more in cells expressing the Y705 mutant. Cathepsins are lysosomal proteases that are essential for the breakdown of the recycled cellular components sequestered by the autophagosomes. Increase in the levels of lysosomal proteases upon deletion of STAT3 is consistent with autophagosome-fusion and autophagy activation.

We then used a catalytic mTOR inhibitor, Everolimus (RAD001), to confirm the role of STAT3 in regulating autophagic activity by mTORC1. Treatment of EV MT330 cells with Everolimus inhibited both mTOR S2448 phosphorylation and pS6K phosphorylation (a downstream target of mTORC1) but had no significant effect on autophagy induction (Figs. 2D-E). STAT3 deletion had no effect on basal mTOR S2448 and S6Rbp phosphorylation S235/236 (Fig. 2D) indicating that STAT3 does not regulate mTORC1 activity in MT330 cells. As expected, the mTOR inhibitor, Everolimus, inhibited mTORC1 activity in these cells (Fig. 2D). LC3-II accumulation in STAT3-KO cells significantly increased in response to Everolimus and Baf compared to Everolimus alone, demonstrating that STAT3 deletion and concomitant mTOR inhibition increase autophagy flux (Figs. 2A-B). These effects were reversed in cells reconstituted with WT STAT3. Expression of either Y705F or S727A mutants (as with the STAT3-KO cells) significantly increased LC3-II/actin ratio in the presence of Everolimus and
Baf (Figs. 2D-E). These results demonstrate that Everolimus enhances autophagic flux in cells lacking STAT3 and those expressing STAT3 mutants.

Since Prom1 activates autophagy through inhibiting mTORC1/2 activity, we examined Prom1 expression in MT330 cells. STAT3-KO increased Prom1 expression, which was reversed by rescue with WT-STAT3 (Fig. 2F). Expression of Y705F and S727 mutants in STAT3-KO MT330 cells also showed increased Prom1 expression, which correlated with high basal autophagy and flux (Fig. 2F). These results suggest that the induction of autophagy in cells lacking STAT3 is in part dependent on Prom1 expression. Reconstitution of WT-STAT3 in these cells completely reversed these effects confirming STAT3-mediated suppression of autophagy.

Transcriptional regulation of autophagy

Since STAT3-KO increased ULK1 expression and decreased p62 expression, we next performed qPCR on several STAT3 target genes, among which BNIP3 and ULK1 are autophagy-related genes. Our data show that STAT3 increases expression of BNIP3 and p62 genes in MT330 cells (Fig. 2G). Consistent with these observations, STAT3-KO reduces expression of these genes and both Y705 and S727 residues are necessary for expression of these genes, demonstrating that cells expressing STAT3 mutants behave like STAT3-KO cells. Furthermore, STAT3 reduces expression of ULK1 gene and STAT3 phosphorylation on both Y705 and S727 residues are necessary for its repression (Fig. 2G). Together, these results demonstrate that STAT3 regulates autophagy in MT330 cells in part through transcriptional regulation of p62, ULK1, and BNIP3.

STAT3-deletion increases autophagic flux in LN229 cells

To validate the role of STAT3 in GBM autophagy regulation, we knocked out STAT3 in another GBM cell line, LN229 cells, by CAS/CRISPR gene editing. Absence of STAT3 protein
in LN229 cells was validated in whole cell extracts by immunoblotting with antibodies to STAT3 (Fig. 3A). STAT3 expression was restored in the STAT3 knockout (KO) cells by transduction with lentiviral vectors encoding either the wild-type (WT) STAT3 or the STAT3 mutants (Y705F and S727A). Similar to MT330 cells, we found that the Y705F mutant protein had slightly higher electromobility compared to S727A mutant and WT-STAT3. Our results demonstrate that STAT3 was phosphorylated on both Y705 and S727 sites and was acetylated on K685 in EV-transduced LN229 cells (Figs. 3A-B). Rescue with WT-STAT3 lentivirus in KO cells restored STAT3 expression and its phosphorylation on both Y705 and S727 residues, and acetylation on K685. Expression of the Y705F mutant in LN229-KO cells eliminated Y705 phosphorylation and significantly decreased S727 phosphorylation (P=0.0029), which was increased in the presence of bafilomycin (P=0.0028) (Figs. 3A-B). Likewise, expression of the S727A mutant in STAT3-KO cells resulted in significantly higher levels of Y705 phosphorylation in the absence of bafilomycin (P=0.0076), without any significant change in the presence of bafilomycin (Figs. 3A-B). However, acetylation of STAT3 at K685 did not significantly change in cells expressing Y705F and S727A mutants. Together, these results indicate that phosphorylation at Y705 and S727 sites are interdependent in LN229 cells.

To validate our finding on the role of STAT3 in autophagy, LN229 cells were treated with or without Baf and examined for the relative levels of LC3-I and LC3-II. While basal levels of LC3-I and LC3-II were low in LN229 cells and increased in response to Baf treatment, deletion of STAT3 significantly increased both basal and Baf-induced accumulation of LC3-I and LC3-II levels when compared to EV cells, demonstrating constitutive autophagic flux (Fig.3C-D). Furthermore, rescue with WT STAT3 reduced the levels of LC3-I and LC3-II in the presence and absence of Baf similar to that of EV cells, confirming that STAT3 blocks
autophagy in LN229 cells. Since both Y705 and S727 sites are required for STAT3 function, we expressed STAT3 mutants in STAT3-KO LN229 cells and studied LC3-II/LC3-I accumulation in response to Baf. As shown in Figs. 3C-D, Baf robustly increased LC3-II accumulation in cells expressing the Y705F and S727A mutants similar to STAT3-KO cells. These results suggest that both STAT3 phosphorylation events are necessary for suppressing basal autophagic flux in GBM cells.

Deletion of STAT3 in LN229 cells increased AMPKα activity (assessed by T172 phosphorylation in the catalytic domain) \(^47\), without altering total AMPKα protein levels in the presence and absence of Baf (Fig. 3E) indicating higher AMPKα activation (Fig. 3F). Expression of the Y705F mutant in STAT3-KO cells also showed AMPKα activation. However, expression of S727A mutant in KO cells and treatment with or without Baf had no effect on AMPKα phosphorylation compared to KO and Y705F expressing lines suggesting that this mutant is incapable of activating AMPKα signaling.

We next examined changes in mTOR phosphorylation in LN229 cells. The mTOR protein and its phosphorylation status at S2481 were unaltered in the KO and lines expressing STAT3 phosphorylation-inactive mutants (Fig. 3G). Furthermore, expression of S6 ribosomal protein (S6Rbp) and its phosphorylation at S235/S236 were also unaltered in KO cells and cells expressing STAT3 mutants. Therefore, autophagy induction in LN229 cells either lacking STAT3 or expressing these STAT3 mutants depends upon AMPKα and ULK1 but is independent of the mTOR pathway.

**Immunolocalization of LC3 and p62**
To confirm autophagy induction in MT330 cells, we performed immunolocalization studies to detect LC3 and p62 puncta formation. Under basal conditions, we observed diffuse LC3 (green) and p62 (red) staining predominantly in the cytoplasm with some nuclear staining (Fig. 4). Treatment of EV cells with a low dose of Baf (1μM) for 24-48h had no effect on LC3 and p62 staining (Fig. 4). These observations are consistent with our data presented in Fig. 2 and demonstrate impaired autophagy in MT330 cells. STAT3-KO cells without bafilomycin treatment showed LC3 and p62 puncta formation. Treatment of STAT3-KO cells with bafilomycin for 24h robustly increased LC3+ and p62 puncta formation and their co-localization, which increased after 48h. Reconstitution of WT-STAT3 in STAT3-deleted cells completely reversed these effects. These observations demonstrate cellular induction of autophagy flux in response to STAT3 deletion.

**Targeting of ULK1 blocks autophagy and induces apoptosis in STAT3-KO MT330 cells**

Because STAT3-KO in MT330 cells induces autophagy flux and concomitantly activates AMPKα/ULK1 signaling, we tested whether pharmacologic inhibition of ULK1 attenuates autophagy. Previous studies have shown that pharmacologic inhibition of ULK1 and ULK2 with MTR68921 blocks autophagy flux \(^{54,55}\), demonstrating the importance of ULK kinase activity in autophagy. Since activated ULK1 phosphorylates ATG14 on S29 and BECN1 on S30 for autophagy \(^{56}\), we measured ATG14 and BECN1 phosphorylation in response to MRT68921 treatment. Our results show that STAT3-KO cells and cells expressing STAT3-mutants contained significantly elevated levels of ATG14 S29 phosphorylation and Beclin1 S30 phosphorylation (Figs. 5A, B). Of note, the phospho-Beclin1 S30 antibody recognized a band with high electrophoretic mobility in STAT3-KO and STAT3 mutant expressing lines (Fig. 5A), which is consistent with our earlier observations with LN229 cells (Fig. 1G) demonstrating
Beclin1 PTMs. The ULK1 inhibitor, MRT68921, completely blocked ATG14 S29 phosphorylation demonstrating the efficacy of this inhibitor in targeting ULK1 and showing that constitutive activation of ULK1 in STAT3-KO and STAT3-mutant expressing lines leads to increased ATG14 phosphorylation on S29 (Fig. 5B). Similarly, MRT68921, significantly reduced Beclin1 phosphorylation in cell lines treated with or without Baf (Fig. 5C), demonstrating that ULK1 regulates Beclin1 activation in STAT3-KO and STAT3-mutant expressing lines. Importantly, MRT68921 significantly inhibited the high levels of ULK1 phosphorylation in STAT3-KO cells and cells expressing STAT3 mutants (Fig. 5D) confirming that Atg14 and Beclin1 are downstream targets of ULK1 signaling in MT330 cells. MRT68921 did not significantly block mTOR S2448 phosphorylation (Fig. 5A). Since mTORC1 contains mTOR phosphorylated predominantly on S2448, these observations suggest that ULK1 inhibition has no significant effect on mTORC1.

MRT68921 did not show any additional effect in EV and WT-STAT3 expressing cells, in which autophagy is inhibited. MRT68921 significantly increased LC3-II accumulation in STAT3-KO cells and in STAT3-KO cells transduced with the Y705F or S727A mutants (Figs. 5A, E). Given that ULK1 inhibition disrupts autophagosome maturation downstream of LC3 conjugation 57, this increase in LC3-II in MRT68921-treated cells could also be interpreted as a decrease of LC3 autolysosomal degradation. Treatment of cells with MRT68921 and Baf failed to increase LC3-II accumulation compared to DMSO-treated controls, suggesting inhibition of autophagy flux. Taken together these results strongly suggest that MRT68921 blocks autophagy flux in cells lacking STAT3 function.

To further elucidate the role of ULK1 inhibition in MT330 cells, we determined whether apoptosis occurred after MRT68921 treatment. We found that caspase-3 was not activated in
DMSO treated control cells. However, MRT68921 was capable of inducing apoptosis in STAT3-KO and STAT3-mutant expressing lines but not in control EV and cells reconstituted with WT-STAT3 (Fig. 5A). Of note, STAT3-KO cells expressing the S727A-STAT3 mutant showed remarkably high levels of active caspase-3 suggesting increased sensitivity to MRT68921-induced apoptosis. In STAT3-KO and STAT3-mutant expressing cells treated with MRT68921 plus Baf, the level of active caspase-3 remained unchanged. These observations demonstrate that autophagy can have a cytoprotective role in STAT3-KO and STAT3-mutant expressing lines, but cell killing was rapidly induced by the addition of the ULK1-dependent autophagy inhibitor MRT6892, suggesting an opportunity for combined therapy.

To further confirm the role of ULK1 in autophagy regulation, we used siRNA to knockdown ULK1 in MT330 cells. Consistent with our data presented in Fig. 2, STAT3-deleted MT330 cells and STAT3-mutant expressing lines contained very high levels of ULK1 expression and phosphorylated ULK1. We found that control scrambled siRNA had no effect on ULK1 expression but ULK1-specific siRNA significantly decreased ULK1 expression in STAT3-KO cells and cells expressing both Y705F and S727A mutants and treated with or without Baf (Figs. 6A-B). As expected, LC3-II accumulation did not increase in cells treated with ULK1 siRNA but significantly decreased in cells treated with ULK1-siRNA plus Baf (Figs. 6A, C). However, LC3-II levels remained unchanged in cells expressing the S727A mutant and treated with ULK1-siRNA plus Baf (Fig. 6C). These observations demonstrate impaired autophagosomal biogenesis and reduced autophagy flux in response to ULK1 knockdown. In summary, these studies indicate that autophagy induction through combining STAT3 and ULK1 inhibition may be therapeutically beneficial for autophagy defective GBM cells.
To investigate whether ULK1 knockdown induces apoptosis, we measured caspase-3 activation. Similar to our observations involving pharmacologic inhibition of ULK1 by MRT68921 (Fig. 5), our data show that ULK1 knockdown robustly increases caspase-3 activation in STAT3-KO cells and cells expressing both phosphorylation-defective mutants in the presence and absence of Baf (Figs. 6A, D). However, the extent of caspase-3 activation was considerably higher in cells expressing the S727A-STAT3 mutant treated with ULK1-siRNA. These observations show that a potential effective combination strategy of dual inhibition of STAT3 and ULK1 activities may promote apoptosis and have a synergistic cytotoxic effect in GBM cells.
Discussion

A key finding of these studies is that constitutive STAT3 phosphorylation represses autophagy flux in GBM cells. KO of STAT3 and the expression of STAT3 phosphorylation-defective mutants in GBM cells increase autophagic activity via upregulation of p62/SQSTM1 degradation, Prom1 expression, LC3 conversion, and lysosomal activity. Loss of STAT3 functional activity increases phosphorylation of AMPKα, TSC2, and ULK1 on multiple sites, all of which were reversed by expression of ectopic WT-STAT3 in STAT3-KO GBM cells. Expression of the Y705F and S727A mutants in STAT3-KO LN229 and MT330 cells robustly increases autophagy flux, confirming that fully functional STAT3 is indispensable for suppressing autophagy in GBM cells.

The role of autophagy in cancer is controversial because it has been reported to both promote and inhibit tumorigenesis \[^{58,59}\]. Autophagy confers drug resistance to radiotherapy and chemotherapy but also slows tumor progression \[^{60}\]. Defective autophagy has been implicated in maintaining the oncogenic properties of GSCs, and autophagy induction through mTOR inhibition suppresses GSC self-renewal and tumorigenicity \[^{61}\]. In contrast, rapid tumor growth in GBM and insufficient nutrient supply from the vasculature contribute to activating autophagy and desensitization of tumor cells to chemotherapy \[^{61}\]. We found that basal autophagy was defective in MT330 cells and STAT3-KO activated autophagy without altering mTOR activity. Inhibition of mTOR in STAT3-KO cells potentiated autophagic flux suggesting that combining STAT3 and mTOR inhibitors may improve GBM responses to treatment. Given these complex relationships, a better understanding of autophagy induction in response to STAT3 inhibition will benefit our understanding of GBM chemoresistance.
STAT3 undergoes various PTMs that act as a molecular switch governing STAT3 activation and localization. Besides its well-known Y705 phosphorylation, STAT3 is phosphorylated on S727, which regulates its mitochondrial localization. The Y705F mutant is used frequently to understand STAT3 functions that depend on tyrosine phosphorylation. Our data demonstrate that the Y705F mutant had slightly higher electromobility compared to WT-STAT3 and S727A mutant. Cells expressing the Y705F mutant are viable and proliferate albeit at a slower rate compared to cells expressing WT-STAT3. Other studies have demonstrated that the STAT3-Y705F mutant can form dimers and the preformed unphosphorylated dimers were present in both stimulated and unstimulated cells. Other STAT3 PTMs include acetylation on K685 by CBP/p300, S-glutathionylation by intracellular oxidative stress, and trimethylation by EZH2. We found that STAT3 is constitutively phosphorylated on Y705 and S727 residues, acetylated on K685, and trimethylated on K180, but these modifications were interdependent upon each other in GBM cell lines. In contrast, STAT3-S727 phosphorylation has been shown to be dependent on STAT3-Y705 phosphorylation in GSCs, but Y705 phosphorylation was independent of S727 phosphorylation, suggesting that STAT3 phosphorylation occurs sequentially in GSCs, but not in differentiated GBM cells. Analysis of how STAT3’s acetylation and trimethylation regulate gene transcription, autophagy pathways, and GBM tumorigenesis will be the subject of future investigation.

STAT3 regulates autophagy through several mechanisms. Nuclear STAT3 regulates autophagy through the transcriptional regulation of pro-autophagy genes such as Beclin1 (BECN1) and anti- or pro-autophagy modulating microRNAs. Our qPCR analyses of STAT3-targets involved in autophagy demonstrate that STAT3 inhibits ULK1 but activates expression of p62 and BNIP3 genes. The BH3 domain containing protein, BNIP3, is regulated
by STAT3 phosphorylation. BNIP3 expression was linked with induction of autophagy and required upregulation of concanavalin-induced JAK2/STAT3 signaling in GBM cells. By contrast, our data demonstrate that STAT3-deletion and expression of the phosphorylation-inactive STAT3 mutants significantly decreased BNIP3 gene expression showing that BNIP3 downregulation correlates with autophagy induction in GBM cells. These differences support the notion that BNIP3 plays diverse roles in GBM autophagy regulation and these roles may be stimuli dependent.

Since enhanced autophagy results in p62 degradation, we expected that induction of autophagy flux and co-localization of LC3+ puncta with p62 upon STAT3 deletion would lead to enhanced degradation of p62. Supporting this, STAT3-KO and Y705 mutant expressing cells reduced p62 protein expression by activation of autophagy flux. The failure of control and WT-STAT3 to eliminate p62 accumulation confirmed deficient autophagy and impaired autolysosomal degradation in GBM cells. In addition, our results showed for the first time that p62 gene expression was significantly downregulated in these lines demonstrating that p62 is transcriptionally regulated by STAT3. Reconstitution of STAT3-KO cells with WT-STAT3 restored p62 gene expression confirming that STAT3 regulates p62 at the transcriptional level.

AMPKα maintains energy homeostasis and plays an important role in autophagy induction. AMPKα negatively regulates mTORC1 through TSC2, activates ULK1 Ser555 and Beclin1 Thr388 phosphorylation, all of which initiate autophagy. ULK1 plays a central role in autophagy by promoting fusion of autophagosomes with lysosomes and phosphorylating multiple autophagy-related targets including Beclin1 and ATG101. Our data show that the AMPKα/ULK1 signaling axis is a key regulator of STAT3-dependent autophagy in GBM cells. AMPKα and ULK1 phosphorylation was low in control EV cells, but was markedly increased in
both STAT3-KO MT330 cells and cells expressing STAT3 mutants. Rescue of KO cells with WT-STAT3 abolished AMPKα T172 phosphorylation confirming STAT3’s role in repressing AMPKα signaling in both LN229 and MT330 GBM cell lines. Control EV MT330 cells were resistant to autophagy induction in response to mTOR-inhibition (using Everolimus). These results demonstrate that the attenuating effect of STAT3 on autophagy induction primarily depends on Y705 phosphorylation and its ability to inhibit AMPKα signaling pathway. Furthermore, our results show STAT3 is a novel suppressor of ULK1 in MT330 cells. Knockout of STAT3 dramatically increases ULK1 protein and gene expression in MT330 cells, which were blocked by expression of WT-STAT3. This is consistent with earlier studies showing STAT3 as a transcriptional suppressor of autophagy through inhibition of ULK1 protein and mRNA levels. Our studies highlight the function of various phosphorylation-inactive STAT3 mutants in GBM lines and our data unambiguously demonstrate that Y705F and S727A mutants differentially regulate AMPKα and ULK1 signaling to activate autophagy. In this context, our data suggest the presence of cellular and molecular heterogeneity between LN229 and MT330 GBM lines. This may account for the observed variability in AMPK signaling in response to STAT3 deletion and expression of STAT3 mutants.

AMPKα activation inhibits mTORC1, which leads to autophagy activation. In addition to regulating mTORC1, AMPKα activation inhibits tumor cell growth by phosphorylating TSC2 on S1387, which in turn inhibits mTORC1 leading to autophagy activation. Our data demonstrate that cells lacking STAT3 and STAT3-KO cells expressing STAT3 mutants have higher amounts of TSC2 T1462 and S1387 phosphorylation. The regulation of TSC2 and mTORC1 by AMPKα has special implications in autophagy regulation. While TSC2 T1462 phosphorylation inhibits its activity leading to mTORC1 activation, AMPKα inhibits mTORC1 at least in part by
phosphorylating and activating TSC2 on S1387. ULK1 S555 phosphorylation is mediated through AMPKα and indicates autophagy activation, whereas other ULK1 sites are targeted by mTOR for autophagy inhibition. Conversely, ULK1 inhibits the kinase activity of mTORC1 to stimulate autophagy. Our findings that AMPKα is activated in STAT3-KO and STAT3-KO GBM cells expressing phosphorylation-defective STAT3 mutants suggest that AMPKα associates with, and directly phosphorylates ULK1 on several sites and this modification is required for maintaining sustained ULK1 activation. This mechanism of ULK1 activation is sufficient to inhibit mTORC1 and activate autophagy. Currently, we do not know how STAT3 regulates AMPKα signaling, but our data suggest that activated AMPKα is able to induce autophagy in STAT3-KO cells through coordination with TSC2-mTORC1 to directly activate ULK1.

Because STAT3 represses autophagy through inhibition of AMPKα/ULK1 signaling, we expected that inhibition of ULK1 signaling by MRT68921 would reduce autophagy in STAT3-KO cells. Our results show that pharmacologic intervention of ULK1 activity by MRT68921 or ULK1 protein knockdown by siRNA decreased autophagy flux and sensitized STAT3-KO and mutant expressing lines to caspase-3-dependent apoptosis. These results demonstrate that autophagy is cytoprotective in STAT3-KO cells and approaches aimed at inhibition of ULK1 cause inhibition of autophagy through inhibition of ULK1-Atg14-Beclin1 signaling and consequently lead to GBM cell death. To our knowledge, this is the first example of the involvement of ULK1 signaling in the regulation of STAT3-dependent autophagy/apoptosis and highlight the potential of targeting of both STAT3 and ULK1 as an applicable therapeutic approach for GBM treatment.
Based on our data, we propose a conceptual model (Fig. 7, left) by which STAT3 represses autophagy and promotes GBM tumorigenesis. STAT3’s PTMs are responsible for the inhibition of autophagy flux in GBM cells. STAT3 PTMs inhibit AMPKα/ULK1 signaling in GBM cells, which in turn causes inhibition of autophagy. This promotes GBM tumor formation and increases chemoresistance. Our data indicate that both Y705 and S727 phosphorylation are essential for autophagy suppression, and Y705 plays a predominant role in the inhibition of AMPKα/ULK1 signaling. Deletion of STAT3 decreases p62 protein and gene expression levels, increases AMPKα activity, and ULK1 gene expression and its activity (Fig. 7, right). Activated AMPKα phosphorylates TSC2, which inhibits mTORC1 activity leading to induction of autophagy. Because STAT3 deletion activates Akt, which inhibits TCS2 via phosphorylation, resulting in mTORC1 activation, we speculate that activation of mTORC1 could phosphorylate and inhibit ULK1. This is counteracted by extensive ULK1 phosphorylation at multiple sites favoring its association with AMPKα. As a result, AMPKα directly phosphorylates ULK1 leading to sustained mTORC1 inhibition required for maintaining autophagy flux in STAT3-KO cells.

STAT3 deletion also increases Prom1-mediated autophagy in GBM cells, through negative regulation of mTORC1 as we found in retinal pigment epithelial (RPE) cells. These molecular events enhance autophagy flux and reduce GBM tumorigenesis. Pharmacologic disruption of mTOR-ULK1 signaling in STAT3-KO cells validates the conceptual model described above. Pharmacologic inhibition of mTORC1 by Everolimus stimulates autophagy flux, whereas inhibition of ULK1 (by MTY68921 and siRNA) inhibits autophagy and induces apoptosis in STAT3-KO cells. Together, these studies demonstrate that STAT3-dependent suppression of autophagy is an essential contributor to GBM biology and that restoration of
autophagy by a combined approach through STAT3 inhibition and mTOR inhibitors may be a novel approach for treatment and overcome chemoresistance in GBM.
Materials and Methods

Reagents

Materials purchased include: Fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA); Enhanced chemiluminescence (ECL) western blot detection system (Perkin Elmer, Inc., Boston, MA); Protease/Phosphatase Inhibitor Cocktail, cleaved active caspase-3 (Asp 175) (Cat# 9664); LC3-I/LC3-II (Cat# 12741); SQSTM1/p62 (Cat# 88588), p-Akt Ser473 (Cat# 4060), HDAC-6 (Cat# 7612), phospho-S6 Ribosomal protein Ser235/236 (Cat# 2211), p-STAT3 Y705 (Cat# 9145), p-STAT3 Ser727 (Cat# 9134), Acetyl-STAT3 Lys685 (Cat# 2523), total-STAT3 (Cat# 9139), p-AMPKα Thr172 (Cat# 2535), Total-AMPKα (Cat# 2793), p-ULK1 Ser757 (Cat# 14202), p-ULK1 Ser555 (Cat# 5869), p-ULK1 Ser638 (Cat# 14205), Total-ULK1 (Cat# 8054), p-TSC2 Ser1387 (Cat# 23402), p-TSC2 Ser1462 (Cat# 3617), Total-Tuberin/TSC2 (Cat# 3990), Beclin-1 (Cat# 3738), and Cathepsin-D antibodies (Cat # 2284) (Cell Signaling Technology, Inc., Beverly, MA); Alexa-Fluor 488 conjugated, Alexa-Fluor 647, and Cy3 conjugated secondary antibodies (Molecular Probes, Eugene, OR); Anti-trimethyl STAT3 Lys180 (Cat # ABE1397), Bafilomycin A1, and Torin-1 (EMD Biosciences/Millipore Corp., Billercia, MA); Temozolomide and ULK1 Inhibitor (MRT68921) (MedChemExpress, Monmouth Junction, NJ). The ULK1 siRNA and transfection reagent were obtained from Santa Cruz Biotechnology (Dallas, TX). All chemicals were of the highest purity commercially available.

Cell Culture

MT330 (UTHSC, Department of Neurosurgery) and LN229 (ATCC CRL-2611) were grown in DMEM containing high glucose, containing 10% fetal bovine serum, and supplemented with 1X antibiotic-antimycotic solution (Gibco, Thermo Fisher Scientific,
Waltham, MA) at 37°C with 5% CO₂, as described previously.  

CRISPR/Cas9-mediated genomic deletion of STAT3 in MT330 and LN229 cells, and the expression of phosphorylation-defective STAT3 mutants

STAT3 was knocked out in both LN229 and MT330 cells as described previously. The STAT3 sgRNA lentiviral set for STAT3 knockout (KO) was commercially obtained from ABM (Richmond, Canada). The E-GFP gRNA sequences were inserted into the pLenti CRISPR V2 lentiviral vector to create the control vector. The gRNA sequences used for creating the lentiviral vectors were selected from the Human GeCKOv2 CRISPR knockout library. Lentivirus was produced by packaging in 293FT cells using protocols described previously. Both MT330 and LN229 cells were transduced with the lentiviral CRISPR/Cas9 vectors to generate stable pools of STAT3 KO cells and selected using 5 μg/ml Puromycin for 48h. After puromycin selection, pools of STAT3 KO cells were maintained without puromycin, and STAT3 deletion validated by western blotting and qPCR. After confirmation of STAT3-deletion, cells were expanded and subsequently used for autophagy experiments.

The constructs for wild-type (WT), Y705F-STAT3, and S727A-STAT3 mutants were cloned into lentiviral vectors with bidirectional promoters driving expression of puromycin resistance and E-GFP (Systems Biosciences, Palo Alto, CA), as described earlier. STAT3-KO cells were subjected to multiple rounds of transduction with lentivirus encoding either WT-STAT3, Y705F or S727A STAT3 mutants until stable cell lines with similar levels of STAT3 protein expression to that of parental GBM cells were isolated as determined by western blotting.

Western blotting

Cell lysates were prepared using mammalian protein extraction buffer (Cell Signaling Technology, Beverly, MA) with 150mM NaCl, 1mM Na₂EDTA and a protease inhibitor cocktail
followed by SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore Bedford, MA, USA) and probed with primary antibodies overnight at 4°C in TBS buffer containing 0.1% Tween-20 and 5% nonfat dry milk (Bio-Rad, Hercules, CA). Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1h and the immunocomplexes were visualized by the ECL detection system (Perkin Elmer, Waltham, MA) quantified on the Azure Biosystems C500 (Dublin, CA). Membranes were stripped and re-probed for actin or GAPDH as loading controls. Representative western blots from three experiments are shown. Densitometric analysis of all western blots was performed using Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html).

**Immunoprecipitation**

GBM cells were rinsed with ice cold PBS and lysed using a cell lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) containing protease and phosphatase inhibitors (Thermo Fisher Scientific, Waltham, MA). The lysates were clarified by centrifugation at 12,500 rpm for 15 minutes at 4°C. The cell extracts containing equal amounts of protein were incubated with STAT3 antibody overnight at 4°C followed by addition of protein A/G agarose beads (Santa Cruz Biotechnology, Dallas, TX) with gentle rocking for 2 hrs. The beads were washed 3 times with lysis buffer and once with PBS, and the immunocomplexes were released by heating in Laemmli sample buffer and analyzed by Western blotting using trimethyl-STAT3 antibody (EMD Biosciences/Millipore Corp., Billerica, MA).

**Immunofluorescence and confocal microscopy**

Cells were cultured in chamber slides (Millipore) to ~70% confluence and washed with PBS. Cells were fixed in 4% paraformaldehyde and methanol, and permeabilized with 1% Triton
X-100. After blocking with 5% goat serum, cells were incubated with anti-rabbit LC3 and anti-mouse p62 antibodies and subsequently stained with Alexa Fluor 488 (goat anti-rabbit) and Alexa Fluor 633 (goat anti-mouse) secondary antibodies, as described previously. DNA was counterstained with Vectashield mounting media with DAPI (Vectra Laboratories). Images were captured on a Zeiss LSM700 laser scanning confocal microscope.

**siRNA transfection**

MT330 cells were grown to 60-70% confluency in 6-well tissue culture plates and siRNA transfection was performed using a protocol available from Santa Cruz Biotechnology. For ULK1 siRNA transfection, the cell monolayer was washed with siRNA transfection medium (Santa Cruz) and the siRNA/transfection reagent mixture (8μl siRNA duplex containing 80 pmols siRNA: 8μl siRNA transfection reagent) was added dropwise on to the cell monolayer and incubated overnight at 37°C in a CO2 incubator. The following day complete growth medium containing 2 times the normal serum and antibiotics was added without removing the transfection mixture. After an additional incubation for 18-24h, the medium was aspirated and replaced with fresh 1X growth medium. After another 24h of incubation, cells were treated with or without 100nM Baf and assayed for autophagy and apoptosis markers. Efficiency of transfection was monitored using FITC-conjugated control siRNA.

**Real-time Quantitative PCR**

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) was used to extract total-RNA from control MT330 EV, STAT3-KO, and cells expressing phosphorylation-inactive STAT3 mutants. Total RNA concentrations were quantified by measuring A260 and A280 using NanoDrop spectrophotometry. Total-RNA (1mg) was reverse-transcribed to cDNA using a kit
from Promega (Madison, WT) and following manufacturer’s instructions. The cDNA was
diluted 1:5 with DNase-free water. Real-time qPCR was performed using an Ariamx Real-Time
PCR system (Agilent Technologies, Santa Clara, CA) with 2.5ml of the cDNA product in a 25ml
reaction mixture containing 1X SYBR® Green Master Mix (Applied Biosystems, Foster City,
CA) and 120nM forward and reverse primers. The primers used for human ULK1 forward (5’-
GGGCAAGTTTCAGTCTCC- 3’), reverse (5’- GCCATTTTCTGGAAAGTCGTA- 3’); BNIP3
forward (5’- CGCAGCTGAAGCACATCC- 3’), reverse (5’ –
CTTGGAGCTACTCCGTCCAG- 3’); p62 forward (5’-GCCTCTGGTTCTGACACTTT-3’),
reverse (5’-GGTGAAGGTGAAGGCATT-3); beta actin forward (5’-
ACCTTCTACAATGAGCTGCG- 3’) and reverse (5’- CCTGGATAGCAACGTACATGG- 3’)
sequences were used. The qPCR conditions were 50°C for 2min, 95°C for 10min, followed by 40
cycles of 95°C for 15sec and 60°C for 1min, as described previously. Each reaction was
performed in triplicate. Samples were analyzed using the comparative ΔCT method. The CT
values of all genes were normalized with beta-actin to calculate relative gene expression.

Statistical analysis

All data were analyzed by GraphPad Prism 9 program (GraphPad Software Inc., San
Diego, CA) and an unpaired 2-tailed Student’s t test was used to assess statistical significance.
Data are expressed as mean ± SE. Experiments were repeated three times, with triplicate samples
for each. Unless otherwise stated, values of *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001
were considered significant.
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Competing Interests

Authors declare they have no competing interests

Author contributions

S.B., E.C., L.M.P., designed research; and S.B., J.Y., C.Y., Y. W., and M.S. performed research; S.B., E.C., and L.M.P. analyzed data; and S.B., E.C., and L.M.P. wrote the paper. All authors reviewed the manuscript.

Data availability

The datasets and STAT3-KO and mutant expressing GBM cells generated during and/or analysed during the current study are available from the corresponding author on reasonable request.
References


FIGURE LEGENDS

**Fig. 1. Genomic deletion of STAT3 activates autophagy flux through AMPKα-ULK1-TSC2 signaling pathways in MT330 cells.** (A) Confluent EV MT330 cells, STAT3-knockout cell line # 2 (KO2), STAT3-knockout cell line # 3 (KO3), STAT3-KO3 rescued with wild-type (WT), and STAT3-KO3 cells expressing Y705F-STAT3 and S727A-STAT3 mutants were exposed to Bafilomycin (Baf, 100nM) for 3h. Untreated (UT) cells served as controls. Total cell lysates were prepared and immunoblotted with indicated antibodies. (B) Quantification of the ratio of phospho-STAT3, acetyl-STAT3, and total-STAT3 from three independent experiments. (C) Cell lysates were also immunoprecipitated (IP) with STAT3 and immunoprecipitates were western blotted (WB) with Tri-methyl Lys180 STAT3. Membranes were stripped and probed for total-STAT3. (D) Quantification of Trimethyl- to total-STAT3. (E) Cell lysates were analyzed for p-AMPKα T172. Blots were stripped and probed for total-AMPKα. (F) Quantification of the ratio of phosphorylated and total AMPKα shown in E. (G) Cell lysates were analyzed for p-ULK1 S555 and S638. Blots were stripped and probed for total-ULK1. (H) Quantification of the ratio of phosho-ULK1 S555 and total-ULK1; ns, not significant. (I) Western blotting of cell lysates with phospho-TSC2 antibodies. Blots were stripped and probed with total-TSC2 antibody. (J) Quantification of the ratio of phospho-T1462- and total-TSC2 and phospho-S1387 and total-TSC2.

**Fig. 2. STAT3-KO activates autophagy through mTOR-independent but Prom1-dependent signaling pathways in MT330 cells.** (A) EV MT330 cells, STAT3-KO, STAT3-KO rescued with WT-STAT3, and STAT3-KO cells expressing Y705F (Y) and S727A (S) STAT3 mutants were grown to confluence and treated with or without 100nM bafilomycin for 3h. Cell lysates were analyzed for LC3-I/II, p62, and CathepsinD. Quantification of (B) LC3-II/actin ratio and
(C) p62/actin ratio. (D) DMSO, Everolimus (RAD001) (10µM), and RAD001 (10µM) +Baf (100nM) for 3h. Cell lysates were analyzed for LC3-I/II, phospho-S6 Ribosomal protein (Rpb) S235/236, and β-actin was used as an internal loading control. (E) Quantification of LC3-II/actin ratio presented in D. (F) EV, STAT3-KO cells, KO3 rescued with WT-STAT3, and KO3 cells expressing Y705F and S727A mutants were grown to confluence and treated with Baf (100nM) for 3h. Cell lysates were analyzed by western blotting using antibodies specific for STAT3, Prom1, and LC3-I/II. (G) Relative gene expression levels of BNIP3, ULK1, and p62 genes. Values of P<0.05 were considered significant.

**Fig.3. STAT3 represses autophagy flux in LN229 cells.** (A) EV LN229 cells, STAT3-knockout (KO), STAT3-KO cells, STAT3-KO cells rescued with WT STAT3, and STAT3-KO cells expressing Y705F-STAT3 and S727A-STAT3 phosphorylation-inactive mutants, were grown to 80% confluency and exposed to Bafilomycin (Baf, 100nM) for 3h or left untreated (UT). Total cell lysates were prepared and immunoblotted with indicated antibodies with β-actin serving as a loading control. (B) Densitometric analysis from n=3 observations of the ratio of phosphorylated and acetylated STAT3 to total-STAT3 shown in A. (C) EV, KO and STAT3 mutant expressing lines with treated with or without 100nM bafilomycin for 3h. Cell lysates were immunoblotted for LC3-I/II with actin as a loading control. (D) Quantification of data shown in C. (E) Cell lysates were analyzed for p-AMPKα Thr172. Blots were stripped and probed for total-AMPKα. (F) Quantification of the ratio of phosphorylated and total AMPKα shown in E. (G) Cell lysates were immunoblotted with the indicated antibodies.

**Fig. 4. Immunolocalization of LC3 and p62 in MT330 cells.** EV MT330 cells, STAT3-KO, STAT3-KO cells rescued with WT-STAT3, were grown on chamber slides, and treated with or
without Baf for 24-48h. Cells were fixed and immunostained for LC3 (green), p62 (red), and counterstained with DAPI (blue), and analyzed by confocal microscopy. Scale bar 20μM.

Fig. 5. Pharmacologic inhibition of ULK1 activity inhibits autophagy and induces apoptosis in STAT3-KO MT330 cells. (A) MT330 EV, STAT3-KO, WT, Y705F-STAT3 (Y), and S727A-STAT3 (S) mutant expressing cells were treated with the ULK1 inhibitor, MRT68921 (20μM), in the presence or absence of Baf (100nM) for 3h. Total cell lysates were immunoblotted with indicated antibodies with β-actin serving as a loading control. Quantification of the ratio of (B) phospho- and total-ATG14; (C) phospho- and total-Beclin1; (D) phospho- and total-ULK1; (E) LC3-II and actin ratios.

Fig. 6. Knockdown of ULK1 expression blocks autophagy and induces apoptosis in STAT3-KO and STAT3-mutant expressing lines. (A) MT330 EV, STAT3-KO, WT, Y705F, and S727A mutant expressing cells were grown to confluence and treated with control or ULK1 siRNA followed by treatment with or without Baf for 3h. ULK1 knockdown was verified by western blotting with ULK1 and phospho-ULK1 and cell lysates were analyzed for LC3, cleaved caspase-3 with β-actin as a loading control. Quantification of the ratio of (C) total-ULK1 and actin; (D) LC3-II and actin; (E) active caspase-3 and actin.

Fig. 7. Schematic showing the molecular cross talk regulating STAT3-dependent autophagy and chemoresistance in GBM. In GBM cells, STAT3 is constitutively activated by phosphorylation, acetylation, and trimethylation. Activation of STAT3 inhibits AMPKα and ULK1 signaling and consequently, blocks autophagy induction. This increases GBM resistance to chemotherapy. Genomic deletion of STAT3 activates AMPKα–ULK1 signaling, which in turn triggers autophagic activity in GBM cells. Activated AMPKα blocks mTORC1 via TSC2
phosphorylation. Furthermore, AMPKα-mediated mTORC1 inhibition impairs mTORC1’s ability to inhibit ULK1. These pathways activate autophagy flux and concomitantly inhibit GBM tumor progression in mice, highlighting the current view that STAT3 inhibition and autophagy induction are the main elements underlying the in vivo antitumor activity of STAT3 deletion. Pharmacologic inhibition of ULK1 (using MRT68921) causes autophagy inhibition and sensitizes STAT3-KO and mutant STAT3 expressing cells to apoptosis, demonstrating that a combinatorial approach involving simultaneous inhibition of STAT3 and ULK1 can open new perspectives for the treatment of GBM. Furthermore, mTOR inhibition by Everolimus potentiates autophagic flux in STAT3-KO and mutant expressing lines suggesting that combining STAT3 and mTOR inhibitors may be exploitable therapeutically for inducing autophagy and decreasing GBM tumorigenicity. \(\rightarrow\), Activation; \(\perp\), inhibition; (P) in red indicates phosphorylation causing inhibition; and (P) in green indicates phosphorylation leading to activation. Boxes in green indicate protein activation and boxes in red indicate inhibition.
Genomic deletion of STAT3 activates autophagy flux through AMPKα-ULK1-TSC2 signaling pathways in MT330 cells. (A) Confluent EV MT330 cells, STAT3-knockout cell line # 2 (K02), STAT3-knockout cell line # 3 (K03), STAT3-KO3 rescued with wild-type (WT), and STAT3-KO3 cells expressing Y705F-STAT3 and
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Figure 3

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Figure 7

Schematic showing the molecular cross talk regulating STAT3-dependent autophagy and chemoresistance in GBM. In GBM cells, STAT3 is constitutively activated by phosphorylation, acetylation, and trimethylation. Activation of STAT3 inhibits AMPKα and ULK1 signaling and consequently, blocks autophagy induction. This increases GBM resistance to chemotherapy. Genomic deletion of STAT3 activates AMPKα–ULK1 signaling, which in turn triggers autophagic activity in GBM cells. Activated AMPKα blocks mTORC1 via TSC2 phosphorylation. Furthermore, AMPKα-mediated mTORC1 inhibition impairs mTORC1’s ability to inhibit ULK1. These pathways activate autophagy flux and concomitantly inhibit GBM tumor progression in mice, highlighting the current view that STAT3 inhibition and autophagy induction are the main elements underlying the in vivo antitumor activity of STAT3 deletion. Pharmacologic inhibition of ULK1 (using MRT68921) causes autophagy inhibition and sensitizes STAT3-KO and mutant STAT3 expressing cells to apoptosis, demonstrating that a combinatorial approach involving simultaneous inhibition of STAT3 and ULK1 can open new perspectives for the treatment of GBM. Furthermore, mTOR inhibition by Everolimus potentiates autophagic flux in STAT3-KO and mutant
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