STAMP2 suppresses autophagy in prostate cancer cells by modulating the integrated stress response pathway

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Running title: STAMP2 effects on autophagy in prostate cancer

Abstract

Six Transmembrane Protein of Prostate 2 (STAMP2) is critical for prostate cancer (PCa) growth. We previously showed that STAMP2 regulates the expression of stress induced transcription factor ATF4, which is implicated in starvation-induced autophagy. We therefore investigated whether STAMP2 is involved in the regulation of autophagy in PCa cells. Here we show that STAMP2 suppresses autophagy in PCa cells through modulation of the integrated stress response axis. We also find that STAMP2 regulates mitochondrial respiration. These findings suggest that STAMP2 has significant metabolic effects through mitochondrial function and autophagy, both of which support PCa growth.

Keywords

prostate cancer; integrated stress response; autophagy; mitochondria; ATF4; eIF2α; STAMP2

Introduction

Six Transmembrane Protein of Prostate (STAMP) family of transmembrane proteins (STAMP1-3, also known as STEAPs – Six Transmembrane Epithelial Antigen of Prostate) have iron reductase activities and are expressed in a range of tissues. STAMP expression is regulated by a variety of stimuli and they are implicated in different metabolic and inflammatory diseases, as well as in cancer [1]. STAMP2 has the highest enzymatic activity among STAMPs, and it is also the most extensively studied: in addition to cancer [2-6], STAMP2 has important roles in metabolic tissues [7-12] and macrophages [13].

We have previously shown that STAMP2 is an androgen regulated gene in prostate cancer (PCa) [2]. Consistent with an important role of STAMP2 in PCa growth, ectopic expression of STAMP2 increased, whereas its inhibition strongly inhibited, PCa cell growth *in vitro* and *in vivo* [3]. STAMP2 is required to maintain the levels of Activating Transcription Factor 4 (ATF4) through its iron reductase activity and the production of reactive oxygen species (ROS). ATF4 has key roles in a number of cellular functions, such as regulation growth, response to stress pathways, and survival [14]. ATF4 is also a major driver of autophagy that links endoplasmic reticulum (ER) stress and nutrient sensing pathways [15].

Autophagy is a catabolic process whereby cellular components are engulfed in double membrane vesicles called autophagosomes. As the autophagosomes mature, they fuse with lysosomes such that their contents are degraded by the hydrolases therein [16]. Autophagy is a homeostatic process that is central in multiple aspects of both normal and pathological conditions. Often referred to as a double-edged sword, autophagy may either counteract or promote carcinogenesis [17, 18]. Even though autophagy may protect cancer cells from the nutrient-deprived, hypoxic, and metabolically unfavorable microenvironment, many cancers have defective autophagy regulators suggesting that these act as tumor suppressors; however, in developed tumors autophagy may play a pro-survival role [19, 20].

The findings on the role of autophagy in prostate cancer are mixed. Androgen signaling, which is the most central proliferative pathway in PCa cells, induce autophagy in PCa cells [21, 22]. However, androgen signaling also inhibits nutrient induced autophagy and autophagic cell death [23, 24]. Furthermore, androgen ablation, a central treatment in hormone responsive PCa, increases autophagy [25]. Consistently, autophagy may be related to treatment resistance in PCa tumors and disease progression to castration resistant prostate cancer (CRPC) [26, 27]. Therefore, there is a need for deeper understanding of the mechanisms that regulate autophagy in PCa.

Here, we have evaluated the potential role of STAMP2 to affect autophagy in PCa cells. Coupled to our recent findings on the role of the integrated stress response in PCa [28], involving eIF2α kinases, eIF2α and ATF4, we present evidence that STAMP2 inhibits autophagic flux in PCa cells through its effects on the eIF2α-ATF4 axis. These data suggest that autophagy acts as a tumor suppressor in PCa cells that express STAMP2.

Materials and methods

Cell culture

HEK293T cells and the human PCa cell lines LNCaP and C4-2B, were purchased from the American Type Culture Collection (Rockville, MD) and cultured according to the supplier's recommendations. For starvation treatments, the cells were washed once in pre-warmed PBS and incubated in EBSS medium (Gibco) for the indicated times.

siRNA transfection

siRNA-mediated knockdown was performed using Lipofectamine RNAiMax (Invitrogen) according to manufacturer's protocol. Reverse transfection was used with the following adjustments: cells were incubated in antibiotic free medium containing a final siRNA concentration of 5 nM and Lipofectamine RNAiMax diluted 1:2000 for a minimum of 48 hours. Sequences are available in Supplementary Information.

Lentivirus transduction and stable cell line selection

Vectors carrying the CDS to be expressed (pGIPZ FLAG and pGIPZ FLAG-STAMP2) were transfected together with a packaging plasmid (pCMV-ΔR8.2) and an envelope plasmid (pCMV-VSV-G) into HEK293T cells using Lipofectamine 3000 (Thermo Fisher Scientific). 48 h and 72 h post transfection conditioned medium was harvested, filtered through a 0.45 µm filter (Millipore) and added to LNCaP and C4-2B cells. 36 h post infection, cells were subjected to selection using 1 µg/ml puromycin for 7-10 days.

Immunofluorescence confocal microscopy

Cells grown on cover slips were washed with PBS, fixed in 4% PFA (pH 7.2) for 15 min, and permeabilized using 0.2% Triton X-100. Cells were blocked with 1% BSA for 30 min before incubation with antiserum against LC3 (MBL, 1:100) at 4°C overnight and incubated with Alexa Fluor 488 goat anti-rabbit secondary antibodies (1:500) (Invitrogen) for 1 h at room temperature. Images were acquired with an Olympus FluoView FV1000 and analyzed with Fiji (ImageJ).

Live confocal microscopy

Cells growing on glass bottom plates were co-transfected with p-EGFP-STAMP2 and mCherry-Mito-7 (mitochondrial localization signal-sequence, MLS, tagged with mCherry fluorescent protein) using Lipofectamine 3000 (Thermo Fisher Scientific). After 48 h incubation images of cells were acquired with an Olympus FluoView FV1000 and processed with Fiji (ImageJ).

Mitochondrial staining

Cells were stained for 30 min according to manufacturer's protocol using a concentration of 10 nM TMRE (Tetramethylrhodamine ethyl ester, Cayman Chemicals). Images were acquired with an Olympus FluoView FV1000 and analyzed with Fiji (ImageJ). TMRE staining were quantified by measuring fluorescent signal using a plate reader.

Seahorse mito stress test assay

A Seahorse XFe24 instrument (Agilent) was used to measure basal cellular respiration rate. Parameters of mitochondrial function were obtained by subjecting cells to a "Mito stress test" assay (Agilent) using the manufacturer's recommended protocol for LNCaP cells. Calculated mitochondrial ATP-linked respiration was also performed according to manufacturer's protocol.

Western analysis

Cells were washed in ice cold PBS and protein was extracted by incubating cells in lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 0.1% Sodium dodecyl sulphate, 0.5% Sodium deoxycholate with 1X Protease inhibitor cocktail [Roche] and Phosphatase inhibitor cocktail [Roche]) for 10 min on ice. 40 µg of protein extract was resolved in a polyacrylamide-SDS gel, blotted to a PVDF membrane and incubated over night at 4°C with antisera against target proteins (listed in Supplementary Information file) in 5% BSA in TBS-0.1% Tween. Subsequently, the membranes were probed with a secondary antibody conjugated with HRP for 1 h in 5% skimmed milk in TBS-0.1% Tween. Membranes were developed using ECL (Bio-Rad). Western images were obtained using a Kodak imaging station 4000R and the band intensities were quantified with Fiji (ImageJ).

Quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from cells using the TRI-reagent (Invitrogen). Transcripts were converted to cDNA by the Superscript II (Invitrogen) reverse transcriptase using oligo (dT) primers. cDNA was quantified by the Lightcycler 480 system (Roche) using the SYBR Green dye (Bimake). The values were normalized to the expression of the reference gene *RPLP0*. All PCR products were analyzed by melting curve analysis. qRT-PCR primer sequences are available in Supplementary Information.

Statistics

All quantified data are presented as means, and all error bars represent standard deviation. Statistical analyses were performed using the Student's t-test, and p-values < 0.05 were considered significant indicated with "*".

Results

STAMP2 suppresses autophagic flux in PCa cells

To assess the potential impact of STAMP2 on autophagic flux, we treated LNCaP cells with either control siRNA or siRNA targeting STAMP2 (Figure S1A). Cells were then kept in normal or amino acid deficient medium (EBSS) in the presence or absence of Bafilomycin A1 (BafA1), a potent inhibitor of lysosomal H^+-ATPase [29], and lipidated LC3-II protein abundance was measured by Western analysis (Figure 1A). Treatment with BafA1 led to a clear and expected increase in LC3-II levels, which was further increased in starved cells compared with fed cells. Similar results were obtained in C4-2B cells (Figure S2B). A representation of autophagic flux was calculated by comparison of LC3-II levels (normalized to GAPDH) in BafA1-treated cells to those of corresponding untreated cells [30]. STAMP2 knockdown led to a significant increase in autophagic flux in both normal and EBSS medium (Figure 1B). In addition, quantitation of the number of endogenous LC3-positive spots by immunofluorescence confocal microscopy showed an increased number of LC3 puncta in STAMP2 knockdown cells compared to control cells (Figure 1C and 1D).

To evaluate these results further, we ectopically expressed STAMP2 in LNCaP and C4-2B cells and evaluated LC3-II protein abundance. In contrast to STAMP2 knockdown cells, In the presence of ectopic STAMP2 expression, there was a decline in LC3-II levels in both starved and fed cells upon BafA1-treatment indicating a decrease in autophagic flux (Figure 2A, 2B and S2C). LC3 puncta formation was assessed in LNCaP cells under the same experimental conditions as above. Compared with control cells, STAMP2 expression led to a decrease in the number of LC3 puncta (Figure 2C and 2D). Together, these results demonstrate that STAMP2 acts as a suppressor of autophagic flux in PCa cells.

STAMP2 depletion disrupts an ATF4-controlled feedback loop in PCa cells

We have previously linked STAMP2 to unfolded protein response (UPR) signaling through its effects on the expression of transcription factor ATF4 [3]. To investigate the signaling events that can mediate STAMP2 effects on autophagic flux in PCa cells, we determined ATF4 expression and components of the integrated stress response pathway (IRS) PERK-eIF2α-ATF4. LNCaP and C4-2B cells were treated with either control siRNA or siRNA targeting STAMP2, maintained in normal or EBSS medium with increasing time, and subjected to Western analysis (Figure 3A and S3A). In LNCaP cells, ATF4 expression markedly decreased upon STAMP2 knockdown in fed cells and at early time points of starvation, consistent with our previous findings [3]. However, in C4-2B cells there was no significant difference in ATF4 levels in fed cells with or without STAMP2 knockdown, but STAMP2 suppression significantly decreased ATF4 levels when starvation was initiated at 0.5 h (Figure S3B). Interestingly, in both PCa cell lines STAMP2 knockdown did not significantly affect ATF4 levels after 1 h starvation, but prolonged starvation (2 and 4 h) quickly depleted ATF4 levels. In the fed state and at all time points of starvation in LNCAP cells, STAMP2 knockdown was distinctly correlated to the expression of the transcription factor EB (TFEB) (Figure S1C), a key factor for modulating autophagy related gene expression [31, 32]. This correlation was similar although less prominent in C4-2B cells (Figure S3C). Similarly, STAMP2 knockdown was significantly correlated with FAM129A expression in both cell lines (Figure S1D and S3D, a target of ATF4 in PCa cells [28]. Unexpectedly, in LNCaP cells, p-eIF2α levels, upstream of ATF4 that normally correlates with its expression, were significantly increased upon STAMP2 knockdown in the fed state and at all time points of starvation (Figure 3C); in contrast, total eIF2α levels were

not affected. Interestingly, STAMP2 suppression did not affect p-eIF2α levels in C4-2B cells (Figure S3E).

To evaluate these results further, we ectopically expressed STAMP2 in LNCaP cells under the same conditions (Figure 3B). As expected, ATF4 expression increased upon ectopic STAMP2 expression in the fed state and at early time points of starvation, which was dramatically reduced by 2 h of starvation (Figure S1E). Consistently, expression of ATF4 target genes TFEB and FAM129A was upregulated at all time points upon ectopic expression of STAMP2 (Figure S1F and S1G). Although not substantial, p-eIF2α level was significantly reduced upon ectopic STAMP2 expression at basal levels and 0.5 h of starvation (Figure 3D), but with prolonged starvation there was no significant difference between the control and STAMP2 expressing cells. These data suggest that STAMP2 has multiple effects on PERK-eIF2α-ATF4 signaling and may positively regulate the established autophagy activating transcription factor TFEB in PCa cells.

STAMP2 affects mitochondrial function in LNCaP cells

Previous work has shown that mitochondrial function is linked to regulation of autophagy [33]. As STAMP2 has been implicated in regulation of mitochondrial function [6, 34], we used ectopic co-expression of EGFP-STAMP2 and mCherry-Mito-7 (with N-terminus mitochondria localization signal) in LNCaP cells to investigate a potential colocalization using live cell confocal imaging. Indeed, EGFP-STAMP2 was localized to some, but not all mitochondria, suggesting that it may have a role in mitochondria function (Figure S4).

To assess a potential role of STAMP2 in mitochondrial homeostasis, we transfected LNCaP cells with either control siRNA or siRNA targeting STAMP2 and stained the cells with tetramethylrhodamine ethyl ester (TMRE), a cell-permeant fluorescent dye that is readily sequestered by active mitochondria and accumulates based on the mitochondrial membrane potential. STAMP2 knockdown led to a significant reduction in TMRE staining, suggesting that STAMP2 is required for normal mitochondrial activity (Figure 4A and 4B). We followed up on these findings by a Seahorse mitochondrial stress assay in LNCaP cells treated with either control siRNA or siRNA targeting STAMP2 (Figure 4C). STAMP2 knockdown led to a significantly reduced basal relative oxygen consumption rate (OCR) as well as a decline in maximal respiratory capacity, which was coincident with a significant drop in the calculated ATP production deduced from the mitochondrial stress assay (Figure 4D).

To evaluate these results further, we ectopically expressed STAMP2 in LNCaP cells under the same conditions and evaluated mitochondrial parameters. Consistent with the knockdown experiments, STAMP2 ectopic expression led to a significant increase in mitochondrial membrane potential, OCR, and calculated ATP production (Figure 5A-D). Together, these data suggest that STAMP2 is required for normal mitochondria function in LNCaP cells.

Discussion

Here we show that STAMP2 suppresses autophagic flux in PCa cells and simultaneously promotes mitochondrial respiration. This occurs despite the fact that STAMP2 expression is positively correlated to the expression of ATF4 and TFEB, both transcriptional regulators of genes involved in autophagy [15, 35].

Consistent with our previous findings, STAMP2 upregulated the ATF4-FAM129A-eIF2α feedback loop [3, 28]. eIF2α is a node for both the UPR and the ISR, and usually drives the activation of ATF4, which in turn controls the expression of genes linked to cellular metabolism and stress relief [36]. eIF2α phosphorylation was recently suggested to be a central event for pharmacological induction of autophagy, including through ATF4 independent mechanisms [37]. In LNCaP cells, our data support a mechanism where hyper-activation of p-eIF2α following STAMP2 suppression leads to ATF4 and TFEB independent upregulation of autophagy. However, we did not observe an excess increase in p-eIF2α levels upon STAMP2 knockdown in the C4-2B cell line, but the increase in ATF4 and TFEB levels were maintained. Moreover, C4-2B cells displayed a somewhat different pattern of ATF4 levels in response to starvation than that of LNCaP cells. These data suggest that there are some nuances in the mechanistic details on the role of STAMP2 in affecting autophagy in PCa cells that could be linked to the intracellular environment or the extracellular cues (e.g. metabolism, UPR, or ROS) that may be related to PCa progression.

Previous publications have shown that dysregulated eIF2α activation can indicate [38], or lead to mitochondrial perturbation [39]; in addition, STAMP2 has been shown to affect mitochondrial function in other tissues [6, 40]. Here we showed that STAMP2 co-localizes with a portion of the mitochondria in LNCaP cells and its expression is positively correlated with mitochondrial membrane potential, respiration, and calculated ATP production. Downregulation of ATP production often translates to an increase in AMPK signaling [41], which activates autophagy through suppression of mTOR1 and activation of ULK1 [42]. Nevertheless, we could not detect any significant and consistent effect of STAMP2 knockdown on these pathways (data not shown). One possible explanation for the change in mitochondrial bioenergetics is that STAMP2 knockdown leads to an overall inhibition of PCa cell proliferation [3] that would indicate a lower energy demand of the cells. Another plausible explanation is the potential role of the eIF2α-ATF4-pathway in the formation of respiratory supercomplexes to regulate mitochondrial respiration independently of AMPK [43].

Previous studies on the potential role of autophagy in PCa cells resulted in mixed findings, with some data indicating the proliferative pathways both inducing and inhibiting autophagy in different settings [21, 22] [23, 24]. As a target of the primary proliferative pathway in PCa cells, androgen signaling, and itself an important factor in PCa growth and survival [3], the data we present here support the view that androgen signaling-mediated STAMP2 expression inhibits autophagy in PCa. This is consistent with the finding that the main mode of treatment in hormone responsive metastatic PCa, androgen ablation, increases autophagy [25]. Our data suggest that STAMP2 suppresses autophagy in PCa cells through affecting the eIF2α–ATF4 pathway. Further work is required to uncover the details of this regulation and its impact on PCa.

Conflicts of interest

The authors declare no conflicts of interest.

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Figure legends

Figure 1

(A) LNCaP cells were treated with control siRNA (siCtrl) or siRNA against STAMP2 (siST2) for 48 h. They were then washed with PBS and incubated in normal growth medium or EBSS starvation medium for 4 h in the presence of BafA1 or vehicle. The cells were harvested and subjected to Western blot analysis probing for LC3 and GAPDH. (B) Quantification of autophagic flux from A, as described in the text. Data represent five independent experiments. (C) Immunoflourescence confocal imaging of LNCaP cells treated with control siRNA (siCtrl) or siRNA against STAMP2 (siST2) for 48 h. After transfection, cells were kept in EBSS medium for 4 h in the presence of BafA1 and stained with antibodies recognizing LC3 and dyed with DAPI. Scale bar = 10μM. (D) Quantification of LC3 puncta from the data presented in C. Data represent three independent experiments and > 200 cells. Statistical representation of the data is described in Materials & Methods.

Figure 2

(A) LNCaP cells were infected with viral vectors for FLAG or FLAG-STAMP2 expression for 48 h. They were then washed with PBS and incubated in normal growth medium or EBSS for 4 h in the presence of BafA1 or vehicle. The cells were harvested and subjected to immunoblotting probing for STAMP2, LC3 and GAPDH. (B) Quantification of autophagic flux from the data presented in A. Data presented are from four independent experiments. (C) Immunoflourescence confocal imaging of LNCaP cells infected with viral vectors for FLAG or FLAG-STAMP2 expression for 48 h. After transfection, cells were kept in EBSS starvation medium for 4 h in the presence of BafA1, and stained with antibodies recognizing LC3 and dyed with DAPI. Scale bar = 10μM. (D) Quantification of LC3 puncta from data presented in C. Data represent three independent experiments and > 200 cells.

Figure 3

(A) LNCaP cells were treated with control siRNA ("-") or siRNA against STAMP2 (siST2) for 48 h. They were then washed with PBS and incubated in normal growth medium (4 h) or EBSS for 0.5 h, 1 h, 2 h, or 4 h. The cells were harvested and subjected to Western blot analysis probing for TFEB, ATF4, FAM129A, phospho (p)- and total (t)-eIF2α, and GAPDH. The images are representative of four independent experiments. (B) LNCaP cells were infected with viral vectors for FLAG ("-") or FLAG-STAMP2 (ST2) expression for 48 h. They were then washed with PBS and given new normal growth medium (4 h) or EBSS for 0.5, 1, 2, or 4 h, as indicated. The cells were harvested and subjected to Western analysis probing for STAMP2 and the same proteins as in A. (C) Quantification of relative p-eIF2α levels (normalized to t-eIF2α and GAPDH levels) from experiments represented by blots in A (D) Quantification of relative p-eIF2α levels (normalized to t-eIF2α and GAPDH levels) from experiments represented by blots in B. All data are representative for at least three independent experiments.

Figure 4

(A) LNCaP cells were treated with control siRNA (siCtrl) or siRNA against STAMP2 (siST2) for 48 h. The mitochondrial membrane potential was then assessed using the TMRE stain. Images representative of > 200 cells. Scale bar = 10 μM. (B) Relative levels of TMRE stain (normalized to siCtrl) from cells in A. (C) Cells were treated as in A. The cells were then subjected to Seahorse analysis using mitochondrial stress testing and the relative oxygen consumption rate (OCR) reflecting different mitochondrial capacities was obtained. (D) OCR

reflecting calculated ATP production rate. All data are representative of three independent experiments.

Figure 5

(A) LNCaP cells were infected with viral vectors for FLAG or FLAG-STAMP2 for 48 h. Mitochondrial membrane potential was then assessed using the TMRE stain. Images representative for > 200 cells. Scale bar = 10 μM. (B) Relative levels of TMRE stain (normalized to FLAG) from cells in A. (C) Cells were treated as in A. Cells were then subjected to Seahorse analysis using mitochondrial stress testing and the relative OCR was obtained. (D) OCR reflecting calculated ATP production rate. All data are representative of three independent experiments.

Figures

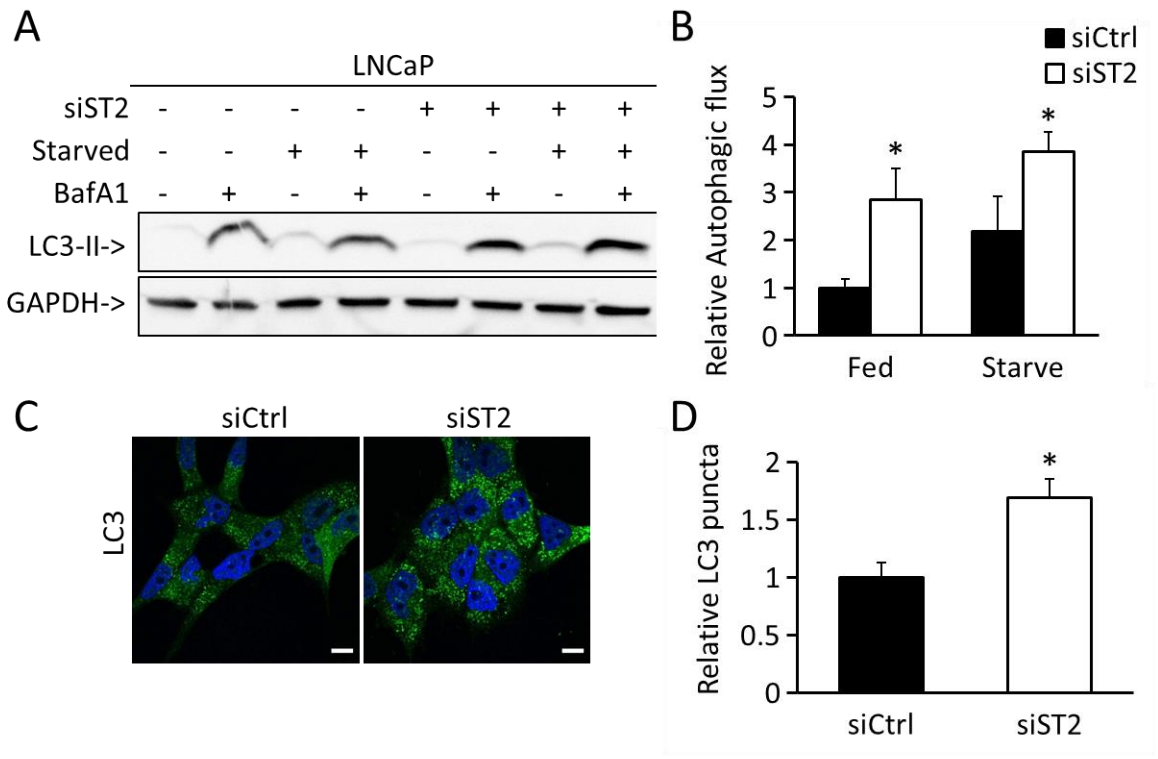

Figure 1

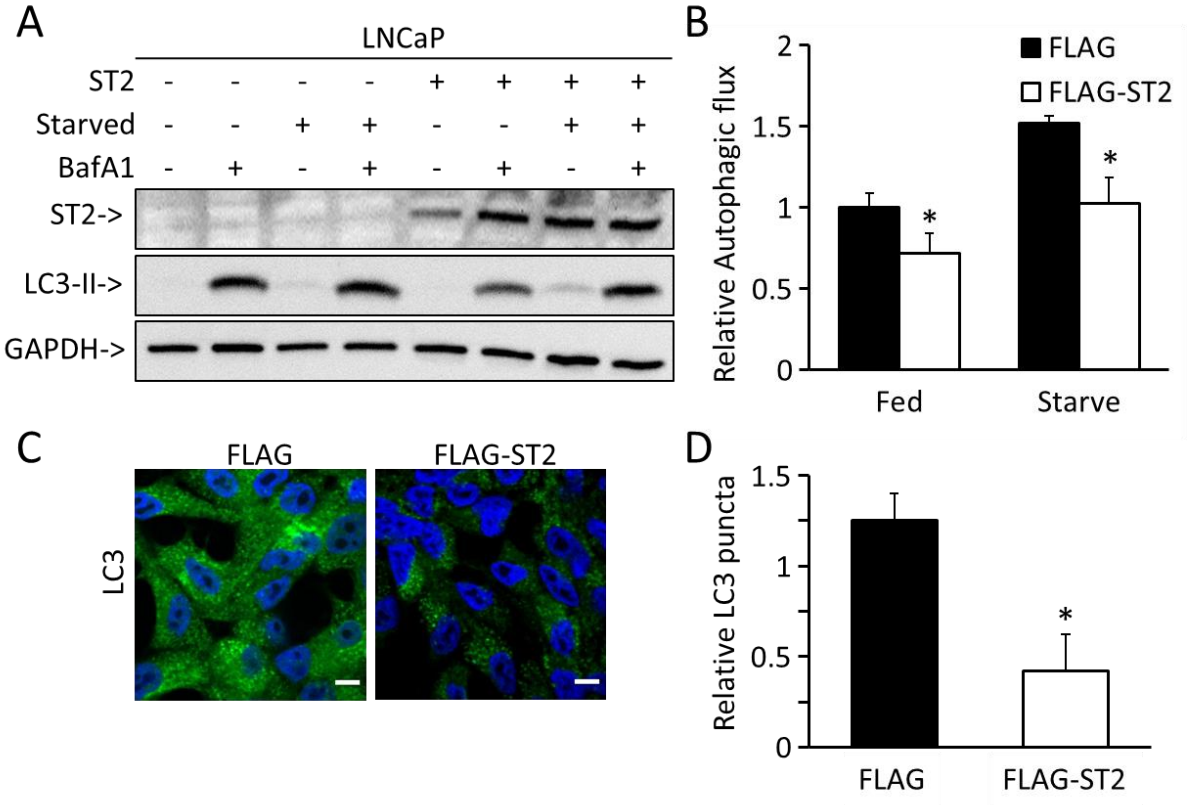
A
LNCaP
ST2 - - - - + + + +
Starved - - + + - - + +
BafA1 - + - + - + - +
ST2->
LC3-II->
GAPDH->
B
FLAG
FLAG-ST2
Relative Autophagic flux
2
1.5
1
0.5
0
*
*
Fed
Starve
C
FLAG
FLAG-ST2
LC3
D
Relative LC3 puncta
1.5
1
0.5
0
*
FLAG
FLAG-ST2

Figure 2

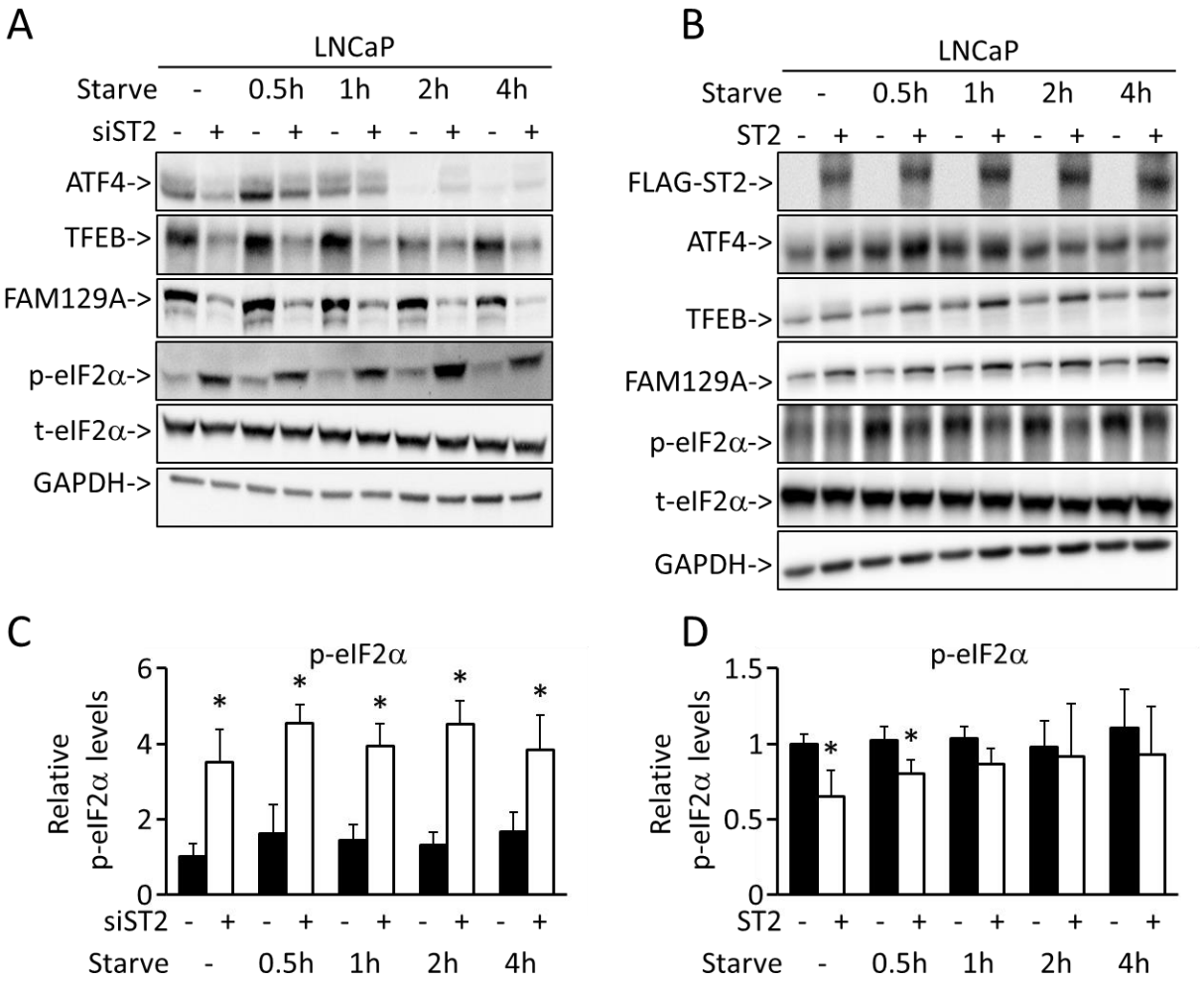
A
LNCaP
Starve - 0.5h 1h 2h 4h
siST2 - + - + - + - + - +
ATF4->
TFEB->
FAM129A->
p-eIF2α->
t-eIF2α->
GAPDH->
B
LNCaP
Starve - 0.5h 1h 2h 4h
ST2 - + - + - + - + - +
FLAG-ST2->
ATF4->
TFEB->
FAM129A->
p-eIF2α->
t-eIF2α->
GAPDH->
C
p-eIF2α
Relative p-eIF2α levels
0
2
4
6
siST2 - + - + - + - + - +
Starve - 0.5h 1h 2h 4h
D
p-eIF2α
Relative p-eIF2α levels
0
0.5
1
1.5
ST2 - + - + - + - + - +
Starve - 0.5h 1h 2h 4h

Figure 3

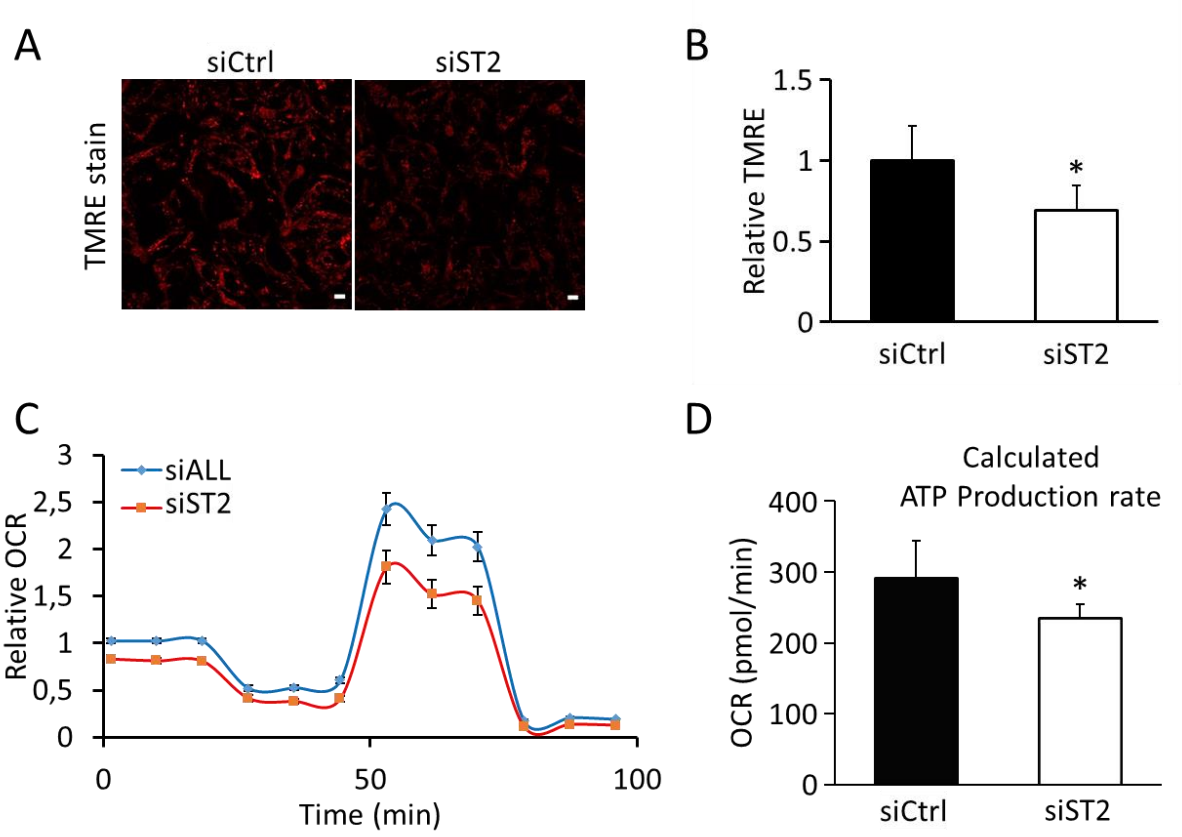
A
siCtrl
siST2
TMRE stain
B
Relative TMRE
1.5
1
0.5
0
*
siCtrl
siST2
C
Relative OCR
3
2,5
2
1,5
1
0,5
0
siALL
siST2
0
50
100
Time (min)
D
Calculated
ATP Production rate
OCR (pmol/min)
400
300
200
100
0
*
siCtrl
siST2

Figure 4

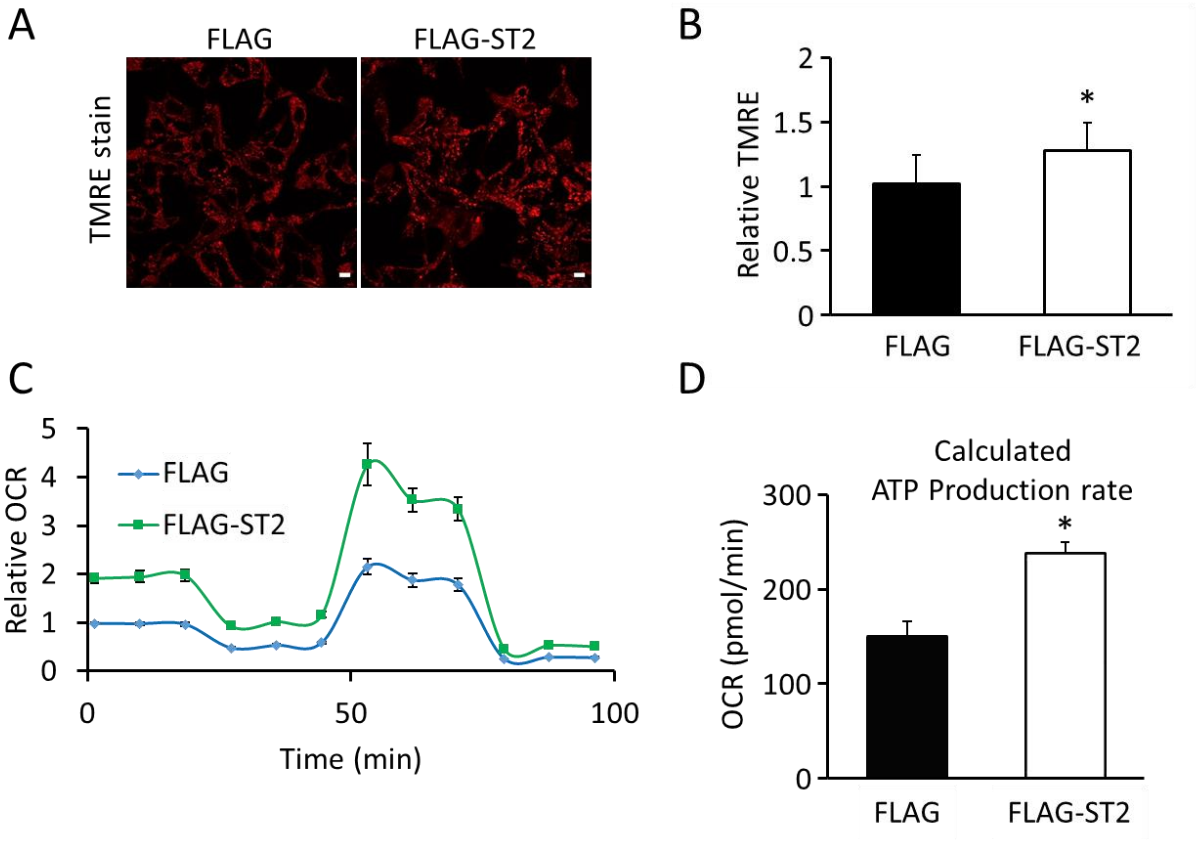
A
FLAG
FLAG-ST2
TMRE stain
B
Relative TMRE
2
1.5
1
0.5
0
*
FLAG
FLAG-ST2
C
Relative OCR
5
4
3
2
1
0
FLAG
FLAG-ST2
0
50
100
Time (min)
D
Calculated
ATP Production rate
OCR (pmol/min)
300
200
100
0
*
FLAG
FLAG-ST2

Figure 5