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Forkhead Box Q1 is a novel regulator of autophagy in breast cancer cells

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Abstract

Forkhead Box Q1 (FoxQ1) transcription factor is overexpressed in luminal-type and basal-type human breast cancers when compared to normal mammary tissue. This transcription factor is best known for its role in promotion of breast cancer stem-like cells and epithelial to mesenchymal transition. The present study documents a novel function of FoxQ1 in breast cancer cells. Overexpression of FoxQ1 in basal-like SUM159 cells and luminal-type MCF-7 cells resulted in increased conversion of microtubule-associated protein light chain 3 beta-I (LC3B-I) to LC3B-II, which is a hallmark of autophagy. Autophagy induction by FoxQ1 overexpression was confirmed by visualization of LC3B puncta as well as by transmission electron microscopy. Expression profiling for genes implicated in autophagy regulation revealed upregulation of many genes, including *ATG4B, ATG16L1, CTSS, CXCR4*, etc. but downregulation of *BCL2L1*, *DRAM1, TNF, ULK2*, etc. by FoxQ1 overexpression in SUM159 cells. Western blotting confirmed upregulation of ATG4B and CXCR4 proteins by FoxQ1 overexpression in both SUM159 and MCF-7 cells. Chromatin immunoprecipitation assay revealed recruitment of FoxQ1 at the promoter of *ATG4B*. Pharmacological inhibition of ATG4B using S130 significantly increased apoptosis induction by DOX in empty vector transfected as well as FoxQ1 overexpressing SUM159 and MCF-7 cells but this effect was statistically significantly lowered by FoxQ1 overexpression indicating the protective role of FoxQ1 on apoptosis. , Treatment of SUM159 cells with S130 and DOX enhanced LC3B-II level in both empty vector transfected cells and FoxQ1 overexpressing SUM159 cells but not in FoxQ1 overexpressing MCF-7 cells. In conclusion, FoxQ1 is a novel regulator of autophagy.

Keywords

FoxQ1; autophagy; breast cancer; chemosensitivity

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CONFLICT OF INTEREST

The authors do not declare any conflict of interest.

1 INTRODUCTION

Breast cancer, which is a complex and heterogeneous malignancy broadly grouped into luminal-type, human epidermal growth factor 2 (Her2)-enriched, and basal-like, is a leading cause of cancer-related deaths in American women.[1,2] The complexity of the pathogenesis of breast cancer is substantiated by involvement of many oncogenic drivers. For example, estrogen receptor is a well-known driver of luminal-type breast cancer, and this subtype is treated with antiestrogens like tamoxifen and aromatase inhibitors.[3,4] A subset of breast cancer patients are characterized by overexpression of Her2 and a humanized antibody (Herceptin) was approved by the United States Food and Drug Administration for these patients.[5] The basal-like (mostly triple-negative) subtype is less frequent accounting for 10-20% of all breast cancers but highly aggressive with poor clinical outcomes.[6–9] Basal-like breast cancer are characterized by lack of estrogen receptor, progesterone receptor, and Her2.[6–9] However, other oncogenic transcription factor drivers of basal-like breast cancer have been identified, including signal transducer and activator of transcription 3, nuclear factor-κB, β-catenin, Krüppel-like factor 5 to name a few.[6–9] Several kinases have also been implicated in promotion of basal-like breast cancers including mitogen-activated protein kinases.[10-12]

The forkhead Box Q1 (FoxQ1) is another transcription factor implicated in the pathogenesis of breast cancer. Overexpression of FoxQ1 has been reported in basal-like and luminal-type human breast cancers when compared to normal mammary tissue.[13,14] In breast cancer, the FoxQ1 is best known for its role in promotion of epithelial to mesenchymal transition (EMT) and maintenance of breast cancer stem-like cells (bCSC).[15–17] The FoxQ1 expression was shown to correlate with high-grade basal-like breast cancers and its expression was also associated with poor clinical outcomes.[15] Knockdown of FoxQ1 in highly invasive breast cancer cells not only decreased EMT phenotype but also reduced invasion capability, and these effects were reversed upon its overexpression.[15] The effects of FoxQ1 overexpression on EMT phenotype was confirmed by another investigative team.[16] Consequently, overexpression of FoxQ1 in a breast cancer cell line promoted pulmonary metastasis.[16] Twist1, Zeb2, and platelet-derived growth factor receptor (PDGFR) α and β were identified as downstream targets of FoxQ1 in breast cancer.[17] These investigators also provided evidence showing that PDGFRα and β can be directly regulated by FoxQ1 and indirectly regulated by FoxQ1/Twist1 axis.[17] Furthermore, PDGFRβ was found to be a more potent mediator of bCSC driven by FoxQ1 than PDGFRα.[17]

Several downstream targets of FoxQ1 have been identified in breast cancer. Initial studies showed that FoxQ1 is a transcriptional repressor of E-cadherin that explains the EMT promotion by FoxQ1.[15] Recently, we performed RNA-seq analysis using empty vector transfected and FoxQ1 overexpressing SUM159 cells to identify novel targets of FoxQ1.[14] Overexpression of FoxQ1 resulted in downregulation of genes associated with cell cycle checkpoints, M phase, and cellular response to stress/external stimuli as evidenced from the Reactome pathway analysis.[14] Overexpression of FoxQ1 resulted in mitotic arrest in SUM159 cells but not in MCF-7 cells.[14] We also showed FoxQ1-mediated regulation of expression of interleukin (IL)-1α, IL-8, vascular endothelial growth factor, and electron transport chain complex I subunits in breast cancer.[14,18] Activity as well as assembly of

complex I were significantly enhanced by ectopic expression of FoxQ1 in breast cancer cells.[18] FoxQ1 overexpression resulted in upregulation of complex I subunits NDUFS1, NDUFS2, NDUFV1, and NDUFV2.[18] Overexpression of NDUFS1 and NDUFV1 resulted in increased cell proliferation.[18] Further examination of the RNA-seq data suggested that FoxQ1 might induce autophagy. This study was undertaken to test whether FoxQ1 promotes autophagy and to determine if FoxQ1 expression affects sensitivity of breast cancer cells to doxorubicin (DOX).

2 MATERIALS AND METHODS

2.1 Reagents and cell lines

DOX was purchased from Selleckchem (Houston, TX) and S130 was from MedChemExpress (Monmouth Junction, NJ). Stock solutions of DOX and S130 were prepared in dimethyl sulfoxide (DMSO). Reagents for cell culture including fetal bovine serum, culture medium, and antibiotic mixture were from Life Technologies-Thermo Fisher Scientific (Walthom, MA). Antibodies against microtubule-associated protein 1 light chain 3 beta (LC3B) and autophagy related 4B cysteine peptidase (ATG4B) were from Cell signaling Technology (Danvers, MA); antibodies against C-X-C motif chemokine receptor 4 (CXCR4) and WD repeat domain phosphoinositide interacting 1 (WIPI1) were from Abcam Walthom, MA); an antibody against autophagy related 16 like 1 (ATG16L1) was from Proteintech Group (Rosemont, IL); anti-FoxQ1 antibody for chromatin immunoprecipitation (ChIP) assay was from Santa Cruz Biotechnology (Dallas, TX); and anti-β-Actin antibody was from Sigma-Aldrich (St. Louis, MO). Annexin V-FITC Apoptosis Detection kit was purchased from BD Biosciences (Franklin Lakes, NJ). SUM159 and MCF-7 cells stably transfected with pCMV6 empty vector (hereafter abbreviated as EV) and the same vector encoding FoxQ1 (hereafter abbreviated as FoxQ1) were cultured as described by us previously.[13] Un-transfected SUM159 and MCF-7 cells were purchased from American Type Culture Collection and cultured as recommended by the supplier. The cells were authenticated by us by short random repeat profiling.

2.2 Western blotting

Western blotting was performed as descried by us previously.[19] The blots were stripped and re-probed with anti-β-Actin antibody for normalization. The change in protein level was determined by densitometric quantitation of the immunoreactive band using UN-SCAN-IT software.

2.3 Immunofluorescence microscopy

The cells were grown on coverslips in 12-well plates and allowed to attach by overnight incubation. The cells were fixed with 4% paraformaldehyde for 1 hour at room temperature, permeabilized with 0.1% Triton X-100 for 10 minutes, blocked with buffer containing 0.5% bovine serum albumin and 0.15% glycine for 1 hour, followed by overnight incubation with anti-LC3B antibody. The cells were treated with 2 μg/mL Alexa Fluor 488 conjugated secondary antibody for 1 hour. Punctate pattern of LC3B was observed using an Olympus FluoView FV1000 confocal microscope at 60x oil objective magnification. At least 19 non-overlapping images were captured from each group for analysis of LC3B puncta.

2.4 Transmission electron microscopy

The cells (2×10^5 cells per well) were plated in 6-well plates and incubated for 16 hours and then washed and fixed with phosphate-buffered saline and 2.5% glutaraldehyde solution, respectively. Sections were imaged with the use of JEOL 1011 transmission electron microscope at ×12,000 magnification. Autophagy vacuoles were quantitated as previously described.[20]

2.5 RNA-seq analysis

The RNA-seq data presented in this study were from our previously published study.[14] The RNA-seq data was submitted to the Gene Expression Omnibus of NCBI (GSE151059).[14]

2.6 Human autophagy RT^2 Profiler™ polymerase chain reaction (PCR) array

Total RNA was extracted from FoxQ1 overexpressing SUM159 and MCF-7 cells and corresponding EV cells (n=3 for each) using the RNeasy® Mini kit (Qiagen) following the instructions provided by the manufacturer. Other details of the PCR array were essentially the same as described previously.[21] The data was analyzed using the web-based software provided by the manufacturer. The threshold cycles were calculated and the genes with values above 35 were considered undetected.

2.7 Analysis of The Cancer Genome Atlas (TCGA) dataset

The breast cancer TCGA data set (n=1097) was analyzed to determine the correlation between expression of *FoxQ1* with that of genes associated with autophagy using the University of California Santa Cruz Xena Browser (https://tcga.xenahubs.net). The correlation coefficient and statistical significance were determined by Pearson's test.

2.8 ChIP assay

The ChIP assay was done as described by us previously[13,14] using Pierce™ Magnetic ChIP kit according to the manufacturer's protocol. Normal IgG (as a negative control) and FoxQ1 antibodies were used for the ChIP assay. The FoxQ1 binding sites in the *ATG4B* promoter were amplified by the RT-PCR (60°C for 1 minute, 40 cycles) with the following region-specific primers: Site 1 and Site 2, forward:5'-ACCAGCGCAGGAAGATACTG-3' and reverse:5'-CTCCCAAAGTGCTGGGATTA-3'; Site 3, forward:5'-CCTAGGGAGAGGAGGACTGG-3' and reverse:5'-GCAGCTGTCACTACCATCCA-3'. Fold enrichment was normalized to the input.

2.9 Cell proliferation assay

Cell proliferation was performed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit from Promega following the supplier's instructions.

2.10 Detection of apoptosis

Apoptotic cell death was assessed using Annexin V Apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. After treatment, harvested cells were suspended in binding buffer with 4 μL Annexin V for 30 minutes at room temperature

in the dark. Samples were diluted with binding buffer and apoptotic cells were analyzed using CytoFLEX flow cytometer.

2.11 Statistical analysis

All data were analyzed using GraphPad Prism (v 7.02). Statistical significance of difference was determined by one-way analysis of variance (ANOVA) with Bonferroni's adjustment or Student's t-test. A P value < 0.05 was considered statistically significant.

3 RESULTS

3.1 FoxQ1 is a novel regulator of autophagy

Figure 1A confirms overexpression of FoxQ1 protein in stably transfected SUM159 and MCF-7 cells when compared to corresponding EV cells. Conversion of LC3B-I to LC3B-II is a hallmark of autophagy.[22] Western blotting data for LC3B protein is shown in Figure 1A. The level of LC3B-II protein was markedly higher in FoxQ1 overexpressing SUM159 cells (3.3-fold higher) and MCF-7 cells (4.0-fold higher) in comparison with corresponding EV cells (Figure 1A). Figure 1B shows LC3B puncta, which is another method to monitor autophagy,[23] in EV and FoxQ1 overexpressing cells. FoxQ1 overexpression resulted in a statistically significant increase in number of LC3B puncta in both SUM159 and MCF-7 cells (Figure 1B). Autophagy induction was confirmed by transmission electron microscopy which showed higher number of double membrane structures in FoxQ1 overexpressing cells than in EV cells, but the difference did not reach statistical significance in SUM159 cells due to large data scatter (Figure 1C). These results indicated that FoxQ1 overexpression increased basal autophagy without any stress or treatment in two human breast cancer cell lines belonging to different subtype.

3.2 Profiling for autophagy related genes

Expression profiling using EV and FoxQ1 overexpressing SUM159 cells showed changes in expression of genes associated with autophagy (Table 1). The genes upregulated by FoxQ1 overexpression included *ATG10, ATG16L1, ATG4B, CTSS, CXCR4, HSPA8, IGF1, MAP1LC3B*, and several other genes listed in Table 1. The RNA-seq data from our previously published study[14] also showed statistically significant up-regulation of *ATG16L1, ATG4B, MAP1LC3B, PTEN, etc.* by FoxQ1 overexpression (Table 1). Expression of several autophagy-related genes was suppressed by FoxQ1 overexpression, including *BCL2L1, DRAM1, MAPK8, TGM2, RPS6KB1, ULK2,* and so forth (Table 1). Down-regulation of only *RPS6KB1* was evident in the RNA-seq data (Table 1). These gene expression changes explain autophagy induction by FoxQ1 overexpression in SUM159 cells. We also conducted autophagy RT profiler PCR using EV and FoxQ1 overexpressing MCF-7 cells. We identified 13 genes that were upregulated and 9 genes were downregulated by FoxQ1 overexpression in MCF-7 cells. Among them, 10 genes were common in both cell lines (Table 2).

3.3 FoxQ1 overexpression resulted in upregulation of ATG4B and CXCR4 proteins

Figure 2A shows western blotting for ATG4B, CXCR4, ATG16L1, and WIPI1 proteins. Expression of ATG4B protein was increased by 5.4-fold and 2.2-fold in FoxQ1

overexpressing SUM159 and MCF-7 cells, respectively, when compared to corresponding EV cells (Figure 2A). Similarly, FoxQ1 overexpression resulted in a marked increase in level of CXCR4 protein in both cell lines compared to corresponding EV cells (Figure 2A). On the other hand, opposite effects were seen for ATG16L1 and WIPI1. Expression of ATG16L1 and WIPI1 was increased by FoxQ1 overexpression in SUM159 cells, but levels of these proteins were lowered in FoxQ1 overexpressing MCF-7 cells (Figure 2A). Further study is required to explain this subtype-dependent differential effect of FoxQ1 on ATG16L1 and WIPI1 expression. Analysis of the breast cancer TCGA showed a positive and significant correlation between expression of *FoxQ1* with that of *CXCR4* (Figure 2B). On the other hand, no correlation was observed between *ATG4B* and *FoxQ1* at the mRNA level. It might be one reason that transcriptional level is not reflected at the protein level.

3.4 ChIP assay revealed recruitment of FoxQ1 at the promoter of *ATG4B*

Figure 3A depicts putative FoxQ1 occupancy sites (GTTTA)[24] at the promoter of *ATG4B*. The FoxQ1 was recruited at both sites 1+2 and site 3 of the *ATG4B* promoter in both SUM159 and MCF-7 cells (Figure 3B,C). These results indicated that FoxQ1 is a direct regulator of *ATG4B* expression in breast cancer cells.

3.5 Effect of ATG4B inhibition on sensitivity to DOX

Next, we determined the effect of autophagy inhibition using ATG4B inhibitor on sensitivity of SUM159 and MCF-7 cells to DOX. S130, at concentrations ranging from 5 to 10 μM, has been utilized to bind to ATG4B and impede its activity, leading to the suppression of autophagy.[25] Initially, we evaluated the effect of S130 on cell proliferation. At 5 μM dose, S130 did not impact cell proliferation but effectively inhibited autophagy in both MCF-7 and SUM159 cells (data not shown). However, doses higher than 5 μM displayed cytotoxic effects in both cell lines (data not shown). We next carried out a dose response experiment with DOX to identify the optimal dose capable of inducing apoptosis in each cell line. The effective dose of DOX in SUM159 and MCF-7 cells was 2 μM and 4 μM, respectively (data not shown). Accordingly, we proceeded to evaluate the effect of FoxQ1 overexpression on Dox-mediated apoptosis in SUM159 and MCF-7 cells in the absence or presence of S130. Figure 4A shows effect of ATG4B inhibition on DOX-induced apoptosis. As expected, DOX-mediated apoptosis induction was evident in both EV and FoxQ1 overexpressing SUM159 and MCF-7 cells. The S130 treatment caused a modest increase in DOX-induced apoptosis in both EV and FoxQ1 overexpressing SUM159 and MCF-7 cell lines. Interestingly, DOX-induced increase in LC3B-II was further enhanced by S130 treatment in both EV cells and FoxQ1 overexpressing SUM159 cells, even though S130 alone did not exhibit complete autophagy inhibition (Figure 4B). This effect was not observed in FoxQ1 overexpressing MCF-7 cells (Figure 4B).

4 DISCUSSION

The present study reveals that FoxQ1 induces autophagy accompanied by upregulation of CXCR4 and ATG4B in human luminal and basal subtype breast cancer cells. Some Fox family members have been shown to either negatively or positively regulate autophagy under different stress and experimental conditions.[26–30] For example, coordinate activation

of autophagy and the proteasome pathway were reported for FoxO transcription factor.[26] Autophagy induction was observed following silencing of FoxK1 and FoxM1.[29,30] To the best of our knowledge, involvement of FoxQ1 in regulation of basal autophagy has not been reported previously.

Autophagy is a multistep process including phagophore formation, elongation, maturation of autophagosome, fusion with the lysosome, and degradation that is regulated by many proteins.[31] We have found common genes changed by FoxQ1 overexpression in both SUM159 and MCF-7 cells. These include *ATG10, ATG4B*, *GABARAP*, *GABARAPL2*, *IGF1*, *LAMP1*, *MAP1LC3B*, *TGM2*, *TNF*, and *ULK2*. Role of the proteins encoded by these genes are include initiation of phagophore formation (*ULK2*), elongation and maturation of autophagosomes (*ATG10, ATG4B, GABARAP, GABARAPL2*, and *MAP1LC3B*), and degradation in the autolysosome (*LAMP1*).[31] Autophagy is generally regulated by diverse external stimuli and signal transduction pathways including mTOR pathway which is downstream of insulin signaling.[31] IGF1 induction by FoxQ1 overexpression could modulate autophagy through mTOR pathway.[31] It has been documented that autophagy and inflammatory/immune responses are closely interconnected.[32] Therefore, inflammatory cytokines (*TGM2* and *TNF*) could mediate this interaction. FoxQ1 overexpression in SUM159 cells resulted in a significant increase in expression of many autophagy-related genes that included ATG family members *ATG4B, ATG16L1*, and *ATG10*. We confirmed upregulation of ATG4B by FoxQ1 overexpression by western blotting. The ATG4B protein levels vary across breast cancer subtypes but has no prognostic significance.[33] On the other hand, higher LC3B and GABARAP protein expression were associated with poor prognosis and with clinicopathological characteristics of aggressive disease for all subtypes of breast cancer.[33] Interestingly, FoxQ1 overexpression also causes an increase in *GABARAP*. These results suggest that breast tumors with FoxQ1 overexpression may have a poor prognosis. Another study indicated a novel association of ATG4B expression with HER2 positive breast cancers and indicated that this subtype may be suitable for ATG4B inhibition strategies.[34] Pharmacological inhibition of ATG4B using S130 significantly augmented sensitivity to DOX of breast cancer cells in our study. This enhancement was attenuated by FoxQ1 overexpression revealing its protective activity against apoptosis. On the other hand, S130 treatment increased level of LC3B-II induced by DOX in both EV cells and FoxQ1 overexpressing SUM159 cells but not in FoxQ1 overexpressing MCF-7 cells. Altered autophagy genes by DOX treatment might antagonize the genes changed by FoxQ1 overexpression in MCF-7 cells. Therefore, further exploration is required to understand this cell line-dependent effects. Because S130 at 5 μM concentration was not a 100% effective autophagy inhibitor, we determined the effect of a well-known autophagy inhibitor chloroquine on sensitivity of SUM159 and MCF-7 cells to DOX. Chemical inhibition of autophagy by chloroquine did not have a meaningful impact on apoptosis induction and/or cell proliferation inhibition by DOX in SUM159 or MCF-7 cells (data not shown).

We found a robust increase in expression of CXCR4 protein upon FoxQ1 overexpression in both SUM159 and MCF-7 cell lines (Figure 2A). Analysis of 13 studies involving a total of 3865 breast cancer patients reported a significant association between CXCR4 expression and lymph node status.[35] On the other hand, no association was found between CXCR4

expression and some clinical parameters, including estrogen receptor status, progesterone receptor status, and Her2 status.[35] The CXCR4 is a G protein-coupled receptor that is expressed on the cell surface of cancer cells.[36] CXCR4 has been reported to play an important role in cell survival, proliferation, migration, as well as metastasis of several cancers including breast cancer.[36] Knockdown of FoxQ1 in MDA-MB-231 cells was shown to reduce its invasion capacity.[15] Overexpression of FoxQ1 was shown to increase migration of breast cancer cell *in vitro* and lung metastasis *in vivo*.[16] Thus, it is possible that CXCR4 functions downstream of FoxQ1 to explain its effect on cell migration and metastasis. There is limited documentation regarding the involvement of CXCR4 in autophagy in breast cancer. Since CXCR4 regulates the PI3K/AKT pathway,[36] it is possible that the induction of CXCR4 through FoxQ1 overexpression might have implications in autophagy.

In conclusion, the present study shows that FoxQ1 is a novel regulator of autophagy by upregulating expression of several autophagy-related proteins including ATG4B and CXCR4. Chemical inhibition of ATG4B has modest effect on DOX-induced apoptosis

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REFERENCES

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023;73:17–48. [PubMed: 36633525]
2. Perou CM, Sørlie T, Eisen MB, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–752. [PubMed: 10963602]
3. Yip CH, Rhodes A. Estrogen and progesterone receptors in breast cancer. Future Oncol. 2014;10:2293–301. [PubMed: 25471040]
4. Zhou Z, Qiao JX, Shetty A, et al. Regulation of estrogen receptor signaling in breast carcinogenesis and breast cancer therapy. Cell Mol Life Sci. 2014;71:1549.
5. Loibl S, Gianni L. HER2-positive breast cancer. Lancet. 2017;389:2415–2429. [PubMed: 27939064]
6. Conway ME, McDaniel JM, Graham JM, et al. STAT3 and GR cooperate to drive gene expression and growth of basal-like triple-negative breast cancer. Cancer Res. 2020;80:4355–4370. [PubMed: 32816914]
7. Yamamoto M, Taguchi Y, Ito-Kureha T, Semba K, Yamaguchi N, Inoue J. NF-κB non-cell-autonomously regulates cancer stem cell populations in the basal-like breast cancer subtype. Nat Commun. 2013;4:2299. [PubMed: 23934482]
8. Quinn HM, Vogel R, Popp O, et al. YAP and β-catenin cooperate to drive oncogenesis in basal breast cancer. Cancer Res. 2021;81:2116–2127. [PubMed: 33574090]
9. Jiang D, Qiu T, Peng J, et al. YB-1 is a positive regulator of KLF5 transcription factor in basal-like breast cancer. Cell Death Differ. 2022;29:1283–1295. [PubMed: 35022570]
10. Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. N Engl J Med. 2010;363:1938–1948. [PubMed: 21067385]
11. Balko JM, Schwarz LJ, Bhola NE, et al. Activation of MAPK pathways due to DUSP4 loss promotes cancer stem cell-like phenotypes in basal-like breast cancer. Cancer Res. 2013;73:6346–6358. [PubMed: 23966295]
12. Gastaldi S, Comoglio PM, Trusolino L. The Met oncogene and basal-like breast cancer: another culprit to watch out for?. Breast Cancer Res. 2010;12:208. [PubMed: 20804567]

13. Kim SH, Kaschula CH, Priedigkeit N, Lee AV, Singh SV. Forkhead Box Q1 is a novel target of breast cancer stem cell inhibition by diallyl trisulfide. J Biol Chem. 2016;291:13495–13508. [PubMed: 27129776]
14. Kim SH, Hahm ER, Singh KB, Singh SV. Novel mechanistic targets of forkhead box Q1 transcription factor in human breast cancer cells. Mol Carcinog. 2020;59:1116–1128. [PubMed: 32754922]
15. Qiao Y, Jiang X, Lee ST, Karuturi RK, Hooi SC, Yu Q. FOXQ1 regulates epithelial-mesenchymal transition in human cancers. Cancer Res. 2011;71:3076–3086. [PubMed: 21346143]
16. Zhang H, Meng F, Liu G, et al. Forkhead transcription factor foxq1 promotes epithelial-mesenchymal transition and breast cancer metastasis. Cancer Res. 2011;71:1292–1301. [PubMed: 21285253]
17. Meng F, Speyer CL, Zhang B, et al. PDGFRα and β play critical roles in mediating Foxq1-driven breast cancer stemness and chemoresistance. Cancer Res. 2015;75:584–593. [PubMed: 25502837]
18. Kim SH, Singh SV. The FoxQ1 transcription factor is a novel regulator of electron transport chain complex I subunits in human breast cancer cells. Mol Carcinog. 2022;61:372–381. [PubMed: 34939230]
19. Xiao D, Srivastava SK, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. Carcinogenesis. 2003;24:891–897. [PubMed: 12771033]
20. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome C and apoptosis in human prostate cancer cells. Cancer Res. 2006;66:5828–5835. [PubMed: 16740722]
21. Hahm ER and Singh SV. Cytoprotective autophagy induction by withaferin A in prostate cancer cells involved GABARAPL1. Mol Carcinog. 2020;59:1105–1115. [PubMed: 32743846]
22. Schaaf MB, Keulers TG, Vooijs MA, Rouschop KM. LC3/GABARAP family proteins: autophagy-(un)related functions. FASEB J. 2016;30:3961–3978. [PubMed: 27601442]
23. Singh B, Bhaskar S. Methods for detection of autophagy in mammalian cells. Methods Mol Biol. 2019;2045:245–258. [PubMed: 30242567]
24. Bieller A, Pasche B, Frank S, et al. Isolation and characterization of the human forkhead gene FOXQ1. DNA Cell Biol. 2001;20:555–561. [PubMed: 11747606]
25. Fu Y, Hong L, Xu J, et al. Discovery of a small molecule targeting autophagy via ATG4B inhibition and cell death of colorectal cancer cells in vitro and in vivo. Autophagy. 2019;15:295–311. [PubMed: 30176161]
26. Zhao J, Brault JJ, Schild A, Goldberg AL. Coordinate activation of autophagy and the proteasome pathway by FoxO transcription factor. Autophagy. 2008;4:378–380. [PubMed: 18227643]
27. Lin Z, Niu Y, Wan A, et al. RNA m6A methylation regulates sorafenib resistance in liver cancer through FOXO3-mediated autophagy. EMBO J. 2020;39:e103181. [PubMed: 32368828]
28. Fitzwalter BE, Thorburn A. FOXO3 links autophagy to apoptosis. Autophagy. 2018;14:1467–1468. [PubMed: 29938591]
29. Wang Y, Sun L, Qiu W, et al. Inhibiting Forkhead box K1 induces autophagy to reverse epithelial-mesenchymal transition and metastasis in gastric cancer by regulating Myc-associated zinc finger protein in an acidic microenvironment. Aging (Albany NY). 2020;12:6129–6150. [PubMed: 32268297]
30. Yu W, Cui X, Wan Z, Yu Y, Liu X, Jin L. Silencing forkhead box M1 promotes apoptosis and autophagy through SIRT7/mTOR/IGF2 pathway in gastric cancer cells. J Cell Biochem. 2018;119:9090–9098. [PubMed: 29953672]
31. Glick D; Barth S; Macleod KF Autophagy: Cellular and molecular mechanisms. J Pathol. 2010;221:3–12. [PubMed: 20225336]
32. Jiang GM, Tan Y, Wang H, et al. . The relationship between autophagy and the immune system and its applications for tumor immunotherapy. Mol Cancer. 2019;18:17. [PubMed: 30678689]
33. Bortnik S, Tessier-Cloutier B, Leung S, et al. Differential expression and prognostic relevance of autophagy-related markers ATG4B, GABARAP, and LC3B in breast cancer. Breast Cancer Res Treat. 2020;183:525–547. [PubMed: 32685993]

34. Bortnik S, Choutka C, Horlings HM, et al. Identification of breast cancer cell subtypes sensitive to ATG4B inhibition. Oncotarget 2016;7:66970–66988. [PubMed: 27556700]

35. Zhang Z, Ni C, Chen W, et al. Expression of CXCR4 and breast cancer prognosis: a systematic review and meta-analysis. BMC Cancer. 2014;14:49. [PubMed: 24475985]

36. Xu C, Zhao H, Chen H, Yao Q. CXCR4 in breast cancer: oncogenic role and therapeutic targeting. Drug Des Devel Ther. 2015;9:4953–4964.

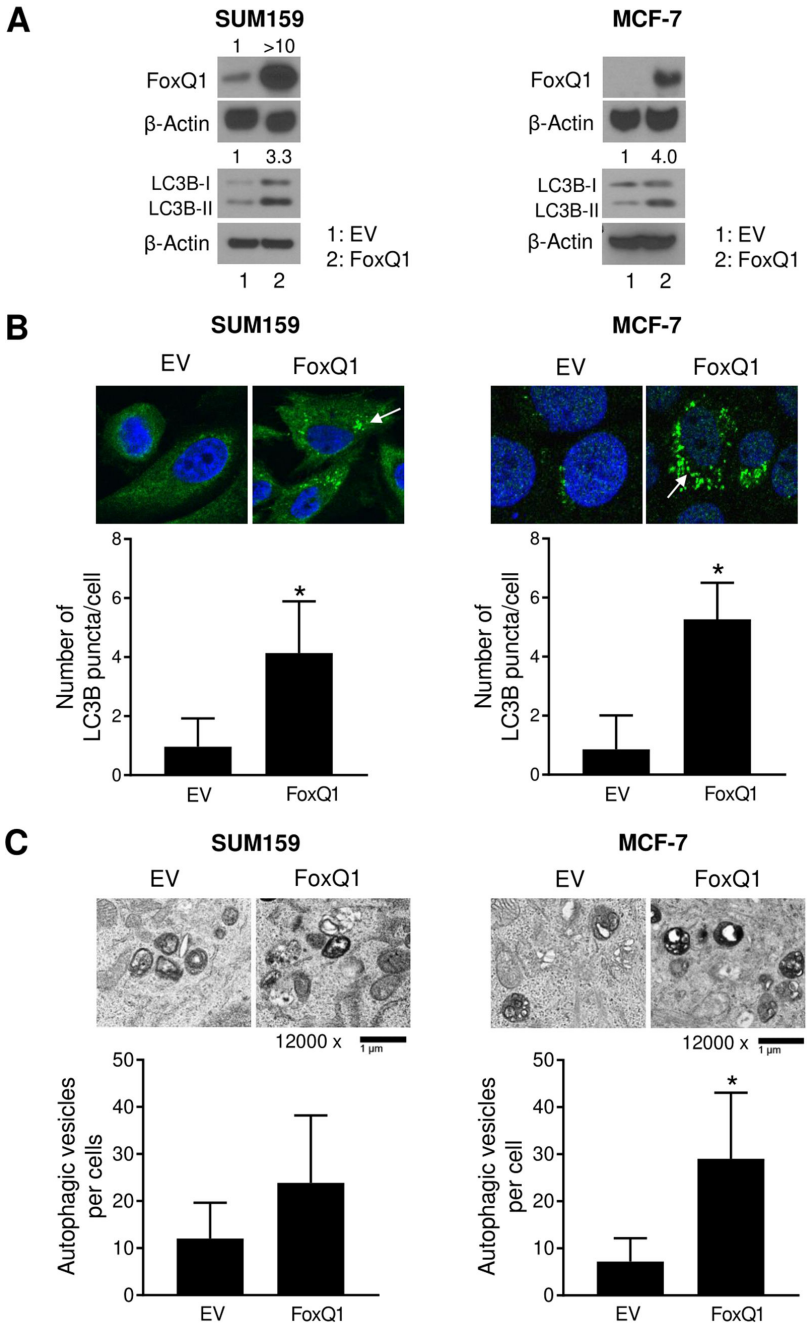

Figure 1.

FoxQ1 overexpression induces autophagy in breast cancer cells. (A) Immunoblotting for FoxQ1 and LC3B-I/LC3B-II expression using lysates from EV or FoxQ1 overexpressing SUM159 and MCF-7 cells. The numbers on top of the bands represent change in FoxQ1 and LC3B-II protein level compared with corresponding EV cells. (B) Representative immunofluorescence microscopy images and quantitation of LC3B puncta (indicated by the arrow) in EV or FoxQ1 overexpressing SUM159 and MCF-7 cells (60x objective magnification). Results shown are mean ± SD (n=19-30). *Statistical significance (P<0.05)

was determined by Student's *t*-test. (C) Representative transmission electron micrographs (12,000 magnification; scale bar = 1 μm) and quantification of the number of autophagic vesicles in EV or FoxQ1 overexpressing SUM159 and MCF-7 cells. Results are shown as mean ± SD (n=6). *Significantly different ($P<0.05$) compared with corresponding EV cells by Student's *t*-test. Consistent results were seen in independent replicate experiments.

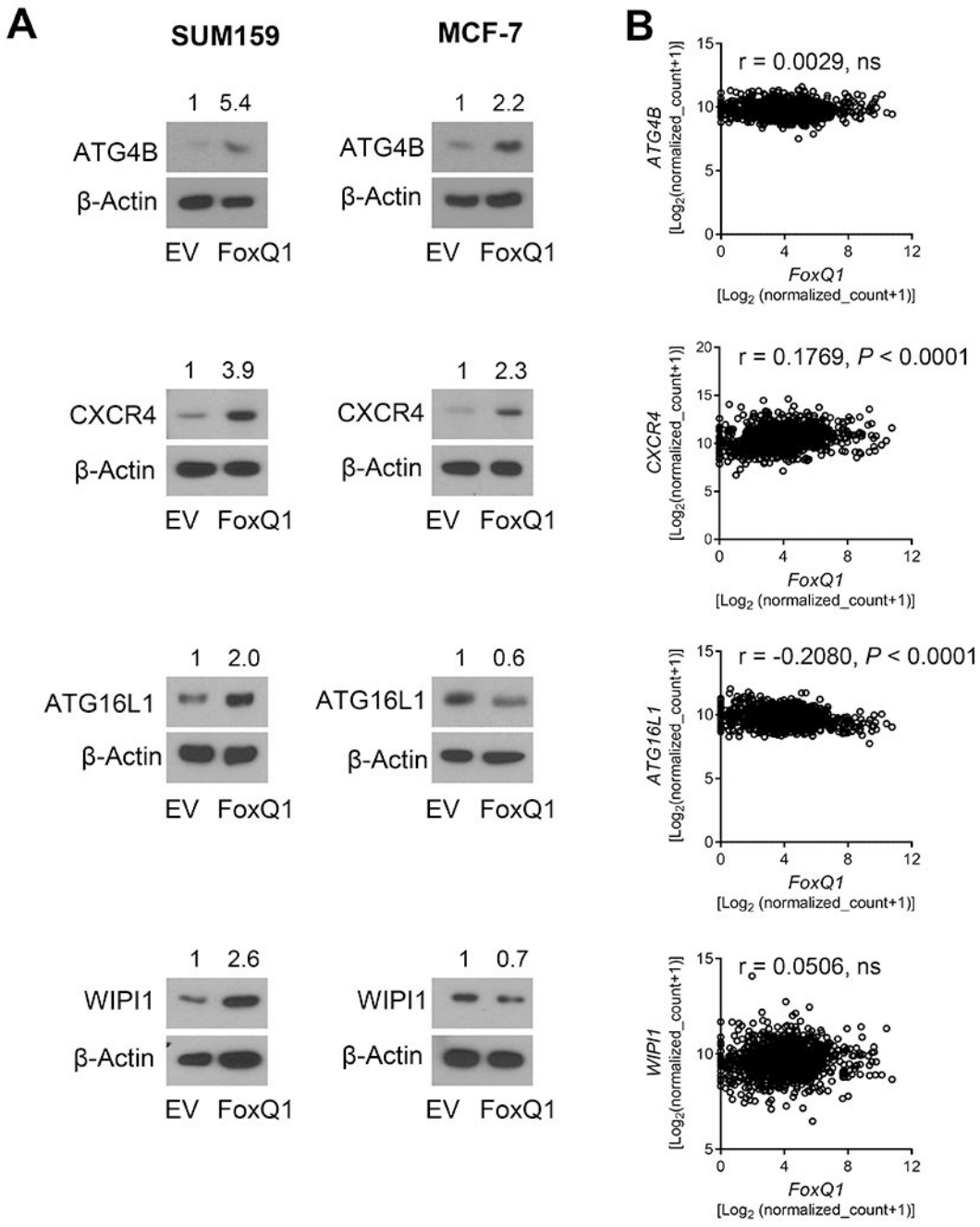

Figure 2.

FoxQ1 overexpression upregulates ATG4B and CXCR4 protein expression in breast cancer cells. (A) Immunoblotting for ATG4B, CXCR4, ATG16L1 and WIPI1 protein expression using lysates from EV or FoxQ1 overexpressing SUM159 and MCF-7 cells. Numbers above bands indicate relative protein expression changes compared with EV cells. Consistent results were seen in independent replicate experiments. (B) Correlation between *FoxQ1* expression with that of *ATG4B*, *CXCR4*, *ATG16L1*, and *WIPI1* in breast cancer TCGA

dataset (n=1097). Pearson test was used to determine the correlation coefficient and statistical significance. ns, non-significant.

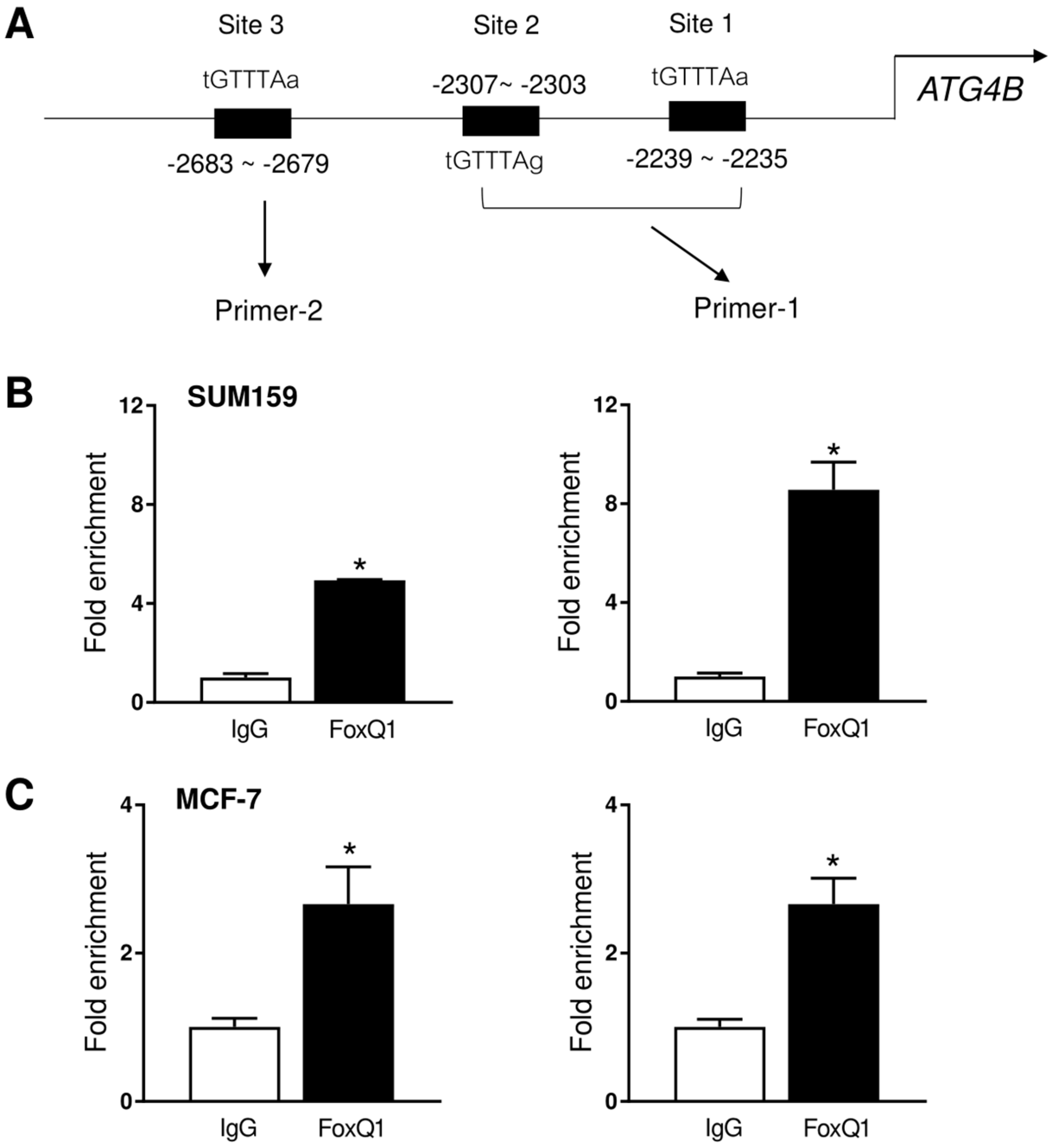

Figure 3.

FoxQ1 is recruited to the promoter of *ATG4B*. (A) Schematic diagram of putative FoxQ1 binding sites at the *ATG4B* promoter. Bar graphs show recruitment of FoxQ1 at the *ATG4B* promoter in SUM159 (B) or MCF-7 (C) cells. Fold enrichment was calculated after normalization to input. Results shown are the mean ± SD (n=3). *Statistical significance (P<0.05) was determined by Student's t-test. Consistent results were seen in independent replicate experiments.

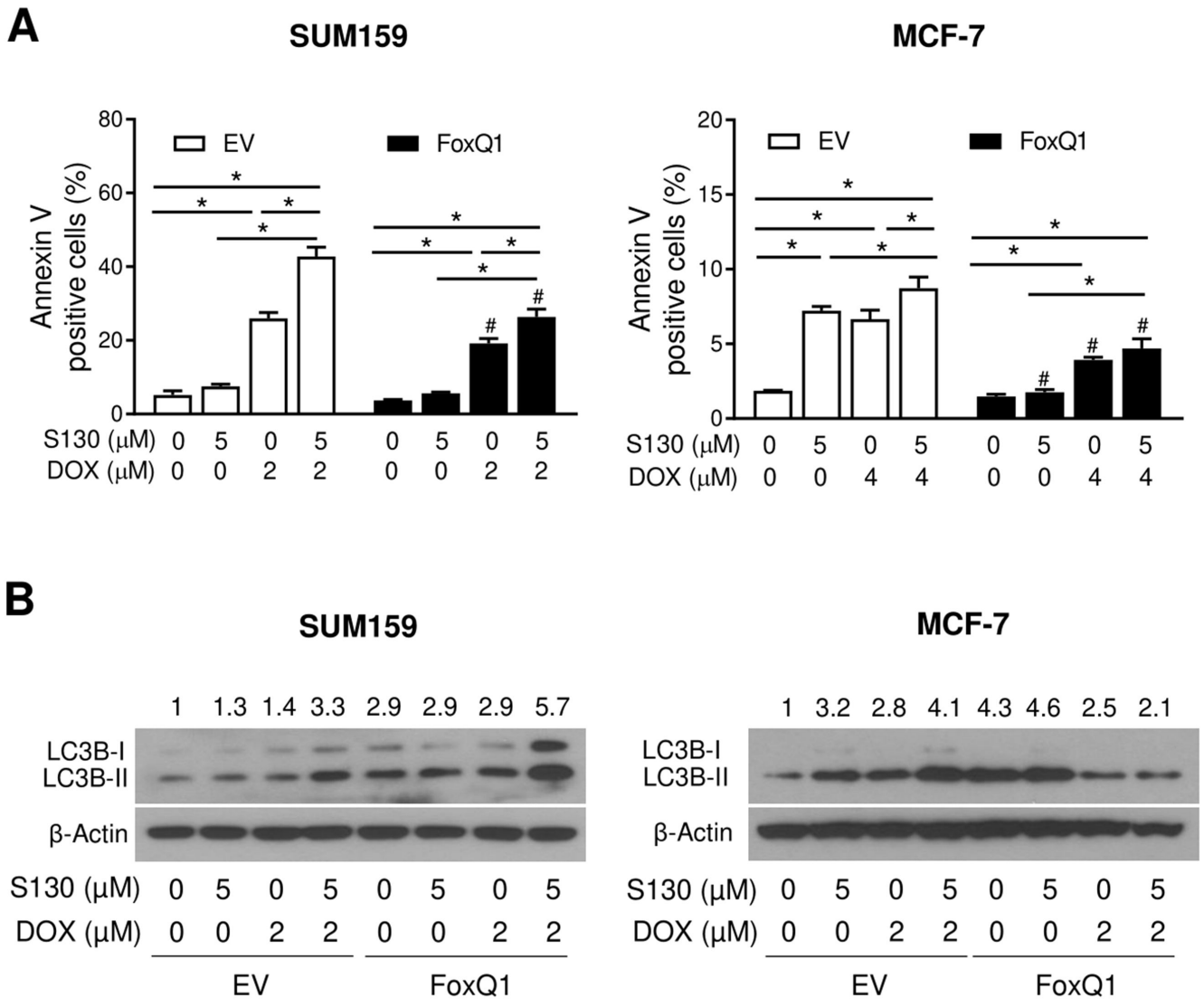

Figure 4.

Chemical inhibition of ATG4B increases DOX-induced apoptosis in human breast cancer cells. (A) Quantitation of apoptotic fraction in EV or FoxQ1 overexpressing SUM159 and MCF-7 cells treated with DMSO or 2~4 μM DOX in the absence or presence of 5 μM S130 for 24 hours (mean ± S.D., n=3). *Significantly different between the indicated group or # between EV and FoxQ1 overexpressing cells at the same dose of each drug by one-way ANOVA followed by Bonferroni's multiple comparison test. (B) Immunoblotting for LC3B-II expression using lysates from EV or FoxQ1 overexpressing SUM159 and MCF-7 cells treated with DMSO or 2 μM DOX in the absence or presence of 5 μM S130 for 24 hours. Numbers above the bands represent fold change in LC3B-II expression level relative to DMSO-treated EV control. Similar results were observed in replicate experiments.

Table 1.

A list of autophagy related gene significantly ($P < 0.05$) altered by FoxQ1 overexpression in SUM159 cells.

Gene name	Autophagy PCR array Fold change	RNA-seq Fold change
ATG10	1.8	1.2 (ns)
ATG16L1	1.4	1.3 (*)
ATG4B	1.6	1.3 (*)
CASP3	1.7	1.1 (ns)
CLN3	1.3	-
CTSD	1.6	0.4 (*)
CTSS	2.1	0.3 (*)
CXCR4	161	-
FADD	1.3	0.9 (ns)
GABARAP	1.7	0.3 (*)
GABARAPL2	1.7	1.0 (ns)
HDAC1	1.7	0.9 (ns)
HSPA8	3	0.8 (*)
IGF1	39.4	3.3 (ns)
LAMP1	1.4	0.6 (*)
MAP1LC3B	1.8	1.1 (*)
NPC1	1.3	0.9 (*)
PIK3R4	1.2	0.9 (*)
PTEN	1.3	1.4 (*)
SNCA	2.3	0.9 (*)
SQSTM1	1.9	0.7 (*)
TGFB1	1.9	1.1 (ns)
WIPI1	1.6	1.2 (ns)
BCL2L1	0.5	1.2 (*)
DRAM1	0.6	1.2 (*)
MAPK8	0.6	0.9 (ns)
NFKB1	0.8	1.1 (ns)
PRKAA1	0.8	1.3 (*)
RPS6KB1	0.7	0.7 (*)
TGM2	0.6	1.4 (*)
TNF	0.3	0.2 (ns)
ULK2	0.5	3.1 (*)

*Abbreviations: Statistically significant compared to EV cells by Student's *t*-test; ns, non-significant; *ATG10*, autophagy related 10; *ATG16L1*, autophagy related 16 like 1; *ATG4B*, autophagy related 4B cysteine peptidase; *CASP3*, caspase 3 apoptosis related cysteine peptidase; *CLN3*, ceroid-lipofuscinosis neuronal 3; *CTSD*, cathepsin D; *CTSS*, cathepsin S; *CXCR4*, chemokine (C-X-C motif) receptor 4; *FADD*, Fas (TNFRSF6)-associated via death domain; *GABARAP*, GABA(A) receptor-associated protein; *GABARAPL2*, GABA(A) receptor-associated protein-like 2;

HDAC1, histone deacetylase 1; *HSPA8*, heat shock 70kDa protein 8; *IGF1*, insulin-like growth factor 1; *LAMP1*, lysosomal-associated membrane protein 1; *MAP1LC3B*, microtubule-associated protein 1 light chain 3 beta; *NPC1*, Niemann-Pick disease type C1; *PIK3R4*, phosphoinositide-3-kinase regulatory subunit 4; *PTEN*, phosphatase and tensin homolog; *SNCA*, synuclein alpha; *SQSTM1*, sequestosome 1; *TGFB1*, transforming growth factor beta 1; *WIPI1*, WD repeat domain phosphoinositide interacting 1; *BCL2L1*, BCL2-like 1; *DRAM1*, DNA-damage regulated autophagy modulator 1; *MAPK8*, mitogen-activated protein kinase 8; *NFKB1*, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; *PRKAA1*, protein kinase AMP-activated alpha 1 catalytic subunit; *RPS6KB1*, ribosomal protein S6 kinase 70kDa polypeptide 1; *TGM2*, transglutaminase 2; *TNF*, tumor necrosis factor; *ULK2*, unc-51-like kinase 2.

Table 2.

A list of autophagy related gene significantly ($P < 0.05$) altered by FoxQ1 overexpression in MCF-7 cells.

Gene name	Autophagy PCR array Fold change
ATG10	1.3 *
ATG4B	1.3 *
BAD	1.5 *
BCL2	3.6 *
FAS	1.5 *
GABARAP	1.9 *
GABARAPL2	1.4 *
HGS	1.3 *
IGF1	5.3 *
LAMP1	2.2 *
MAPK8	1.6 *
MAP1LC3B	1.7 *
TNFSF10	1.5 *
ATG16L1	0.8 *
CXCR4	0.3 *
DAPK1	0.2 *
TGM2	0.8 *
TMEM74	0.6 *
TNF	0.4 *
ULK1	0.7 *
ULK2	0.8 *
WIPI1	0.8 *

* Abbreviations: Statistically significant compared to EV cells by Student's *t*-test; *ATG10*, autophagy related 10; *ATG4B*, autophagy related 4B cysteine peptidase; *BAD*, BCL-2 associated agonist of cell death; *BCL2*, B-cell CLL/lymphoma 2; FAS, Fas (TNF receptor superfamily, member 6); *GABARAP*, GABA(A) receptor-associated protein; *GABARAPL2*, GABA(A) receptor-associated protein-like 2; *HGS*, hapatocyte growth factor-regulated tyrosine kinase substrate; *IGF1*, insulin-like growth factor 1; *LAMP1*, lysosomal-associated membrane protein 1; *MAPK8*, mitogen-activated protein kinase 8; *MAP1LC3B*, microtubule-associated protein 1 light chain 3 beta; *TNFSF10*, tumor necrosis factor super family, member 10; *ATG16L1*, autophagy related 16 like 1; *CXCR4*, chemokine (C-X-C motif) receptor 4; *DAPK1*, death-associated protein kinase 1; *TGM2*, transglutaminase 2; *TMEM74*, Transmembrane protein 74; *TNF*, tumor necrosis factor; *ULK1*, unc-51-like kinase 1; *ULK2*, unc-51-like kinase 2; *WIPI1*, WD repeated domain phosphoinositide interacting 1.