

Article

WBP2 Attenuates Metformin Response in HER2-Positive Breast Cancer Cells by Repressing AMPK Activation and Inducing a Lower AMP:ATP Ratio State Through Enhanced ATP Production

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Highlights

What are the main findings?

- WBP2 inhibits the metformin response of HER2+ breast cancer cells.
- WBP2 represses metformin-induced AMPK activation while concomitantly decreasing AMP:ATP ratio through promoting glycolytic capacity and mitochondria respiration.

What are the implications of the main findings?

- WBP2 is a potential biomarker for predicting response and facilitates repurposing of metformin for cancer therapy.



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Abstract

Metformin is an antidiabetic drug that has been tested widely as an anti-cancer agent. However, data from clinical trials have been mixed. Evidence for metformin's efficacy in HER2+ breast cancer exists. Hence, we evaluated whether WBP2, a HER2-coamplified gene, can regulate the response of HER2+ breast cancer to metformin. Identification of biomarkers for predicting metformin response has implications in repurposing metformin for precision oncology. The effect of WBP2 on breast cancer response to metformin was studied using in vitro and mouse models. The mechanism of WBP2 on metformin-induced AMPK activation was elucidated, and its co-expression with p-AMPK was examined in clinical specimens using IHC. RNA-seq analyses were performed to elucidate WBP2's mechanism in energy metabolism. WBP2 inhibited the metformin response of HER2+ breast cancer in vitro and in vivo. These effects were concomitant with WBP2-mediated repression of

metformin-induced AMPK activation and mTOR inhibition in HER2+ breast cancer cells, a lower AMP:ATP ratio state, and enhanced glycolytic capacity and mitochondria respiration. Analysis of HER2-positive breast cancer samples supports the negative correlation between WBP2 expression and activated AMPK observed in vitro. RNA-seq analysis revealed the potential mechanism of WBP2 in regulating ATP production processes and preferential effect of WBP2 on metformin response in HER2+ breast cancer. This study reported a novel role of WBP2 in cancer metabolism and energetics that contributes new insights into the molecular etiology of cancer. WBP2 may be a biomarker for patient stratification, paving the way towards repurposing metformin for precision oncology.

Keywords: WW-domain binding protein 2; metformin; HER2-positive breast cancer; drug repurposing; biomarker

1. Introduction

Breast cancer (BC) is the second most common cancer and second leading cause of cancer-related mortality [1]. It can be classified into four molecular subtypes, with HER2-overexpressed and triple-negative breast cancer (TNBC) being the most aggressive and prone to drug resistance [2]. Despite advancement in early detection and targeted therapy for BC treatment, drug resistance and disease recurrence remain a major clinical problem. Identification of drug targets and repurposing of existing drugs for BC treatment are avenues to address these challenges.

Increasing evidence has shown that metabolic disease, particularly type 2 diabetes (T2D), has a significant association with BC risk and prognosis. Epidemiological studies have demonstrated that T2D is associated with a 20–27% increase in risk of BC in women and worse disease outcomes compared to non-diabetic patients [3–6]. Metformin, the first-line drug for T2D, has emerged as a potential drug for cancer prevention and therapy. In some observational studies, metformin treatment in diabetic patients correlated with reduced BC incidence and improved survival [7–9]. Metformin treatment was also shown to improve the prognosis of patients with HER2-positive BC (HER2+ BC) [10,11]. However, the MA.32 randomized clinical trial failed to demonstrate that metformin could significantly improve survival outcomes in BC patients [12]. An exploratory study conducted on the MA.32 trial found that HER2+ BC patients with the rs11212617 allele treated with metformin achieved improved survival outcome [12]. This shows that molecular stratification of patients may be an important component in precision therapy involving metformin. It is conceivable that there exist other biomarkers that could aid in the selection of BC patients who would better benefit from metformin treatment. A potential avenue for the discovery of biomarkers is through the elucidation of molecular regulators and effectors in the anti-cancer effects of metformin.

Metformin exerts many of its beneficial effects through the activation of AMP-activated protein kinase (AMPK). In normal states, AMPK is a central regulator of cellular energy homeostasis by sensing AMP:ATP levels and responds to metabolic stress by activating catabolic pathways and inhibiting anabolic processes to maintain ATP for survival [13,14]. In cancer, AMPK generally acts as a tumor suppressor, inhibiting several pro-tumorigenic pathways, inducing cell cycle arrest, and promoting catabolic metabolism, all of which limit cancer cell survival and growth [15]. Activation of AMPK is a key mode of action of metformin as an anti-cancer drug.

WBP2 (WW domain-binding protein 2) is a well-established oncogene that plays a role in many human cancers [16]. It has been demonstrated to exert its oncogenic function via the

regulation of oncogenic and tumor-suppressing pathways such as the ER [17], EGF [18,19], Wnt [20], NFkB [21] and Hippo signaling cascades [19,22,23]. WBP2 has been demonstrated to regulate the response of cancer cells to chemotherapy and targeted therapeutics. For example, WBP2 was found to confer resistance to doxorubicin in the luminal subtype of BC cells [24,25]. WBP2 is a HER2-coamplified gene and was demonstrated by our group to regulate the response of HER2-positive BC cells to trastuzumab in vitro, in vivo and, in a retrospective study, putatively through its role in modulating cell surface HER2 [26].

Given the demonstrated roles of WBP2 in regulating cancer cellular responses, including that of HER2-positive breast cancer cells to anti-cancer drugs, and coupled with the better prognosis of patients with HER2+ BC in association with metformin treatment, we investigated the role of WBP2 in regulating the response of HER2+ BC cells to metformin to examine whether WBP2 may be a determinant of the cancer cellular response to metformin. In this study, WBP2 was discovered to confer resistance to metformin via inhibition of AMPK, putatively in a cellular energetics-dependent manner.

2. Materials and Methods

2.1. Reagent

In-house monoclonal mouse anti-WBP2 (clone 4D2A1) was produced via service from GenScript (USA). Anti-HER2 (#2165), Anti- β -actin (#4967), Anti-phospho-AMPK α -Thr172 (#2535), Anti-AMPK α (#2432), Anti-phospho-mTOR-Ser2448 (#5536), Anti-mTOR (#2983), Anti-phospho-S6 ribosomal protein-Ser235/236 (#2211) and Anti-S6 ribosomal protein (#2212) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-V5 (V2260) antibody was purchased from Sigma-Aldrich (Burlington, MA, USA). Anti-GADPH (MA5-15738) antibody was purchased from Invitrogen, Thermo Fisher Scientific (Waltham, MA, USA). Goat Anti-mouse-IgG-HRP (#31430) and Goat Anti-rabbit-IgG-HRP (#31460) antibodies were purchased from Pierce, Thermo Fisher Scientific (USA). Metformin (D150959) was purchased from Sigma-Aldrich (Burlington, MA, USA). WBP2 siRNAs, Luciferase siRNA and scramble siRNA were purchased from Invitrogen, Thermo Fisher Scientific (USA). The siRNA sequences were listed in Table S1. WBP2 overexpression plasmid (pLenti-puro-V5-WBP2) and knockdown (shRNAs) constructs (pLKO-puro-shWBP2) were previously described [18,20].

2.2. Cell Lines, Culture Conditions and Transient Transfection

Human breast cancer cell lines SK-BR-3, BT-474, ZR-75-30, MDA-MB-453, MDA-MB-231, MDA-MB-468, BT-549 and BT-20 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), and were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, Life Technologies Corporation, San Diego, CA, USA) supplemented with 10% (*v/v*) Fetal Bovine Serum (FBS) (HyClone-Cytiva, Washington, DC, USA) and 1% (*v/v*) penicillin–streptomycin (Biological Industries, Sartorius, Cromwell, CT, USA). All cell lines were authenticated using short tandem repeat DNA profiling (Tsingke Biotech, China). For stable plasmid expression of shRNA or proteins of interest, transfected cells were selected with 0.5 μ g/mL puromycin (Invitrogen, Waltham, MA, USA) for BT-474 and SK-BR-3 cells. The cells were selected for 2–3 weeks before use and expansion. For transient expression, cells were reverse-transfected with siRNA or protein-expressing plasmid using the jetPRIME transfection reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's recommendations.

2.3. Cell Viability Assay

The cells were plated on a 96-well cell culture plate (VWR International, Radnor, PA, USA) until 80–90% confluency on the day of the assay. On the second day, the cells

were treated with various doses of metformin (0–80 mM) and incubated for three to five days. Cell viability was measured by using CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer protocol. Absorbance was read using Synergy H1 microplate reader at 490 nm (BioTek Instruments, Winooski, VT, USA). IC₅₀ values were then calculated using the GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA).

2.4. Immunoblotting Analysis

Cell lysis and Western blot analysis were performed as described previously [20,26]. Briefly, following treatment, cells were washed with PBS twice and lysed using Radioimmuno-precipitation assay (RIPA) lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) containing protease and a phosphatase inhibitor cocktail (Pierce, Thermo Fisher Scientific, USA). Concentration of the protein lysates was estimated using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to manufacturer protocol. An equal amount of protein (20–50 µg) was resolved in polyacrylamide gel and transferred onto a polyvinylidene difluoride fluoride (PDVF) membrane (GE healthcare, Chicago, IL, USA). The membranes were blocked with 1% BSA in TBST (Biowest, Bradenton, FL, USA) for 1 h and probed with their respective primary antibodies at 4 °C overnight. Next, the primary antibodies were removed, and membranes were incubated with their respective horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Chemiluminescent detection was performed by adding Western Bright ECL HRP substrate (Advansta, San Jose, CA, USA) or Amersham ECL Select Western Blotting Detection Reagent (Cytiva, Washington, DC, USA) using ChemiDoc[™] Touch Gel Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The images were processed and analyzed using Image Lab software (Bio-Rad Laboratories, USA).

2.5. Breast Cancer Tumor Xenograft Model

The animal experiment was performed in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. Eight-week-old female athymic nude mice ($n = 6–7$) were purchased from InVivos (InVivos, Singapore). The mice were first implanted with 0.72 mg, 60-day release, 17β-estradiol pellets (Innovative Research, USA) for two days. Next, BT-474 cells stably expressing vector control or WBP2 (1×10^7 in 200 µL of DPBS and Matrigel, 1:1; mixture) were injected subcutaneously into the mammary fat pad of the mice. The mice were then observed for tumor growth. When the tumor size grew to between 100 and 150 mm³, the mice were distributed equally into groups of 6–7, keeping the average tumor size similar between the groups. Next, the groups were allocated into a treatment regime of 250 mg/kg metformin (Sigma-Aldrich, USA) or saline (control) by daily intraperitoneal injection (IP) for three weeks. The mice were observed and tumor sizes were measured twice weekly with calipers; tumor volumes were calculated using the following formula: volume = (width² × length)/2. The maximal tumor size permitted by the Ethics Committee is 1.5 cm for mice, and this was not exceeded throughout the course of our study. At the endpoint, the mice were sacrificed, and tumors were harvested to measure the tumor volume. Representative images of the tumors were also imaged.

2.6. AMP/ATP Measurement

Cells were treated with or without metformin (10 mM). After 48 h, cells were lysed, and AMP and ATP were measured using an adenine nucleotide assay kit (Cat #: A-125) (Biomedical Research Service Center, University at Buffalo, Buffalo, NY, USA) according to the manufacturers' instructions. Luminescence was read using a Synergy H1 microplate reader (BioTek Instruments, USA). AMP and ATP were quantified from the same cell

lysates using identical extraction volumes, and AMP:ATP ratios were calculated within each sample prior to normalization to control condition.

2.7. Seahorse XF-24 Metabolic Flux Analysis

The extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a Seahorse XF-24 extracellular flux analyzer (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's recommendations. The cells were plated at 20,000 cells per well in Seahorse XF-24 plates. Before analysis, the cell culture medium was replaced with Seahorse XF Base Medium containing 10 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine (pH 7.4) for the mito stress assay and Seahorse XF Base Medium containing 2 mM L-glutamine (pH 7.4) for the glycolysis stress assay and were incubated at 37 °C for 1 h in a non-CO₂ incubator. For the mito stress test, oligomycin (1 µM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 2 µM), and antimycin/rotenone (1 µM/1 µM) were sequentially injected, and for the glycolysis stress assay, glucose (10 mM), oligomycin (1 µM), and 2-deoxy-glucose (2-DG) (50 mM) were sequentially injected. All data were normalized to total cellular protein per well (BCA assay; Thermo Fisher Scientific, USA) measured from replicate wells lysed immediately after the Seahorse run. Proton leak, ATP production, and basal, maximal, and spare respiratory capacity were calculated from OCR data, while glycolysis, glycolytic capacity, and glycolytic reserve were derived from ECAR profiles. Data was analyzed by Seahorse XF-96 Wave software (Agilent Technologies, USA) and GraphPad Prism 10 software (GraphPad Software, USA).

2.8. Immunohistochemistry Analysis

Breast tumor tissues from patients with HER2-positive invasive ductal carcinoma who received surgery between 2019 and 2023 with clinical and histopathological information were obtained from Peking University People's Hospital, China ($n = 31$). All 31 patients were of Han ethnicity, female, and aged between 42 and 90 years (mean age $\pm$ SD, 60.8 ± 13.0 years). The tumors were graded and staged following the tumor-node-metastasis (TNM) staging. Detailed clinical data were not available. The freshly resected tumors were snap-frozen and stored in liquid nitrogen. Specimens were obtained with protocol approval by the Institutional Review Board of Peking University People's Hospital.

Tumors were fixed in 4% PFA, embedded in paraffin and cut into 4 µm thick serial sections for IHC. IHC was performed using the BOND Polymer Refine Detection kit (DS9800) (Leica Biosystem, Nussloch, Germany) following the manufacturer protocol on a BOND RX Fully automated research stainer (Leica Biosystem, Germany). Slides were deparaffinized using BOND Dewax Solution (AR9222) (Leica Biosystem, Germany). Antigen retrieval was performed using BOND Retrieval Solution 2 (ER2, pH 6.0) (AR9640) (Leica Biosystem, Germany) at 100 °C for 20 min. Next, endogenous peroxidase blocking was done using hydrogen peroxide from the detection kit for 5 min. Slides were then incubated with the primary antibody against WBP2 (in-house monoclonal mouse anti-WBP2 (clone 4D2A1) at 1:2000 dilution) or p-AMPK α (Thr172) (Anti-phospho-AMPK α -Thr172 (#2535) at 1:200 dilution) for 30 min at room temperature. Detection was carried out using the BOND Polymer Refine Detection kit with the polymer-based HRP secondary antibody system for 10 min and visualized with DAB chromogen incubation for 10 min. Counterstaining to visualize the nucleus was done by hematoxylin staining for 5 min. The slides were then dehydrated and mounted with coverslip using neutral balsam resin (G8950) (Solarbio Life Science, Beijing, China).

WBP2- and p-AMPK α (Thr172)-stained slides were digitalized by scanning at $\times 20$ magnification using a KF-BIO-PRO-020 digital slide scanner (KFBIO, Yuyao, China), generating high-resolution whole-slide images. Digital image analysis was performed using QuPath open-source software (version 0.6.0) [27]. Tumor and stromal segmentation via pixel classification, positive cell detection, and H-score quantification were conducted following published QuPath workflows [28,29]. Staining intensity for each cell was classified as negative (0), weak (1+), moderate (2+), or strong (3+) using optimized DAB density thresholds. The H-score was calculated for each region of interest (ROI) using the formula

$$\text{H-score} = (1 \times \% \text{cells } 1+) + (2 \times \% \text{cells } 2+) + (3 \times \% \text{cells } 3+)$$

Yielding a range of 0–300. The H-score for tumor compartment was calculated and exported from QuPath for downstream analysis.

2.9. RNA Sequencing Analysis

Cell samples were prepared and sent for next-generation RNA sequencing with Novogene Technology (Beijing, China). Total RNA was extracted from the cells, and RNA library preparation was done using the mRNA library preparation kit (poly A enrichment). Clustering of the libraries was performed and sequencing was done on the Novaseq-PE150 illumina system. Raw FastQ files were processed through the Fastp software and mapped to reference genome using HISAT2. FeatureCounts was used to count the read numbers mapped to each gene. And then, the FPKM of each gene was calculated based on the length of the gene and the read count mapped to this gene. Differential gene expression analysis was performed on all expressed genes using DESeq2. The threshold of significant differential expression was set at p -value ≤ 0.05 . Differentially expressed genes were further analyzed, and heatmaps of the selected genes were generated using GraphPad Prism 10 software (GraphPad Software, USA). KEGG pathway enrichment analysis was performed with the web tool ShinyGO 0.85 by providing all common downregulated DEGs as the input for the experiment [30].

2.10. Statistical Analysis

All in vitro experiments were performed in triplicate, and the results were presented as mean $\pm$ SD. The comparisons between two groups for IC₅₀ values were determined by Student's t test, as the values were continuous variables with normal distribution. To compare multiple groups of samples, a one-way ANOVA was performed with post hoc Tukey correction to determine statistical differences in the pairwise comparison for the AMP:ATP ratio and seahorse assays, as their variables were continuous and normally distributed. For in vivo experiments, the data represents mean $\pm$ SEM, and the significance of differences or associations was evaluated using a Mann–Whitney U test, as the sample size was small and tumor sizes were often skewed. For the retrospective IHC study, the data was represented as medians with interquartile ranges, and the significance of differences or associations was evaluated using a Mann–Whitney U test and Spearman correlation, as H-scores are often skewed and not normally distributed. p -values of <0.05 were considered statistically significant and expressed as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. All statistical analyses were performed using GraphPad Prism 10 (GraphPad Software, USA).

3. Results

3.1. Metformin Inhibited HER2+ BC Cells More than HER2- BC Cells

HER2-positive/overexpressing BC has been reported to exhibit increased sensitivity to the anti-cancer effects of metformin [31]. Therefore, comparative studies using a panel of HER2-positive and HER2-negative BC cell lines were subjected to escalating doses of

metformin in the millimolar range, as guided by existing cancer studies [32–34]. The BC cell lines used displayed variable responses to the anti-cancer effect of metformin, as shown by the IC₅₀ values (Figure 1A). Consistent with a published report [31], BC with HER2 overexpression tends to be more sensitive to metformin. Next, we probed the expression of WBP2 and HER2 in the panel of BC cell lines to confirm the HER2 and WBP2 expression status in these cell lines (Figure 1B). The HER2 expression of the cell lines was as expected.

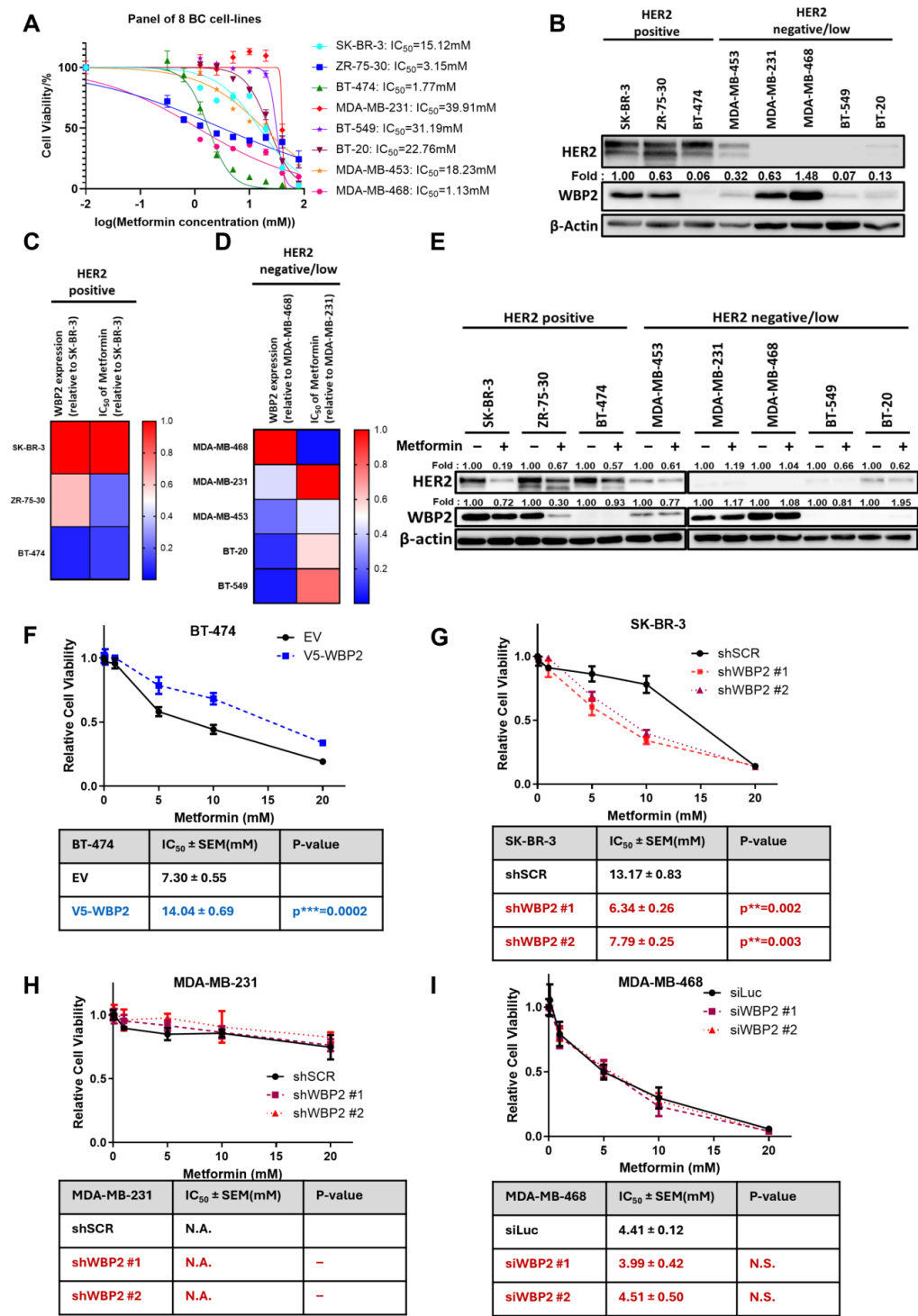


Figure 1. WBP2 expression preferentially inhibits metformin’s anti-cancer efficacy in HER2-positive breast cancer cells. (A) A cell viability assay was performed on a panel of eight

breast cancer cell lines—SK-BR-3, ZR-75-30, BT-474, MDA-MB-453, MDA-MB-231, MDA-MD-468, BT-549 and BT-20—following 3 days of metformin treatment with varying doses using CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega). (B) Immunoblotting analysis showing HER2, WBP2 and β -actin expression in a panel of eight breast cancer cells. Fold change represented refers to WBP2 signals normalized to β -actin and relative to SK-BR-3. (C) Heatmap representing WBP2 expression and IC₅₀ of HER2-positive cells. WBP2 expression fold change is relative to SK-BR-3. IC₅₀ fold change is relative to SK-BR-3. (D) Heatmap representing WBP2 expression and IC₅₀ of HER2-negative/low cells. WBP2 expression fold change is relative to MDA-MB-468. IC₅₀ fold change is relative to MDA-MB-231. (E) Immunoblotting analysis showing HER2, WBP2 and β -actin expression in a panel of eight breast cancer cells treated with 10 mM metformin for 48 h. Fold change represented is normalized to β -actin and relative to untreated control. (F–I) Cell viability assay was performed in breast cancer cell lines with WBP2 overexpressed or knocked down following 5 days of treatment with varying doses of metformin using CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega). (F) WBP2 overexpressed in BT-474. (G) WBP2 knockdown in SK-BR-3. (H) WBP2 knockdown in MDA-MB-231. (I) WBP2 knockdown in MDA-MB-468. IC₅₀ was calculated by non-linear regression using GraphPad Prism 10. The data represent mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$, NS = not statistically significant; NA = not applicable. EV = Empty Vector control. SCR = Scrambled control. Luc = Luciferase control. Statistical analysis was performed using unpaired Student's *t*-test.

The relative IC₅₀ and expression of WBP2 in the BC cell lines are summarized in a heatmap in Figure 1C (HER2-positive) and Figure 1D (HER2-negative) to compare the association between WBP2 expression and metformin response. In the HER2+ BC cells, WBP2 expression was observed to be positively associated with the IC₅₀ value, where higher-WBP2-expressing cells (SK-BR-3, IC₅₀ = 15.12 mM) were more resistant to metformin compared to low-WBP2-expressing cells (BT-474, IC₅₀ = 1.77 mM). On the contrary, there was no clear association between WBP2 expression and metformin response in the HER2-negative/low BC cell line.

Since WBP2 and HER2 expression was associated with cellular response to metformin, specifically where HER2+ BC cells with a high level of WBP2 were more resistant to metformin, a drug–protein interaction between metformin and WBP2 is conceivable. Hence, we re-examined the protein expression of HER2 and WBP2 upon metformin treatment. Metformin was shown to downregulate HER2 expression in all HER2-expressing cell lines (Figure 1E). This is consistent with previous studies [31,35]. Interestingly, WBP2 was observed to be downregulated by metformin only in HER2+ BC (SK-BR-3 and ZR-75-30) but not in HER2- BC (MDA-MB-468 and MDA-MB-231) (Figure 1E). This raises the possibility that metformin exerts its anti-cancer effect on HER2+ BC cells by inhibiting the WBP2 oncogene via modulators that are mobilized only in HER2+ BC cells. However, elucidating the mechanism of the HER2 effect of metformin on WBP2 is not in the purview of this study. Together, these results suggest that WBP2 and HER2 expression was associated with an anti-cancer response of cells to metformin.

3.2. WBP2 Expression Inhibits the Response of BT-474 and SK-BR-3 HER2+ BC Cells to Metformin

Since metformin treatment downregulated WBP2 expression, it is conceivable that WBP2 as an oncogene exerts an inhibitory effect on metformin's anti-cancer effect on HER2+ BC cells. To test this, a cell viability assay was performed in HER2+ BC cell lines, BT-474 and SKBR-3, and HER2- BC cell lines, MDA-MB-231 and MDA-MB-468 (triple-negative subtype). WBP2 was overexpressed in BT-474 or knocked down using two different shRNAs in SK-BR-3 and MDA-MB-231 and two different siRNAs in MDA-MB-468 to add robustness to the study design. These different BC cells were then treated with an increasing dose of metformin, and the cell viability was analyzed to determine their IC₅₀. Overexpression of WBP2 in HER2-positive BT-474 cells inhibited the metformin-induced

anti-cancer effect, and the IC_{50} was increased by 1.92-fold compared to the vector control (IC_{50} : 14.04 mM vs. 7.30 mM, respectively, $p = 0.0002$) (Figure 1F). Consistently, silencing of WBP2 in HER2-positive SK-BR-3 cells resulted in an enhanced response to metformin by ~2.08-fold compared to the control shRNA (IC_{50} : 6.34 mM or 7.79 mM for WBP2 knockdown vs. 13.17 mM for control, $p = 0.002$) (Figure 1G). In contrast, silencing of WBP2 in HER2-negative BC cells, MDA-MB-231 and MDA-MB-468, did not alter the cells' response to metformin (Figure 1H,I). These results suggest that WBP2 antagonizes metformin's action, and it abates the metformin-induced anti-cancer effect in the HER2-positive BC cell lines but not the HER2-negative BC cell lines tested in this study.

The reason behind WBP2's regulation of metformin's effect between HER2-positive and HER2-negative BC cells is unclear, but it could be due to HER2's role in activating/enhancing WBP2's oncogenic property via its dimerization with EGFR in EGFR signaling, putatively conferring resistance to cell death [26].

3.3. WBP2 Overexpression Inhibits the Anti-Tumor Response of Metformin In Vivo

To examine whether the inhibition of metformin response by WBP2 in the cell line model could be recapitulated in a mouse xenograft model, BT-474 cells stably expressing WBP2 or vector control were injected into the mammary fat pad of athymic nude mice. When the size of tumors reached 100–150 mm³, the mice were divided into two groups and treated with metformin (250 mg/kg) or saline by daily intraperitoneal (IP) injection for three weeks (Figure 2A). As shown in Figure 2B,C, treatment with metformin reduced the growth rate of the BT-474 vector tumor by 67% as compared to the saline control. On the contrary, when metformin treatment was applied to the BT-474 tumor stably expressing WBP2, the tumor size reduced by only ~29% compared to the saline treatment control in the WBP2-expressing tumor (Figure 2C). This represents an approximately 2.3-fold attenuation ($p = 0.035$). The in vivo mouse xenograft experiment confirms the in vitro findings that WBP2 inhibits metformin-induced suppression of HER2+ BC.

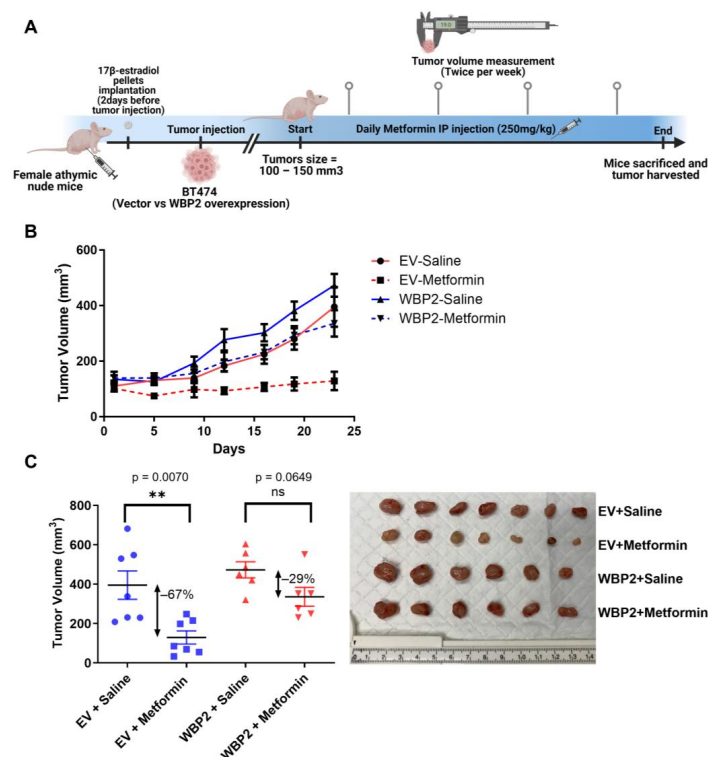


Figure 2. WBP2 expression repressed tumor response to metformin in vivo. (A) Schematic diagram of the workflow of the breast cancer mouse xenograft model. BT-474 cells expressing WBP2 or vector

(1×10^7 in 200 μL of DPBS and Matrigel 1:1 mixture) were injected into the mammary fat pad of female athymic nude mice ($n = 6\text{--}7$ each group, total 26 mice) post implantation of 17β -estradiol pellets (Innovative Research, Sarasota, FL, USA). Once the tumor size reached 100–150 mm^3 , the mice were divided equally into groups, keeping the average tumor size similar between the groups, and treated with metformin (250 mg/kg) or saline (control) by intraperitoneal (IP) injection daily for three weeks. The tumor size was measured twice weekly with calipers and tumor volumes were calculated as follows: $\text{volume} = \frac{(\text{width}^2 \times \text{length})}{2}$. The tumors were harvested and measured at the end of the experiment. (B) Tumor growth over time after the start of metformin treatment represented by line graph. (C) Endpoint tumor size represented by dot plot and tumor images. Mean percentage reduction in tumor size by metformin treatment was calculated for tumors expressing WBP2 or vector. Data are represented as the mean; error bars = SEM; $n = 6/7$. ** $p < 0.01$; ns = not statistically significant. EV = Empty Vector. Statistical analysis was performed using GraphPad Prism 10 using the Mann–Whitney U test.

3.4. WBP2 Represses the Metformin-Induced AMPK Pathway and Associated mTOR Activation in HER2+ BC Cells

A key mode of action mediating metformin's anti-tumor response is through the activation of AMPK signaling pathway. Metformin activates AMPK by increasing the AMP:ATP ratio, which induces phosphorylation and activation of AMPK at the Thr172 site [36,37]. Does the inhibition of the anti-cancer response to metformin by WBP2 in HER2+ BC cells work via AMPK? To answer this question, an overexpression study was performed in the low-WBP2-expressing BT-474 cells, and silencing of WBP2 was done in the high-WBP2-expressing SK-BR-3 cells. These cells were treated with metformin, and the phosphorylation of AMPK at Thr172 was analyzed by immunoblotting. Our preliminary data identified the IC_{50} range of metformin in BC to be between 1 and 40 mM (Figure 1A); 10 mM metformin was chosen for mechanistic assays as it is within this range and has also been used by other studies on the activation of AMPK signaling [38,39].

Treatment with metformin in both BT-474 and SK-BR-3 cells significantly increased the phosphorylation of AMPK at Thr172 (Figure 3A), while the elevated expression of WBP2 in BT-474 cells significantly reduced the metformin-induced AMPK Thr172 phosphorylation level (Figure 3A). Consistently, the silencing of WBP2 in SK-BR-3 cells promoted AMPK activation (Figure 3B). Collectively, these results show that WBP2 antagonizes metformin-induced AMPK activation.

Considering that the mTOR pathway is a key downstream effector pathway that is inhibited by metformin through the activation of AMPK, through which metformin exerts its anti-cancer effect on cellular anabolism [40,41], we examined whether the inhibition of metformin-induced AMPK activation by WBP2 affects the downstream mTOR pathway. WBP2 in SK-BR-3 or BT-474 cells was silenced and overexpressed, respectively, and cells were treated with metformin. The phosphorylation of mTOR at Ser2448 was probed as it is the predominant activating phospho-site for mTORC1, which is associated with regulating growth and nutrient signaling [42]. Furthermore, phosphorylation of S6 ribosomal protein was examined as the downstream target of mTOR signaling. Consistent with our hypothesis, metformin treatment significantly reduced the phosphorylation of mTOR and its downstream S6 ribosomal protein, while the silencing of WBP2 further suppressed the phosphorylation of mTOR and S6 (Figure 3D). Conversely, overexpression of WBP2 in the BT474 cells partially attenuated metformin-induced suppression of mTOR phosphorylation at Ser2448. However, restoration of S6 phosphorylation was not observed under these conditions (Figure 3C). This likely reflects the fact that S6 phosphorylation represents a terminal and threshold-dependent readout that is subject to AMPK-dependent and AMPK-independent suppression by metformin [43]. S6 phosphorylation, attenuated by metformin,

may occur through an AMPK-independent pathway; hence, the restoration of mTOR activity by WBP2 overexpression may not be enough to restore S6 phosphorylation. Moreover, partial restoration of ~20% upstream mTOR phosphorylation by WBP2 overexpression (Figure 3C) may be insufficient to overcome dominant inhibitory inputs by metformin. Nevertheless, the knockdown of WBP2 was able to further reduce S6 phosphorylation significantly, by close to 90%, compared to the shSCR control (Figure 3D). Taken together, this evidence suggests that WBP2 could counteract the anti-tumor role of metformin through the AMPK-mTOR axis.

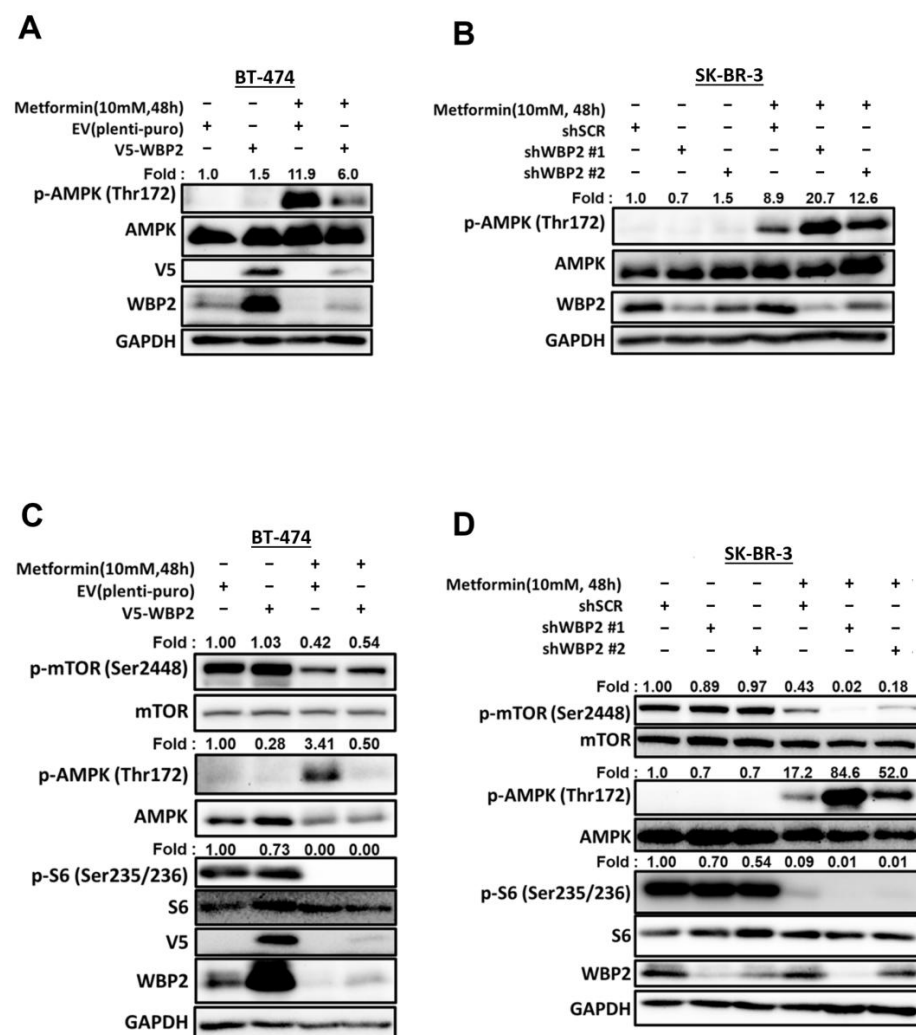


Figure 3. WBP2 represses metformin-induced AMPK pathway and its associated mTOR inhibition. (A,B) Immunoblotting analysis showing AMPK activation and WBP2 expression in cells treated with or without 10 mM metformin for 48 h. (A) WBP2 was overexpressed in BT-474 cells using WBP2-expressing plasmid or vector control. (B) WBP2 knocked down in SK-BR-3 cells with two different shRNAs or a shSCR control. (C,D) Immunoblotting analysis showing AMPK-mTOR signaling components and WBP2 expression in cells treated with or without 10 mM metformin for 48 h. (C) WBP2 was overexpressed in BT-474 cells using WBP2-expressing plasmid or vector control. (D) WBP2 knocked down in SK-BR-3 cells with two different shRNAs or a shSCR control. GAPDH was used as a loading control. EV = Empty Vector control. SCR = Scrambled control. Fold change represented was normalized to total protein and relative to non-treated EV/shSCR controls.

3.5. WBP2 Expression Induced a Lower AMP:ATP Ratio State Through Enhanced ATP Production

Since metformin indirectly activates AMPK via regulating AMP:ATP ratio by disrupting complex I of mitochondria [36,37], the influence of WBP2 expression on regulating

bioenergetic shift as a potential mechanism through which WBP2 regulates AMPK activation was examined. The measuring and reporting of the AMP:ATP ratio is appropriate because AMP and ATP competitively bind to the regulatory sites on the AMPK γ -subunit to regulate AMPK activity; hence, this effect is dependent on the AMP:ATP ratio rather than absolute nucleotide levels [43].

First, the effect of WBP2 expression on the cellular energy status in BC cells was investigated by measuring the AMP:ATP ratio. The AMP:ATP ratio was observed to decrease by ~2-fold in WBP2-overexpressing BT-474 cells compared to vector control in the absence of metformin. When metformin was added to vector control BT-474 cells, the AMP:ATP ratio increased by ~3-fold compared to untreated cells, and this increase was inhibited by WBP2 overexpression (Figure 4A). Consistently, SK-BR-3 cells under metformin treatment showed an increased AMP:ATP ratio by ~7-fold as compared to untreated control, and the knockdown of WBP2 further increased the ratio to ~14-fold, while re-expression of WBP2 reduced the elevated AMP:ATP ratio to ~5-fold (Figure 4B). This effect of WBP2 was also observed in SK-BR-3 cells not treated with metformin, where knockdown of WBP2 increased AMP:ATP ratio by 1.5-fold as compared to the shRNA control, and re-expression of WBP2 decreased the ratio to below baseline. Taken together, these data suggest WBP2 expression induced a higher energy state, shifting the equilibrium to a state with higher ATP.

Next, recognizing that WBP2 altered the energy balance to a higher bioenergetic state, we questioned the role of WBP2 in regulating energy-producing processes such as mitochondria respiration and glycolysis. To evaluate the effect of WBP2 on mitochondria respiration, we performed the Seahorse XF Cell Mito Stress test in BT-474 cells stably overexpressing WBP2 and WBP2 knockdown SK-BR-3 cells with the treatment of metformin. Mitochondrial function was analyzed by direct measurement of the oxygen consumption rate (OCR) of the cells upon treatment with different drug compounds. Compared to vector control cells, WBP2-overexpressing BT-474 exhibited significant increase in basal respiration and ATP production, suggesting enhanced mitochondria energy production. Upon FCCP injection, the maximal respiration rate was elevated significantly in WBP2-overexpressing BT-474, leading to an increase in mitochondria spare respiratory capacity (Figure 4C). Consistently, in SK-BR-3 cells with WBP2 silencing, a general decrease in basal respiration, ATP production, maximal respiration rate and mitochondria spare respiratory capacity compared to siRNA control was observed, but only the latter two parameters were found to be statistically significantly reduced (Figure 4D). In both BT-474 and SK-BR-3 control or WBP2 knockdown/overexpression cells, metformin treatment was observed to attenuate all the mitochondria respiration parameters in the Seahorse assay, which is consistent with previous reports [44,45]. Collectively, our data suggests that WBP2 enhances the mitochondria's oxidative respiratory capacity in BC cells.

To evaluate the effect of WBP2 on glycolytic functions, the Seahorse XF Glycolysis Stress Test was performed by measuring the extracellular acidification rate (ECAR) of BC cells with knocked down or overexpressed WBP2, with and without metformin treatment. In all the vector control, WBP2-overexpressing, siRNA control and WBP2-silenced cells, metformin induced an increase in glycolysis compared with untreated cells (Figure 4E,F). This is consistent with a previous study where metformin was shown to induce glycolysis as a compensatory effect for its inhibition on mitochondrial oxidative respiration [46]. No significant change was observed for basal glycolysis (ECAR after glucose addition) of WBP2-overexpressing BT-474 and WBP2-silenced SK-BR-3 as compared to their controls. In contrast, an increase in the glycolytic capacity (maximal ECAR with oligomycin treatment) of the WBP2-overexpressing BT-474 cells and a reciprocal decrease when WBP2 was silenced in SK-BR-3 cells was observed in metformin-untreated cells, although the data were not statistically significant. Notably,

glycolytic reserve was elevated significantly when WBP2 was overexpressed in BT474 cells, and WBP2-silenced SK-BR-3 cells demonstrated lowered glycolytic reserve (Figure 4E,F). These results demonstrate that WBP2 confers metabolic flexibility to BC cells by upregulating glycolytic functions in response to mitochondria stress. Collectively, the data support a role of WBP2 in mitochondrial respiration that is responsible for the bulk of ATP generation. The observed effect of WBP2 on the cellular ATP/AMP ratio may be due in part to WBP2-mediated ATP increase via mitochondrial respiration.

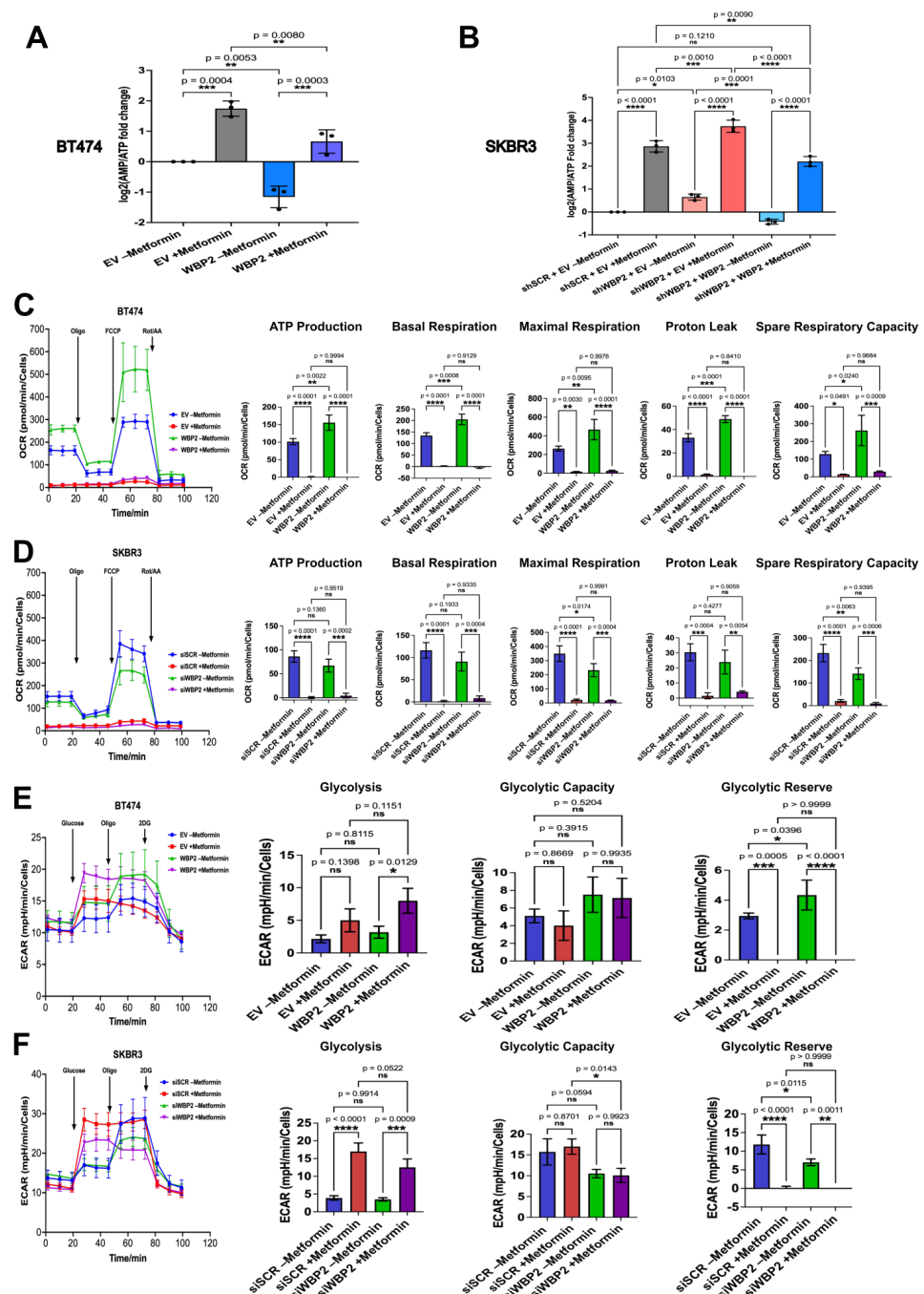


Figure 4. WBP2 enhanced cellular energy status and metabolic function in breast cancer cells. (A,B) AMP and ATP were measured from the same samples in breast cancer cells treated with or without 10 mM metformin for 48 h, and ratios were calculated prior to normalization to control conditions. The AMP/ATP ratio fold change was calculated relative to non-treated EV/shSCR controls. Data presented was log₂ of fold change. (A) WBP2 was overexpressed in BT-474 cells using WBP2-expressing

plasmid or vector control. (B) WBP2 was knocked down in SK-BR-3 cells using shWBP2 or shSCR control and re-expressed using WBP2-expressing plasmid or vector control. (C,D) Mitochondrial respiration was assessed by measuring oxygen consumption rate (OCR) using the Seahorse XF Mito Stress Test in breast cancer cells treated with or without 10 mM metformin for 48 h. ATP production, basal respiration, maximal respiration, proton leak and spare respiratory capacity were calculated. (C) WBP2 was overexpressed in BT-474 cells using WBP2-expressing plasmid or vector control. (D) WBP2 was knocked down in SK-BR-3 cells using siWBP2 or siSCR control. (E,F) Glycolytic function was assessed by measuring extracellular acidification rate (ECAR) using the Seahorse XF Glycolysis Stress Test in breast cancer cells treated with or without 10 mM metformin for 48 h. Glycolysis, glycolytic capacity and glycolytic reserve were calculated. (E) WBP2 was overexpressed in BT-474 cells using WBP2-expressing plasmid or vector control. (F) WBP2 was knocked down in SK-BR-3 cells using siWBP2 or siSCR control. EV = Empty Vector control. SCR = Scrambled control. The data represent mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns = not statistically significant. Statistical analysis was performed using GraphPad Prism 10 using one-way ANOVA followed by a post hoc Tukey test.

3.6. WBP2 Expression Is Negatively Correlated with p-AMPK(Thr172) Expression in BC Patients with Invasive Ductal Carcinoma

The finding that WBP2 negatively regulates AMPK activation, corroborated by the observation that WBP2 does so putatively via regulation of cancer metabolism and cellular energetics, provided the basis for investigating the relationship between WBP2 expression and activated AMPK in clinical cancers to assess the clinical relevance of their relationships. To this end, we analyzed the expression of WBP2 and p-AMPK (Thr172) in an exploratory cohort of 31 HER2+ invasive ductal carcinoma samples (Supplementary Table S2) via IHC of serial sections, so that the comparison of WBP2 and activated AMPK would be as accurate as possible. Both WBP2 and p-AMPK antibodies have been duly proven to be specific through various controls such as peptide competition and phenformin/phosphatase treatment (Supplementary Figure S1). The IHC scores for WBP2 and p-AMPK are provided in the Supplementary Data (Supplementary Data S1). Representative IHC images of the differential expression of WBP2 and p-AMPK across different patients are shown in Figure 5A. Consistent with existing reports, WBP2 and p-AMPK were distributed in both the cytosol and nucleus [19,47]. Spearman correlation analysis revealed that WBP2 and p-AMPK had a negative association, with a correlation coefficient (Spearman ρ) of -0.3597 ($p = 0.0469$) (Figure 5B). Further analysis via stratification of tumor grades demonstrated that the negative association between WBP2 and p-AMPK is significant in Grade 3 BC patients, with a correlation coefficient (Spearman ρ) of -0.4895 ($p = 0.0334$), but absent in Grade 2 BC patient (Figure 5C,D). Stratification using tumor stage did not achieve statistical significance (Supplementary Figure S2). Nevertheless, the moderate correlation observed reflects tumor heterogeneity, and while the negative association between WBP2 and p-AMPK in the clinical samples supports the experimental observation that WBP2 negatively regulates AMPK activation, this is probably not the case for all HER2+ BCs.

Next, we assessed the interplay between oncogenic and tumor suppressive signaling in the tissue samples by examining the ratio of WBP2/p-AMPK H-scores. The assumption was that a change in this ratio would reflect a shift between oncogenic WBP2 and tumor suppressive p-AMPK activity. In this cohort, we observed higher WBP2 expression in Grade 3 (median = 149.7) compared to Grade 2 tumors (median = 92.42), while a higher WBP2/p-AMPK ratio was observed in Grade 3 (median = 0.900) compared to Grade 2 tumors (median = 0.500) (Figure 5E), although the data did not achieve statistical significance. Interestingly, when the patients were stratified by tumor (T) stage, the ratio increased significantly from stage 1 (median = 0.514) to stage 2 (median = 0.900) ($p = 0.0284$), with WBP2 expression also significantly higher in stage 2 patients (median = 151.6) compared

to stage 1 (median = 92.42) ($p = 0.0141$) (Figure 5F). The increase in the WBP2/p-AMPK ratio in higher tumor (T) stages of the patients suggest that progression of HER2+ BC is associated with a dominance of WBP2 oncogenic activity over AMPK tumor suppressor.

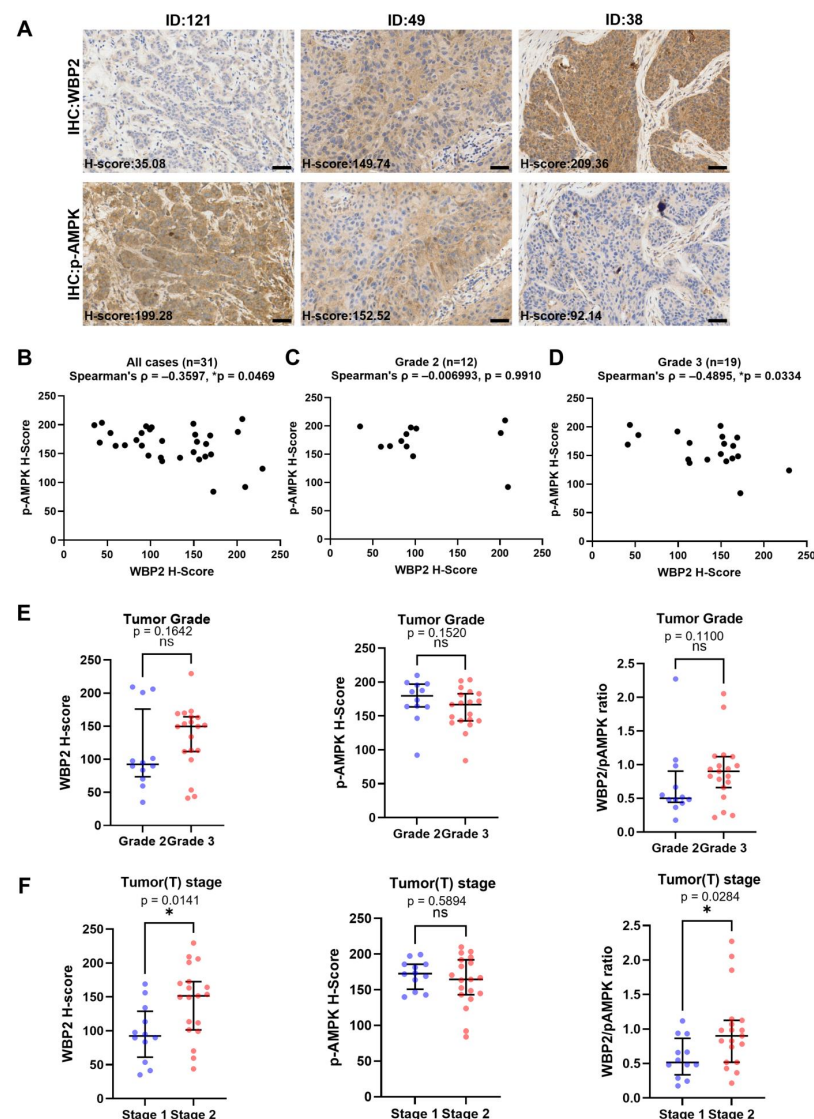


Figure 5. Expression of WBP2 and p-AMPK in HER2+ invasive ductal carcinoma breast cancer tissue. (A) Representative images of WBP2 and p-AMPK expression in IDC samples with varying expression from three different patients (ID: 121, 49 and 38). Scale bar = 50 μ m. (B) Spearman's correlation of H-scores of WBP2 and p-AMPK in all IDC cases and (C,D) patients separated by tumor grades. (E) Comparison of H-score of WBP2, p-AMPK and the ratio of H-score of WBP2/pAMPK in breast cancer tissue of different tumor grades. Medians and interquartile ranges are shown. (F) Comparison of H-scores of WBP2 and p-AMPK and the ratio of H-scores of WBP2/pAMPK in breast cancer tissue of different tumor stages. Medians and interquartile ranges are shown. * $p < 0.05$; ns = not statistically significant. Statistical analysis was performed using GraphPad Prism 10 using the Mann–Whitney U test.

Collectively, WBP2 enhanced mitochondrial energy production and glycolytic reserve, suggesting that WBP2 induced a higher bioenergetic state by promoting ATP production. The consequential reduction in AMP:ATP ratio would in turn dampen the activation of AMPK and affect metformin sensitivity. The negative correlation between WBP2 expression and activated AMPK was also observed to be clinically relevant in at least a significant portion of cases, suggesting that WBP2 can alter cancer's metabolic state and flexibility and affect the efficacy of metformin in HER2+ BC patients.

3.7. Transcriptomic Analysis Revealed That WBP2 Modulates a Network of Genes Regulating Energy Metabolism

To elucidate how WBP2 regulates glycolysis and mitochondrial respiration, we sought a comprehensive snapshot of its potential mechanisms of action. Given that WBP2 is a transcriptional co-regulator, we performed RNA-seq on biological duplicates of SK-BR-3 cells with silenced WBP2 using two independent shRNAs and compared them to a scrambled (shSCR) control. The silencing of WBP2 was effective as analyzed by Western blotting (Figure 6A). Following differential expression gene analysis, a Venn diagram was generated to identify the common genes that are affected by the 2 WBP2-specific shRNAs. Next, genes consistently up- or downregulated by both WBP2-specific shRNAs were identified, thereby increasing the robustness of the data used for subsequent analysis (Figure 6B–D). A total of 281 and 179 genes were downregulated and upregulated, respectively, following WBP2 knockdown. The former set of downregulated genes following WBP2 knockdown was selected for further processing as they should represent WBP2 co-expressed genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed using ShinyGO v0.85 and, interestingly, a major group of genes was “metabolic pathways”, while “cancer”-related genes also featured quite prominently (Figure 6E).

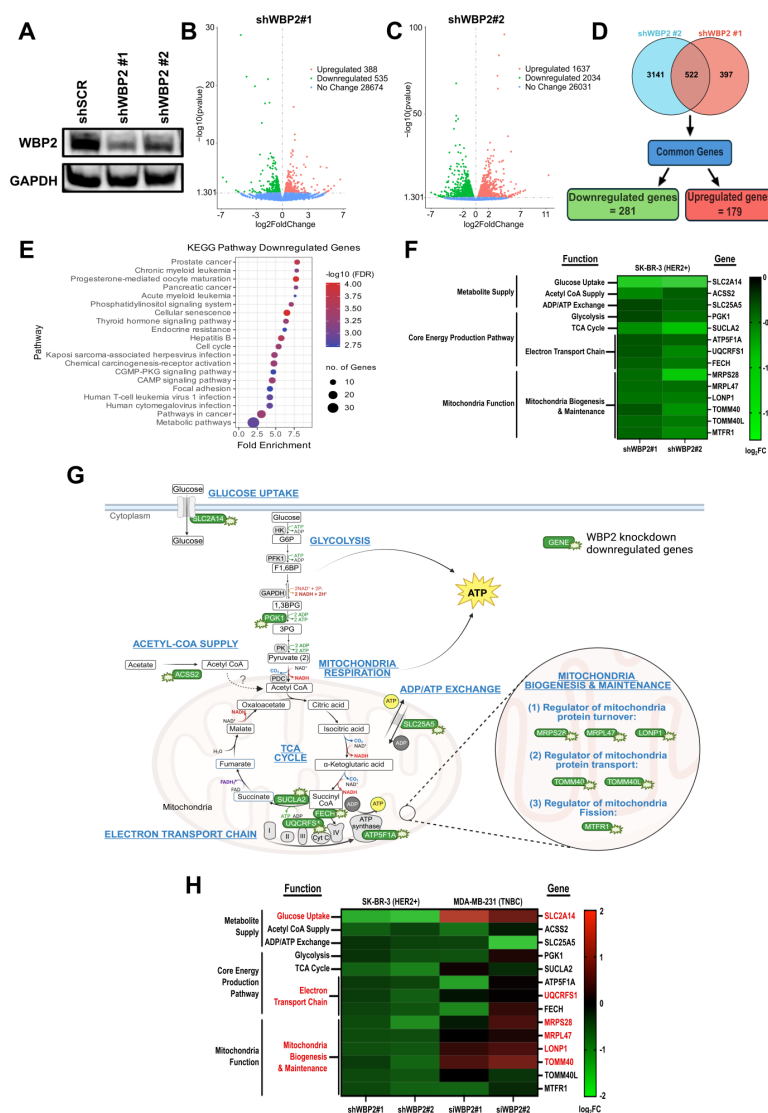


Figure 6. RNA sequencing analysis reveals WBP2 regulating a network of genes regulating energy metabolism. RNA sequencing was performed in WBP2 knockdown SK-BR-3 cells with two different

shRNA (Sequence #1 and #2) compared to shSCR control. (A) Immunoblotting showing WBP2 expression was silenced in WBP2 knockdown SK-BR-3 cells. (B,C) Volcano plot of differentially expressed genes (DEG) identified between WBP2 knockdown and control SK-BR-3 cells. Each point represents a gene plotted according to $\log_2$ fold change (x -axis) and $-\log_{10}$ (p -value) (y -axis). Downregulated gene expression is highlighted in green, upregulated gene expression is highlighted in red, and blue dots represent the gene expression with no change. (B) DEGs of knocked down WBP2 with shWBP2#1 compared to shSCR. (C) DEGs of knocked down WBP2 with shWBP2#2 compared to shSCR. (D) Venn diagram showing DEG between shWBP2#1 and shSCR (red) and DEG between shWBP2#2 and shSCR (blue). The area that intersects represents the common gene sets that were regulated following WBP2 knockdown. (E) KEGG pathway enrichment analysis of common downregulated DEGs. The bubble plot displays significantly enriched pathways ranked by Fold Enrichment, with bubble size representing gene count and color indicating $-\log_{10}$ (FDR). (F) Heatmap showing the expression profiles of 14 selected WBP2-downregulated genes involved in energy metabolism and mitochondria function across WBP2 knockdown SK-BR-3 cells. Color intensity expression values represent the $\log_2$ FC. (G) Schematic pathway mapping of selected WBP2-downregulated genes summarizing the potential multi-pronged mode of action of WBP2 in regulating energy metabolism. Selected DEGs affected by WBP2 knockdown are highlighted in green. Functions are labelled in Blue. Created with BioRender.com (H) Heatmap comparison of selected 14 WBP2-downregulated gene expression profiles shown in (F) with that from MDA-MB-231 (TNBC) cells with WBP2 knockdown extracted from another RNA-seq data set. The genes and their respective functions that display differences in the trend of regulation by WBP2 knockdown between the two cell types are highlighted in red. Color intensity expression values represent the $\log_2$ FC.

Next, genes that are related to energy metabolism and mitochondria were selected via online interrogation and manual curation and are presented as a heatmap in Figure 6F. The selected genes were found to fall within several pathways/functions that regulate energy production, such as glucose uptake (e.g., *SLC2A14*), glycolysis (e.g., *PGK1*), acetyl-coA supply (e.g., *ACSS2*), ADP/ATP transport (*SLC25A5*), Tricarboxylic Acid (TCA) cycle (e.g., *SUCLA2*), Electron Transport Chain (ETC) (e.g., *ATP5F1A*), and mitochondria machinery (e.g., *MTFR1*). The functions of these potential direct mediators of WBP2 are summarized in Table 1. These genes may offer insights into the potential mechanisms through which WBP2 influences cellular energetics and AMPK regulation, as illustrated in Figure 6G, which depicts the multi-pronged mode of action of WBP2 in bioenergetic regulation.

Table 1. Selected candidate downregulated genes from RNAseq analysis upon WBP2 knockdown in SK-BR-3 cells.

Category of Function	GENE	Protein Name	Function	Description of Cellular Function	Examples of Implications in Cancer
Metabolite supply	SLC2A14	Solute carrier family 2, facilitated glucose transporter member 14	Glucose Uptake	Cellular uptake of hexoses such as glucose and fructose	High expression is correlated with poorer prognosis in Gastric Adenocarcinoma [48]
	ACSS2	Acyl-CoA synthetase short-chain family member 2	Acetyl-CoA Supply	Catalyzes the reaction of acetate to acetyl-CoA	High expression in 40% of breast invasive ductal carcinoma [49]
	SLC25A5	ADP/ATP translocase 2 (Solute carrier family 25 member 5)	ADP/ATP Exchange	Inner mitochondrial membrane transporter involved in the exchange of ADP and ATP between cytosol and mitochondrial matrix	Suppression of siRNA in human breast cancer cells induced apoptosis and inhibits tumor growth in vitro and in vivo [50]

Table 1. Cont.

Category of Function	GENE	Protein Name	Function	Description of Cellular Function	Examples of Implications in Cancer
Key energy production pathway	PGK1	Phosphoglycerate kinase 1	Glycolysis	Catalyzes the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate (1,3-BPG) to ADP, producing ATP and 3-phosphoglycerate (3-PG)	Involved in multiple human cancers [51]
	SUCLA2	Succinyl-CoA ligase [ADP-forming] subunit β	TCA Cycle	Catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA	Promotes ketolysis and liver tumor growth [52]
	ATP5F1A	ATP synthase F1 subunit alpha	Electron Transport Chain	Synthesizing ATP from ADP and inorganic phosphate using the proton motive force generated by the ETC	Phosphorylation promotes prostate cancer [53]
	UQCRCF1	Ubiquinol-Cytochrome C Reductase, Rieske Iron-Sulfur Polypeptide 1	Electron Transport Chain	Involved in electron transfer from ubiquinol to cytochrome c and pumps protons across the inner mitochondria membrane	Required for growth and migration of TNBC cells [54]
	FECH	Ferrochelatase	Electron Transport Chain	Enzyme in heme biosynthesis, where heme is important for proper function of ETC complexes (cytochromes)	Expression correlates with prognosis and tumor immune microenvironment in clear cell renal cell carcinoma [55]
Mitochondria Function	MRPS28	28S ribosomal protein S28, mitochondrial	Mitochondria-Protein Translation	Mitochondrial small ribosomal subunit essential for translating mitochondria-encoded proteins	One of the 6 genes in prognostic biomarker panel in breast cancer [56]
	MRPL47	39S ribosomal protein L47, mitochondrial	Mitochondria-Protein Translation	Mitochondrial large ribosomal subunit essential for translating mitochondria-encoded proteins	Upregulated in HCC [57]
	LONP1	Lon Peptidase 1	Mitochondria-Protein Quality Control	Degrades misfolded/damaged proteins in the mitochondrial matrix	Inhibition of LONP1 induces proteotoxic stress and suppresses tumor progression [58]
	TOMM40	Translocase of Outer Mitochondrial Membrane 40	Mitochondria-Protein Import	Mediates the import of nuclear-encoded mitochondrial proteins	TOMM40 knockdown led to decreased PHB1 levels and increased ROS accumulation in tumor tissue, thus repressing tumor progression [59]
	TOMM40L	Translocase Of Outer Mitochondrial Membrane 40 Like	Mitochondria-Protein Import	Mediates the import of nuclear-encoded mitochondrial proteins	Elevated levels in malignant tissues compared to adjacent tissues, with heightened TOMM40L expression correlating with unfavorable prognostic outcomes [60]
	MTFR1	Mitochondrial fission regulator 1	Mitochondrial Fission	Promotes mitochondrial fission	MTFR1 phosphorylation-activated adaptive mitochondrial fusion is essential for colon cancer cell survival during glucose deprivation [61]

What might be the mechanisms that account for WBP2's effect on metformin response in HER2+ but not HER2- BC cells? To examine this closer, the effect of WBP2 silencing on the expression of selected genes in Figure 6F was compared to the same set of genes in MDA-MB-231 TNBC. This was done by extracting the data from another RNA-seq dataset of MDA-MB-231 cells with and without WBP2 knockdown using siRNA. The genes that are regulated by

WBP2 knockdown in HER2+ but not in HER2- BC cells are potential factors that might explain the differences in WBP2's action in response to metformin. As can be seen in Figure 6H, the genes exhibiting non-congruent expression pattern upon WBP2 knockdown between the two datasets are *SLC2A14* (affecting glucose uptake) and genes in the ETC: *UQCRCF1*, *MRPS28*, *MRPL47*, *LONP1* and *TOMM40* (involved in mitochondria biogenesis/maintenance). The data suggests that the selectivity of WBP2's effect on metformin response invokes metabolic processes at the level of glucose uptake and mitochondria respiration (affecting ETC and mitochondria maintenance). A more in-depth and extensive investigation involving a larger panel of cell lines would provide a clearer picture.

Collectively, the RNA-seq data provides an unbiased overview of WBP2-regulated metabolic pathways, generates mechanistic hypotheses implicating WBP2 in bioenergetic regulation and may underlie its preferential modulation of metformin response. Nonetheless, these candidate targets require validation at the mRNA and protein levels, and functional studies are needed to determine whether they mediate WBP2's effects on cellular energy metabolism.

4. Discussion

Our findings demonstrated that WBP2 expression inhibited metformin-induced anti-cancer response preferentially in the HER2+ BC cells in the limited in vitro study performed. This regulation is associated with WBP2's inhibitory effect on AMPK activation, which was concomitant with a higher bioenergetic state. A limitation of this study is that while our data demonstrates clear associations, they do not establish direct causal relationships. Due to the intrinsically bidirectional relationship between AMPK activity and metabolic flux, it is technically challenging to establish a definitive upstream–downstream hierarchy using endpoint-based assays. While our proposed working model (Figure 7) suggests WBP2 influences mitochondrial respiratory function and ATP production to modulate AMPK activity, alternative scenarios, such as WBP2 acting directly on AMPK first, cannot be excluded. Preliminary molecular docking may provide some clues as to the plausible mode of action of WBP2. Furthermore, the candidate gene/s identified from our transcriptomic analysis that potentially mediate WBP2's effect on mitochondrial functions need to be validated to achieve mechanistic insights.

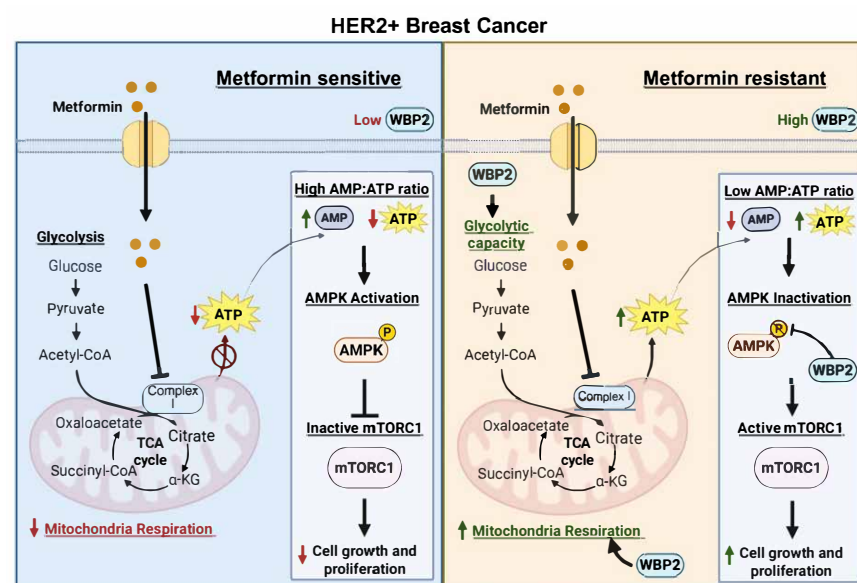


Figure 7. Proposed model for the mode of action of WBP2 on metformin in HER2+ BC cells. Left panel: Under low-WBP2 conditions, metformin acts on Complex I in the mitochondria to inhibit

mitochondria respiration and ATP production. This causes an increase in the AMP:ATP ratio, resulting in activation of AMPK and inactivation of the downstream mTORC1 pathway to antagonize cell growth and proliferation. Right panel: Under high-WBP2 conditions, ATP production from mitochondria respiration was enhanced, while glycolytic capacity was elevated. The resulting drop in AMP:ATP ratio could potentially suppress the metformin-induced AMPK activation, thereby allowing the mTORC1 pathway to promote cell growth and proliferation. Green arrow represent enhanced, Red arrow represent reduced. Created with BioRender.com.

4.1. A Key Limitation of This Study Is the Use of Supraphysiological Concentrations of Metformin In Vitro

While millimolar doses are commonly required to elicit measurable AMPK activation in cell culture, these concentrations exceed plasma levels achievable in patients (approximately 5–30 μ M) [34,62] and may induce non-specific metabolic stress. Consequently, the data obtained may not be physiologically relevant. Therefore, future studies incorporating low doses and long exposure of metformin treatment regimes, which have been shown to induce cytotoxic effects in cancer and preferentially target cancer stem cells [63], would clarify how WBP2 regulates mitochondria function, AMPK activity and metformin response within a more physiological context.

4.2. What About the Selectivity of WBP2's Action in HER2+ BC?

The observation of the preferential impact of WBP2 on metformin response in HER2+ BC cells expands upon existing studies, where metformin treatment was reported to provide better prognosis and improved survival in patients with HER2+ BC [10,11]. The metformin anti-cancer effect in HER2+ BC could be due to its direct downregulation of HER2 protein [31,35], which was also observed in our study. On the other hand, the plausible selective inhibitory role of WBP2 on metformin response in HER2+ BC cells tested could be due to an intricate link between WBP2 and HER2 proteins. Indeed, WBP2 was demonstrated in our previous studies to be activated following EGFR/HER2 signaling pathway activation [18,26]. It is therefore conceivable that high HER2 expression in HER2+ BC cells renders WBP2 to become more active, resulting in stronger inhibition of metformin response.

However, we concede that the number of cell lines used in our study is not sufficient to robustly claim that WBP2's modulation of metformin response is selective to HER2+ BC. Breast cancer is highly heterogeneous, with substantial variability not only in HER2 expression status but also in metabolic baseline, molecular composition, genetic mutations and epigenetic status across molecular subtypes. These factors can strongly influence the selectivity of WBP2 in response to metformin in the cells independently of HER2 signaling. Hence, future studies leveraging a broader panel of breast cancer cell lines would increase the robustness of this conclusion. Furthermore, the use of HER2 gain- and loss-of-function approaches will be essential to rigorously investigate the selectivity of WBP2 dependency to metformin response in HER2 BC cells.

Lastly, the preliminary comparative analysis of a subset of RNA-seq data following WBP2 KD in HER2+ and TNBC cells highlighted a few potential alternative/complementary mechanisms that could explain the selective effect of WBP2 on metformin response in HER2+ BC cells tested, other than the HER2 receptor status. These include its regulation of metabolic processes at the level of glucose uptake and mitochondria respiration by regulating ETC proteins and mitochondria maintenance proteins. Future follow-up studies should unravel and clarify the mechanism and hence identify more molecular determinants behind the response of BC cells to metformin.

4.3. Mode of Action of WBP2 on Metformin in HER2+ BC

This study demonstrated that WBP2 inhibits metformin-induced phosphorylation/activation of AMPK. This is in contrast to the reported activation of AMPK by WBP2 in hepatocytes [64]. This could be due to the different molecular soils that exist in cancer and non-cancer cells. To start, the expression level of WBP2 is low in normal cells compared to cancer cells. As a transcription coregulator, the differential expression of WBP2 means that the presence of different transcriptomes/proteomes in normal and cancer cells and the differential interaction of these proteins with WBP2 are likely to determine how WBP2 acts on AMPK. This reiterates the importance of cell-type-specific molecular compositions as rich fields for mining biomarkers for prediction of drug response.

This study highlights that WBP2 represses metformin and its target gene AMPK with an accompanied alteration in cellular energetics such as ATP/AMP ratio. This is the first report of WBP2's function in regulating energy-producing processes such as glycolytic capacity and mitochondria respiration in BC. Our data support the role of WBP2 in yet another hallmark of cancer [65]—metabolic reprogramming—in addition to other cancer hallmarks, such as sustaining proliferative signaling, activating invasion, and metastasis, that WBP2 has already been implicated in [16].

WBP2 is a recognized transcriptional coactivator that enhances oncogenic signaling pathways, including YAP/TAZ [22,66] and EGFR/PI3K/AKT [18,19]. These pathways are known to regulate metabolic processes transcriptionally, providing a plausible mechanistic basis for WBP2's regulation of metabolic genes. Our RNAseq data further reveals WBP2-regulated candidate genes in associated metabolic processes such as glucose uptake, acetyl-coA supply, glycolysis, mitochondria respiration, and maintenance as potential modes of its pleiotropic actions. These processes can be broadly categorized into three groups, namely metabolite supply, core energy production, and mitochondria biogenesis/maintenance.

WBP2 may modulate the metabolite substrate supply to drive energy production by regulating the *SLC2A14* glucose transporter and *ACSS2* acetyl coA synthase, which control glucose uptake [67] and acetyl-coA production, respectively. WBP2 may also directly influence steps in the core energy production pathway (glycolysis/mitochondria respiration) by regulating key genes involved, such as *PKG1* (Phosphoglycerate kinase 1 in the glycolysis pathway, which transfers a phosphate group from 1,3-bisphosphoglycerate to ADP, producing ATP and 3-phosphoglycerate); *SUCLA2* (Succinyl-CoA ligase [ADP-forming] subunit β in the TCA cycle, converting succinyl-CoA from succinate); and *ATP5F1A* (ATP synthase F1 subunit alpha in the ETC, involved in ATP synthesis). Lastly, WBP2 may be involved in driving energy production through enhancing the function of the mitochondria, the central organelle responsible for oxidative respiration and ATP production. WBP2 may drive mitochondria biogenesis by modulating fission and fusion processes through regulating *MTFR1* while maintaining mitochondria function through regulating genes involved in mitochondria protein production and proteostasis, such as *MRPS28*, *MRPL47* and *LONP1*. Therefore, the regulation of these key metabolic genes by WBP2 could provide clues to its mode of action on metformin by driving energy production.

4.4. WBP2 Is a Candidate Predictive Biomarker for Metformin Response

Our study supports the notion of WBP2 as a biomarker for metformin response in BC, where high WBP2 expression may predict against the anticancer efficacy of metformin in HER2+ BC due to its suppressing role on AMPK activation. Along with the reported role of WBP2 in resistance to chemotherapy [24,25] and targeted therapeutics [26], our data highlights the potential role of WBP2 as a determinant of drug response and hence a candidate biomarker for patient stratification.

Our IHC analysis of WBP2 and p-AMPK in HER2+ BC tissues provides supportive evidence linking WBP2 expression to AMPK signaling in a clinical context. However, we acknowledge the cohort size is limited, and future studies involving larger cohort size will increase the robustness of the relationship between WBP2 and AMPK activation. Future investigations incorporating larger cohort with critical clinical information, including metformin exposure, diabetic status, treatment history, and patient outcomes, will be essential to evaluate whether WBP2 expression can inform patient stratification or therapeutic response to metformin. These studies would set the foundation for the plausible use of WBP2 to guide metformin-based cancer treatment to facilitate the success of repurposing metformin for precision cancer therapy.

4.5. WBP2 Offers a Therapeutic Opportunity for Management of HER2+ BC with Metabolism-Targeting Drugs

WBP2 confers metabolic plasticity to HER2+ BC by enhancing both glycolytic and mitochondria respiration capacity. This may cause cancer cells to be less sensitive to drugs that target cancer metabolism, such as mitochondrial TCA-targeting agents (CPI-613/devimistat), which are in Phase III clinical trials for cancer [68]. This exposes a therapeutic opportunity in WBP2-high, HER2+ BC, where combination of WBP2 and cancer metabolism inhibitors can enhance efficacy or overcome resistance and induce a metabolic crisis in tumor cells. In the same vein, unraveling the mechanism of WBP2's inhibition of the efficacy of metformin or its equivalent in HER2+ BC would uncover more therapeutic vulnerabilities, open up as combinatorial therapy approach and enhance cancer metabolism-targeting drug strategies.

5. Conclusions

In conclusion, WBP2 was demonstrated to preferentially suppress the anti-cancer response to metformin in HER2-positive BC cells by regulating AMPK and cellular energetics. While not yet clinically actionable, our data raises the possibility that WBP2, in conjunction with HER2 status, may inform future biomarker-driven strategies to stratify patients for metformin repurposing.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/cells15040381/s1>. Figure S1: Specificity of p-AMPK(Thr172) and WBP2 antibodies for IHC staining. Figure S2: Spearman's correlation of H-score of WBP2 and p-AMPK patients separated by tumor(T) stages. Data S1: Raw H-score and patient information; Table S1: List of siRNAs used in the study. Table S2: Characteristics of breast cancer patients with Invasive Ductal Carcinoma.

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The animal experiment was performed in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore (IACUC R17-0335(A)19, approval date: 19 March 2020).

Informed Consent Statement: Informed consent for participation was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Abbreviations

The following abbreviations are used in this manuscript:

BC	Breast cancer
T2D	Type 2 diabetes
HER2+ BC	HER2-positive breast cancer
AMPK	AMP-activated protein kinase
WBP2	WW domain-binding protein 2
IP	Intraperitoneal
OCR	Oxygen Consumption Rate
ECAR	Extracellular acidification rate
KEGG	Kyoto Encyclopedia of Genes and Genomes
TCA	Tricarboxylic Acid
ETC	Electron Transport Chain
ACSS	Acyl-CoA synthetase short-chain family member 2
PGK1	Phosphoglycerate kinase 1
MTFR1	Mitochondrial Fission Regulator 1
LONP1	Lon Peptidase 1

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