Fatty Acid Binding Protein 4 Alters Obesity Associated Cancer Metabolism via Lipid Desaturation and Redox Signaling

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Dedication

I dedicate this thesis to my parents, who taught me to never stop challenging myself.

Abstract

Background: An association of obesity with cancer incidence and worsened clinical outcomes has been established. Circulating and local levels of fatty acid binding protein 4 (FABP4), a lipid chaperone and adipokine, have been correlated with degree of obesity and metabolic dysfunction, and more recently with breast and pancreas cancer prognosis. FABP4 transcriptional regulation has been linked to cellular redox status, with nuclear factor (erythroid-derived 2)-like 2 (Nrf2) being an upstream regulator of this balance. We hypothesized FABP4 modifies fatty acid saturation indices in cancer, driving an altered redox status, and ultimately inducing tumor proliferation.

Methods: Panc1 pancreatic adenocarcinoma and MCF7 breast cancer cells were treated with recombinant FABP4, R126Q (FABP4 point mutant), and HTS01037 (FABP4 inhibitor.) Cell growth and proliferation was assessed. Targeted lipidomic analysis was performed in the same conditions. Whole cell reactive oxygen species (ROS) was quantified via Amplex Red assay. Nrf2 activity was quantified via antioxidant response element luciferase assay, and cell proliferation with chemical inhibitor (brusatol) was assessed. Untargeted gene expression profiles after FABP4 or HTS treatment were studied via RNA sequencing. C57BL/6J FABP4 knockout (AKO) and littermate wild type (WT) mice were injected with Pan02 and E0771 cells, murine pancreas and breast cancer cell lines. Tumor volume and progression was evaluated.

Results: Panc1 and MCF7 cells treated with recombinant FABP4 demonstrated increased proliferation relative to control and point mutant protein treatment. This increase was abolished with HTS treatment. Unsaturated/saturated fatty acid ratio was decreased with FABP4 treatment and increased with HTS treatment. ROS levels were decreased and

Nrf2 activity was concurrently increased with exposure to exogenous FABP4. Nrf2 gene expression profile was upregulated with FABP4 treatment, independent of ER stress. Tumor progression was significantly decreased in AKO mice.

Conclusions: FABP4 induces a shift in the fatty acid saturation index of tumor cells, activating Nrf2 expression and decreasing intracellular ROS, independent of ER stress, allowing for aggressive tumor proliferation.

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ALDH1A1-Aldehyde dehydrogenase 1 family member A1

ARE- Antioxidant response element

CSC- Cancer stem cell

CYP1A1- Cytochrome P450 family 1 subfamily A member 1

DEG- Differentially expressed gene

DNL- De novo lipogenesis

ER- Endoplasmic reticulum

FABP4- Fatty acid binding protein 4

FFA- Free fatty acid

HTS01037- FABP4 inhibitor

Nrf2- Nuclear factor erythroid 2-related factor 2

PDAC- Pancreatic adenocarcinoma

R126Q- Mutant FABP4, arginine for glutamine single substitution

ROS- reactive oxygen species

SFA- Saturated fatty acid

UFA- Unsaturated fatty acid

INTRODUCTION

Obesity has long been appreciated as a driver of metabolic disease. More recently, obesity has been implicated in the pathogenesis of a variety of cancers, particularly notable in breast and pancreatic cancer (PDAC).[1] The diagnosis of obesity is associated with a higher risk of diagnosis of breast cancer, higher risk of advanced disease at diagnosis, and increased hazard of death after diagnosis [2,3] Additionally, indicators of worsened metabolic disease, such as type II diabetes mellitus and metabolic syndrome, have demonstrated similar adverse relationships with breast cancer outcomes.[4] Pancreatic cancer is associated with not only obesity, but is often preceded by new onset type II diabetes.[5]

In search for a link between obesity, metabolic disease, and cancer, fatty acid binding protein 4 (FABP4, alternatively called adipocyte protein 2, aP2) has been suggested as a molecule of interest.[6] FABP4 is an intracellular lipid chaperone that facilitates fatty acid flux and is the one of the most abundant proteins in mature adipocytes.[7] Circulating levels of FABP4 have been noted to be higher in obese subjects, and levels correlate with BMI, metabolic syndrome, and inflammatory markers.[8] Since its first description as a fatty acid chaperone, however, it has been implicated in a diverse array of cellular processes aside from simply shuttling lipids.[7]

FABP4 has also been linked to the development and progression of a number of obesity related cancers.[6] Women with breast cancer demonstrate higher circulating serum levels of FABP4 and higher FABP4 expression levels in the breast stroma, as compared to healthy controls.[9,10] Higher expression of FABP4 in breast cancer specimens is also significantly correlated with recurrence and disease free survival.[11]

Higher FABP4 expression, as measured by immunohistochemistry, is associated with both progression and prognosis in PDAC.[12]

Mechanistic studies have describe increased proliferation, invasiveness, and stemness of breast cancer cells in response to exogenous FABP4 treatment.[10,13] Additionally, tumor progression is significantly decreased in whole body and macrophage specific FABP4 knockout models.[10,14] Potential mechanisms from these investigations of FABP4 in a variety of obesity related cancers have implicated interleukin-6/Stat-3 dependent pathways, regulation of aldehyde dehydrogenase, FOXM1 pathways, and altered fatty acid metabolism, among others.[13–15]

FABP4 has previously been studied in the setting of bariatric (weight loss) surgery, evaluating its role in the robust weight loss and metabolic improvements exhibited postoperatively.[16] An acute decrease in both serum and adipose tissue levels of FABP4 after bariatric surgery has been noted.[16,17] Interestingly, bariatric surgery has also been associated with a protective effect for breast cancer development, progression, and mortality.[18,19] Data for PDAC is more limited, likely due to its significantly lower prevalence, however evidence from pre-clinical studies have suggested similar results.[20] Evidence has shown that alterations in redox signaling play a significant role in the deleterious inflammatory and metabolic effects of FABP4 in both macrophages and adipose tissue.[21,22] Finally, it has been suggested that these effects are mechanistically linked via altered intracellular lipid desaturation levels, as mediated by a selective sequestration of lipids by FABP4.[22]

The goal of this study was to reconcile the redox mechanisms noted after bariatric surgery with the FABP4 induced stem cell like properties reported in breast cancer. In

this study, the effects of exogenous FABP4 on the redox status of cancer cells is evaluated, lipidomics are utilized to characterize changes in tumor cell lipid saturation indices after these treatments, gene signatures associated with these changes are evaluated, and finally modulation of these potential pathways is investigated.

METHODS

Cell culture

MCF-7 breast cancer cells and E0771 murine breast cancer cells were maintained in DMEM (Invitrogen) with 5% fetal bovine serum (FBS) and 2uL of insulin/mL of media. T47D cell lines were maintained in RPMI (Invitrogen) with 5% FBS and insulin. Panc1 and Pan02 cells were maintained in DMEM with 10% FBS.

Cell proliferation analysis

Cells were seeded in 6-well plates at a concentration of $2x10^5$ cells/mL in serum-starved media containing 0.1% FBS and 2uL of insulin/mL of media. Cells were cultured for 24 hours and baseline cell counts performed utilizing automated cell counter (Countess, Thermofisher). Media was aspirated and replaced the same composition media containing an additional reagent of: recombinant FABP4, 100 ng/mL, R126Q (R-single point mutant FABP4), 100ng/mL, FABP5, 100ng/mL, HTS01037 at 30uM, brusatol at 20nM, or vehicle solvent at the same volume. Cell counts were then performed at time points 24hr and 48hr.

Reactive Oxygen Species Analysis

Hydrogen peroxide level was assayed utilizing an Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen, Carlsbad, CA), per manufacturer's instructions.

Nrf2 Reporter Assay

Antioxidant response element (ARE) luciferase reporter vector and constitutively active Renilla luciferase vector (BPS Bioscience) were transfected into MCF-7 cells utilizing Lipofectamine (Invitrogen) in standard OMEM media. After 24 hours of

incubation media was aspirated and replaced with FABP4, HTS, or solvents, and serum starved media, as described earlier. After 24 hours of incubation, dual luciferase reporter assay (Promega) was performed utilizing a luminometer per manufacturer's instructions. ARE reporter activity was normalized to cell number by Renilla activity.

Quantitative RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was performed by using iScript (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Quantitative reverse transcription (qRT)-PCR amplification utilized a Bio-Rad CFX 96 real-time system with SYBR green Supermix. TATA-binding protein (TBP) was used as an internal control to normalize expression.

RNA Sequencing

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified utilizing RNeasy kit (Qiagen) per manufacturer's instructions. Total RNA samples were converted to Illumina sequencing libraries using Illumina's Truseq RNA sample preparation kit per manufacturer's instructions. After quality check, sequencing was performed on utilizing HiSeq 2500 with 50bp at 220M reads.

Lipidomics

After cell plating and treatment, cells were centrifuged and lysed, and total protein content was determined by standard BCA assay. Targeted lipidomics were quantitatively performed utilizing high performance liquid chromatography. Lipid species were normalized to total cell protein.

Mice

All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. Male C57BL/6J WT and AKO mice were fed ad libitum a high-saturated-fat (lard) diet (F3282; BioServe, Flemington, NJ) for 12 weeks after weaning. At week 12-14 of high fat diet, mice were injected with E0771 cells ($5x10^6$) in the mammary fat pads. Tumor volume was measured three times weekly with calipers. Mice were euthanized at endpoints of >2cm^3, tumor ulceration, metastases, or the end of study period at 35 days.

Statistical analysis

Values are reported as mean ±standard error (SEM) for figures and mean ±standard deviation (SD) for the text. Differences were compared using a Student's t-test or analysis of variance (ANOVA) with Sidak's post hoc multiple comparisons test. Survival was evaluating utilizing Kaplan-Meier method and log-rank tests. Above statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, California, USA).

For RNAseq analysis, 50bp FastQ paired-end reads (n=23.6 Million per sample) were trimmed using Trimmomatic (v 0.33) enabled with the optional "-q" option; 3bp sliding-window trimming from 3' end requiring minimum Q30. Quality control checks on raw sequence data for each sample were performed with FastQC. Read mapping was performed via Hisat2 (2.1.0) using the Human UCSC genome (hg38) as reference. Gene quantification was done via Cuffquant for FPKM values and Feature Counts for raw read counts. Differentially expressed genes were identified using the edgeR (negative binomial) feature in CLCGWB (Qiagen, Valencia, CA) using raw read counts.

The generated list was filtered based on a minimum 2X Absolute Fold Change and FDR corrected $p < 0.05$. Pathway analysis was performed in Ingenuity Pathway Assist (IPA) using fold change and FDR corrected values.

RESULTS

Increased cancer proliferation in response to exogenous FABP4 requires lipid binding

Increasing concentrations of exogenous FABP4 present in the cell media (20, 100, and 200ng/mL) resulted in increased cell proliferation (Fig 1A.) We then utilized a mutant of FABP4, R126Q, to evaluate the contribution of FABP4's lipid binding to this increase in cell proliferation in both Panc1 and MCF7 cells. The single residue substitution of arginine for glutamine of the R126Q mutant alters the hydrophobic cavity of FABP4 in a manner which hinders its ability to bind free fatty acids, while maintaining the overall chemical structure of FABP4, theoretically allowing any lipid exclusive allosteric binding or transcriptional related activities.[23] Cell proliferation increased only with wild type recombinant FABP4, and was not affected by treatment with mutant R126Q (Fig 1B-D.) Finally, we demonstrated the effects of inhibition of endogenous FABP4 via its chemical inhibitor HTS01037, with a decrease in cell proliferation noted (Fig 1E.) Photographs obtained from light microscopy highlight not only the differences in cell density, but a more phenotypic change in MCF7 cell size and shape, which we did not quantify or investigate further (Fig 1F.)

FABP4 knockout decreases in vivo tumor growth

In a C57/Bl6 high fat diet mouse model, growth of the murine breast cancer line E0771 and pancreatic cancer cell line Pan02 was significantly decreased in the FABP4 knockout (AKO) cohort (Fig 2A, 2C.) Overall survival was not significantly different but trended towards worsened survival in the FABP4 wild type (WT) cohort (Fig 2B.) It should be noted that, while not statistically significant, AKO mice characteristically

demonstrated lower body weight, and lost more body mass towards the end of the survival period (Fig 2D.)

Untargeted RNA Sequencing reveals downregulated Nrf2 pathways with HTS treatment

To identify target genes offering insight into the possible mechanisms of these phenotypic changes described, untargeted RNA sequencing data from FABP4, HTS, or control treated MCF-7 cells were compared. Differentially expressed genes (DEGs) were identified by a fold change (FC) > 2 and corrected p-value <0.05 (Fig 3A.) The most robust changes were noted with HTS treatment, with 3,241 DEGs as compared to control and 3225 DEGs as compared to FABP4. Taking into account the already perturbed metabolic and oncogenic signaling of MCF-7 cells, transcriptomic analysis of FABP4 treated cells vs control treatment revealed only 32 DEGs. Overlapping DEGs in these sets with divergent changes and known functional outputs were notable for ALDH1A1 (aldehyde dehydrogenase 1, and CYP1A1 (cytochrome P450 family 1 subfamily A member 1.)

Pathway analysis identified numerous cancer and metabolism related pathway changes, most interesting in redox state pathways was a significant increase in Nrf2 signaling (Fig 3B.) Table 1 provided fold change of known Nrf2 targets correlated with cancer prognosis and their representation in our dataset, with a significant portion being upregulated comparing FABP4 vs HTS treatment.[24]

In silico analysis demonstrates FABP4 correlation with ALDH1A1, and Nrf2 signaling

In silico analysis was performed utilizing of the TCGA BRCA dataset to investigate correlation of expression of FABP4 with downstream targets in clinical specimens as well as evaluated their prognostic significance. Figure 3C demonstrates a

significant correlation of FABP4 expression with ALDH1A1, as well as GPx3, a downstream target of Nrf2. Similar correlations of FABP4 with Nrf2 targets were noted with a large majority of those DEGs noted in Fig 3C, although GPx3 was the most significant. Upregulation of both ALDH1A1 and GPx3 were significantly associated with worsened survival.

FABP4 treatment decreases reactive oxygen species, upregulates Nrf2 Activity

FABP4 treatment resulted in significantly decreased intracellular ROS, as measured by relative abundance of H2O2 (Figure 4A, 4B.) This decrease in ROS is concurrently associated with a significant upregulation of Nrf2 activity, as measure by antioxidant response element (ARE) reporter assay (Fig 4C.) HTS treatment significantly increases cellular ROS, while decreasing Nrf2 Activity. Relating this to previous proliferation experiments, chemical inhibition of Nrf2 via brusatol abrogated the proliferative effects of FABP4 in MCF7 cells (Fig 4D.)

Lipid desaturation index decreases with FABP4 treatment, increases with inhibitor

Investigating of the relationship of FABP4 in lipid partitioning, targeted lipidomic analysis of whole cell intracellular lipid contents was performed after various treatments. FABP4 treatment most significantly altered saturated fatty acid levels (Fig S2,) leading to significantly decreased desaturation indices (Fig 5A.) This shift in desaturation indices was most notable in the palmitoleic to palimitic acid (16:1/16:0), oleic to steric acid (18:1/18:0), and docosahexaenoic (DHA) to stearic acid (22:6/18:0) ratios.

Markers of ER stress are increased with FABP4 treatment, independent of ROS

Utilizing protein extracts from the above experiments, downstream signals of the endoplasmic reticulum (ER) stress pathways were quantified. Protein levels of binding

immunoglobulin protein (BiP) and CCAAT-enhancer-binding protein homologous protein (CHOP) were increased after FABP4 treatment, notably in the presence of decreased ROS, as measured previously (Fig 5B.)

DISCUSSION

Previous studies have demonstrated that FABP4 can influence both tumor cell proliferation and in vivo tumor progression.[25,26] Those findings were recapitulated here in breast cancer and provided novel evidence of this in PDAC. The proposed mechanisms of FABP4's downstream effects in cancer, however, have been diverse and variable.[9,15,25] The data presented here suggests a unifying factor amongst these mechanistic frameworks: an FABP4 driven alteration of intracellular lipid saturation indices. FABP4 has a distinct lipid binding profile, and exogenous FABP4 causes a shift in lipid saturation indices within breast cancer cells. This shift alters cellular redox status via Nrf2 activity, independent of endoplasmic reticulum (ER) stress, and promotes the expression of stem-like markers.

Metabolic reprograming in cancer cells has long been appreciated, however the majority of the literature has focused on alterations of glucose and glutamine metabolism, such as the Warburg effect.[27] Taking into account that adipose tissue makes up over 50% of the breast stroma, it is not surprising that the importance of fatty acid metabolism in cancer pathogenesis has also become apparent.[28–30] In PDAC, high fat diet induced obesity not only increases peri-pancreatic fat mass but additionally inflammation in the KRASG12D murine model.[31] Adipocytes surrounding malignant breast tissue express a characteristic metabolic phenotype; these adipocytes demonstrate increased lipolysis and decreased lipid pools as compared to their non-malignant counterparts, lending to an overall smaller size.[30] Indeed, breast cancer cells not only increase uptake of free fatty acids when co-cultured with adipocytes, but the malignant cells reciprocally stimulate

lipolysis in the adipocytes.[32] Further, co-culture of breast cancer cells with adipocytes increases both proliferation and migration.[32]

The mechanistic framework behind this adipocyte-cancer relationship is undoubtedly multifactorial, including increased energy substrate availability, altered adipocyte signaling, and increased inflammation, among other possible explanations.[33] The intersection of two of these pathways, altered adipocyte signaling and concomitant intracellular lipid pool modifications, is addressed here. Simply supplementing with breast cancer cells with exogenous fatty acids results in an increase in cell proliferation with unsaturated fatty acid (UFA), and pro-apoptotic effects are noted with saturated fatty acid (SFA) supplementation.[34] Interestingly, the pro-apoptotic effects of palmitate (SFA) supplementation are abrogated by concurrently providing oleate (UFA.)[34–36] FABP4 has been implicated in FFA availability in cancer, with an increased expression of FABP4 associated with an increased transfer of FFA from adipocytes to breast tumor cells.[15] The intracellular FABP4 protein of these cells is localized at tumor-adipocytes interface.[15] Further, FABP4 induced expression of ADLH1A1, a stem cell marker in breast cancer, is dependent upon concurrently providing palmitic acid (16:0, SFA), an effect which is absent with only oleic acid (18:1, UFA) supplementation.[25]

With evidence that the relative abundance of UFAs and SFAs may act as a metabolic scale, altering tumor proliferation and phenotype, the intracellular "desaturation index" of tumor cells, or the UFA/SFA ratio, has been further investigated.[34–36] In ovarian cancer, higher desaturation indices (UFA/SFA) have been associated with enhanced sphere formation and increased ALDH1A1 expression, while inhibition of SCD1 (stearoyl-CoA desaturase 1) reverses these observations.[37] Further,

evaluation of lipid and metabolite profiles of ovarian cancer specimens stratified into high vs low FABP4 expression found overall an increase in unsaturated fatty acids in those samples with high FABP4 expression.[38] Untargeted lipidomics of membrane lipids present in breast cancer specimens have also suggested that altered UFA/SFA ratios and products of de novo lipogenesis (DNL) are associated with tumor aggressiveness and hormone receptor status.[39]

It is clear from the studies highlighted here that the relationship of fatty acid pool composition and tumor phenotype is more complex than those effects noted from simple FFA supplementation. It is demonstrated here that the desaturation index of breast cancer cells decreased with exogenous FABP4 supplementation. Findings here correlate with those of exogenous supplementation data, while contradicting those data from studies focusing on desaturation index. Two possible reasons for these disparate findings can be suggested. First, while de novo synthesis of fatty acids is certainly an important process to factor into cancer metabolism, the contribution of glucose and glutamine to overall metabolic turnover is significantly less than supplied FFA in breast cancer.[32,40] The breast cancer milieu is predominantly adipocytes, offering a steady supply of exogenous FFA.[27] I could be posited that, in comparison to more desmoplastic cancers such as pancreatic cancer, the role of de novo lipogenesis in breast cancer would be much less. Additionally, the interface of hormone sensitive elements and breast cancer cell lines, notably estrogen receptor status, significantly affects lipid uptake, partitioning to beta-oxidation or storage, and sensitivity to palmitate induced toxicity.[34,39]

These findings also agree with previous studies of FABP4 and its relationship with lipid profile. FABP4 is often cited as an indiscriminate lipid chaperone, with a wide

array of non-selective binding affinities.[7] More detailed investigations of FABP4, however, have demonstrated selectivity amongst FFAs (i.e. UFA vs SFA), with these affinities likely being altered by intracellular metabolic status.[41] Previous work has indicated that FABP4 drives a selective alteration of the available intracellular FFA pool, with loss of FABP4 in macrophages resulting in an elevated intracellular abundance of monounsaturated fatty acids (C16:1 and C18:1).[22] The initial hypothesis of this study, linking FABP4 to the downstream effector pathways was based upon this finding, and evidence supporting this hypothesis from lipidomics is provided, demonstrating a decrease in the desaturation ratio.

Further delving into the mechanism of increased proliferation in response to FABP4, the redox status of cancer cells in an FABP4 rich environment is evaluated. Previous data in macrophages demonstrated that deletion of FABP results in lower ROS production, an increase in antioxidant protein expression, and decreased inflammatory cytokine production.[42] Data from 3TL-31 adipocytes and tumor associated endothelial cells found contrasting results, an increase in ROS levels after treatment with small interfering RNA against FABP4 or FAB4 knockdown.[43,44] Redox status in cancer metabolism has historically been characterized in the setting of increased aerobic glycolysis (Warburg effect) and an associated increase in oxidative stress, as indicated by increased reactive oxygen species (ROS) production.[45] This elevation of intracellular ROS can lead to DNA damage, increased in oncogenic pathway signaling, and overall increased tumorogenesis.[46] With a greater appreciation for tumor heterogeneity and the existence of distinct tumor cell subpopulations, however, it has become apparent that specific populations, such as cancer stem cells (CSCs), exhibit notably altered cellular

processes and metabolism, as compared to the overall tumor population.[46] This metabolic reprogramming has been associated with distinctly lower levels of ROS and a metabolic shift favoring oxidative phosphorylation.[47] Further, alterations of CSC metabolism have been linked to their unique ability for self-renewal as well as chemotherapy and radiation resistance.[46] Interestingly, the authors of the study investigating redox status in differentiating adipocytes hypothesized that the increase of FABP4 expression during pre-adipocyte differentiation is crucial to adipogenesis, playing a cytoprotective role by decreasing oxidative stress and endoplasmic reticulum (ER) stress.[43]

Here, evidence is provided that exogenous FABP4 treatment leads to significantly decreased levels of intracellular ROS in breast cancer cells, while treatment with a chemical inhibitor of FABP4 significantly increased intracellular ROS. This finding in breast cancer correlates with that of differentiating adipocytes, which we suggest may be a correlate to the metabolic environment of CSCs.[43] Others have demonstrated that redox signaling modulates the equilibrium of breast CSC populations in response to metabolic stressors, which we perturb here with exogenous FABP4.[47] This redox alteration in CSCs is mediated by changes in Nrf2 (nuclear factor (erythroid-derived 2)-like 2) pathway signaling.[47]

Nrf2 is a redox sensitive transcription factor which, at basal conditions, is sequestered in the cytoplasm, bound with its repressor protein Keap 1 (Kelch like ECH associated protein.)[48] Upon exposure to oxidative stress, the cysteine residues on Keap1 are oxidized and the complex dissociates, allowing nuclear translocation and binding of Nrf2 to the antioxidant response element (ARE).[48] Transcripts from this promoter

include antioxidant enzymes (SOD1, HO-1), phase 2 detoxifying enzymes, and drug transporters.[48] These synthesized proteins allows for better handling or ROS, as well as evasion of the cytotoxic effects of chemotherapy and radiation.[49] Evidence from the pathway analysis of gene signatures associated with FABP4 treatment presented here suggests the decrease in ROS we noted earlier is associated with an upregulation of Nrf2 pathways. Upregulated Nrf2 activity as measured by ARE reporter assay was confirmed and also demonstrated decreased activity with HTS treatment. It is further demonstrated that chemical inhibition of the Nrf2 pathway abolished the proliferative effects of FABP4 in MCF7 cells.

While the literature regarding FABP4 and redox homeostasis and inflammation is rather robust, sparse evidence as to the role of Nrf2 signaling in these pathways is present. A recent study investigating the precise mechanism of intracellular FABP4 uptake, a still debated topic, corroborates our Nrf2 findings.[50] Their group demonstrate an increased Nrf2 nuclear translocation after treatment of endothelial cells with exogenous FABP4.[50] They go on to establish that this mechanism of uptake is reliant upon an interaction of FABP4 with cytokeratin 1 (CK1, KRT1), a membrane receptor-like protein present on a number of cell membranes, including breast cancer.[51] Finally, it is noted that this uptake, as well as nuclear translocation of Nrf2 and FABP4, are significantly influenced by the profile of free fatty acids (FFA) present in the media. All FFAs increased nuclear translocation of Nrf2, however this effect is most pronounced with media containing the saturated fatty acid (SFAs) palmitate (C16:0), as compared to the unsaturated fatty acids (UFAs).[51]

The deleterious effects of FABP4 in atherosclerosis and insulin resistance have been linked to a protection from SFA induced apoptosis via ER stress and redox pathways.[52] Expanding from this, the findings of decreased desaturation ratio, or increased relative abundance of SFAs in FABP4 treated cells, with increased proliferation and decreased ROS in breast cancer, may be summed via altered ER stress pathways. The endoplasmic reticulum serves as a compartment for modification of proteins prior to release in the cytoplasm, providing an environment to synthesis, maturation, folding, and quality control.[53] More recently, the ER has been appreciated as a key site for integration of cell stress signaling.[52] In response to various insults, notably here palmitate and SFAs, ER stress increases through a variety of pathways, leading to improperly folded proteins and activation of the UPR, or unfolded protein response.[53] This, in turn, leads to varying downstream effects depending on the cell type of interest.[43,52] The effects of FABP4 in this pathway vary significantly as well.[43,52] FABP4 knockdown increases palmitate induced ER stress in macrophages by upregulated DNL and increasing UFA synthesis, with the opposite findings noted in adipocytes.[43,52] These findings mirror the opposing changes induced by FABP4 in ROS among macrophages versus adipocytes.[43,44] Integrating these signals, ER stress is typically associated with increased ROS, particularly in cancer metabolism.[53] UPR activation secondary to significant proliferation is typically associated with increased cellular stress, and can be noted in cancer.[53] Upregulated Nrf2 signaling with downregulated ER stress initially may seem disparate in the setting of normal tissue, however has been recently described as a hallmark metabolic shift of cancer initiating cells.[54] Thus we propose FABP4 is inducing

ER stress via SFA in an already perturbed tumor environment, leading to downstream Nrf2 upregulated, independent of ROS levels.

Finally, the limitations of this study must be noted. The most influential source of FFA in cancer metabolism (i.e. exogenous FFA vs DNL produced lipids) is yet to be settled. The model presented here describes only the effects of exogenous FFA; further work investigating the concurrent inhibition of DNL and characterization of FABP4's influence would certainly provide further insight. Next, while a novel mechanism of FABP4s effects in cancer metabolism is presented via modifying desaturation levels, the pathway from FABP4 to increased Nrf2 activity must be defined with more granularity. Evidence is provided that ER stress is likely involved, however further study of the precise mechanisms is crucial. Finally, while these phenotypic results are also demonstrated in PDAC, further investigation cancer specific mechanisms must be undertaken.

In conclusion, the proliferative effects of exogenous FABP4 in breast cancer alter the FFA pool via decreased desaturation index, correlating with increased Nrf2 activity and decreased ROS, independent of elevated markers of ER stress. This pathway dysregulation is correlated with increased markers of stemness, and may provide a novel avenue of targeted breast cancer therapeutics.

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Figure 1.

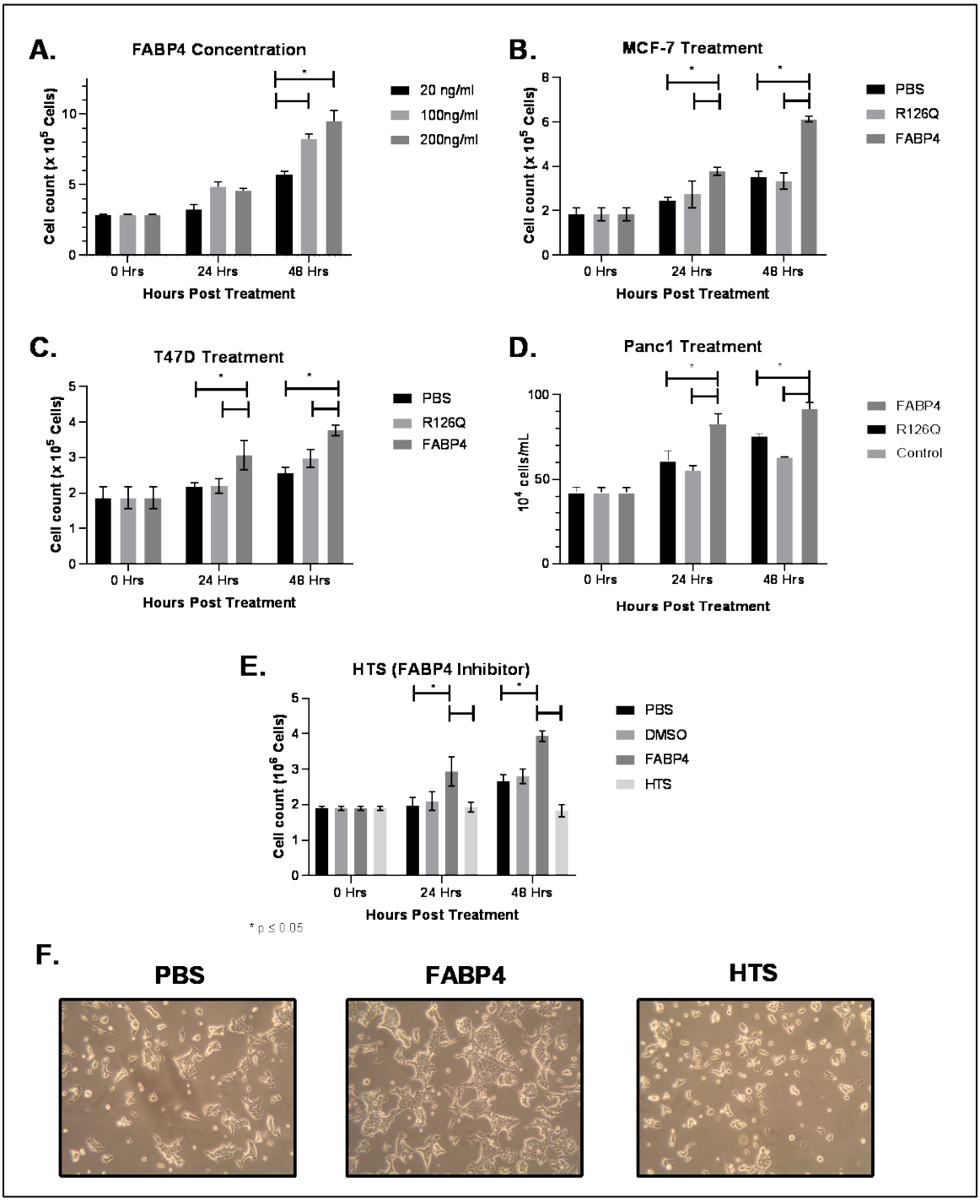

Figure 1. Cell proliferation with exogenous FABP4, mutant R126Q, or HTS01037. Breast cancer cell lines A-C,E. Pancreas cell lines D. Photomicrographs of phenotypic changes F.

Figure 2.

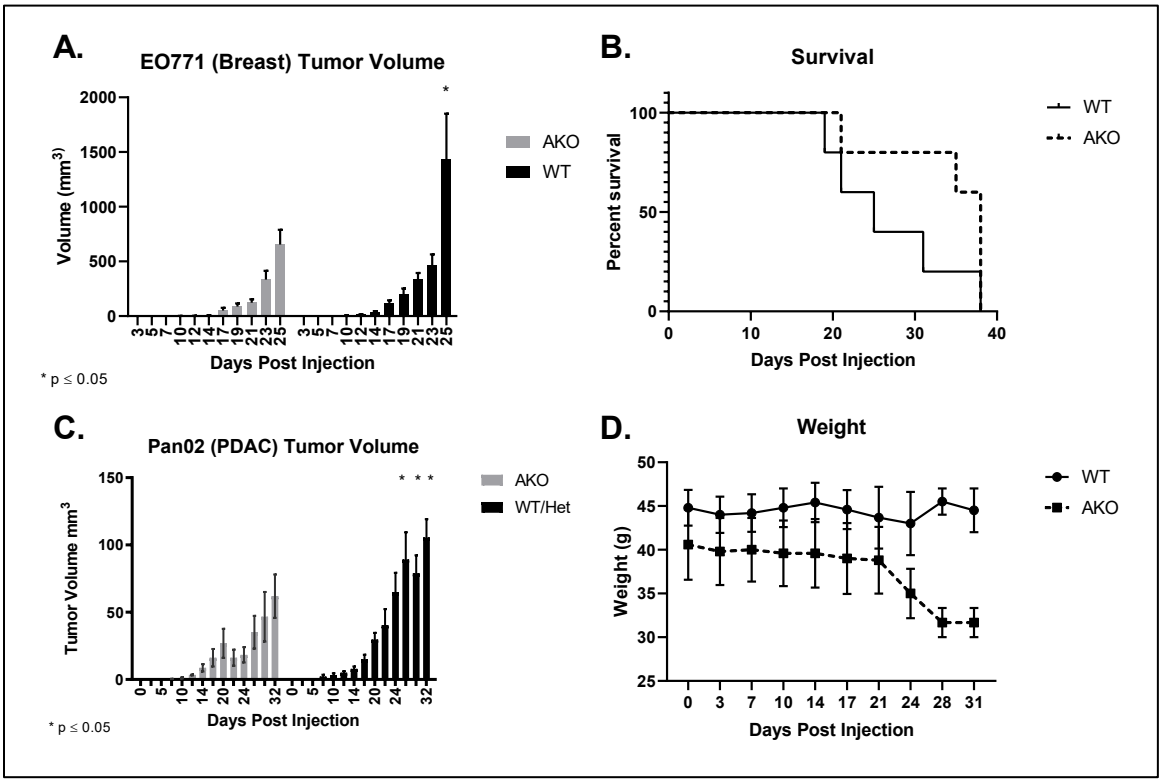

Figure 2. In vivo tumor progression models. Breast cancer progression A. Pancreatic cancer progression B. Survival and weight outcomes for breast cancer C-D.

Figure 3.

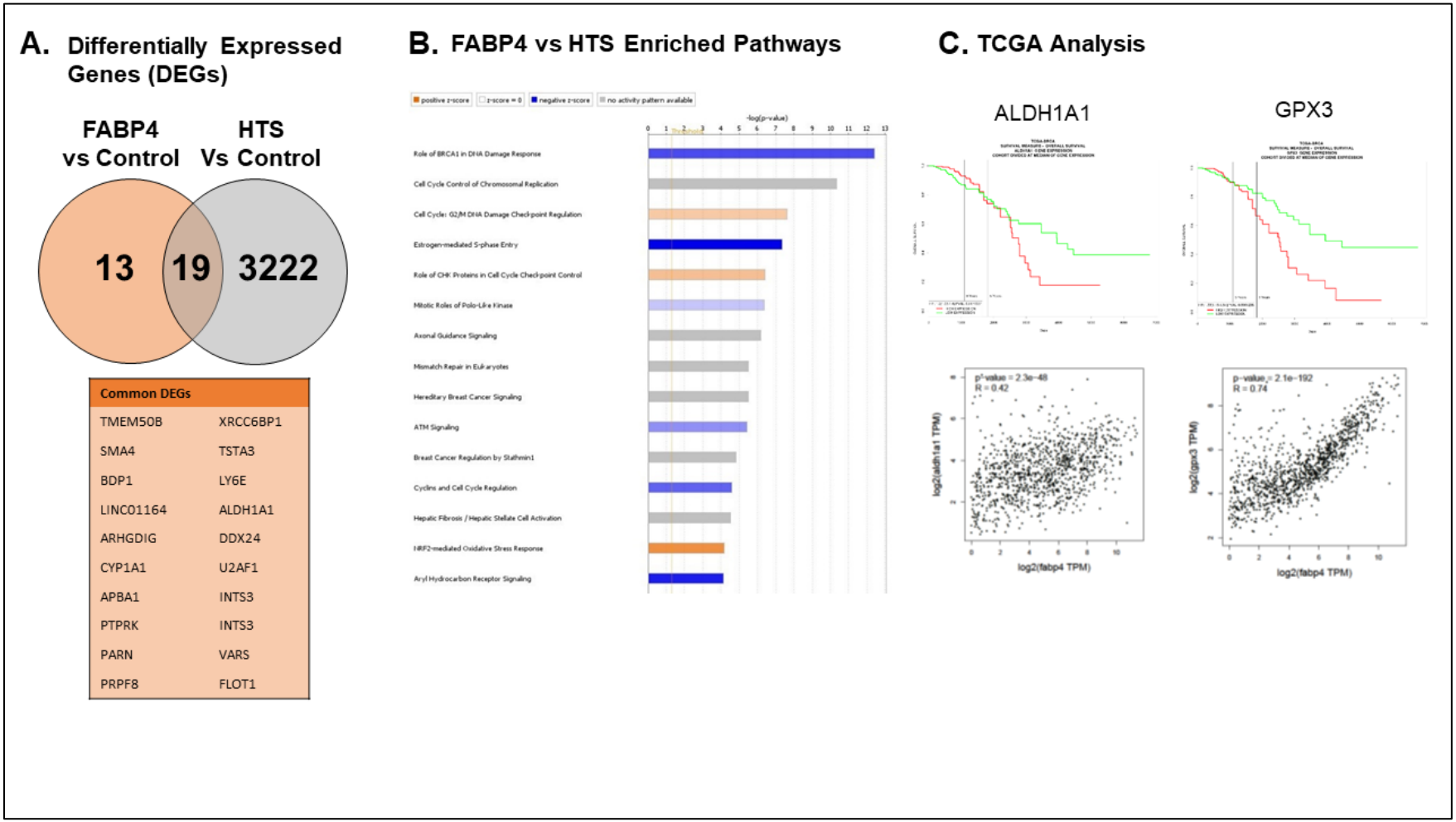

Figure 3. RNA Sequencing results. A-Differentially expressed genes. B- FABP4 vs HTS pathway analysis. C- TCGA correlation and prognostic analysis.

Table 1.

Gene	FABP4 vs HTS Fold Change	Accession no.	Gene name
Glutathione pathway genes			
GCLM	4.26594	NM_002061.1	Glutamate-cysteine ligase, modifier subunit
GCLC	4.145201	NM_001498.1	Glutamate-cysteine ligase, catalytic subunit
GSR	NS	NM_000637.1	GSR
SLC7A11	27.65054	NM_14331.1	Solute carrier family 7, (cationic amino acid transporter, y+ system)
SLC7A11-AS1	22.72613		member 11
MGST1	NS	AV705233	Microsomal glutathione S-transferase 1
GSTM4	NS	NM_000850.1	Glutathione S-transferase M4
GPX2	6.250539	NM_002082.1	GPX 2 (gastrointestinal)
GPX3	8.895754	NM_02084.2	GPX 3 (plasma)
Thioredoxin pathway genes			
TXN	2.207802	NM_003329.1	Thioredoxin
TXNRD1	5.361862	NM_003330.1	TXNRD1
NADPH regenerating enzymes			
G6PD	3.90102	NM_000402.1	Glucose-6-phosphate dehydrogenase
ME1	6.194583	NM.002395.1	ME1
Other antioxidants			
NQO1	NS	NM_000903.1	NAD(P)H dehydrogenase, quinone 1
HMOX1	12.71654	NM_002133.1	Heme oxygenase (decycling) 1
PRDX1	3.344414	NM_002574.2	PRDX 1
Drug transporters			
ABCC1	2.044955	NM_004996.2	ATP-binding cassette, subfamily C (CFTR/MRP), member 1
ABCC2	6.896341	NM_000392.1	ATP-binding cassette, subfamily C (CFTR/MRP), member 2

Table 1. Nrf2 prognostic gene signature differentially expressed genes.

Figure 4.

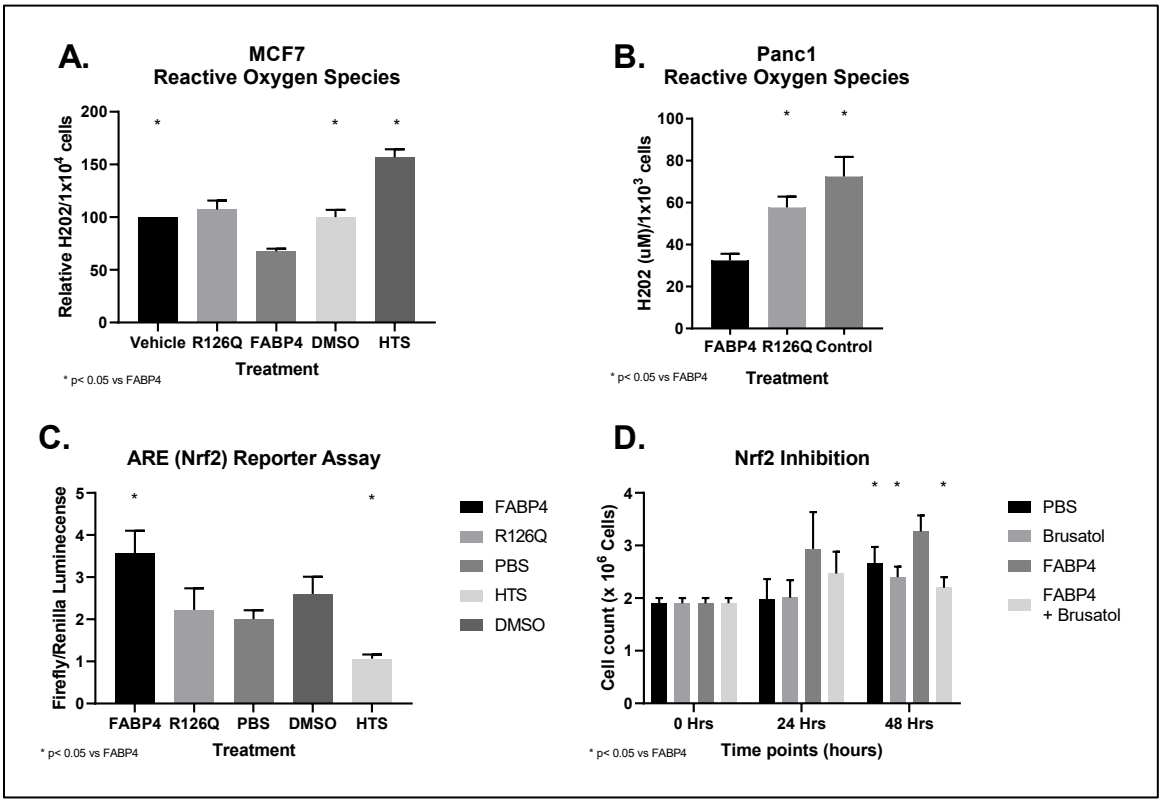

Figure 4. Reactive oxygen species and Nrf2 modulation. A, B- ROS after treatment in breast and pancreas cancer, respectively. C- Nrf2 reporter Assay. D- Proliferation with Nrf2 inhibitor.

Figure 5.

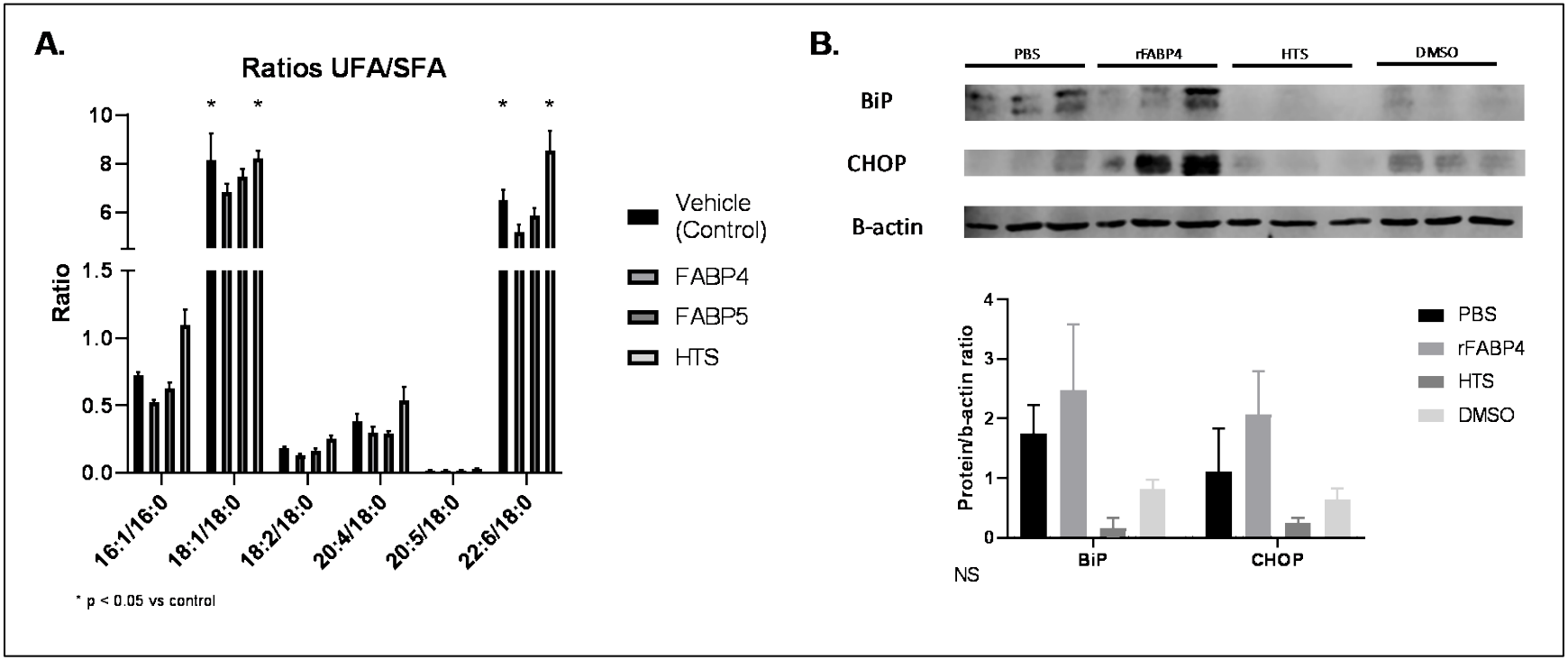

Figure 5. Desaturation ratio and ER stress markers. A- UFA/SFA ratio after MCF7 cell treatment. B- ER stress western blot after MCF7 cell treatment.