Identification of Putative Fatty Acid Binding Protein 4 Receptors on Breast Cancer Epithelial Cells

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Abstract

Fatty acid binding protein 4 (FABP4) is a small 15-kDa cytoplasmic lipid carrier protein regulating fatty acid trafficking and metabolism. It is one of the most abundant proteins in mature adipocytes and can be secreted into the extracellular environment upon lipolytic stimulus, functioning as an adipokine. Elevated circulating FABP4 levels have been associated with obesity-related and inflammation-related diseases. Recent studies have indicated FABPP4 as a potential biomarker in cancer diagnosis and prognosis. FABP4 upregulation or exogenous FABP4 (eFABP4) administration has been found to promote cancer growth, invasion and metastasis, while FABP4 inhibition reduced cancer progression. Understanding the molecular mechanism underlying FABP4 effects in cancer is critical for developing anticancer drugs. Although exogenous FABP4 has been found to exert pro-tumorigenic effects, there is no known FABP4 receptor that transduces its signaling into intracellular responses. Identifying a FABP4 receptor would be significant for targeting FABP4 in cancers. Furthermore, although fatty acids have been found to be essential in FABP4 function, the mechanisms explaining their collaboration remain largely unknown. This thesis aims to identify putative FABP4 receptors on cancer cell plasma membrane to understand FABP4 signaling in breast cancer cells. Our work reveals that eFABP4 binds to the extracellular domain of desmoglein 2 to mediate breast cancer epithelial cell growth via an ERK-NRF2 axis, suggesting desmoglein 2 as a FABP4 receptor. We also find that fatty acids enhance the interaction between FABP4 and desmoglein 2, which may explain why non-fatty acid binding mutants of FABP4 abolish FABP4 effects and support the role of fatty acids in FABP4 signaling. In conclusion, our

findings provide new insights into the mechanism of FABP4 in the development and progression of obesity-associated cancers.

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List of Abbreviations

1,8-ANS, 1-anilinonaphthalene 8-sulfonic acid

AKO, FABP4 knockout or null

ALDH1A1, aldehyde dehydrogenase 1 family member A1, also known retinaldehyde dehydrogenase 1

ARE, antioxidant response element

CAA, cancer-associated adipocyte

CYP1A1, cytochrome P450 family 1 subfamily A member 1

DSC, desmocollin

DSG, desmoglein

EC, extracellular cadherin domain

ECM, extracellular matrix

EMT, epithelial-mesenchymal transition

ERK, extracellular signal-regulated kinase ½

pERK, phosphorylated ERK

eFABP4, exogeneous/extracellular/circulating FABP4

FA, fatty acid

FFA, free fatty acid

FABP, fatty acid binding protein

HMOX1, heme oxygenase 1

HUVEC, human umbilical vein endothelial cells

NRF2, nuclear factor E2-related factor 2

ROS, reactive oxygen species

SOD1, superoxide Dismutase 1

TME, tumor microenvironment

UPS, unconventional protein secretion

Preface

Fatty acid binding protein 4 (FABP4, A-FABP4 or aP2) is a small 15-kDa cytoplasmic fatty acid (FA) carrier protein involved in intracellular fatty acid trafficking and regulation of insulin sensitivity [1]. The basic structure of the FABP4 is 10 antiparallel β-strands capped with a helix-loop-helix motif, forming a flattened barrel. The interior cavity is for fatty acid binding [2]. It is highly expressed in adipocytes and detectable in macrophages upon inflammatory stimuli [1].

As one of the most abundant proteins in mature adipocytes, it can be secreted into the extracellular environment through unconventional protein secretion (UPS) [3], which represents a process by which proteins are secreted independent of the ER or Golgi apparatus in a cell type-dependent manner [3-6]. The recent discovery of extracellular FABP4 (eFABP4) opens new perspectives into its role as an adipokine, a circulating adipose-derived cytokine, regulating a wide range of physiological functions [5].

As an adipocyte-derived protein, elevated circulating FABP4 levels have long been associated with obesity-related diseases, such as type 2 diabetes [7-9], and cardiovascular diseases [10-13]. Studies have shown that eFABP4 acting on metabolic tissues, such as fat, liver, and skeletal muscle, leads to lipid abnormalities, impairment of insulin signaling and reduced glucose uptake [14]. Administration of antibodies targeting FABP4 corrects a diabetic phenotype of obese mice by lowering fasting blood glucose, improving glucose metabolism, increasing insulin sensitivity, and reducing fat mass and

liver steatosis [15]. Loss of FABP4 showed improved metabolism and reduced risk of coronary disease and type 2 diabetes in animal models [16, 17].

FABP4 is also a link between obesity and inflammation. It plays a proinflammatory role in the development of inflammation-related disorders, such as allergic asthma and atherosclerosis [18, 19]. Previous work from our laboratory has demonstrated that FABP4 is involved in the activation of macrophage inflammation, polarizing macrophages from the classically activated M1 phenotype to the alternatively activated M2 [20-22]. Targeting FABP4 uncoupled obesity from inflammation in macrophages [20, 22]. In addition to macrophages, FABP4 also showed proinflammatory effects in human umbilical vein endothelial cells (HUVECs) and eosinophils [18, 23]. Loss or inhibition of FABP4 protected the organism against inflammation-related disorders, such as atherosclerosis and allergic airway inflammation [18, 19].

Whereas FABP4 has been associated with complex and reciprocal crosstalk between obesity, metabolic disorders and inflammation, emerging studies are proposing a role of FABP4 in cancers. Elevated FABP4 has been reported in a wide variety of cancers, including breast cancer [24-27], ovarian cancer [28-30], pancreatic cancer [31, 32], colon [33] and colorectal cancer [34], bladder cancer [35], cervical cancer [36], glioblastoma [37], hepatocellular carcinoma [38], non-small cell lung cancer [39], acute myelocytic leukemia [40], oral squamous cell carcinoma [41], and cholangiocarcinoma [42], correlating with proliferation, progression and invasion of cancers. Research suggested that FABP4 reprogrammed metabolic and inflammatory profiles in cancer that favors

tumor development and progression. The molecular mechanism underlying FABP4's role in cancers might provide therapeutic implications for cancer treatment.

FABP4 can bind hydrophobic ligands to regulate lipid trafficking and metabolism. By interacting with intracellular hormone-sensitive lipase (HSL) and peroxisome proliferator-activated receptor gamma (PPAR-γ) in adipocytes or macrophages, FABP4 is involved in regulating lipolysis of fatty acids [43, 44]. FABP4 has been implicated in intracellular free fatty acids (FFA) availability, with FABP4 expression positively associated with the transfer of FFA from adipocytes to tumor cells [28]. FABP4 could also enhance fatty acids uptake in T cells [45]. Previous studies from our lab have indicated FABP4 as a biomarker for de novo lipogenesis, driving a selective alteration of the available intracellular free fatty acids pool, while loss of FABP4 resulting in an elevated intracellular abundance of monounsaturated fatty acids in macrophages [20, 22]. To further investigate fatty acid binding of FABP4 in its function, our lab generated a non-fatty acid binding FABP4 mutant, R126Q, and showed that R126Q did not have any effect as FABP4 [20, 31, 46]. FABP4 inhibitors, which also disrupted fatty acid binding, showed beneficial pharmacological effects for atherosclerosis and diabetes treatments [20, 33]. Additionally, studies showed that fatty acids cooperated with FABP4 in its pro-metabolic and pro-inflammatory effects [25, 47]. With studies supporting collaboration between fatty acids and FABP4 in pathological conditions, the mechanisms underlying their cooperation, however, remains largely unknown.

Predominantly expressed in adipocytes and macrophages, serum/circulating FABP4 (eFABP4) has been reported to have functions in other cells [5]. It has been shown to regulate glucose uptake, lipid accumulation and insulin signaling in hepatocytes [5], pancreatic β-cells [48], and cardiomyocytes [14]. In human umbilical vein endothelial cells (HUVECs), eFABP4 exhibited pro-oxidative and pro-inflammatory effects [23]. eFABP4 secreted by adipocytes directly targeted tumor cells cocultured with adipocytes [25], with increased proliferation, invasiveness, and stemness in cancer cells [25, 49]. These results suggest a model where eFABP4 binds to cell surface protein(s) to mediate cellular uptake and/or intracellular signaling. However, there are no FABP4 receptors identified till now.

The aim of this work is to 1) identify putative FABP4 receptors on cancer cell plasma membrane, 2) investigate FABP4 signaling in breast cancer cells, and 3) characterize the role of lipid binding in FABP signaling.

Part 1 Overviews FABP4 in the development, progression and invasion of cancer. Part 2 “Desmoglein 2 Functions As A Receptor for Fatty Acid Binding Protein 4 in Breast Cancer Epithelial Cells” identified a putative FABP4 receptor on breast cancer cells.

Our work revealed desmoglein 2 as a possible receptor for eFABP4 that stimulated breast cancer growth through an ERK/NRF2 signaling axis, and fatty acid binding to FABP4 may be a key determinant in its signaling, providing insight into the mechanism of eFABP4 as a driver of obesity-related cancers.

Part 1 FABP4 in Cancer Progression

1.1 Abstract

Fatty acid binding protein 4 (FABP4) is a lipid chaperone that regulates fatty acid trafficking and metabolism. Predominately expressed in adipocytes and macrophages, it can also be secreted into the extracellular environment by adipocytes, functioning as an adipokine and regulating a wide range of pathological processes. FABP4 expression has been found in various types of cancer and is associated with cancer risk and prognosis. This review highlights the crucial role of FABP4 in the development, progression and invasion of cancer, as well as the underlying mechanisms involved.

1.2 FABP4 as a link between obesity with cancer

1.2.1 Obesity and Cancer

Obesity, which is defined as abnormal or excessive fat accumulation in adipose tissue, has been a severe health issue. According to the latest obesity epidemiology released by the World Health Organization, obesity affects over a billion people worldwide in 2022, with this number continuing to rise [50, 51]. Obesity can lead to adipocyte hypertrophy, inflammation, hypoxia, angiogenesis, extracellular matrix (ECM) remodeling, fibrosis and stress responses in adipose tissue, which can trigger adipose tissue dysfunction [52]. Those alterations further increase the risk of obesity-associated metabolic complications, such as changes in steroid hormone and adipokine production, insulin resistance, dyslipidemia, inflammation, type 2 diabetes, cardiovascular disease, and cancers.

With emerging epidemiological studies showing the association between obesity and various types of cancers, the cross-talk between adipose tissue and cancer cells is receiving significant attention. Studies have indicated that obese patients have an increased risk of cancer and show poor cancer outcomes [53-57]. Body mass index (BMI), a simple index used to measure body fat and classify obese, is positively correlated with cancer incidence [58]. Specifically, in men, higher BMI is associated with an increased risk of thyroid, colon, renal, esophageal adenocarcinoma, and rectal cancers and malignant melanoma; while in women, it is linked to postmenopausal breast, endometrial, gallbladder, esophageal adenocarcinoma, renal, pancreatic, thyroid, and colon cancer. In both sexes, the relationship is observed in multiple myeloma, leukemia, and non-Hodgkin lymphoma [59, 60]. Moreover, obesity is also linked to an increased risk of overall mortality and recurrence in cancers [59-63].

The proposed mechanisms underlying obesity-related cancer include insulin resistance, hyperinsulinemia, hyperglycemia, glucose intolerance, oxidative stress, inflammation and adipokine production [25, 28, 49, 53]. Fatty acids released by adipocyte lipolysis may fuel energy requirements for the growth of certain cancers. Adipose tissue is also a large endocrine organ that produces adipokines, such as leptin and adiponectin, which have been implicated in cancer development and progression by regulating lipid metabolism, inflammation, angiogenesis and cell proliferation [53, 64-67]. Adipocyte infiltration in the tumor microenvironment has been reported in multiple cancers, and in-tumoral adipocytes, termed cancer-associated adipocytes (CAA), exhibit an activated phenotype. Studies have shown that adipokines secreted by CAAs created a pro-tumorigenic

microenvironment, contributing to cancer development, progression and invasion [54, 68-71]. Fatty acid binding protein 4 (FABP4), among the adipokines secreted by adipocytes, has frequently been associated with various types of cancers **(Table 1)**.

1.2.2 FABPs and Cancer

Fatty acid binding proteins (FABPs) are a family of lipid chaperones that facilitate fatty acids uptake and trafficking. To date, at least nine types of FABPs have been identified and named after the tissue they were originally isolated from, including liver FABP (FABP1), intestine FABP (FABP2), heart FABP (FABP3), adipocyte FABP (FABP4), epidermal FABP (FABP5), Ileal FABP (FABP6), brain FABP (FABP7), myelin FABP (FABP8), testis FABP (FABP9) [72]. These cytosolic proteins are approximately 15 kDa and share a similar structure: a fatty acid binding pocket surrounded by antiparallel β-strands that form a flattened barrel [2]. FABPs are increasingly implicated in many types of cancers (Table 1), with FABP4 being the most intensively studied. Despite their tissue-specific expression, FABPs have also been detected at high levels in the cerebrospinal fluid, plasma, or urine of cancer patients [35–39].

Table 1. FABP family in cancers

FABP family	Other names	Cancer
FABP1	L-FABP, liver FABP, Z protein, hepatic FABP	colorectal cancer [73], renal cell carcinoma [74]
FABP2	I-FABP, intestinal FABP, gut FABP	breast cancer [75], colon cancer [76]
FABP3	H-FABP, heart FABP, muscle FABP	non-small cell lung cancer [39], renal cell carcinoma [74],

		bladder cancer [74], gastric carcinoma [77]
FABP4	A-FABP, adipose FABP, aP2, ALBP	breast cancer [24-27], ovarian cancer [28-30], pancreatic cancer [31, 32], colon [33] and colorectal cancer [34], bladder cancer [35], cervical cancer [36], glioblastoma [37], hepatocellular carcinoma [38], non-small cell lung cancer [39], acute myelocytic leukemia [40], oral squamous cell carcinoma [41], cholangiocarcinoma [42]
FABP5	E-FABP, epidermal FABP, mal-1, KFABP, skin FABP, psoriasis-associated FABP, PA-FABP, C-FABP	breast cancer [24], renal cell carcinoma [74], lung adenocarcinoma [78], colorectal cancer [79, 80], prostate cancer [81-84], oral squamous cell carcinoma [85], intrahepatic cholangio carcinoma [86], pancreatic adenocarcinoma [87], bladder cancer [88], triple negative breast cancer [89, 90], and hepatocellular carcinoma [91]
FABP6	IL-FABP, Ileal FABP, I-BABP, gastrotropin	colorectal cancer [34], renal cell carcinoma [74], bladder cancer [74], prostate cancer [74]
FABP7	B-FABP, brain FABP, BLBP	prostate cancer [92], renal cell carcinoma [74, 93] , glioblastoma [94, 95], melanoma [96, 97]
FABP8	M-FABP, myelin FABP, myelin P2	N/A
FABP9	T-FABP, testis FABP, TLBP, PERF15	N/A

1.3 FABP4 in Cancers

FABP4 is one of the most abundant proteins in mature adipocytes, constituting approximately 1% of all soluble proteins in adipose tissue. Upon lipolytic stimuli, increased intracellular Ca^{2+} levels, or hypoxia, FABP4 can be secreted into the extracellular environment via unconventional protein secretion (UPS), which represents a process by which proteins are secreted independent of the ER or Golgi apparatus in a cell type-dependent manner [3-6]. The recent discovery of extracellular FABP4 (eFABP4) has opened new perspectives into its role as an adipokine, a circulating adipose-derived cytokine, regulating a broad range of physiological functions [5].

In obese people, serum FABP4 level increase 10-20 fold [7-9]. Elevated circulating FABP4 levels have been linked to obesity-related diseases, such as type 2 diabetes [7-9], cardiovascular diseases [10-13], and allergic asthma [18]. Additionally, emerging studies are revealing associations between FABP4 and various types of cancers, including breast cancer [24-27], ovarian cancer [28-30], pancreatic cancer [31, 32], colon [33] and colorectal cancer[34], bladder cancer [35], cervical cancer[36], glioblastoma [37], hepatocellular carcinoma [38], non-small cell lung cancer [39], acute myelocytic leukemia [40], and oral squamous cell carcinoma[41], cholangiocarcinoma [42]. These findings provide new insights into the interplay between obesity and cancers.

1.3.1 FABP4 positively associates with cancer progression

Circulating FABP4 levels have been suggested as a potential biomarker for cancer diagnosis. Studies have demonstrated that serum FABP4 levels were elevated in breast

cancer patients, and circulating FABP4 released by adipose tissue directly targeted tumor cells, enhancing tumor stemness in breast cancer [24, 25]. In colorectal cancer patients, serum levels of FABP4 were also observed to be higher before surgery, and significantly decreased after the operation [34].

Elevated expression of FABP4 in tumor tissues is a strong risk factor for the development and progression of cancers. In breast cancer, high expression of FABP4 in cancer cells was significantly associated with low overall survival of patients [26]. The expression of FABP4 was positively correlated with the invasive capacity of ovarian cancer cells, with FABP4 upregulation in all omental metastases in ovarian cancer [28, 29]. Furthermore, ectopic expression of FABP4 in ovarian cancer cells resulted in increased migration and invasion of cancer cells [30]. Upregulation of protein FABP4 and transcription level of FABP4 mRNA was also reported in colon and colorectal cancer tissues with high grade of invasion and migration of cancer cells [33, 34]. Similarly, FABP4 overexpression in colon cancer cells significantly enhanced epithelial-mesenchymal transition (EMT) [33].

Increased FABP4 expression was also highly associated with poor overall survival and advanced tumor metastasis stage in cervical cancer [36], glioblastoma [37], non-small cell lung cancer [39], and pancreatic ductal adenocarcinomas patients, [32] severing as an independent risk factor for cancer prognosis. Significantly higher expression of FABP4 was also found in the tumor tissue of oral squamous cell carcinoma patients [41]. Moreover, acute myeloid leukemia patients with higher FABP4 expression in primary cells

showed shorter lifespans [40]. These findings highlight the potential clinical significance of FABP4 as a prognostic biomarker in cancer.

The role of FABP4 in cancer progression has also been validated in mouse models. Mice injected with cells ectopically expressing FABP4 had a significantly higher tumor weight, as well as enhanced distant nodules metastasis in ovarian cancer [29]. In a mouse model of colon cancer, FABP4 expression in host mice enhanced metastasis [33], and in breast cancer, it increased aggressiveness [25]. These preclinical studies provide compelling evidence for the oncogenic role of FABP4 and suggest its potential as a therapeutic target in cancer treatment.

Furthermore, in vitro studies have demonstrated that endogenous/extracellular FABP4 (eFABP4) alone is sufficient to potentiate cell growth in vitro. The addition of eFABP4 increased cell proliferation in a dose-dependent manner in acute myelocytic leukemia and pancreatic cancer [31, 40], providing additional evidence for the contribution of FABP4 to tumor progression. However, the effect of FABP4 on tumor progression in bladder cancer and hepatocellular carcinoma is controversial. A long-term clinical follow-up showed that loss of FABP4 was associated with higher mean age, higher stage, and metastasis in bladder cancer, correlated with shorter event-free survival and overall survival in those patients [35]. Supportively, Boiteux et al reported FABP4 loss in poor prognosis high-grade bladder cancer, compared to tumors with good prognosis [98]. However, Uehara et al found that the administration of eFABP4 promoted prostate cancer cell invasion, and these promoting effects were reduced by FABP4 inhibition in vitro as well as in animal

models [47]. Similarly, in hepatocellular carcinoma patients, FABP4 expression was downregulated in tumor tissue, with a significantly better recurrence-free survival and overall survival rate in the FABP4-high group [38]. Overexpression of FABP4 suppressed proliferation and migration of hepatocellular carcinoma cell lines, and in vivo, FABP4-overexpressing cells injected into mice showed inhibited growth, with smaller and lighter tumor weight [38]. Conversely, Laouirem et al found that addition of eFABP4 potentiated cell viability and proliferation in hepatocellular carcinoma cell lines, and this effect was also significantly reversed by FABP4 inhibition in both cell lines and xenografted mice [99].

1.3.2 FABP4 deficiency inhibits cancer progression

Studies have demonstrated that FABP4 deficiency led to the inhibition of tumor proliferation, migration and invasion in cancers, providing further support for the role of FABP4 in enhancing cancer progression, FABP4-deficient cancer cells exhibited significant suppression of growth in breast cancer [25], ovarian cancer [29], pancreatic cancer [31], cervical cancer [36], glioblastoma [37], and oral squamous cell carcinoma [41]. In these studies, FABP4-deficient cells displayed inhibited proliferation in vitro, as well as smaller tumor weight and fewer metastasis nodules in mouse models. Additionally, *FABP4* ablation led to decreased proliferation *in vitro*, reduced leukemic disease burden and induced leukemia regression *in vivo* [40]. These findings suggest that FABP4 may represent a potential therapeutic target for the treatment of various cancers.

In mouse models, the role of FABP4 in cancer has been further elucidated. FABP4-null mice showed a significant reduction in tumor growth and metastasis in colon cancer and breast cancer model [26]. In an intraperitoneal model of ovarian cancer, *Fabp4*$^{-/-}$ mice exhibited decreased tumor burden compared to wild type mice [28]. Additionally, absence of FABP4 in mice significantly reduced tumor weight and improved overall survival in pancreatic cancer xenograft [31]. These findings support the notion that host-derived FABP4 plays a critical role in tumor progression.

1.4 Fatty Acids Binding of FABP4 in Cancers

In cancer cells, fatty acid metabolism was reprogrammed to not only fuel tumor growth, but also serve as secondary messengers for oncogenic signaling [100]. FABP4 interacted with intracellular hormone-sensitive lipase (HSL) and peroxisome proliferator-activated receptor gamma (PPAR-γ), leading to the release of free fatty acids by adipocytes, to support tumor growth [28, 33, 43, 44]. As a lipid chaperone, FABP4 can remodel the cellular lipidome of cancer cells, promoting tumor growth and metastasis. Studies have shown that FABP4 expression was positively associated with the transfer of free fatty acids (FFAs) from adipocytes to tumor cells cocultured with adipocytes in ovarian cancer and cholangiocarcinoma [28, 42]. Additionally, FABP4 can also regulate fatty acid uptake in cancer-infiltrated immune cells [45].

Breast cancer cells cocultured with adipocytes showed significantly increased levels of saturated FAs, including palmitate and stearate [101]. Further studies found that it was

saturated palmitic acid, not unsaturated oleic acid, that cooperated with eFABP4 in promoting tumor growth by enhancing tumor cell stemness in breast cancer cells [25]. Conversely, in ovarian cancer, the most abundant fatty acid in cancer cells cocultured with adipocytes was the unsaturated linoleate, which was associated with an increased risk of developing ovarian cancer [30]. In prostate cancer cells, the unsaturated oleic acid was found to corroborate FABP4 effect as well [47, 102]. Although FABP4 does not have selectivity for saturated or unsaturated FAs, this variation in fatty acid preference might be attributed to different cancer types.

As a lipid chaperone, fatty acids bind to FABP4 with their lipid carboxylate buried into a large water-filled central cavity and their omega methyl group oriented towards the protein surface [103-105]. To explore the role of lipid binding in FABP4-mediated cell proliferation, our lab generated a non-fatty acid binding FABP4 mutant, R126Q. We found that this mutant did not show any effect on cancer cells or macrophages [20, 31, 46], indicating that the conformational change due to lipid binding might be essential for FABP4 function. The collaboration between FABP4 and fatty acids in cancers might also be explained by this conformational change.

1.5 FABP4 inhibitors as therapeutic targets in cancer

As the link between elevated FABP4 levels and pathophysiological disorders became more apparent, there has been growing interest in the synthesis and discovery of FABP4 inhibitors. Novel FABP4 inhibitors, such as BMS309403 and HTS01037, have been

identified for their ability to interact with the FABP4 lipid binding pocket and outcompete fatty acids [106]. The pharmacological benefits of BMS309403 in atherosclerosis and metabolic syndrome have been reported in numerous studies [107, 108].

Previous studies have reported the potential of FABP4 inhibitors in blocking lipid accumulation and proliferative effects of FABP4 in ovarian cancer cells [28, 30]. FABP4 inhibition showed a significant reduction in metastasis in xenografted mouse models of ovarian cancer, colon cancer and cholangiocarcinoma [28, 30, 33, 42]. The collaborative effects of FABP4 and oleic acid were also reduced by FABP4 inhibitor in tumor growth and metastasis of prostate cancer [47]. Moreover, FABP4 inhibitors showed promising results in enhancing the anti-tumor effects of chemotherapy drugs and tyrosine kinase inhibitors (TKI) by inhibiting tumor growth and recurrence in breast cancer [109]. Additionally, FABP4 inhibitors increased the sensitivity of ovaria cancer cells to carboplatin, the most used chemotherapy drug against epithelial ovarian cancer, as evidenced by a decrease in tumor volume and metastatic nodules, as well as the restoration of omental tissue architecture [30].

1.6 FABP4 in tumor microenvironment

Tumor microenvironment (TME) is the dynamic environment surrounding the tumor body. The communication between the tumor and TME is critical in the initiation, development, and progression of cancer. Although the composition of the TME varies in different types of cancers, it mainly consists of blood vessels, extracellular matrix and tumor stromal cells. The tumor stromal cells are a heterogeneous cell system comprised of various types of

cells, including endothelial cells, fibroblasts, adipocytes and immune cells [68, 110]. Studies have shown that FABP4 contributed to a pro-tumorigenic TME.

1.6.1 FABP4 in adipocytes

Adipocytes are one of the most common cells in TME of cancers that grow near adipose tissue, such as breast cancer and ovarian cancer. The close interaction between tumors and adipocytes remodeled adipocytes, which became favorable for tumor progression and were called cancer cancer-associated adipocytes (CAAs). The typical features of CAAs have been characterized in differentiation, catabolism, secretoma, senescence and immunomodulator [111]. Using proteomics, Mukherjee et al identified FABP4 upregulation as a critical regulator of lipid responses in ovarian cancer cells and cholangiocarcinoma cocultured with adipocytes [30, 42]. Nieman et al found that FABP4 was highly expressed in ovarian cancer cells at the adipocyte-cancer cell interface compared to distant from their interface [28]. In vitro, FABP4 expression was upregulated in cancer cells cocultured with adipocytes, while ovarian cancer cells cocultured with *Fabp4*$^{-/-}$ adipocytes exhibited dramatic inhibition of lipid accumulation and invasion [28]. Colon cancer cells cocultured with adipose tissue extract also showed significantly enhanced the invasion and migration, as well as energy and lipid metabolism, which were reversed with FABP4 inhibitors [33]. Taken together, these studies suggested the importance of FABP4 expression in adipocytes for tumor progression.

1.6.2 FABP4 in immune cells

Immune cells commonly infiltrate tumors and are major components of TME. Tumor-infiltrating immune cells (TIICs) have been used as biomarkers for diagnosis and indicators of immunotherapy and prognosis [112]. Macrophages in the TME are defined as tumor-associated macrophages (TAMs). Studies have shown that TAMs promoted cell proliferation, invasion, metastasis, and drug resistance in cancers [113]. Previous work from our laboratory has demonstrated that FABP4 is involved in the activation of macrophage inflammation, polarizing macrophages from the classically activated M1 phenotype to the alternatively activated M2 [20-22]. Targeting FABP4 uncoupled obesity from inflammation in macrophages [20, 22]. Other groups have also shown that FABP4 increased FA transport and oxidation in macrophages, leading to increased cellular stress, senescence, and death, while FABP4 inhibition suppressed cell death by disrupting FA-induced ceramide production [114]. Hao et al found that FABP4 was significantly upregulated in a subset of TAMs, and the overexpression of FABP4 in TAMs promoted breast cancer growth through IL6/STAT3 pathway [26].

The infiltration of memory T cell subset CD8+Trm cells was positively correlated with antitumor immune responses and improved prognosis in patients with cancer. FABP4 played a critical role in the maintenance, longevity and function of CD8+ Trm cells by enhancing fatty acid uptake [45], as these cells rely on exogenous fatty acid uptake for their survival. In gastric cancer, tumor cells with significantly higher FABP4 expression outcompeted Trm cells for lipid uptake, thus inducing apoptosis of Trm cells [115]. Anti-cancer drugs have been developed to block programmed death-ligand 1 (PD-L1), which

inhibits T cell activation by binding to programmed death-1 (PD-1) on T cell surface. Lin et al found that PD-L1 blockade enhanced anti-tumor immunity by increasing FABP4 expression in Trm cells, while decreasing FABP4 expression in gastric tumor cells, thereby enhancing fatty acid uptake and survival of Trm cells [115].

1.6.3 FABP4 in endothelial cells

Endothelial cells play a crucial role in cancers by regulating the exchange of nutrients and oxygen, as well as mediating angiogenesis and metastasis [116]. In HCC, FABP4 was upregulated in endothelial cells with an upregulated angiogenesis gene signature [99]. Treatment with FABP4 inhibitor BMS309403 in xenografted mice led to decreased endothelial FABP4 levels and reduced tumor volume [99]. Similarly, targeting endothelial FABP4 by siRNA in ovarian tumor xenografts increased FA oxidation and ROS production in endothelial cells, reduced microvessel density in xenografts, and led to a reduction in tumor weight and number of tumor nodules [117, 118]. Endothelial FABP4 has been suggested as a point of convergence for cancer, angiogenesis, and metabolic disease, as pro-angiogenic and metabolic signaling DLL4-NOTCH, and oncogenic signaling FOXO1 were found to co-regulate gene transcription of *FABP4* in endothelial cells [118].

1.7 FABP4 Signaling in Cancer

Numerous studies have provided evidence supporting the involvement of FABP4 in the initiation, progression, and invasion of cancers. Additionally, intracellular signaling

mechanisms associated with FABP4 have also been investigated, revealing its potential role in activating oncogenic signaling pathways.

Mitogen-activated protein kinase (MAPK) cascades are extensively studied signaling pathways in cancer, where constitutively active ERK phosphorylation promoting cancer proliferation. Notably, in breast and pancreatic cancer, eFABP4 has been found to promote tumor growth by activating MAPK signaling cascades [31, 49]. Inhibition of FABP4, which blocked its proliferative effects, has been shown to decrease phosphorylated MAPK in breast cancer, pancreatic cancer and oral squamous cell carcinoma [31, 41, 49]

The activation of PI3K/AKT pathway has also been found to contribute to cancer progression. Studies have shown that FABP4 can activate the PI3K/Akt pathway in breast cancer, prostate cancer, and cervical cancer, with FABP4 silencing leading to downregulation of p-AKT [36, 47, 49].

STAT3 is known to be involved in cancer as a convergence point of several major oncogenic signaling pathways. Hao et al reported that FABP4 enhanced the stemness of breast cancer by activating the IL-6/STAT3/ALDH1 pathway [25]. In AML, FABP4 was found to enhance aggressiveness via the NFκB/STAT3/DNMT1 cascade [40]. Furthermore, in HCC, the expression of p-STAT3 was found to be correlated with FABP4-mediated proliferation and invasion [38].

Studies have indicated the significant role of oxidative stress in cancer progression. FABP4 has been found to potentiate pancreatic cancer proliferation through NRF2-mediated reactive oxygen species (ROS) signaling [31]. Additionally, FABP4 loss-mediated inhibition of cell proliferation was accompanied by downregulated ROS generation and lipid peroxidation in ovarian cancer [30].

Downregulation of cell adhesion increases invasiveness and metastatic activity in solid cancers. Proteomics analysis identified adhesion and cell junction pathway as pertaining to FABP4-regulated metastasis in ovarian cancer [29]. Inhibition of migration and invasion by FABP4 silencing in cervical cancer cells was accompanied by elevated expression of E-cadherin and downregulated expression of N-cadherin and Vimentin in cervical cancer [36]. Epithelial-mesenchymal transition (EMT) is also implicated in cancer metastasis, with FABP4 inducing EMT in various cancer types [33, 42]. eFABP4 has been shown to promote EMT via the activation of AKT/GSK3β/Snail pathway and reorganization of the actin cytoskeletons in cervical squamous cell carcinoma [119]. Altered expression of Snail was also found to correlate with FABP4-associated invasion in HCC [38]. Furthermore, FABP4 silencing led to decreased levels of EMT-associated genes, such as matrix metallopeptidase 2 (MMP-2), Wnt10b, MMP-9, resulting in reduced metastasis glioblastoma [37]. Wnt10b knockdown also reduced metastasis, while double knockdown of Wnt10b and FABP4 abolished this effect, indicating Wnt10b involved in FABP4 mediated metastasis [37].

Although FABP4 expression levels in cancer cells have been linked to cancer progression, the molecular mechanisms regulating its expression remain poorly understood. Notably, studies have shown that CD36 inhibition blocked adipocyte-induced FABP4 upregulation and FABP4 mediated-EMT in ovarian cancer, while FABP4 inhibition did not affect adipocyte-mediated CD36 expression, suggesting that CD36 regulates downstream FABP4 signaling [120]. Additionally, miR-409-3p expression in ovarian cells has been shown to significantly downregulate FABP4 expression, leading to inhibited tumor growth and metastasis [29]. The inhibitory effects of miR-409-3p on FABP4 and tumor proliferation could be rescued by FABP4, indicating miR-409-3p may act as an upstream regulator of FABP4 [29]. Furthermore, specific PPARγ agonists were reported to up-regulate FABP4 expression in bladder cancer [121].

1.8 FABP5 and cancer

FABPs are a family of proteins that share a similar tertiary protein structure and demonstrate high-affinity binding of fatty acids. Given the vital role of fatty acids in processes such as membrane phospholipid synthesis, signaling pathways, and energy production, the dysregulation of fatty acid metabolism has been linked to cancer progression [122, 123]. FABPs have been implicated in multiple types of cancer as lipid chaperones, with FABP4 being the most extensively studied (**Table 1**).

FABP5 is a protein that has 52% sequence similarity with FABP4 and contains a functional nuclear localization signal. It is also expressed in adipocytes and macrophages

[124]. Interestingly, FABP4 loss resulted in compensatory induction of FABP5 in adipocytes, suggesting that they may have overlapping functions [125].

Elevated FABP5 levels have also been observed in several cancer types, including breast cancer [24], renal cell carcinoma [74], lung adenocarcinoma [78], colorectal cancer [79, 80], prostate cancer [81-84], oral squamous cell carcinoma [85], intrahepatic cholangio carcinoma [86], pancreatic adenocarcinoma [87], bladder cancer [88], triple negative breast cancer [89, 90], and hepatocellular carcinoma [91], making it a promising prognostic or therapeutic biomarker for cancers. FABP5 has been shown to increase tumor growth, invasion and metastasis in various cancers, while FABP5 silencing significantly inhibited these effects. Proposed molecular mechanisms underlying FABP5 effects included nuclear translocation of fatty acids, activation of PPARγ and PPARβ/δ, EMT and reduction of epidermal growth factor receptor (EGFR) [78, 81-85, 126, 127].

1.9 Conclusion and Perspective

FABP4 has emerged as a link between obesity and cancer due to its regulatory role lipid metabolism, inflammation, angiogenesis and cell proliferation. Extensive studies have shown that FABP4 was upregulated in cancers and exerted pro-tumorigenic effects in cancer cells. Studies have demonstrated that FABP4 levels had clinical significance in cancer risk and prognosis, and that inhibition of FABP4 alleviated tumor progression. These findings suggested that FABP4 represents a promising therapeutic target for cancer, and that a better understanding if its role in cancer may offer a novel strategy for

cancer treatment. Despite multiple proposed mechanisms explaining FABP4 effects in cancer, none have been conclusive. Therefore, further studies on FABP4 intracellular signaling are warranted. Notably, no studies have been conducted on FABP4 receptors on the cell membrane, rendering eFABP4 signaling transduction to intracellular signaling entirely unknown. Identifying a FABP4 receptor would be of great significance for targeting FABP4 in cancers. Moreover, fatty acids have been found to be essential in FABP4 signaling, but the molecular mechanisms underlying their association remain largely unknow. Therefore, further studies to investigate the role of fatty acids in FABP4 signaling are critical to understand the association between obesity and cancer.

Part 2 Desmoglein 2 Functions As A Receptor for Fatty Acid Binding Protein 4 in Breast Cancer Epithelial Cells

CONTRIBUTIONS

Keith M. Wirth produced **Figure 1** (1A, 1B, 1C and 1E), **Figure 2** and **Figure 3** (2C, 2D, 2H and 2I). And Joseph M. Muretta produced **Figure 6** (6A and 6B). Mass spectrometry analysis was conducted by Todd W Markowski.

ABSTRACT

Fatty acid binding protein 4 (FABP4) is a secreted adipokine linked to obesity and progression of a variety of cancers. Obesity increases extracellular FABP4 (eFABP4) levels in animal models and in obese breast cancer patients compared to lean healthy controls. Using MCF-7 and T47D breast cancer epithelial cells, we show herein that eFABP4 stimulates cellular proliferation in a time and concentration dependent manner while the non-fatty acid-binding mutant, R126Q, failed to potentiate growth. When E0771 murine breast cancer cells were injected into mice, FABP4 null animals exhibited delayed tumor growth and enhanced survival compared to injections into control C57Bl/6J animals. eFABP4 treatment of MCF-7 cells resulted in a significant increase in phosphorylation of extracellular signal-regulated kinase 1/2 (pERK), transcriptional activation of nuclear factor E2-related factor 2 (NRF2) and corresponding gene targets ALDH1A1, CYP1A1, HMOX1, SOD1 and decreased oxidative stress, while R126Q treatment did not show any effects. Proximity-labeling employing an APEX2-FABP4 fusion protein revealed several proteins functioning in desmosomes as eFABP4 receptor candidates including desmoglein, desmocollin, junction plankoglobin, desomoplankin and cytokeratins. AlphaFold modeling predicted an interaction between eFABP4 and the extracellular cadherin repeats of DSG2, and pull-down and immunoprecipitation assays confirmed complex formation that was potentiated by oleic acid. Silencing of Desmoglein 2 in MCF-7 cells attenuated eFABP4 effects on cellular proliferation, pERK levels, and ALDH1A1 expression compared to controls.

IMPLICATIONS

These results suggest desmosomal proteins, and in particular Desmoglein 2, may function as receptors of eFABP4 and provide new insight into the development and progression of obesity-associated cancers.

INTRODUCTION

Whereas 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 breast and pancreatic cancer [71]. 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 [61, 62]. Additionally, indicators of worsened metabolic disease, such as type II diabetes mellitus and metabolic syndrome, have demonstrated similar adverse relationships with breast cancer outcomes [63]. Whereas a number of potential mechanisms have been proposed that link adipose tissue to cancer (e.g., changes in adipokine secretion, fatty acids as fuels for cancer growth, increased local inflammation potentiated by tumor necrosis factor a and interleukin-6, none have been conclusive [71].

In search of a mechanistic 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 [128]. FABP4 is a small, 15-kDa fatty acid carrier protein predominantly expressed by adipocytes, but also found at low levels in macrophages and some endothelial cells. In adipocytes is involved in intracellular fatty acid trafficking and functions to mediate lipolysis [2] while in macrophages, it regulates fatty acid metabolism and leukotriene synthesis [129]. In response to lipolytic stimuli, FABP4 is secreted by adipocytes via a non-canonical mechanism linked to activation of SirT1 and autophagy [3]. Elevated circulating FABP4 levels have been linked to obesity

[7], type 2 diabetes [8, 9], cardiovascular diseases [10-13], and allergic asthma [18]. Conversely, loss or inhibition of FABP4 exhibits reduced risk of coronary disease and type 2 diabetes [16, 17, 130], and protection from inflammation-related disorders [16, 18, 19]. FABP4 has also been implicated in the development and progression of a number of obesity related cancers [128]. 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 [24, 25]. Higher expression of FABP4 in breast cancer specimens is also significantly correlated with recurrence and disease-free survival [131]. Additionally, tumor progression is significantly decreased in FABP4 knockout models [25, 26].

The recent discovery of extracellular FABP4 (eFABP4) provided new insight into a role for FABP4 as an adipose-derived cytokine (adipokine) [5]. eFABP4 has been shown to regulate glucose synthesis in hepatocytes [5] and release of insulin from pancreatic β-cells [48]. In human umbilical vein endothelial cells (HUVECs), eFABP4 exhibited pro-oxidative and pro-inflammatory effects [23] while in cardiomyocytes, eFABP4 increased intracellular lipid accumulation that led to impairment of the insulin signaling and reduced insulin-stimulated glucose uptake [14]. In cancer cells, studies have described increased proliferation, invasiveness, and stemness of cancer cells in response to eFABP4 treatment [25, 49]. Potential mechanisms have implicated IL-6/Stat-3 dependent pathways, regulation of aldehyde dehydrogenase, FoxM1 pathways, and altered fatty acid metabolism [25, 26, 28]. These results suggest a model where eFABP4 binds to cell surface protein(s) to mediate cellular uptake and/or intracellular signaling. Herein, we

present results suggesting eFABP4 binds to the extracellular domain of desmosomal proteins to mediate breast cancer epithelial cell growth via an ERK-NRF2 axis.

METHODS

Animal Care and Use

All experimental procedures using animals were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee. Male C57BL/6J wild type (WT) and whole body FABP4 null (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 >2 cm^3, tumor ulceration, metastases or the end of study period at 35 days.

Cell Culture and Proliferation Analysis

MCF-7, E0771 and T47D were purchased from the American Tissue Culture Collection (ATCC; Manassas, VA) and all cells were quality assured to be mycoplasma free and verified. MCF-7 breast cancer cells and E0771 murine breast cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 µg/mL insulin and 1 mM penicillin-streptomycin in a humidified incubator at 37° C with 5% CO_2. T47D cell lines were maintained in RPMI (Invitrogen) with 5% FBS and 2 µg/mL insulin. To assess the effect of FABP4 on cell growth, cells were seeded in 6-well plates at a concentration of $2x10^5$ cells/mL in media containing 5% FBS and 2 µg/mL insulin. Cells were cultured for 24 hours, and baseline cell number determined utilizing an automated cell counter (Countess, Thermo Fisher) or CyQUANT cell proliferation assay kit (C7027, Invitrogen) according to the manufacturer's protocol. Media

was aspirated and cells were washed with PBS. Cells were treated with 20-200 ng/mL eFABP4, R126Q, FABP5, 20 nM brusatol, or vehicle in media containing 0.1% FBS and 2 µg/mL insulin. Cell counting was determined at 24 or 48 hours post addition. Live or Dead cell viability was determined using LIVE/DEAD Viability/Cytotoxicity Kit (L3224, Invitrogen) according to the manufacturer's protocol.

NRF2 Reporter Assays

The antioxidant response element (ARE) luciferase reporter vector and constitutively active Renilla luciferase vector (BPS Bioscience) were transfected into MCF-7 cells utilizing Lipofectamine (Invitrogen). After 24 hours, the media was aspirated and replaced with media containing 100 ng/mL FABP4. After 24 hours of incubation, dual luciferase reporter assay (Promega) was performed utilizing a luminometer per the manufacturer's instructions and ARE reporter activity was normalized to Renilla activity.

H_2O_2 Assay

Hydrogen peroxide (H_2O_2) quantification was determined using the Amplex Red hydrogen peroxide/peroxidase assay kit (A22188, Invitrogen) according to the manufacturer's protocol with modification. Briefly, cells were scraped into phosphate buffered saline pH 7.4 and inactivated at 95° C for 10 min. After removal of cell debris, 50 µL of extract was incubated with 50 µL of substrate solution for 30 minutes and the fluorescence was measured using a microplate reader with excitation at 540 nm and emission at 590 nm.

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. The primer sequences were ALDH1A1 forward: CTTACCTGTCCTACTCACCGATTTG, ALDH1A1 reverse: TCCTTATCTCCTTCTTCTACCTGGC; CYP1A1 forward: GATTGAGCACTGTCAGGAGAAGC, CYP1A1 reverse: ATGAGGCTCCAGGAGATAGCAG; SOD1 forward: CTCACTCTCAGGAGACCATTGC, SOD1 reverse CCACAAGCCAAACGACTTCCAG; HMOX1 forward: CCAGGCAGAGAATGCTGAGTTC, HMOX1 reverse: AAGACTGGGCTCTCCTTGTTGC; TBP forward: AGCGGTTTGCTGCGGTAATC, TBP reverse: ACTGTTCTTCACTCTTGGCTCCTG, DSG2 forward: AGGGAAGCACAGCATGACTCCT, DSG2 reverse: CCTTTCCGCAATGGCACATCAG; DSC1 forward: CAGAGTCAAGATGGCTTCCCAG DSC1 reverse: GTTCTCAAGTCGCCAGTGTGTTG; DSC2 forward: CACAGAAGCTCCTGGAGATGAC, DSC2 reverse: GATGGTCTCCTGACCTCCGTTT. CYP1A1, SOD1, HMOX1, DSG2, DSC1 and DSC2 primers were from ORiGENE.

Generation of Recombinant Proteins

The ascorbate peroxidase 2 (APEX2) cDNA was obtained from Addgene (Plasmid #49386) and the APEX2-FABP4 and APEX2 sequences modified to contain unique restriction sites permissive for insertion were obtained by commercial gene synthesis and

subcloned into pRSET-A vector (#3990, Addgene) and transformed into DH5α E. coli. Sequence verified clones were transformed into E. coli BL21(DE3)-pLysS for expression. Recombinant FABP isoforms (FABP4, R126Q-FABP4, or FABP1) were expressed in E. coli as N-terminal His-tagged or GST-tagged fusion proteins as previously described [132]. His-tagged recombinant proteins were purified using Ni^{+2} affinity chromatography while GST-tagged proteins were purified using glutathione-sepharose chromatography. Each recombinant protein was delipidated using Lipidex-1000 resin and stored at -80° C until use.

1,8-ANS binding Assay

To measure the binding affinity of recombinant proteins for fatty acids, the 1-anilinonaphthalene 8-sulfonic acid (1,8-ANS) binding assay was utilized as previously described [133]. Briefly, a fixed concentration of FABP4, APEX2, or APEX2-FABP4 (10 μM) in assay buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM dithiothreitol, pH 7.4) was mixed with 1,8-ANS for 30 minutes at room temperature and the fluorescence intensity recorded using an excitation wavelength of 370 nm and emission wavelength of 470 nm.

In Vitro Peroxidase Activity

To evaluate the peroxidase activity of APEX2 and APEX2-FABP4, 3 pmol of recombinant proteins were mixed with peroxidase substrate (#34094, Thermo Fisher Scientific) for 10 minutes at room temperature and then spotted on nitrocellulose membrane for chemiluminescence detection.

Affinity Capture of Proximity-labeled Proteins

The culture media from MCF-7 cells at 70%-80% confluence was aspirated and 500 μM biotin-SS-tyramide (#914916, Sigma) pre-mixed with fresh growth media was added. After 15 minutes, cells were treated with 500 nM recombinant APEX2 or APEX2-FABP4 for 10 minutes at which time 1 mM H_2O_2 was supplied for 1 min to initiate oxidation and biotinylation of target proteins. The reaction was terminated by the addition of quench buffer (10 mM sodium azide, 10 mM sodium ascorbate, and 5 mM Trolox in phosphate-buffered saline) and cells immediately washed 3 times with phosphate-buffered saline and lysed (8M Urea, 0.4M Tris pH 8.0, 20% acetonitrile supplemented with protease inhibitors). Lysates were sonicated and centrifuged at 14 000 rpm for 10 min at 4° C. The bicinchoninic acid (BCA) assay was used to determine the protein content. To capture the biotinylated proteins, Streptavidin Magnetic Beads (#88817, Thermo Fisher Scientific) were pre-washed 6 times (2 times with phosphate buffered saline, one time with 1M KCl, one time with 0.1M Na_2CO_3, 1.6M Urea, 80mM Tris pH 8.0, 4% acetonitrile, and 2 times with phosphate buffered saline). Lysates were diluted 5-fold with phosphate buffered saline plus protease inhibitors and incubated with pre-washed beads overnight at 4° C. After capture, beads were washed 6 times and proteins eluted in 200 mL 50 mM Tris pH 8.0, 1.5 M Urea and 20 mM dithiothreitol at room temperature for 0.5 hours. The elution and pull-down steps were repeated three times and the eluates pooled for mass spectrometry analysis.

Mass Spectrometry Analysis

For each sample, 5 mg was incubated with 50 mM chloroacetamide in 50 mM ammonium bicarbonate and incubated at room temperature for 15 min. Following modification, trypsin was added to each sample and incubated at 37° C overnight. The resultant peptides were desalted using an MCX stage tip (https://doi.org/10.1021/ac026117i) and eluted sequentially using 5% trifluoroacetic acid followed by 50% acetonitrile in water (v/v) and finally 100% acetonitrile. Peptides were dried and reconstituted in sample buffer (97.99:2:0.01, water:acetonitrile:formic acid) and analyzed on an Orbitrap Fusion (Thermo Scientific, Waltham, MA) liquid chromatography-mass spectrometry system in data dependent acquisition mode. Data was collected from 15 dependent scans on the Orbitrap Fusion.

The MS/MS data was then analyzed using Sequest (Thermo Scientific in Proteome Discoverer 2.4.0305) and the human Universal Proteome UP0000005640 protein sequence database with canonical sequence as the target after concatenation with the common lab contaminants protein sequences as per https://www.thegpm.org/crap/. Scaffold (version 4.11, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications reported by Sequest.

AlphaFold Modeling

AlphaFold-multimer [134, 135], an AI-based program capable of predicting structures of multi-protein complexes based on the amino-acid sequences was used to predict interactions between human FABP4 (UniProt P15090) and extracellular cadherin domains (EC) of human DSG2 (UniProt Q14126). All calculations were performed at the

University of Minnesota Super Computing institute (MSI) high-performance computing center using a non-Docker-based AlphaFold implementation model. AlphaFold calculations were subsequently performed on MSI's NVIDIA V100, and A100 GPU nodes. The highest ranked AlphaFold predictions for each set of interaction predictions were evaluated using PyMOL.

Generation of DSG2-silenced MCF-7 cells

The lentivirus-based RNA interference (RNAi) vector pLKO1 was used for siRNA-mediated DSG2 knockout in MCF-7 cells. siRNA transfection was performed using Lipofectamine 3000 (#L3000001, Invitrogen) according to the manufacturer's protocol. Recombinant lentiviruses were packaged in 293FT cells and harvested 48 hours post-transfection. Lentiviruses were then transduced into MCF-7 cells and after a 48 hr recovery, selected using 2 µg/mL puromycin. Stable knockdown cell lines were generated from a heterogeneous pool of puromycin-resistant MCF-7 cells and were cultured in DMEM supplemented with 5% FBS, 2 µg /mL insulin, 1 µg /mL puromycin and 1 mM penicillin-streptomycin in a humidified incubator at 37° C with 5% CO_2. The degree of silencing was evaluated using quantitative PCR and Western blotting.

ERK Phosphorylation Assays

MCF-7 cells were seeded in 6-well plates at a concentration of 2x 10^5 cells/mL in media containing 5% FBS, 2 µg/mL insulin and 1 µg /mL puromycin. Cells were cultured for 24 hours and replaced with DMEM supplemented with 0.1% FBS. After 24 hours, cells were washed and treated with 20, 100 or 200 ng/mL FABP4 in DMEM supplemented with 0.1%

FBS for the indicated times. Cells were lysed in RIPA buffer (50 mM Tris pH7.4, 150 mM NaCl, 0.1% SDS, 0.5 % sodium deoxycholate, 1% Triton-X100) with protease (#539134, Millipore) and phosphatase inhibitors (#P5726, #P0044, Millipore) and subject to Western blotting.

Co-immunoprecipitation

MCF-7 cells were lysed in extraction buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.4) containing the protease inhibitor cocktail Millipore #539134 and allowed to incubate at 4° C for 2 hours with gentle rotation. A detergent solubilized extract was obtained following centrifugation at 100,000g and used for immunoprecipitation. To immunoprecipitate the DSG2-FABP4 complex, 10µg of anti-DSG2 monoclonal antibody (sc80663, Santa Cruz) was bound to Dynabeads™ Protein G (#10003D, Invitrogen), pelleted by centrifugation, and washed with extraction buffer. Subsequently, the soluble cell lysate was incubated with antibody bound Dynabeads overnight at 4° C. Next, the DSG2-antibody bead complex was recovered by centrifugation, washed and incubated with 1µM FABP4 (pre-incubated with 10µM oleate) for 2 hours at 4° C. The protein-bead complex was subsequently washed, and bound proteins eluted using 4% SDS. The composition of the eluted proteins was evaluated by Western blotting.

Glutathione S-transferase (GST) Pull-down Assays

GST-tagged FABP4 or R126Q (2 µM) was immobilized to glutathione-sepharose resin in the presence or absence of 20 µM oleate for 1 hour in wash buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl). The sample was pelleted by brief centrifugation at 500 x g and the

unbound proteins removed by washing. 0.2 μM His-DSG2 EC3-4 (MBS2033234) was incubated with the resin with or without His-R126Q (0.2 μM, 2 μM, or 10 μM). The resin was then pelleted and washed as before. All steps above were performed at 4° C. The captured proteins were eluted by heating in SDS-PAGE sample loading buffer for 5 minutes and evaluated by immunoblotting.

Western Blot

Samples for analysis were separated using by 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad) for Western blotting. After pre-incubation with blocking buffer (#927-60001, Li-Cor Biosciences), membranes were incubated with primary antibody overnight at 4° C followed by washing with 0.1% Tween-20 in phosphate-buffered saline 3 times and incubated with secondary antibody conjugated to Li-Cor IRDye for 1 hour and visualized using Odyssey infrared imaging (Li-Cor Biosciences). The primary antibodies used were DSG2 (ab150372, Abcam; sc-80663, Santa Cruz), DSC1(sc-398590, Santa Cruz), DSC2(AF4688, R&D systems) and β-actin (A2228, Sigma). Antibodies against p44/p42 MAPK (ERK) (#9102), Phospho-p44/42 MAPK (ERK) (Thr202/Tyr204) (#9106), AKT (#9272), Phospho-Akt (Ser473) (#9271) and GADPH (#5174) were obtained from Cell Signaling Technology.

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 Students' 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. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, California, USA).

Data Availability

The data generated in this study are available within the article and its supplementary data files. Some raw data for this study were generated at the University of Minnesota Center for Mass Spectrometry and Proteomics. Derived data supporting the findings of this study are available from the corresponding author upon request.

RESULTS

eFABP4 has Proliferative Effects on MCF-7 Cells and T47D Breast Cancer Epithelial Cells. Obesity is a positive risk factor for development of breast cancer and circulating levels of eFABP4 are increased ~5-fold as a function of elevated body mass [7, 63]. To address mechanistic relationships between eFABP4 and progression of breast cancer, MCF-7 or T47D breast cancer cells that lack endogenous FABP4 were treated with eFABP4, and cell proliferation was assessed. MCF-7 and T47D breast cancer cell lines are classified as luminal A (ER+, PR+/-, HER2-) [136]. To assess the necessity of fatty acid binding in FABP4 effect, we utilized a non-fatty acid-binding mutant of FABP4, R126Q, to evaluate the contribution of FABP4's lipid binding to cellular proliferation in MCF-7 cells. The single residue substitution of glutamine for arginine of the R126Q

mutant disrupts ion pairing between the carboxylate head group of the fatty acid and the basic side chain such that the affinity for FFA is reduced by over 100-fold [137].

Importantly, cell proliferation increased only with wild type recombinant eFABP4 but was not stimulated by the non-fatty acid binding FABP4 mutant R126Q in either MCF-7 (**Figure 1A**) or T47D cells (**Figure 1C**). Furthermore, increasing concentrations of eFABP4 present in the cell media (20, 100, and 200 ng/mL) resulted in significantly increased cell proliferation in a time and concentration dependent manner (**Figure 1B**). Additionally, the potentiation of proliferation was specific for eFABP4; FABP1 that adopts the same fold as FABP4 and binds fatty acids similarly [2], did not have any effect on MCF-7 cell growth (**Figure 1E**), indicating that fatty acid binding to FABP4 was essential to its function. Other phenotypic changes in cellular appearance such as cell size or shape were not affected by eFABP4.

Figure 1

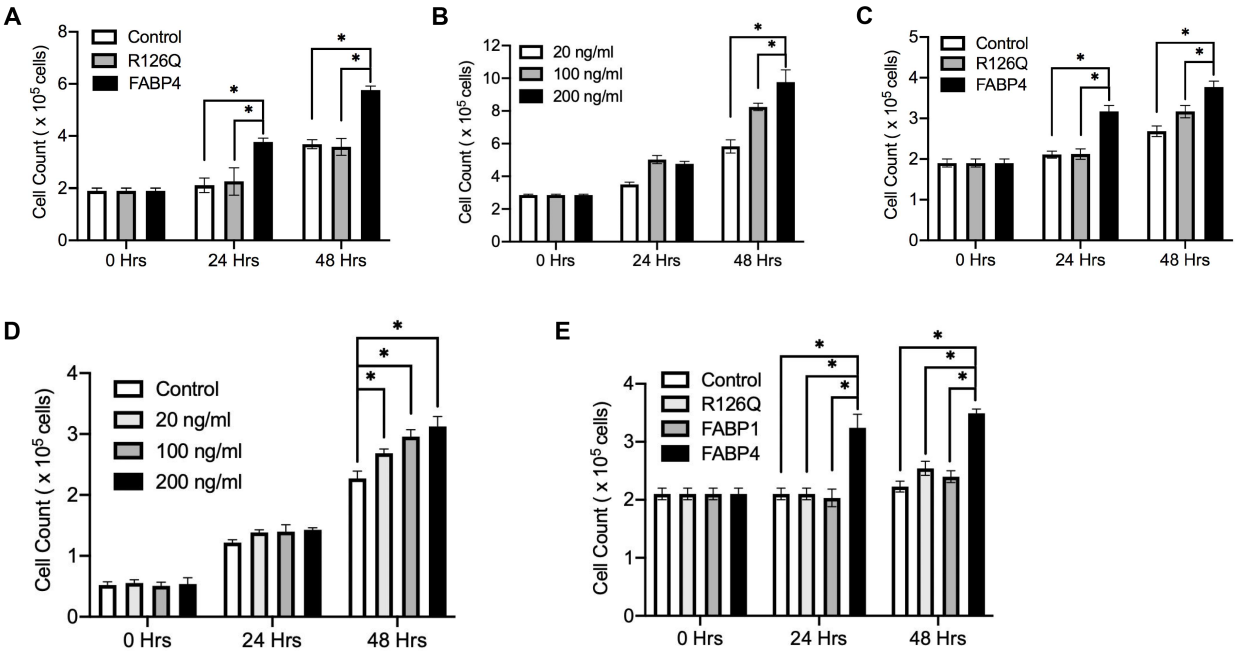

Figure 1. Proliferative effects of eFABP4 on breast cancer cell culture

*A) Effect of FABP4 or mutant R126Q on MCF-7 proliferation. B) Effect of varying concentrations and time of eFABP4 on MCF-7 proliferation. C) Effect of FABP4 or mutant R126Q on T47D proliferation. D) Effect of varying concentrations and time of eFABP4 on E0771 proliferation. E) Effect of FABP1 compared to FABP4 and R126Q on MCF-7 cell growth. Results are shown for one experiment that was repeated 3 times. *, $p < 0.05$.*

FABP4 null mice restrict tumor growth and enhance survival. Similarly, when E0771 cells were treated with varying concentrations of eFABP4 (20, 100, and 200 ng/mL) cell proliferation was increased in a time and concentration dependent manner (**Figure 1D**). Moreover, eFABP4 treatment at all concentrations modestly increased cell viability (~ 20%) under the same conditions (Supplementary Figure S2B). In vivo, using a high fat diet mouse model, growth of the murine breast cancer line E0771 was significantly delayed when E0771 cells were implanted into the FABP4 null mouse (designated as AKO) (**Figure 2A**) compared to growth in wild type C57Bl/6J mice. This coincided with significantly potentiated overall survival in the AKO cohort (**Figure 2B**). AKO mice characteristically demonstrated a trend towards lower body weight and reduced body mass towards the end of the survival period.

Figure 2

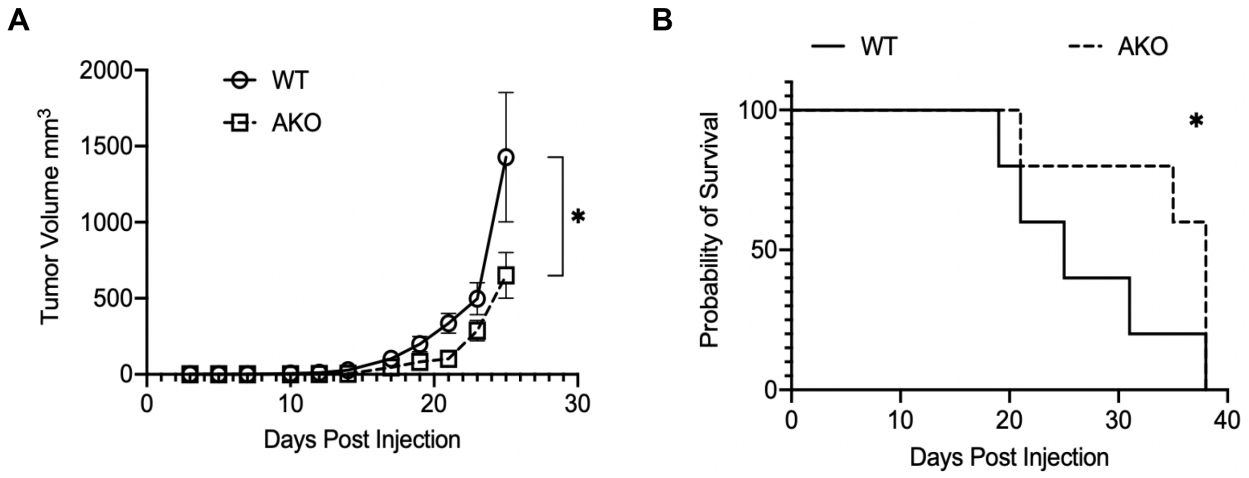

Figure 2. FABP4 null (AKO) mice injected with E0771 murine breast cancer showed decreased tumor volume and enhanced survival

*A) Tumor volume over time. B) Survival curves of FABP4 -/- null (AKO) mice vs. wild type (WT) mice. N = 20, 10 per group. *, $p < 0.05$.*

eFABP4 activated the ERK-NRF2 pathways in MCF-7 cells. The mitogen-activated protein kinases (MAPK) signaling plays an important role in cancer proliferation [138]. Activating mutation of this pathway, which is hallmarked by a high prevalence of BRAF and NRAS mutations, occurs in over 40% of all human cancers [139, 140]. The hyperactivation of MAPK contributes to constitutive ERK phosphorylation, resulting in potentiated cellular proliferation [141]. Treatment of MCF-7 cells with eFABP4 significantly increased the phosphorylated ERK (pERK) while R126Q did not (**Figure 3A** and **3B**).

The NRF2 pathway is of primary importance in maintaining redox homeostasis. Under basal conditions, NRF2 is degraded in the cytoplasm via KEAP1 but in response to stressed conditions, NRF2 is activated and translocates to the nucleus and initiating the transcription of various antioxidant genes thereby reducing reactive oxygen species (ROS) levels [142]. As an emerging concept, reductive stress is defined as a condition characterized by excess accumulation of reducing equivalents (e.g., NADH, NADPH, GSH), suppressing the activity of endogenous oxidoreductases. Recent studies show NRF2 as a controller of reductive stress [143] and NRF2 activation promotes cancer growth [144, 145], metastasis [146] and drug resistance [147, 148]. NRF2 is an ERK phosphorylation target [149-151] and the translocation of NRF2 to the nucleus is activated by ERK phosphorylation [152, 153] while ERK inhibitors attenuate NRF2 activity [154]. ERK/NRF2 signaling pathway has been reported to promote tumor growth in a variety of cancers [144, 155-157], protecting cancer cells from oxidative injury [155, 158]. We used the antioxidant response element (ARE) luciferase reporter gene system and found that

eFABP4 treatment resulted in significantly increased NRF2 transcriptional activation (**Figure 3C**). Aldehyde dehydrogenase 1 (ALDH1A1) is one of downstream antioxidant genes of NRF2, which catalyzes the irreversible oxidation of a wide range of aldehydes to their corresponding carboxylic acid and was linked to breast cancer invasiveness [159]. In line with the activation of NRF2, ALDH1A1 was upregulated about 2-fold following eFABP4 treatment (**Figure 3D**). Similarly, other NRF2 targets such as cytochrome P450 family 1 subfamily A member 1(CYP1A1), heme oxygenase 1(HMOX1) and superoxide Dismutase 1 (SOD1) [160] were also upregulated specifically with FABP4 but not R126Q. Accordingly, the downstream ROS level was significantly decreased in eFABP4 treated MCF-7 cells (**Figure 3H**). However, none of these effects were observed in R126Q-treated MCF-7 cells (**Figure 3A-3H**), indicating that fatty acid binding to FABP4 was essential to its function. As an alternate experimental strategy towards interrogation of NRF2 on MCF-7 growth, chemical inhibition of NRF2 with brusatol abrogated the proliferative effects of FABP4 in MCF-7 cells (**Figure 3I**). In aggregate, these results demonstrated that FABP4 stimulated MCF-7 growth through NRF2 signaling axis.

Figure 3

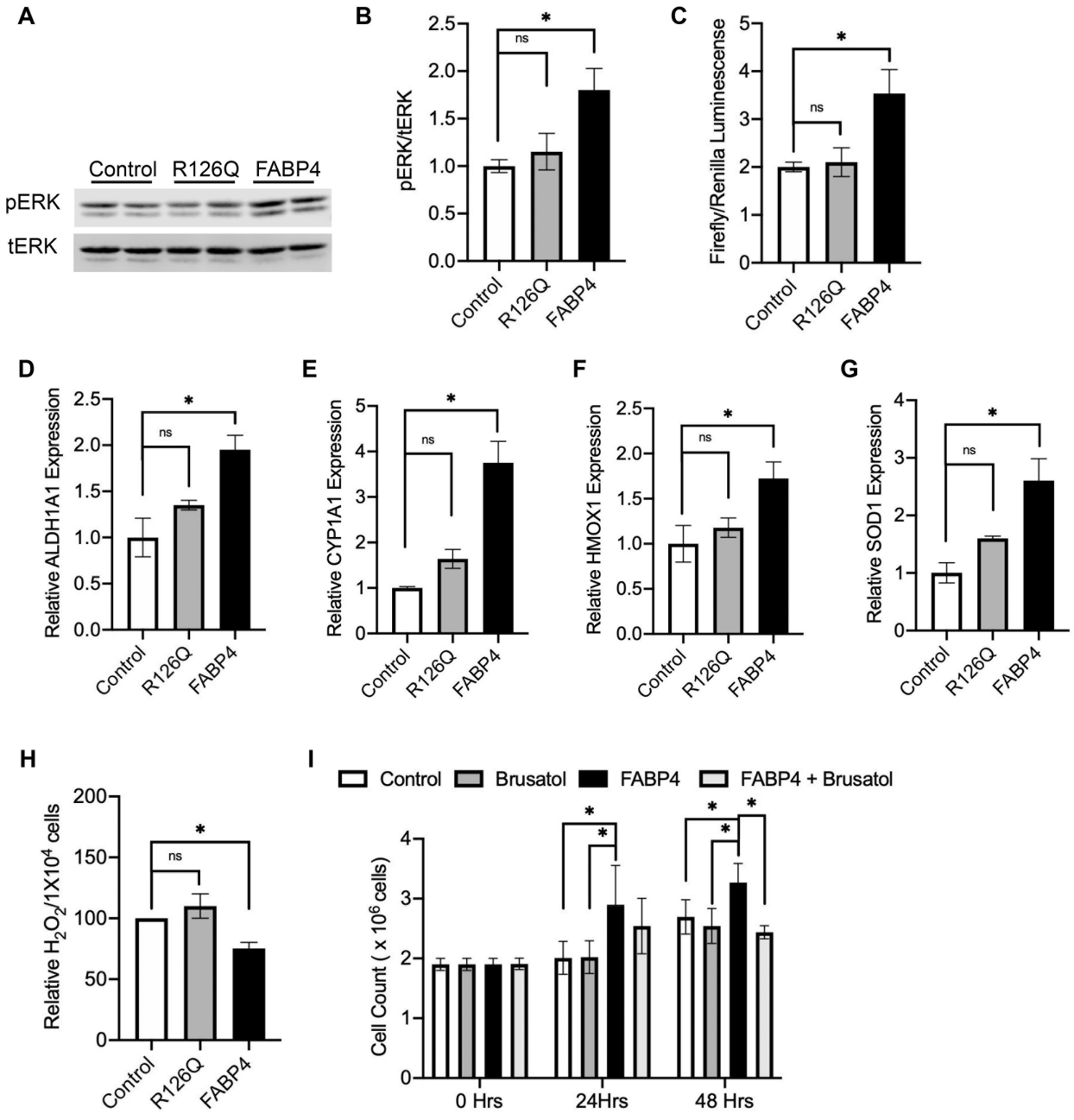

Figure 3. FABP4 mediated breast cancer growth was dependent on the ERK/NRF2 pathway

A) MCF-7 cells were treated with 100 ng/mL FABP4 or R126Q for 5 minutes and pERK levels were evaluated using western blot. B) Quantification of pERK level in A). Bands of pERK were normalized to the level of tERK in the same samples. C) MCF-7 cells were treated with 100 ng/mL FABP4 or 100 ng/mL

*R126Q for 48 hours and the NRF2 activity was evaluated using the antioxidant response element (ARE) luciferase reporter gene system. D-G) mRNA expression of ALDH1A1, CYP1A1, HMOX1, and SOD1 were evaluated using qRT-PCR (normalized to TBP) following treatment of MCF-7 cells with 100 ng/mL FABP4 or 100 ng/mL R126Q for 48 hours. H) Levels of H_2O_2 were measured following treatment with 100 ng/mL FABP4 for 48 hours in MCF-7 cells. I) MCF-7 proliferation after chemical inhibition of NRF2 with 20 nM brusatol. Results are shown for one experiment that was repeated 3 times. *, $p < 0.05$. ns, nonsignificant.*

Proximity labeling of MCF-7 cells to identify a putative eFABP4 receptor. Proximity labeling has been used successfully to identify intracellular and extracellular binding partners in a variety of contexts [161-163]. The experimental design makes use of fusion proteins between an experimental ligand (protein) and ascorbate peroxidase (APEX2) that when incubated with a suitable substrate and oxidant, produces a short-lived, diffusible radical that non-specifically adds to the side chains of proteins in a proximity-based manner. In our design we utilized biotin-that was disulfide linked to tyramide that when oxidized by ascorbate peroxidase in the presence of hydrogen peroxide produces a biotinylated phenoxyl radical. Avidin-based capture coupled with mass spectrometry analysis produces a protein dataset from which to interrogate functional interactions. To that end, we adopted an APEX2-FABP4 (along with APEX2 alone control) study design to identify putative eFABP4 binding partners.

Recombinant proteins were expressed in E. coli as His-tagged fusion proteins as previously described [137]. The purity of His-tagged recombinant proteins FABP4, APEX2 and APEX2-FABP4 is shown in **Figure 4A**. To assess the fatty acid binding affinity of recombinant proteins, we utilized the 1,8 ANS binding assays on the His-tagged fusions [133]. As shown in **Figure 4B**, APEX2-FABP4 and FABP4 exhibited the same lipid-binding activity while APEX2 was essentially devoid of any ligand binding. Moreover, APEX2-FABP4 retained peroxidase activity that was qualitatively similar to that of APEX2 alone (**Figure 4C**).

Figure 4

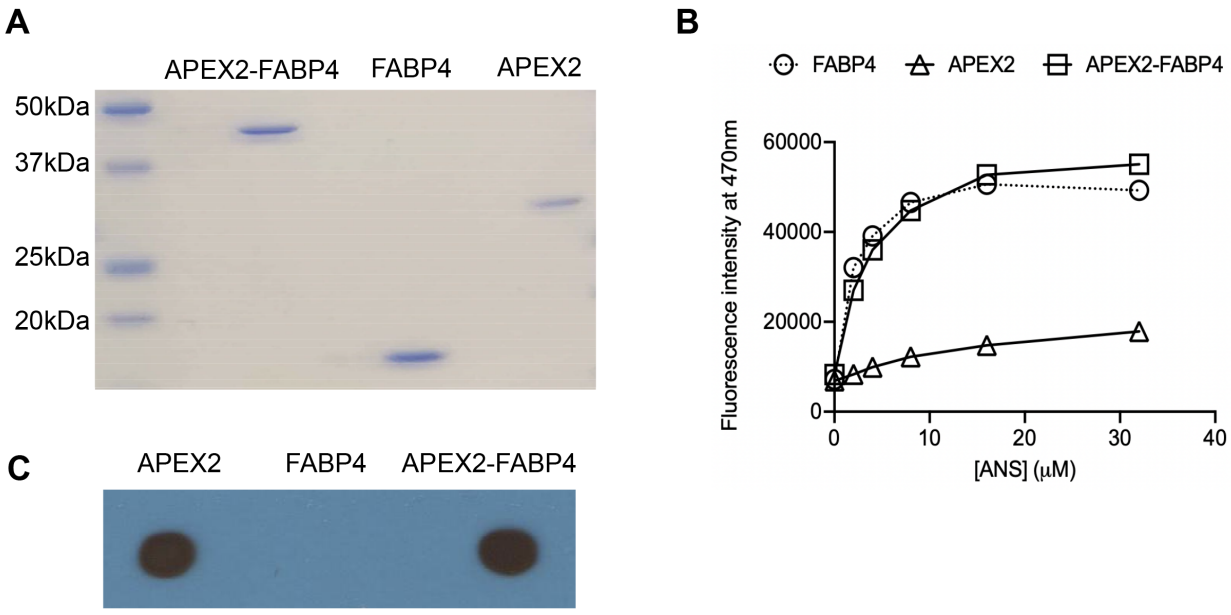

Figure 4. Recombinant protein APEX2-FABP4 was highly functional

A) Recombinant proteins APEX2-FABP4, FABP4 and APEX2 expressed in E. coli were analyzed by SDS-PAGE stained with Coomassie blue. B) Fatty acid binding affinity of recombinant proteins was assessed with ANS binding assay. (C) Peroxidase activity of recombinant proteins were detected with luminol-based enhanced-chemiluminescence HRP substrate and monitored on X-ray film.

APEX2-FABP4 and APEX2 control protein was added to MCF-7 cells in the presence of biotin S-S tyramide for 10 minutes and hydrogen peroxide added for 1 minute as an oxidant for the peroxidase activity. Following modification, the reaction was quenched, and the cells rapidly washed and lysed. Biotinylated proteins were captured using avidin-sepharose and proteins identified using mass spectrometry. There were 361 proteins identified using either APEX2 or APEX2-FABP4 treated MCF-7 cells and the results are presented as the ratio of ions detected by APEX2-FABP4 compared to that detected by APEX2 alone (**Figure 5**). Surprisingly, among the top APEX2-FABP4 captured proteins we identified several proteins functioning in desmosomes as potential FABP4 receptor candidates including desmoglein (DSG), desmocollin (DSC), junction plankoglobin (JUP), desomoplankin (DSP) and several cytokeratins (CK) (**Figure 5**).

Figure 5

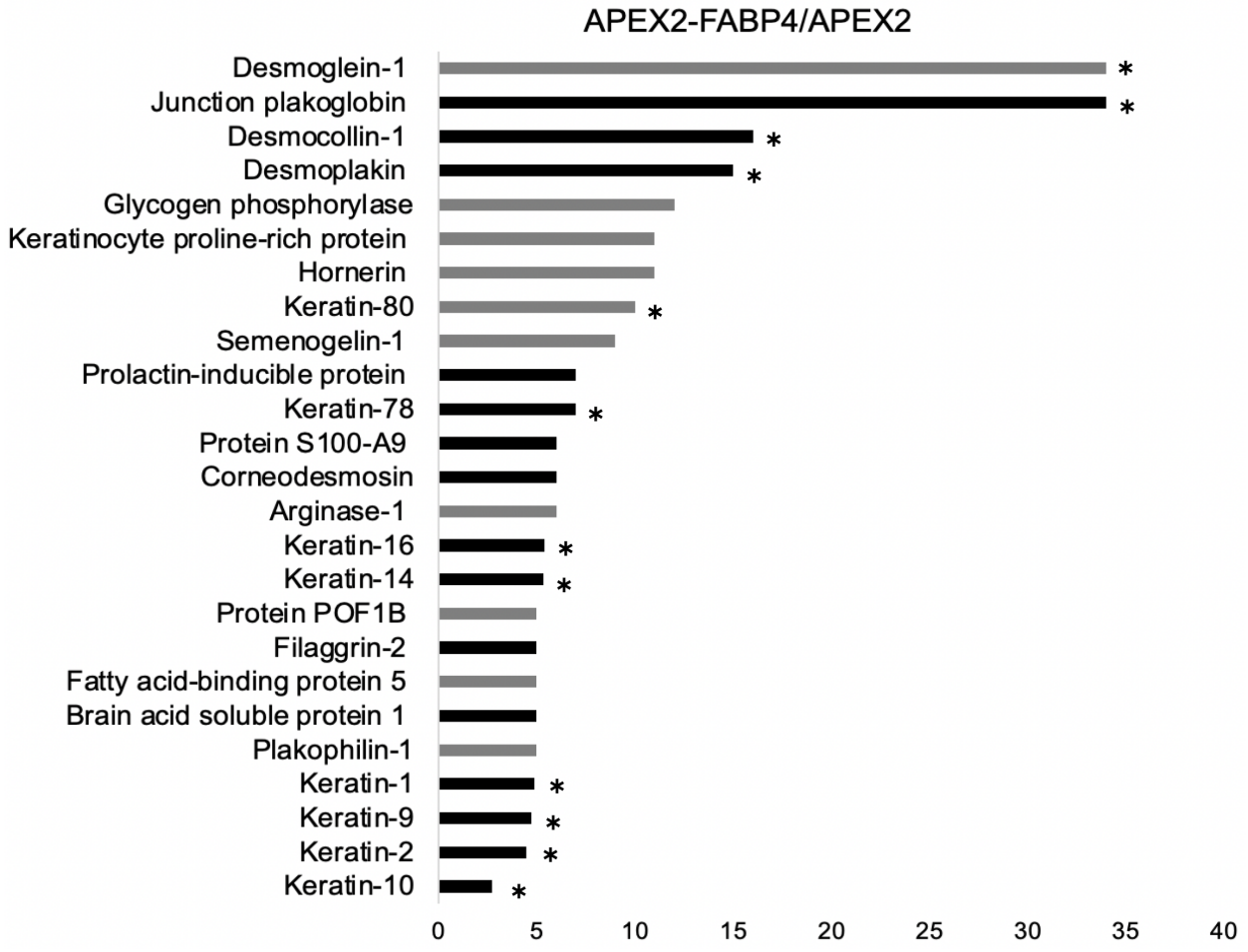

Figure 5. Top FABP4 receptor candidates identified by proximal labeling

MCF-7 cells were treated with biotin-SS-phenol and APEX2-FABP4 or APEX2 followed by H_2O_2 to initiate biotin labeling. The biotinylated proteins were enriched using streptavidin magnetic and analyzed by mass spectrometry. The top FABP4 receptor candidates reported in mass spectrometry was normalized to APEX2. Bars in grey mean the output was "0" in APEX2 but put in "1" instead for normalization calculation. Asterisks showing the candidates are components of desmosome complex.

Downregulation of desmosomal cadherins promote cancer progression

Desmosomes are adhesive intercellular junctions that connect cells by desmosomal cadherins. Three major components of desmosome are desmosomal cadherins desmoglein and desmocollin, the armadillo family proteins plakoglobin and plakophilin, and the plakin family protein desmoplakin [164]**.** Keratin filaments interact with desmosomes to form networks subjacent to the membrane [165]. Importantly, our proximity labeling strategy identified over a dozen members of the desmosomal complex (**Figure 5**). Because the biotin-phenoxyl radical can diffuse after activation (even within the 1-minute labeling time frame), it is not unreasonable to identify several members of the desmosomal protein family given their close physical proximity. However, since the extracellular cadherin domains (EC) of DSG and DSC may be most likely to interact with eFABP4, we further analyzed these two classes of proteins in MCF-7 cells.

DSG and DSC are transmembrane proteins with five extracellular domains, a single-pass transmembrane domain, and a cytoplasmic domain. The five extracellular domains are composed of four extracellular cadherin repeats (EC1–4) and one extracellular anchor domain [166]. DSG and DSC isoforms are differentially expressed in epithelial cells while DSG2 and DSC2 are widely expressed in all desmosome-containing tissues [166] [167]. Previous studies have shown an association between desmosomal cadherins and cancers with reduced expression of desmosomal cadherins being observed in various tumors. In contrast, overexpression of desmosomal cadherins inhibit cancer progression [167]. Paradoxically, in MCF-7 cells, DSG2, DSC1 and DSC2 were detectable at both the mRNA (**Supplementary Figure S1A-C**) and protein level (**Supplementary Figure**

S1D-G), while DSG isoforms 1, 3 or 4 were not immunologically detectable (results not shown) despite being identified by mass spectrometry. Based on relative abundance in MCF-7 cells, we chose to focus on DSG2 for further analysis.

AlphaFold modeling predicted eFABP4 interaction with the ECs of DSG2. Hypothesizing that desmosomal cadherins serve as FABP4 receptors to transduce intracellular signaling, we employed AphaFold to model FABP4 interaction with ECs of desmosomal cadherin DSG2. AlphaFold is an artificial intelligence-based program that performs predictions of protein structure [168]. Two confidence measures were used to evaluate the reasonableness of AlphaFold predictions. One is a per-residue measure of local confidence termed predicted Local Distance Difference Test (pLDDT), while the second is Predicted Aligned Error (PAE), that reports AlphaFold's expected position error between individual alpha-carbons in the model. A consistently low PAE suggests AlphaFold is confident about the relative domain positions.

Modeling the interaction between FABP4 (UniProt P15090) and each individual EC (1 through 4) of DSG2 (UniProt P15090), AlphaFold predicted interactions (based on pLDDT and PAE metrics) between FABP4 and each EC, with the highest confidence values focused on EC3 and EC4 (**Figure 6A and 6B**). When AlphaFold was challenged with defining an interaction between FABP4 and an EC3-EC4 fusion, the prediction placed FABP4 onto EC3 (**Figure 6C**). Interestingly, structural modeling placed the orientation of EC3 onto a key proximal region of FABP4. This region is referred to as the "portal" and is characterized as the fatty acid ligand entry-exit site [2]. Within the portal region lie two

determinants for fatty acid binding: helix alpha2 and the side chain of F57 (**Figure 6C**). The side chain of F57 affects ligand affinity, but not selectivity while helix alpha2 participates in protein-protein interaction with other lipid binding proteins such as the adipocyte hormone-sensitive lipase [105, 169, 170].

Co-immunoprecipitation and GST pull-down assays demonstrated the interaction between FABP4 and DSG2. To corroborate the physical interaction between FABP4 and DSG2 identified by proximity labeling, we performed co-immunoprecipitation of FABP4 to DSG2 bound to Dynabeads. As shown in **Figure 6D**, His-FABP4 was co-immunoprecipitated with DSG2 detergent extracted from MCF-7 cells. To demonstrate that the interact was direct, GST pull-down assays using GST-FABP4 and His-DSG2 EC3-4 further confirmed direct interaction between eFABP4 and the EC3-4 region of DSG2 (**Figure 6E, 6F**). The DSG2-FABP4 interaction was potentiated in the presence of oleic acid (C18:1) (**Figure 6E, 6F**) and consistent with this, the non-fatty acid binding mutant FABP4 mutant, R126Q, exhibited reduced interaction with FABP4. In competition assays, high levels of R126Q could compete with lipid bound FABP4 on DSG2 (**Figure 6E, 6F**). These results in sum suggest a direct interaction between eFABP4 and DSG2 and that complex formation is potentiated by fatty acids.

Figure 6

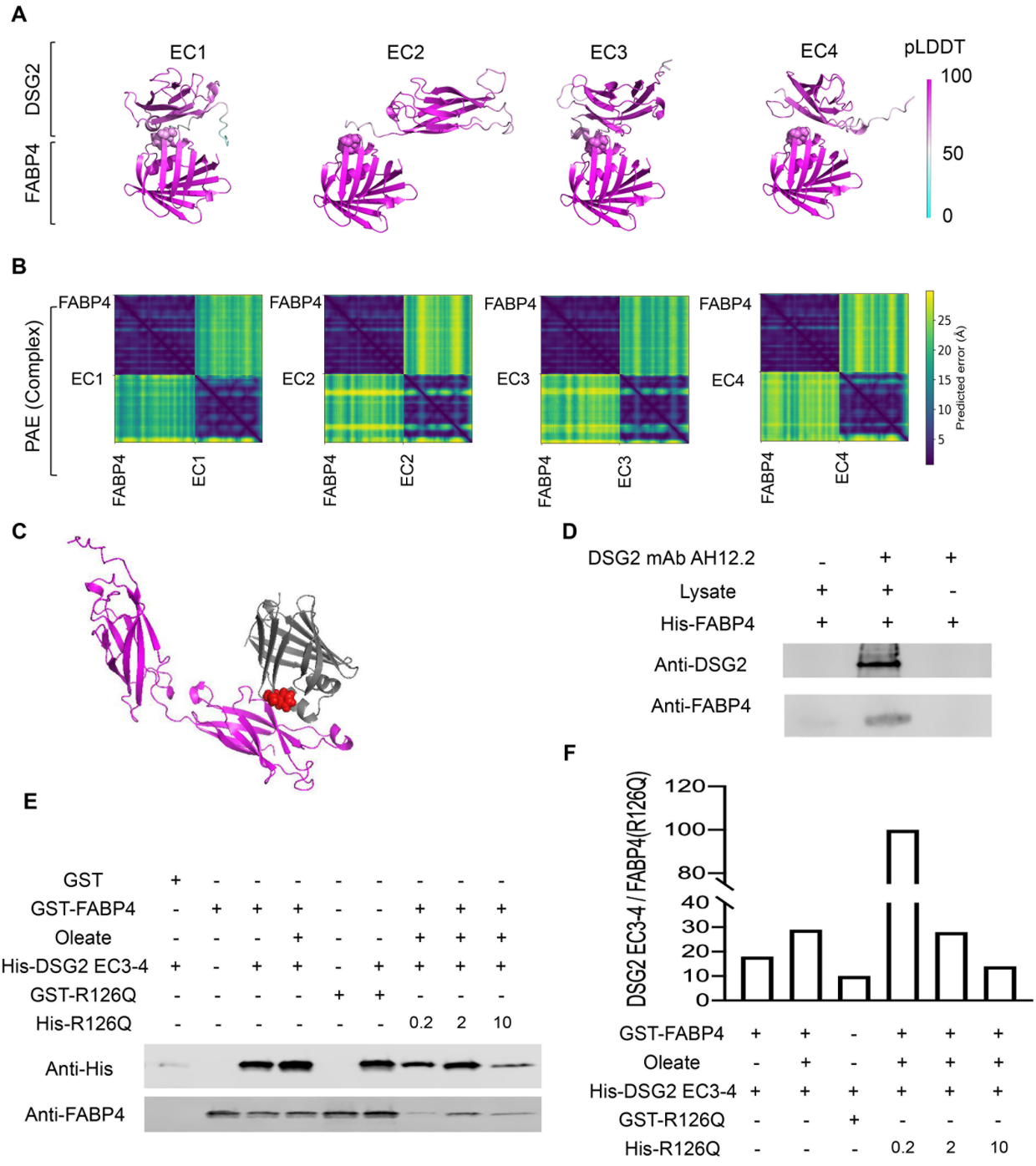

Figure 6. FABP4 interacted with ECs of DSG2

A) AlphaFold predicted the interaction between FABP4 and each EC(1 through 4) of DSG2 individually. Top panel shows ribbon cartoons of the complex colored by AlphaFold's pLDDT parameter.

Each residue in the sequence is color-coded based on the model confidence score. Model confidence from high to low: magenta (100), white (50), cyan (0). B) PAE distance plots show the distance error map. Blue is 0 angstroms error, yellow is 30 angstroms error. C) Docking of F57 (red) of FABP4 (grey) and DSG2 EC3-4 (magenta). The native structures of FABP4 (grey), and DSG2 EC3-4(magenta) are represented as ribbons. D) MCF-7 cell lysates were incubated with DSG2 antibody bound Dynabeads as described in Methods 1μM FABP4 (pre-incubated with 10 μM oleate) was added to the protein-beads complex for 2 hr. and complexed proteins captured by centrifugation. Proteins in the FABP4-DSG2 complex were eluted and evaluated by immunoblotting. E) GST-tagged FABP4 or R126Q was immobilized to glutathione resin in the presence or absence of 20 μM oleate followed by incubation with 0.2 μM His-DSG2 EC3-4. Immunoblotting was performed to analyze the eluted proteins after incubation. F) Quantification of the results from panel E where the level of DSG2 EC3-4 was normalized to GST-FABP4 or GST-R126Q.

eFABP4 mediated cell proliferation is abolished in DSG2 silenced MCF-7 cells. To assess the functional role of desmosomal cadherins in eFABP4 mediated cell growth, we used an RNA silencing strategy to create MCF-7 cells with decreased expression of DSG2. Two stable DSG2 knockdown cell lines were generated (**Figure 7A and 7B**) and silencing of DSG2 had no effect on the cellular levels of DSC1 or DSC2 (**Supplementary Figure S1H**). Consistent with prior work on the effect of Desmoglein mutations and cancer [167, 171], silencing of DSG2 resulted in increased cellular proliferation (**Figure 7C**). However, in such DSG2 silenced cell lines, the proliferative effect of eFABP4 was completely abolished compared to control siRNA-transduced MCF-7 cells that retained eFABP4 stimulation (**Figure 7C**).

Consistent with the growth effects, while the basal level of pERK was significantly higher in DSG2 knockdown compared to control siRNA-transduced MCF-7 cells, eFABP4 failed to increase pERK levels further (**Figure 7D and 7E**). Similarly, while the basal level of ALDH1A1 mRNA was also significantly higher in DSG2 knockdown compared to control siRNA-transduced MCF-7 cells, there was no additional up-regulation of ALDH1A1 mRNA expression with eFABP4 treatment (**Figure 7F**). The efficiency of DSG2-silencing is shown in **Supplementary Figures S1I.** Addition of eFABP4 had no significant impact on the level of DSG2, DSC1 or DSC2 mRNA or protein expression in wild type MCF-7 cells (**Supplementary Figures S1A**). Taken together, these results point to DSG2 as a possible receptor for eFABP4 that stimulates breast cancer proliferation through the ERK/NRF2 pathway.

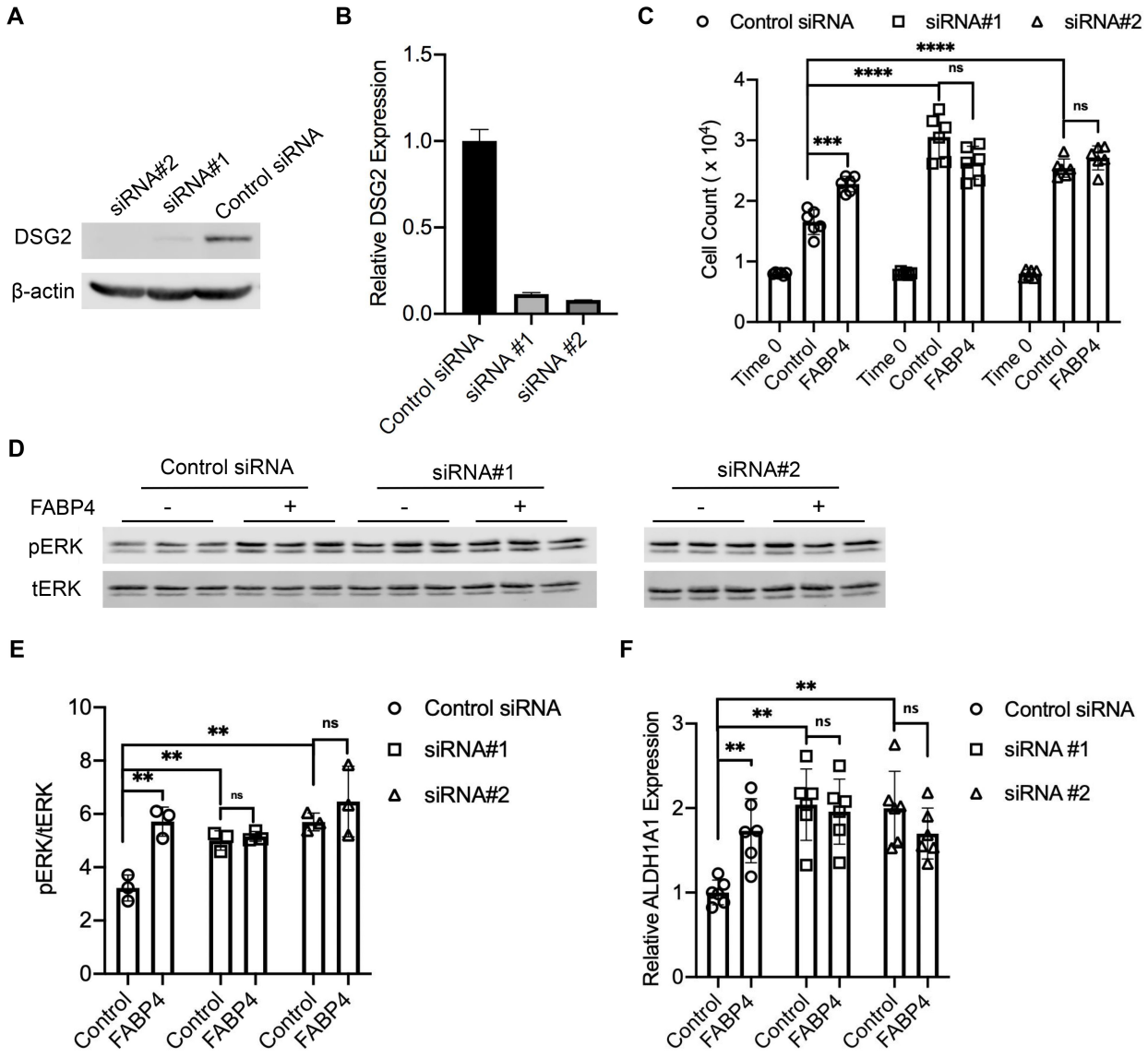

Figure 7. Loss of DSG2 abolished FABP4 mediated cell proliferation through ERK/NRF2 pathway in MCF-7 cells

A) Two DSG2 knockdown (DSG2 siRNA#1 and DSG2 siRNA#2) MCF-7 cell lines were made. B) mRNA level of DSG2 expression in two DSG2 knockdown cells lines. C) Cells with stable knockdown of DSG2 were treated were treated with 200 ng/mL FABP4 for 48 hours and cell numbers were detected using CyQUANT Cell Proliferation Assay Kit. D) DSG2 knockdown cells were treated with 200 ng/mL FABP4 for 5 minutes and pERK level was evaluated using western blot. E) Quantification of pERK level in D). F)

*Cells with stable knockdown of DSG2 were treated with 200 ng/mL FABP4 for 48 hours and mRNA expression level of ALDH1A1 was evaluated using qRT-PCR. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, nonsignificant.*

Discussion

Obesity is frequently associated with cancer progression and adipose tissue contributes to this process by producing metabolic, endocrine and paracrine factors that impact tumor growth. The proposed mechanisms, however, have been diverse and varied [25, 28, 49]. On one hand, fatty acids released by adipocyte lipolysis may fuel energy requirements for growth of certain cancers (e.g., ovarian) while alternatively, adipokines may potentiate tumorigenesis through endocrine or paracrine signaling. Of the various adipokines that may be tumor regulators, eFABP4, a lipid carrier, has received considerable attention. eFABP4 has been found to influence both tumor cell proliferation and in vivo tumor progression in several systems [25, 49]. Previous studies from our laboratory revealed that eFABP4 stimulated the proliferation of pancreatic cancer cells via activation of the NRF2 pathway [31] and the ERK/NRF2 signaling axis is reported to promote tumor growth in a variety of cancers [144, 155-157]. Our studies employing MCF-7 cells and in vivo in the FABP4 null mouse model suggested that eFABP4 treatment stimulated cell proliferation (**Figure 1**) and tumor progression (**Figure 2**) in breast cancer. More specifically, eFABP4 increased pERK level, activated NRF2 transcription, up-regulated expression of NRF2 downstream antioxidant gene ALDH1A1, and lead to reduced ROS level (**Figure 3**). In addition, NRF2 inhibition abolished the proliferative effect of eFABP4 in MCF-7 cells (**Figure 3F**). These findings indicated that in MCF-7 cells, eFABP4 likely stimulates cell proliferation through the ERK/NRF2 signaling axis.

More than 90% of cancers originate from epithelial tissues [172]. Desmosomes are vital for the structure and function of cells in epithelial sheets, not only forming intercellular

adhesion to ensure tissue integrity but also acting as signaling platforms involved in the regulation of cell proliferation, differentiation, migration, morphogenesis, and apoptosis [167, 171]. Dysfunction or destabilization of desmosome complexes is appreciated as a driver of cancer progression in multiple systems [171]. For example, in Wnt signaling, desmosome destabilization can provoke the release of JUP to activate oncogenic β-catenin signaling [173-175]. The MAPK pathway has also been identified as a potential system regulated by desmosomal proteins [176, 177]. ERK is normally kept inactive by its regulator Erbin. Desmosomal cadherins DSGs or DSCs capture Erbin via their cytosolic domains thereby inhibiting ERK activation, whereas disruption of the DSC-DSG interaction or loss of DSCs or DSGs activates ERK signaling [176-178]. In our DSG2 knockdown MCF-7 cells, the basal level of pERK and ALDH1A1 mRNA were significantly higher than control siRNA-transduced cells (**Figures 7D-F**) leading to increased cell growth. Moreover, eFABP4 failed to potentiate cell proliferation, pERK level or ALDH1A1 mRNA expression in DSG2-silenced cells (**Figures 7D-F**).

Despite the various effects of eFABP4 on hepatocytes, pancreatic β-cells, macrophages, cardiomyocytes, vascular endothelial cells and cancer cells [5, 14, 23, 25, 48, 49], potential receptors for FABP4 have not been identified. In the current study, we utilized proximity labeling involving an APEX2-FABP4 fusion protein and identified desmosomal proteins including DSC, DSG, JUP, PKP and CK, as eFABP4 receptor candidates (**Figure 5**). Consistent with this proposition, Masana and colleagues previously reported that eFABP4 uptake by endothelial cells was dependent on cytokeratin 1 (CK1), as CK1

silenced cells showed significantly reduced uptake of eFABP4 and reduced activation of NRF2 [27, 74].

A major advance in the analysis of eFABP4-DSG2 interaction was the introduction of AlphaFold modeling as a tool towards interaction prediction. We focused our modeling on the extracellular domains of DSG due to their known effect in mediating cellular adhesion via heterotypic association with DSC proteins and the availability of high-resolution crystal structures [179]. Molecular modeling suggested the interaction between FABP4 and ECs of DSG2, likely EC3, and GST pull-down assays demonstrated their direct interaction in vitro with a EC3-EC4 fusion protein (**Figure 6**) that was potentiated by oleic acid. Furthermore, loss of DSG2 completely abolished eFABP4 effects in MCF-7 cells (**Figure 7**). It is unknown if eFABP4 affects heterotypic association between DSG and DSC proteins, but a working model suggests that eFABP4 binding to DSG (and possibly DSC) would negatively regulate desmoglein-desmocollin association thereby potentiating ERK-dependent signaling.

AlphaFold predicts an interaction between FABP4 and the EC3 domain of Desmoglein 2 involving an F57 interface along with helix alpha2. Fatty acids bind to FABPs with their lipid carboxylate buried into a large water-filled central cavity and their omega methyl group oriented towards the protein surface. F57 is important for fatty acid binding to FABP4 as it undergoes a rotation of almost 180 degrees from an inward orientation to an outward orientation coincident with lipid binding [103-105]. Mutants of FABP4 that substitute amino acids with smaller side chains at position 57 exhibited reduced fatty acid

affinity, but no change in selectivity suggesting that the bulky Phe side chain effectively serves to seal the lipid internally. **Supplementary Figure S2A** shows that the predicted orientation of the F57 side chain in EC1-4 is in the apo FABP4 form suggesting that upon fatty acid binding the F57 sidechain rotates into the outward position to possibly mediate interaction with DSG2. Consistent with this, GST-based pull-down assays revealed a robust increase in FABP4-DSG2 association in the presence of oleate (**Figure 6E and 6F**). The predicted inward orientation of F57 suggests that fatty acid binding to FABP4 may be a key determinant in its interaction with desmosomal cadherins and provide an explanation why R126Q failed to stimulate cancer growth.

FABP4 also forms a physical complex with the intracellular hormone-sensitive lipase (HSL) of adipocytes to mediate lipolysis of fatty acids [44]. FABP4 physically associates with HSL on the surface of intracellular lipid droplets and the interaction is mediated by two ion pairs forming a charged quartet, one involving D17 to R30 and the second D18 to K21 [170]. D17, D18 and K21 reside on helix alpha1 while R30 is on helix alpha2 and binding to HSL involve pair switching in which the FABP4 basic residues form ion pairs with corresponding acidic sidechains on HSL [170]. Fatty acids potentiate FABP4-HSL interaction as measured by titration microcalorimetry and the non-fatty acid binding mutant, R126Q does not associate with HSL to any measurable extent [44]. Inspection of the region of EC3 predicted to interact with helix alpha2 reveals a cluster of acidic amino acids (Glu-Val-Asp-Tyr-Glu-Glu^{344}) in close proximity. Future work will determine if such ion pairing exists between FABP4 and EC3.

In summary, our findings reveal DSG2 as a possible receptor for eFABP4 that stimulates breast cancer growth through an ERK/NRF2 signaling axis. Although our current studies emphasize DSG2, it should be stressed that proximity labeling suggested DSC1 may also be an FABP4 interaction target. Identification of a cluster of proteins involved in desmosomal biology implicates this complex as a critical element for FABP4 association and provides insight into the mechanism of eFABP4 as a driver of obesity-related cancers.

Acknowledgements

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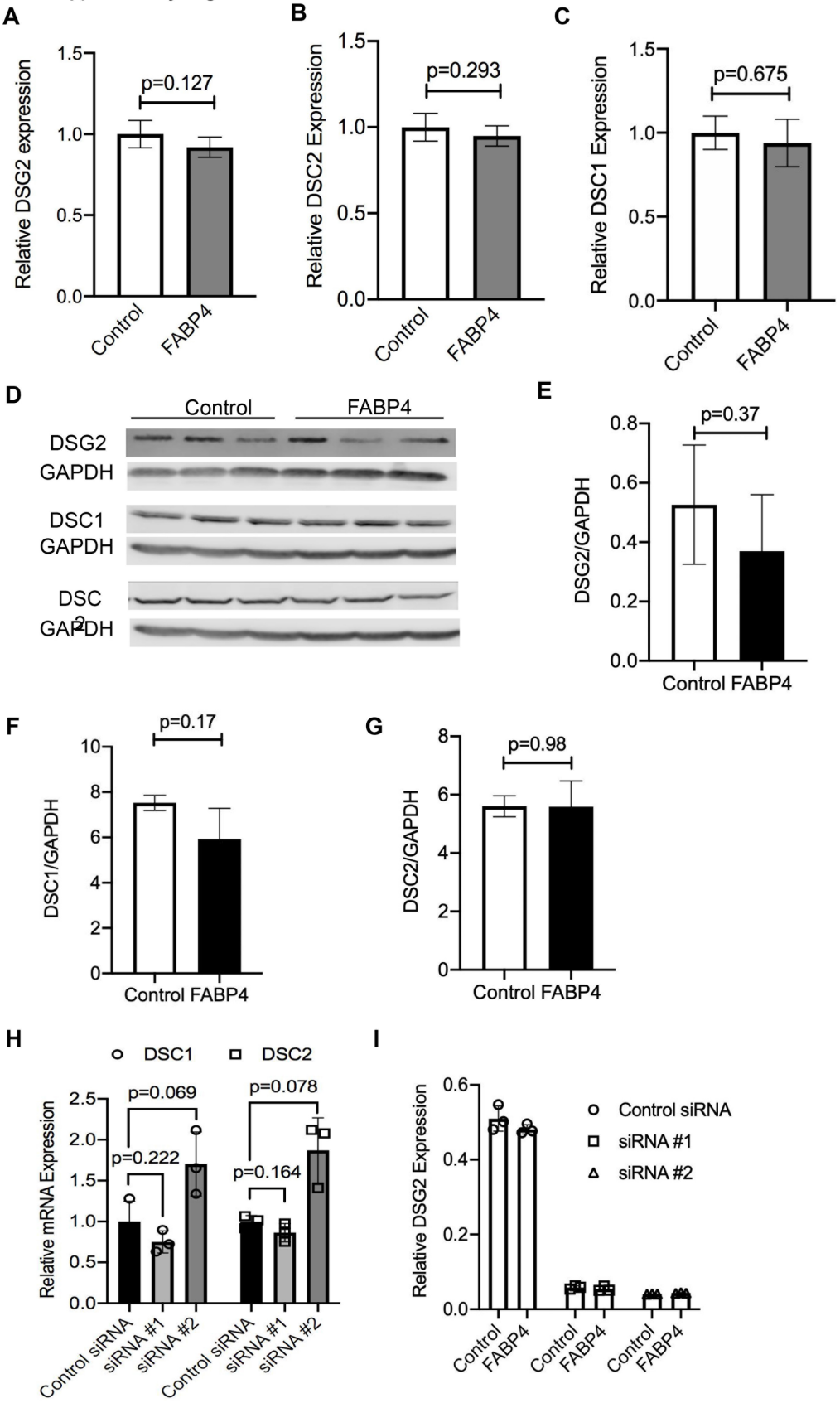
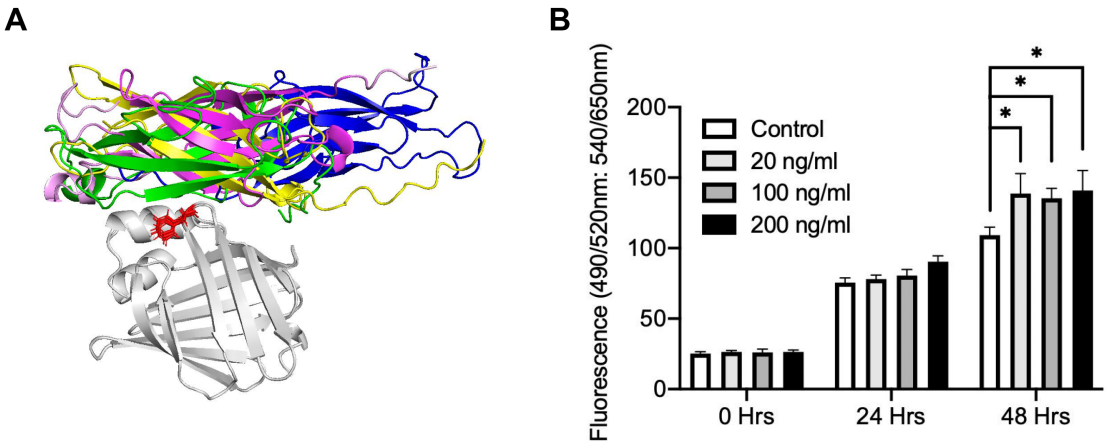
Supplementary Figures

Supplementary Figure S1



Supplementary Figure S1. DSC and DSG isoforms expressed in MCF-7 cells. A-C) MCF-7 cells were treated with 200 ng/mL FABP4 for 48 hours and mRNA expression levels of DSG2, DSC1 and DSC2 were evaluated using qRT-PCR. D) MCF-7 cells were treated with 200 ng/mL FABP4 for 48 hours, protein levels of DSG2, DSC1 and DSC2 were evaluated using western blot. E-G) Quantification of DSG2, DSC1 and DSC2 level in D). Bands of DSG2, DSC1 and DSC2 were normalized to the level of GAPDH in the same samples. Results are shown for one experiment that was repeated 3 times. H) mRNA level of DSC1 and DSC2 expression in two DSG2 knockdown cell lines. I) Corresponding mRNA level of DSG2 in Figure 7F was evaluated using qRT-PCR.

Supplementary Figure S2



Supplementary Figure S2. Predicted orientation of the F57 side chain in the apo-FABP4 form. AlphaFold predicted the F57(red) side chain in interaction with EC1-4 of DSG2 is oriented inwardly towards its interior cavity. The native structures of FABP4 (grey), EC1(green), EC2(blue), EC3(magenta), and EC4 (yellow) are represented as ribbons. B) E0771 cell viability using the ratio of calcein AM to ethidium homodimer-1 fluorescence. *, $p < 0.05$.

Final Perspectives

eFABP4 has been reported in several cell types. Although Masana's group showed that eFABP4 uptake in endothelial cells was dependent on Cytokeratin 1, there has no studies on FABP4 receptors on the plasma membrane of epithelial cells. Our work suggested DSG2 as a putative FABP4 receptor on the breast cancer cell membrane, with validated interaction in vivo and cellular FABP4 signaling abolished in DSG2 silenced cells. However, we identified a family of desmosome proteins as putative receptors. As DSGs and DSCs share similarities in both sequence and structure, further studies are warranted to investigate their roles. Previous studies have suggested that destabilization of desmosome facilitates cancer progression. We proposed that the interaction between FABP4 and desmosomal cadherins disrupted desmosomal complexes, thus promoting cancer growth. Further studies are needed to unravel the FABP4-desmosome interaction complex. Since the expression of desmosomal cadherins is tissue-specific, more studies on cells that do not express desmosomal cadherin family members or cannot assemble desmosomes but show FABP4 effects would also be interesting.

Dysregulation of fatty acid metabolism plays crucial role in cancer progression. FABP4, as a fatty acid carrier, has been found to regulate fatty acid uptake, saturated and unsaturated fatty acids balance and lipid metabolism. The effects of FABP4 in inflammation and cancer were abolished when a non-fatty acid binding mutant of FABP4 was used. Besides, previous studies demonstrated the interaction between FABP4 and fatty acids in cancer. However, the molecular mechanism underlying the roles of fatty acids in FABP4 signaling remains largely unknown. Our study showed that oleate

increased the interaction between FABP4 and DSG2 in vivo. The question is whether this interaction can be translatable in intracellular signaling or in vitro experiments. Additionally, it has been observed that saturated and unsaturated fatty acids have opposite effects on cancer growth in different cancers. This raise the question of whether these differences are due to their impact on FABP4 interaction with receptors.

Our study has demonstrated that eFABP4 promoted tumor growth through DSG2/ERK/NRF2 signaling axis. The MAPK pathway has been found to act as a signaling platform, integrating extracellular signals and initiating intracellular physiological responses. In our case, eFABP4 signaling was transduced into cellular proliferation via this pathway. Additionally, the MAPK pathway was also identified as a potential system regulated by desmosomal cadherins. ERK is normally kept inactive by its regulator Erbin. However, desmosomal cadherins DSGs or DSCs capture Erbin via their cytosolic domains, thereby inhibiting ERK activation. Disruption of the DSC-DSG interaction or loss of DSCs or DSGs activates ERK signaling. Therefore, our findings suggested an eFABP4/DSG2/ERK signaling axis that transduces extracellular signals to cellular responses on the cell membrane. Further investigations are warranted to identify other molecules involved in this process. Furthermore, as binding to specific lipids could regulate the activation of MAPK signaling proteins, it would be interesting to study if FABP4 activates MAPK signaling by manipulating specific lipids availability.

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