

**THE IMPACT OF FAM84B EXPRESSION ON BREAST CANCER
TUMORIGENESIS AND TAMOXIFEN RESISTANCE**

THE IMPACT OF FAM84B EXPRESSION ON BREAST CANCER
TUMORIGENESIS AND TAMOXIFEN RESISTANCE

By

MARC RAMKAIRSINGH, B.Sc. Honours

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AUTHOR: Marc Ramkairsingh, B.Sc. (McMaster University)

SUPERVISOR: Dr. Damu Tang

SUPERVISORY COMMITTEE: Dr. Peter Whyte
Dr. Andre Bedard

EXTERNAL DEFENCE
COMMITTEE MEMBER: Dr. Geoff Werstuck

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LAY ABSTRACT

Breast cancer (BC) is the second most common malignancy in the world and it accounts for 15% of female deaths due to cancer every year. Although hormonal therapy with tamoxifen (TAM) is a commonly used treatment for the disease, a recurring problem is that tumours eventually develop resistance to the drug. We are interested in investigating the role of FAM84B in BC tumorigenesis and the development of TAM resistance in these tumours. FAM84B has been shown to have higher expression in esophageal squamous cell carcinoma than in normal tissue and the protein was associated with increased tumour growth. Similar studies in prostate cancer have shown that FAM84B is associated with progression of the disease. The results of our analyses suggest that FAM84B may have a possible role in promoting events associated with enhancing the viability of MCF7 cells, leading to increased rates of growth and division. In addition, FAM84B may also function to direct events associated with the early developmental stages of BC. We did not, however, observe any impact of altered FAM84B expression on the development of TAM resistance in BC. Further research involving improved *in vitro* and *in vivo* studies, along with an examination of FAM84B's impact on various oncogenic molecular signalling pathways, will help improve our understanding of the protein's role in BC tumorigenesis.

ABSTRACT

Breast cancer (BC) is the second most common malignancy in the world and it accounts for 15% of female deaths due to cancer every year. The development of these tumours is regulated by the activities of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Tamoxifen (TAM) is frequently used to treat patients with ER+ BC; however, a recurring problem is the development of resistance and the mechanisms leading up to this event remain unclear. FAM84B is reported to be associated with the development of various cancers such as esophageal squamous cell carcinoma and prostate cancer. The function of the protein is unknown; however, insight towards its mechanism of action has been made through the discovery of its structural similarities with the H-Ras-like suppressor (HRASLS) subfamily of enzymes. We hypothesize that FAM84B upregulation enhances BC tumorigenesis and facilitates the development of TAM resistance in this disease. We observed that both overexpression and knockdown of FAM84B had little effects on cell proliferation; however, the latter reduced the ability of MCF7 cells to form isolated colonies. We performed similar analyses using a FAM84B mutant with deletion of its HRASLS domain and we observed that MCF7 cells expressing this protein showed higher rates of cell proliferation and increased ability to form isolated colonies compared to cells with baseline expression of wild-type FAM84B. These results suggest that FAM84B regulates BC cell proliferation through a complex manner. An analysis of patient-derived BC tissue revealed that FAM84B expression was associated with BC at early stages than later stages, which suggests a possible role of the protein in directing

events associated with the early developmental stages of the disease. Additional analyses demonstrated that overexpression and knockdown of FAM84B had little impact on TAM-derived cytotoxicity of MCF7 cells. We observed higher expression of FAM84B in TAM-resistant MCF7 cells in comparison to TAM-sensitive cells, while TAM-resistant and TAM-sensitive xenograft tumours showed similar levels of FAM84B expression. This suggests that the contributions of FAM84B in BC tumorigenesis and resistance to TAM are complex; alternatively, FAM84B may not play a major role in either events. Future studies will be needed to clarify the effects of FAM84B on BC tumorigenesis and progression.

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LIST OF ABBREVIATIONS

BC	Breast Cancer
ER	Estrogen Receptor
ERE	Estrogen-Responsive Element
PR	Progesterone Receptor
PRE	Progesterone-Responsive Element
HER2	Human Epidermal Growth Factor Receptor 2
MAPK	Mitogen-Activated Protein Kinase
T	Tumour Size
N	Number of lymph nodes with cancer cells
M	Metastatic status of the cancer
SERM	Selective Estrogen Receptor Modulator
SERD	Selective Estrogen Receptor Downregulator
TAM	Tamoxifen
4-OH TAM	4-hydroxytamoxifen
FAM84B	Family with Sequence Similarity 84 Member B
PC	Prostate Cancer
EV	Empty Vector
ESCC	Esophageal Squamous Cell Carcinoma
IHC	Immunohistochemistry
HRASLS	H-Ras-like suppressor

LRAT	lecithin:retinol acyltransferase
H23	Histidine 23
H35	Histidine 35
C113	Cysteine 113
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle's Medium
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
VSV-G	Vesicular Stomatitis Virus Glycoprotein
GP	Gag-Pol
shFAM84B	FAM84B shRNA Plasmid
shCtrl	shRNA Control Plasmid
TAM-R	Tamoxifen-Resistant
TBS-T	Tris-Buffered Saline
TMA	Tissue Microarray
PBS	Phosphate-Buffered Saline
DMSO	Dimethylsulfoxide
ANOVA	Analysis of Variance
SEM	Standard Error Mean
ETD	Early Tumour Development
LTD	Late Tumour Development

DECLARATION OF ACADEMIC ACHIEVEMENT

Although I was the major contributor for the work presented in this thesis, it required contributions from multiple individuals.

Dr. Damu Tang and I designed the experiments and conceptualized the overall approach to the project. Yanzhi Jiang performed the multiple alignment analysis of FAM84B, LRAT, and the members of the HRASLS subfamily of enzymes. She also deleted the HRASLS domain from FAM84B and subcloned the resulting gene into a pBABE plasmid. Dr. Xiaozeng Lin assisted in the generation of the MCF7 and HCC1954 stable lines that required lentiviral-based methods of transfection. Dr. Diane Ojo provided the MCF7 xenograft tumour slides used for the *in vivo* portion of the analysis and Maryem Seliman provided the slides used for the tissue microarray analysis. I performed the experiments, analyzed the data, and prepared figures and tables for this dissertation.

CHAPTER 1: INTRODUCTION

1.1 Breast Cancer Epidemiology

Breast cancer (BC) is the second most common malignancy in the world with approximately 1.7 million diagnoses and 500,000 deaths per year; it represents about 25% of all newly identified cancers every year (1, 2, 3). Among women, BC is the most common cancer and it accounts for 15% of deaths due to cancer every year. (1, 3). In the United States, 12.4%, or one in eight women, are expected to develop BC at some point in their life (1).

The disease shows higher prevalence in developed countries in comparison to developing countries, which is likely a reflection of differences in the availability and use of screening programs such as mammography (1, 2, 3). In terms of mortality, developing countries experience higher death rates due to BC than developed countries, which is likely a result of improved treatment programs and early detection of the disease in developed countries (1, 2, 3).

Although women between the ages of 40 and 80 represent the majority of BC diagnoses, the incidence rate for the disease has been steadily increasing for females of all age groups throughout the past three decades (3). Fortunately, the five-year relative survival rate has improved dramatically from 74.8% for women diagnosed in 1975-1977 to 90.8% for women diagnosed in 2006-2012 (3).

While our overall knowledge of BC etiology has improved greatly over time, it remains quite limited; as a result, the incidence rate of the disease is predicted to reach 3.2

million diagnoses per year by 2050 (*1*). Thus, it is critical to accelerate research on BC to discover more effective treatments and methods of screening.

1.2 Breast Cancer Classification

1.2.1 Classification according to location in the breast

BC refers to a group of mammary cells that proliferate uncontrollably, resulting in the formation of a tumour that can grow into and invade nearby tissues (4, 5). The majority of BCs are adenocarcinomas, which are tumours that form in glandular cells (4). The disease is characterized into different types based on the origin of the mammary cells. Lobular carcinoma starts in the lobules, which are the glands that produce milk; whereas, ductal carcinoma starts within the ducts that direct milk towards the nipple. Either types of BC can be non-invasive, in which they are described as carcinoma in situ, or they can be invasive (4, 5).

1.2.2 Classification based on molecular differences

BC is heterogeneous and can be further classified into different molecular subtypes according to the expression status of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Activation of these receptors leads to signalling cascades that promote the transcription of genes involved in increased cell proliferation, migration, angiogenesis, and inhibition of apoptosis, which are all properties that facilitate tumour growth and development (4, 5, 6, 7, 8).

The ER exists as two isoforms, ER α and ER β , which have different tissue expression patterns in humans and other animals (9, 10). The genomic mechanism of ER action occurs when estrogen diffuses into the cell and binds to the ligand-binding domain

of a nuclear ER protein. This results in the subsequent formation of an ER dimer which is recruited to promoter regions known as estrogen-responsive elements (EREs), where they bind to coactivator proteins (9, 10). This results in transcription of genes coding for cyclin D1, insulin-like growth factor I receptor, vascular endothelial growth factor, and others (7, 8). This hormone response usually takes place over the course of a couple of hours; in contrast, the more rapid nongenomic ER mechanism occurs within seconds or minutes (9). In this case, estrogen binds to membrane-associated ER proteins which leads to the activation of various oncogenic signal transduction pathways, including AKT and mitogen-activated protein kinase (MAPK) signalling (9, 10).

The PR also has two isoforms, PR-A and PR-B, which are translated from the same gene and can participate in homo- and heterodimerization interactions (11, 12). Similar to the ER genomic mechanism, the PR is activated upon binding of progesterone to its ligand-binding domain. This results in dimerization of the receptor and recruitment into the nucleus where it binds to progesterone-responsive element (PRE) promoter sequences. Further interactions with coactivator proteins lead to transcription of mitogenic genes that stimulate the proliferation of mammary cells (11, 12). The PR also demonstrates ligand-dependent nongenomic actions in which it interacts with the Src Homology 3 domain of c-src tyrosine kinase, resulting in rapid downstream activation of the oncogenic MAPK pathway (13, 14).

HER2 plays an important role in regulating the growth, division, and repair of healthy breast tissue (8). Although there is no known ligand that interacts with this

receptor (15), its activation is facilitated by heterodimerization upon the binding of other proteins in its family, such as HER3. This leads to intracellular signalling cascades via AKT and MAPK / ERK mediated pathways to induce transcription of target genes (15, 16). Mutations to the HER2 gene are typical in breast cancer and the result is usually overexpression of the receptor. This causes increased activity of the downstream signalling pathways, which results in increased growth and proliferation of tumours (8, 15, 16).

A more sophisticated system classifies BC into three categories based on the expression status of the ER, PR, and HER2. Luminal BC cells are characterized by positive expression of the estrogen receptor (ER+) and variable expression of the progesterone receptor (PR+/-). They are further divided into two groups based on the expression status of HER2: Luminal A cells are negative for the expression of HER2 (HER-); whereas, Luminal B cells are positive for HER2 expression (HER2+) (17). As a result of differential HER2 expression, Luminal B cells are typically more aggressive and associated with worse prognosis for patients in comparison to Luminal A cells (17, 18). HER2+ BC represents around 20-30% of BC tumours (15, 19) and it is characterized by positive HER2 expression (HER2+) and negative ER expression (ER-) (17). These tumours are very aggressive and are associated with high rates of recurrence (17, 18, 19), which is why patients who are diagnosed with HER2+ BC have worse prognosis than patients with Luminal BC (17). Lastly, triple-negative BC represents around 10-17% of all BC tumours (20) and they are characterized as negative for the expression of the three main receptors (ER-, PR-, and HER2-) (17, 20, 21). These are very aggressive tumours

that grow and metastasize at a rapid rate, and are very difficult to target for treatment due to the lack of receptor availability (20, 21, 22). As a result, the prognosis of patients diagnosed with triple-negative BC is significantly worse in comparison to patients with other BC molecular expression profiles (20, 22).

1.2.3 Classification according to clinical stage of the disease

Upon diagnosis of BC doctors and clinicians assign a stage to the disease to describe how far it has progressed in terms of the size of the primary tumour and how far it has spread to other parts of the body (4, 23). One classification system assigns tumours to one of five stages; in general, the higher the stage number, the more the tumour has grown and spread (4, 23).

BCs in stage 0 are only present in the area of the breast from which development began and invasion into nearby tissue has not occurred; these tumours are carcinoma in situ (4, 23). The prognosis for patients diagnosed with BC this early in development is favorable with a five-year survival rate of 100% (24).

Stage 1 describes BC that is smaller than 2 cm in size and may or may not have spread to nearby lymph nodes. Stage 2 describes BC that is between 2 cm and 5 cm in size and may or may not have spread to 1-3 lymph nodes under the arm or inside the chest around the breastbone. These two stages are sometimes combined into one category known as early invasive BC (4, 23). Patients diagnosed with BC in this category also

have favorable five-year survival rates; the rate is 98.8% for tumours localized to the breast and 85.5% for tumours that have spread to nearby lymph nodes (24).

BCs in stage 3 includes those that are 5 cm or larger in size and may or may not have spread to 4-9 lymph nodes that are under the arm, inside the chest around the breastbone, or above the collarbone. Growth into the muscles of the chest wall or skin is another characteristic of stage 3 tumours. Stage 4 describes BC that has metastasized to other locations in the body (4, 23). The prognosis for patients diagnosed with BC at these two stages is significantly less favorable in comparison to BC diagnosed early in development; the five-year survival rate is 57% for stage 3 BC (25) and only 27.4% for tumours at stage 4 (24).

The more common classification system is the TNM staging system (4). The three letters refer to the three components used to describe the primary tumour; each letter is paired with a number or the letter X to describe the severity of the tumour with respect to that component. The letter T refers to the size of the tumour. TX means the tumour cannot be measured and T0 means the tumour cannot be found. T1, T2, T3, etc. describe tumours of increasing size. The letter N refers to the number of nearby lymph nodes that contain cancer cells. NX means the number of cancer cells in the lymph nodes cannot be measured and N0 means that there are no cancer cells present in the lymph nodes. N1, N2, N3, etc. refer to increasing numbers of lymph nodes that contain cancer cells. The letter M refers to whether or not the BC has metastasized to other parts of the body. MX

means that metastasis cannot be measured, M0 means that metastasis has not occurred, and M1 means that metastasis has occurred (23).

1.3 Treatments for Breast Cancer

There are several treatment options available for individuals who suffer from BC. Surgery is usually the primary option for patients diagnosed with BC at any of the first four stages (25). Patients with early-diagnosed BC (stages 0-2) undergo breast-conserving surgery to completely remove the primary tumour and a small portion of healthy surrounding tissue. This lowers the risk of recurrence and maintains a breast that is more or less aesthetically acceptable (4, 25). For patients with stage 3 BC the tumour is usually very aggressive and the risk of recurrence is very high; thus, mastectomy is usually necessary to remove the entire breast and reduce the risk of any further complications (4, 25). Radiation therapy uses high-energy beams to destroy cancer cells and it is often paired with surgery as the primary method of treatment (4, 25). It can be used to shrink the tumour prior to surgery or to kill any cancer cells that remain following the procedure (4). Adjuvant therapies such as hormonal therapy, targeted therapy, and chemotherapy are often applied following the primary treatment to minimize the risk of recurrence (25).

Hormonal therapy takes advantage of the fact that most BC cells require hormone receptor signalling to facilitate their growth and division. For instance, patients with ER+ BC can take drugs that block the binding of estrogen to the ER and inhibit its downstream intracellular signalling effects as a result (4, 8, 17). Some of these drugs function as selective estrogen receptor modulators (SERMs), which bind to the ER's ligand-binding domain with high affinity and triggers dimerization of the receptor. The dimer is recruited into the nucleus where it binds to an ERE promoter sequence; however, in contrast to

estrogen, the binding of a SERM to the ER usually results in subsequent binding of corepressor proteins that inhibit further transcription (26, 27). Other drugs function as selective estrogen receptor downregulators (SERDs), which also bind to the ER's ligand binding domain with high affinity. These drugs usually have long, bulky side chains which sterically hinder dimerization of the receptor, leading to increased ER degradation and reduced transcription of estrogen-responsive genes (27). ER+ patients may also take drugs that inhibit the function of Aromatase, which is an enzyme that synthesizes estrogen in parts of the body other than the ovaries. This treatment is only effective in post-menopausal women because their ovaries are no longer producing estrogen (4, 28).

Targeted therapy involves the use of drugs that specifically target and inhibit the growth of HER2+ BC (4). The most commonly used drug for this type of treatment is Trastuzumab, which is a monoclonal antibody directed against HER2 (4, 15, 19, 25, 29). Adjuvant use of this drug has been shown to decrease recurrence rates and improve the overall survival of HER2+ BC patients after a four-year follow-up analysis (30). The antitumor function of Trastuzumab is not completely understood; however, proposed mechanisms include inhibition of HER2 dimerization and antibody-dependent cell-mediated cytotoxicity (15, 29).

Chemotherapy involves the use of cytotoxic drugs that target cancer cells and other rapidly dividing cells (4). Similar to radiation therapy, these drugs can be used to shrink the tumour prior to surgery or to kill cells that remain after the procedure. Since these drugs travel through the bloodstream to reach their targets, they have the added

benefit of killing any cells that have metastasized from the primary tumour; thus, this type of treatment is particularly helpful for patients diagnosed with stage 4 BC (4, 25). In addition, chemotherapy is the main adjuvant therapy used during the treatment of patients with triple negative BC because these tumours lack expression of the ER, PR, and HER2, which renders hormonal and targeted therapies ineffective (22).

1.4 Tamoxifen Resistance in Breast Cancer

Tamoxifen (TAM) has been used over the past 40 years as a SERM for adjuvant hormonal therapy of patients with ER+ BC (6, 7). It is metabolized in the liver into 4-hydroxytamoxifen (4-OH TAM) and *N*-desmethyltamoxifen. 4-OH TAM is the metabolite which carries out the active role of competitively inhibiting the ER because it has a much greater affinity for the ER's ligand-binding domain than TAM (31).

Similar to other SERMs, the antagonistic activity of TAM in ER-mediated transcription is dependent on the tissue microenvironment; for instance, while TAM functions as an ER antagonist in the breast, it is an ER agonist in bone and the uterus (9, 32). This phenomenon results from a combination of factors that include different promoter elements that regulate estrogen-sensitive genes in these tissues (9, 32), as well as differential recruitment of coactivator and corepressor proteins due to varying ratios of these proteins in different tissues (9, 31, 32). This rises an issue in which the use of TAM for adjuvant treatment in one tissue could possibly stimulate the onset of hyperplasia in another tissue (32).

Nonetheless, a recurring problem with adjuvant use of TAM is that approximately 30% of treated patients develop resistance to the drug and the mechanisms by which this occurs have been the focus of much research (6, 7, 31). Changes to the function of the ER have been shown to play an important role in TAM resistance; for instance, mutations can lead to active ER signalling in the absence of ligand binding (31). It is also possible for the cells to adapt to the lack of ER-mediated transcription by upregulating non-genomic

ER signalling via the AKT and MAPK pathways (31). The cells may also alter the balance of coregulatory proteins by increasing the expression of coactivator proteins and decreasing the expression of corepressor proteins (31).

1.5 The Oncogenic Role of FAM84B

1.5.1 Identification of FAM84B within the 8q24 locus

The 8q24 chromosomal locus is frequently amplified in a variety of cancers including ovarian (33, 34), prostate (33, 35, 36, 37), and colorectal cancers (33, 37, 38). This is particularly associated with the presence of MYC within this region, which is the most commonly amplified gene in cancer (33, 39, 40). The MYC protein is a well-known downstream effector of various signalling pathways that regulate key events in tumour development, such as immortalization, transformation, uncontrolled cell proliferation, and angiogenesis (40). It was also shown to collaborate synergistically with Ras to induce cyclin E-dependent kinase activity and E2F gene expression, which facilitates progression through the G1/S checkpoint of the cell cycle (41). The gene for Family with sequence similarity 84 member B (FAM84B) is located at the 8q24.21 locus. It resides at the centromeric end of a 1.2 Mb gene desert with MYC bordering the telomeric end (33, 42). The proximity between these two genes suggests a possible connection between FAM84B and MYC (42); however, while the tumorigenic properties of MYC are well established, studies of FAM84B's role in cancer development have been quite limited in comparison.

FAM84B displays widespread intracellular localization and it was shown to form intramolecular interactions with other FAM84B molecules based on analyses using co-immunoprecipitation and immunofluorescence (42, 43). A yeast two-hybrid analysis revealed that FAM84B interacts specifically with α 1-catenin (43), which is a component of the cadherin / catenin complex that is essential for the initiation of cell-cell adhesion

(44). This is supported by the observation that the genes for FAM84B and α 1-catenin showed concurrent, yet independent, amplification in 15% of tumours from a cohort of prostate cancer (PC) patients (33).

1.5.2 *Oncogenic activity of FAM84B in various cancers*

Studies of PC revealed that FAM84B expression is elevated in primary PC tumours in comparison to normal tissue and that it is associated with the progression to metastatic castration resistant PC (33). It was also shown that upregulation of FAM84B expression in DU145 cells enhanced various *in vitro* oncogenic properties of the cell line, such as its invasiveness in matrigel and its ability to form colonies in soft agar (42). The *in vivo* portion of this study demonstrated that xenograft tumours grew significantly faster when they were initiated by DU145 cells overexpressing FAM84B in comparison to DU145 cells expressing an empty vector (EV) (42).

Studies of esophageal squamous cell carcinoma (ESCC) revealed that FAM84B expression was significantly higher in several ESCC cell lines in comparison to non-neoplastic human esophageal cell lines (45). Immunohistochemistry (IHC) analyses of ESCC tissue samples showed that FAM84B expression was much higher in cancerous tissue than in matched normal tissue (45, 46). Knockdown of FAM84B expression was shown to reduce the growth, migration, and invasiveness of two ESCC cell lines (46), while also delaying the growth of xenograft tumours in comparison to tumours that were initiated by ESCC cells with baseline expression of FAM84B (45). It was also shown that

decreased serum FAM84B protein was associated with a higher chance of ESCC patients achieving pathological complete response to neoadjuvant chemoradiation therapy (45).

The studies of PC and ESCC provide valuable evidence towards a role of FAM84B in promoting tumour development in various tissues; however, the mechanisms by which the protein functions remain unclear. It was shown that AKT activation was elevated in xenograft lung metastases derived from FAM84B-overexpressing DU145 cells in comparison to lung metastases derived from DU145 cells expressing an EV (42). This occurred alongside a significant reduction in the expression of the pro-apoptotic protein BAD for the DU145 FAM84B derived lung metastases in comparison to the DU145 EV derived tumours (42). This suggests a possible mechanism for FAM84B as a trigger for the oncogenic AKT pathway by enhancing cell survival through a reduction of apoptotic signalling. Further analysis using these DU145 cell-derived xenograft tumours showed that in reference to FAM84B elevation, several other pathways were enriched as a result of nearly 5000 differentially expressed genes (42). These pathways include those that regulate Golgi-to-ER retrograde transport, and others which decrease activity of the tricarboxylic acid cycle and the respiratory electron transport chain.

1.5.3 Structural similarities of FAM84B with the HRASLS subfamily of enzymes

The H-Ras-like suppressor (HRASLS) subfamily of enzymes consists of five members (HRASLS1-5) in humans which demonstrate phospholipid metabolizing activities *in vitro*, including phospholipase A_{1/2} and O-acyltransferase activities (47). Four

members have also been reported to function as class II tumour suppressors *in vitro* based on their ability to repress H-Ras-mediated oncogenic signalling (42, 47). The family shares a homologous lecithin:retinol acyltransferase (LRAT) domain that contains the active site of the enzymes, which is characterized by a catalytic triad formed by histidine 23 (H23), histidine 35 (H35), and cysteine 113 (C113), as numbered in HRASLS2, with C113 being the catalytic residue (Fig. 1A) (42). The exception to this is HRASLS1, in which H35 is replaced by an asparagine residue; nevertheless, the catalytic triad remains crucial to the enzyme's phospholipid metabolizing activity (42, 47).

A structural analysis of FAM84B showed that the region between residues 119 and 212 is homologous to the LRAT domain that is conserved among the HRASLS family (Fig. 1A) (42). The two histidine residues of the HRASLS catalytic triad are conserved in FAM84B; however, the catalytic cysteine residue is missing, which suggests that FAM84B is unlikely to possess phospholipid metabolizing activities (42). Due to its high conservation among FAM84B and the HRASLS family, the 119-145 sub-region of FAM84B was tentatively named the HRASLS domain (Fig. 1A). A FAM84B mutant with a deletion of the HRASLS domain was then constructed (Fig. 1B) to investigate whether or not this region is a crucial component of FAM84B's oncogenic activity in prostate cancer (42). The study showed that removal of this region significantly reduced the invasiveness of DU145 cells *in vitro* and it significantly reduced the ability of the cells to grow in soft agar.

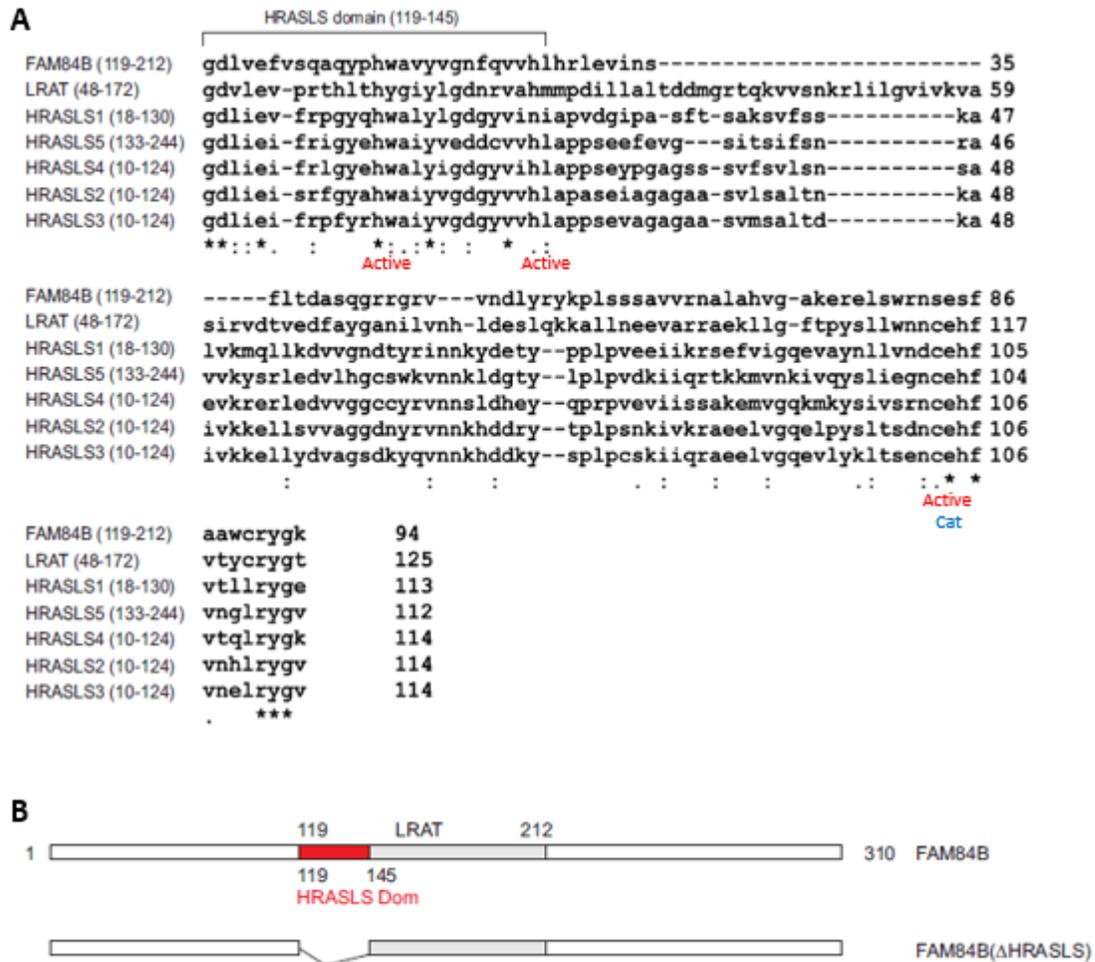


Figure 1. Structural similarities between FAM84B and the HRASLS subfamily of enzymes. (A) The amino acid sequences of FAM84B, LRAT, and HRASLS1-5 were aligned to determine regions of significant homology. A period (.) or colon (:) underneath aligned residues indicate their similarities. An asterisk (*) indicates residues that are conserved among all sequences. The three residues that make up the catalytic triad of the HRASLS active site are highlighted, as well as the FAM84B sub-region that was tentatively named the HRASLS domain. (B) A schematic representation of FAM84B's

structure with its LRAT and HRASLS domains highlighted. Construction of the FAM84B(Δ HRASLS) mutant was achieved via deletion of the 119-145 sub-region (42).

1.6 Central Hypothesis and Specific Aims

1.6.1 Purpose of Thesis

Since FAM84B may serve as a potential target for the development of novel anti-cancer therapy, it is necessary to have a clear understanding of the protein's role in the development of cancers that have not been thoroughly researched so that a holistic interpretation of its tumorigenic potential can be established. The main purpose of this thesis is to investigate the impact of FAM84B expression on BC development and progression. Since resistance to endocrine therapy is a recurring problem in BC patients (6, 7, 31), we are also interested in the role of FAM84B expression in the development of TAM resistance.

1.6.2 Main Thesis Question

Does FAM84B expression have an impact on BC tumorigenesis and the development of TAM resistance?

1.6.3 Main Thesis Hypothesis

FAM84B upregulation enhances BC tumorigenesis and it facilitates the development of TAM resistance in this disease.

1.6.4 *Specific Objectives*

Objective 1: Determine how FAM84B expression impacts various oncogenic properties of BC

We will examine the effect of FAM84B overexpression or knockdown on BC cell proliferation, migration, invasion, and colony formation *in vitro*.

Expectations: FAM84B overexpression will result in increased BC cell proliferation, migration, invasion, and colony formation *in vitro*; whereas, FAM84B knockdown will have the opposite effects.

Objective 2: Determine which parts of FAM84B's structure are crucial for its function

We will create mutated versions of the FAM84B protein in which specific structural regions are deleted. We will express these mutants in BC cells and examine the resulting effects on cell proliferation, migration, invasion, and colony formation *in vitro*.

Expectations: BC cells expressing FAM84B mutants with deletions to crucial structural regions will display reduced cell proliferation, migration, invasion, and colony formation in comparison to BC cells expressing wild-type FAM84B.

Objective 3: Determine how FAM84B expression impacts BC development and progression

We will examine the levels of FAM84B expression in patient-derived tumour tissue that represent various stages of BC.

Expectations: Increasing FAM84B expression will directly correlate with increasing stage of BC.

Objective 4: Determine how FAM84B expression impacts TAM resistance in BC

- 1) We will examine the levels of FAM84B expression in ER+ breast cancer cells that are either resistant or sensitive to TAM.

Expectations: FAM84B expression will be higher in TAM-resistant ER+ breast cancer cells in comparison to TAM-sensitive ER+ breast cancer cells.

- 2) We will examine the levels of FAM84B expression in xenograft BC tissue that are either resistant or sensitive to TAM.

Expectations: FAM84B expression will be higher in TAM-resistant BC tissue in comparison to TAM-sensitive BC tissue.

- 3) We will examine the impact of TAM treatment on the survival of ER+ breast cancer cells that have varying levels of FAM84B expression.

Expectations: Higher amounts of cell survival will be observed for cells that have increasing levels of FAM84B expression.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture and generation of stable cell lines

MCF7, T47D, HCC1954, and 293T cells were purchased from American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (MCF7 and 293T) and Roswell Park Memorial Institute (RPMI) 1640 (T47D and HCC1954) media supplemented with 10% Fetal Bovine Serum (FBS) (Sigma Aldrich) and 1% Penicillin-Streptomycin (Thermo Fisher Scientific). Generation of MCF7, T47D, and HCC1954 cells with stable expression of FAM84B or FAM84B(Δ HRASLS) was performed through retroviral methods. Briefly, 293T cells were transiently co-transfected with plasmids for the viral packaging proteins vesicular stomatitis virus glycoprotein (VSV-G) and gag-pol (GP), along with either an empty pBABE plasmid vector (EV), a pBABE vector with subcloned FAM84B, or a pBABE vector with subcloned FAM84B(Δ HRASLS). After 72 hours the resulting viral particles were harvested from the supernatant of the 293T plates and used to infect parental MCF7, T47D, or HCC1954 cells. Selection of stably transfected cells was performed using 1 μ g/mL of puromycin. Knockdown of FAM84B in MCF7 and HCC1954 cells was achieved through stable expression of a FAM84B shRNA plasmid (shFAM84B) and a knockdown control plasmid (shCtrl) via lentiviral methods. This process is essentially the same as the retroviral method with a few exceptions. The VSV-G and GP viral packaging genes were each subcloned into a separate lentiviral pMD2.G plasmid and a third lentiviral plasmid coding for rev was required. These three plasmids were co-transfected along with either a

pool of three shFAM84B lentiviral plasmids (Santa Cruz) or a pool of three shCtrl plasmids (Santa Cruz). Selection of stably transfected cells was performed using 1 µg/mL of puromycin.

2.2 Generation of TAM-resistant cells

TAM-resistant MCF7 cells (MCF7 TAM-R) were produced by culturing MCF7 cells in phenol-red free DMEM media supplemented with 1 µM of 4-OH TAM (Sigma Aldrich) for 12 months (6, 7).

2.3 Western Blot Analysis

Cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 µg/mL leupeptin, and 10 µg/mL aprotinin. 50 µg of protein from the cell lysate was separated on a 10% SDS-PAGE gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Blocking was performed with 5% skim milk for one hour at room temperature, followed by incubation with a primary antibody at 4°C overnight. Membranes were washed in 1X tris-buffered saline (TBS-T) and then incubated with a horseradish peroxidase-conjugated secondary antibody for one hour at room temperature. Membranes were washed again in 1X TBS-T and signals were then developed using an

ECL Western Blotting Kit (Amersham). The intensities of the resulting protein bands were quantified using ImageJ software (National Institutes of Health). The primary antibodies were anti-FAM84B (1:1000; Proteintech), anti-M2-Flag (1:2000), and anti- α -tubulin (1:1000). The secondary antibodies were anti-rabbit (1:3000; Santa Cruz Biotechnology) and anti-mouse (1:3000; GE Healthcare).

2.4 Immunohistochemistry Analysis

Slides were prepared from xenograft tumours treated with and without TAM. The tissue microarray (TMA) slides were prepared from a patient cohort representing different types of BC at various clinical stages (US Biomax). Both sets of slides were deparaffinized in xylene and washed in an ethanol series. Antigen retrieval was facilitated by heat treatment in a food steamer for 20 minutes using sodium citrate buffer (1.47 g sodium citrate, 0.25 mL Tween dissolved in 500 mL of ddH₂O) (pH = 6.0). The slides were blocked for one hour in 1X phosphate-buffered saline (PBS) containing 1% BSA and 10% normal goat serum (Vector Laboratories) followed by incubation with an antibody specific for FAM84B (1:350 for the xenograft slides; 1:600 for the TMA slides) overnight at 4°C. A biotinylated anti-rabbit secondary antibody (1:200) and Vector avidin-biotin complex reagent (Vector Laboratories) were subsequently incubated with the slides according to the manufacturer's instructions. The chromogen reaction was carried out with diaminobenzidine (Vector Laboratories) for a specific time limit (45 seconds for the xenograft slides; 3.5 minutes for the TMA slides) and counterstaining was performed

using hematoxylin (Sigma Aldrich). The slides were scanned using a ScanScope and then analyzed using ImageScope software (Aperio). For each slide, staining intensity values obtained from ImageScope were converted into an H-Score using the formula [H-Score = (% Positive) x (intensity) + 1] and each resulting H-Score was normalized through background subtraction.

2.5 Colony Formation Assay

Specific numbers of cells (ranging from 100 to 1000) were seeded into individual wells of a 6-well plate, which was then incubated for two weeks with media change every three to four days. The cells were fixed in a solution containing 2% formaldehyde and 0.2% glutaraldehyde for 20 minutes followed by the addition of a crystal violet solution (0.5% crystal violet, 20% methanol, 150 mM NaCl) for 30 minutes. The plate was then rinsed in water and dried before images were taken.

2.6 Cell Proliferation Assay

2×10^4 cells were seeded into individual wells of 6-well plates, which were separated based on the concentration of FBS in the growth media. One plate had media with 2% FBS, the second plate had media with 5% FBS, and the third had media with 10% FBS. Every two to three days following initial seeding one well in each plate was trypsinized and its cells were resuspended in a predetermined volume of media in a separate

microcentrifuge tube. A hemocytometer was used to measure the total number of cells in each tube. Growth media in each well was changed every three to four days.

Quantification of peak cell density for each individual cell proliferation assay was achieved by averaging the peak cell numbers that were counted for each repetition of the experiment.

2.7 Analysis of cytotoxicity due to TAM treatment

10^5 MCF7 cells were seeded into individual wells of a 6-well plate and cultured for 48 hours in phenol-red free growth media. The media was replaced by serum-free media containing either no additional treatment (blank well), Dimethylsulfoxide (DMSO) (1:1000, mock treatment), or TAM dissolved in DMSO at concentrations of 0.5 μ M, 1 μ M, 3 μ M, or 5 μ M. The plates were incubated for 48 hours followed by replacement of media with normal serum-containing media and culturing of the cells for 96 hours. The cells were fixed in a solution containing 2% formaldehyde and 0.2% glutaraldehyde for 20 minutes followed by the addition of a crystal violet solution (0.5% crystal violet, 20% methanol, 150 mM NaCl) for 30 minutes. The plate was then rinsed in water and dried before images were taken.

2.8 Cell Invasion Assay

24-well invasion chamber inserts (8 µm pore size) with and without matrigel (Corning) were placed into individual wells of a 24-well plate. Serum-free media was added to the outside and inside of the inserts followed by incubation of the plate for 2 hours. Cells were centrifuged at 1000 rpm for 5 minutes and resuspended in serum-free media; this process was repeated three times. The media outside the inserts was then replaced with normal serum-containing media, while the media inside the inserts was replaced with 5×10^4 cells in serum-free media. The plate was incubated for 48 hours followed by removal of non-invasive cells using a cotton swab and three washes with serum-free media. The remaining cells were fixed in a solution containing 2% formaldehyde and 0.2% glutaraldehyde for 10 minutes followed by the addition of a crystal violet solution (0.5% crystal violet, 20% methanol, 150 mM NaCl) for 20 minutes. The plate and inserts were then rinsed in water and dried before images were taken using a ScanScope. Images were analyzed using ImageScope software (Aperio) and quantification of percent cell invasion was calculated using the formula [% cell invasion = (number of cells invading matrigel insert / number of cells invading control insert) x 100].

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software. Statistical tests used were the 2-tailed Student's t-test with and without Welch's correction, one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison post-hoc test, and two-way

analysis of variance with Bonferroni's post-hoc test. A value of $p < 0.05$ is considered statistically significant. Data are presented as mean \pm Standard Error Mean (SEM).

CHAPTER 3: RESULTS

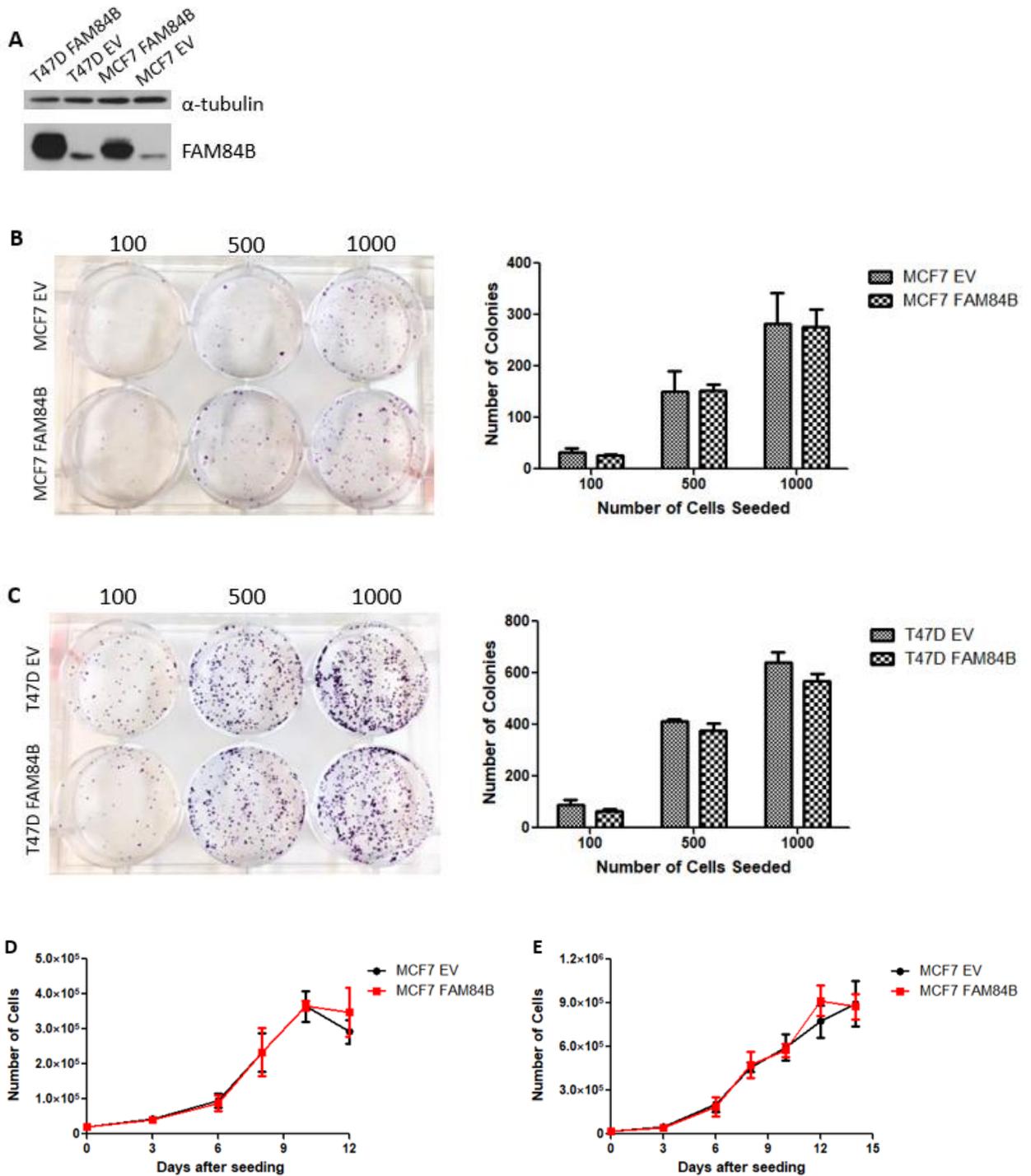
3.1 FAM84B overexpression has little impact on BC colony formation and cell proliferation

To examine the impact of FAM84B overexpression on *in vitro* oncogenic properties of BC, we stably overexpressed the protein in MCF7 and T47D cells. The results of the transfection was confirmed via western blot analysis with α -tubulin expression being used as the loading control (Fig. 2A). The FAM84B bands for the two stable lines (MCF7 FAM84B and T47D FAM84B) are clearly more intense than their EV counterparts, indicating successful transfection.

The colony formation assay was used to measure the impact of FAM84B overexpression on the ability of MCF7 and T47D cells to form isolated colonies *in vitro*. Although the number of colonies in each of the three seedings were slightly higher for MCF7 FAM84B cells than MCF7 EV cells, the differences were not statistically significant (Fig. 2B). Similar results were observed for the T47D cells in which we did not observe any significant differences between the EV and FAM84B cells for each of the three seedings. (Fig. 2C).

The effect of FAM84B overexpression on MCF7 cell proliferation in three different concentrations of FBS-supplemented DMEM media was measured and the results are shown in Figure 2 D-F. For each concentration we did not observe any significant differences between MCF7 EV and FAM84B cell growth. Similarly, we did not observe any significant differences between the peak cell densities of these two cell

lines for each FBS concentration, although there was a trend towards a higher MCF7 FAM84B peak cell density when the cells were cultured in 10% DMEM (Fig. 2G).



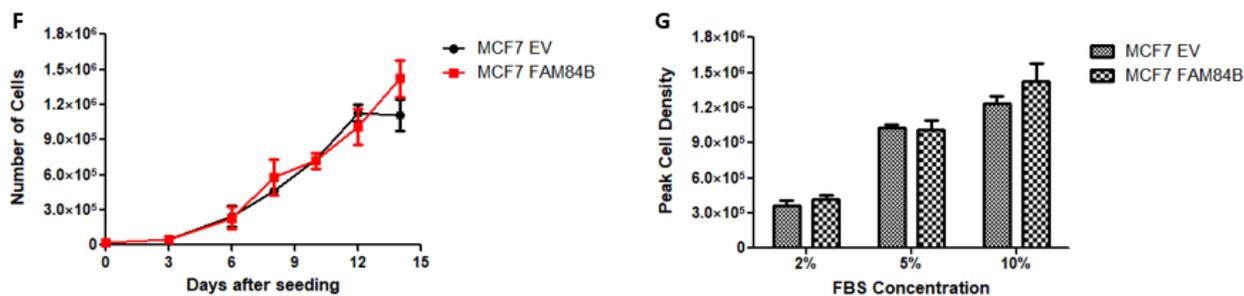


Figure 2. *FAM84B* overexpression has little impact on BC colony formation and cell proliferation. (A) Stable transfection of MCF7 and T47D cells with *FAM84B* was examined via western blot analysis using antibodies for *FAM84B* and α -tubulin. (B-C) 100, 500, and 1000 MCF7 and T47D EV and *FAM84B* stable cell lines were seeded and cultured for 2 weeks to allow for colony formation. The experiments were repeated 3 times and typical images from single repeats are shown. Quantification of the number of colonies for each seeding are displayed with means and standard error graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV and *FAM84B* cells. (D-F) 2×10^4 MCF7 EV and *FAM84B* cells were cultured for 2 weeks and counted every 2-3 days. The cells were grown in DMEM media supplemented with either 2% (D), 5% (E), or 10% (F) FBS. The x-axis represents the number days after initial seeding of cells and the y-axis represents the number of cells that were counted. The experiments were repeated 3 times and means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV and *FAM84B* cells. (G) Average peak cell densities reached by MCF7 EV and *FAM84B* cells were compared for each cell proliferation assay representing a different concentration of FBS-supplemented DMEM media; means with standard error are

graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV and FAM84B cells.

3.2 Knockdown of FAM84B reduces colony formation and has little impact on cell proliferation for MCF7 cells

To further investigate the impact of altered FAM84B expression on *in vitro* oncogenic properties of BC, we stably knocked down expression of the protein in MCF7 cells which was verified through western blot analysis (Fig. 3A). In comparison to cells transfected with a knockdown control plasmid (MCF7 shCtrl) and parental cells with baseline FAM84B expression (MCF7), cells transfected with the shFAM84B plasmid (MCF7 shFAM84B) showed significantly reduced expression of FAM84B; thus, confirming success of the transfection.

Our analysis of the impact of FAM84B knockdown on MCF7 colony formation showed that there were no significant differences between control and FAM84B knockdown cells when 100 and 500 cells were seeded. However, when 1000 cells were seeded we noticed that the knockdown cells formed significantly less colonies than the control cells (Fig. 3B).

Figures 3 C-E show the results of our analysis of the impact of FAM84B knockdown on MCF7 cell proliferation in different concentrations of FBS-supplemented DMEM media. We did not observe any significant differences between the growth patterns of MCF7 shCtrl and shFAM84B cells when cultured in 2% and 5% DMEM. When cultured in 10% DMEM, the control cells showed a trend towards higher rates of cell proliferation compared to the knockdown cells after day 12; however, the difference was also not significant. Examination of the peak cell densities for these experiments

showed that the control and knockdown cells grew to similar numbers when cultured in 2% DMEM (Fig. 3F). We also noticed that the control cells showed a trend towards higher peak numbers than the knockdown cells when cultured in 5% and 10% DMEM; however, both of these differences were not statistically significant.

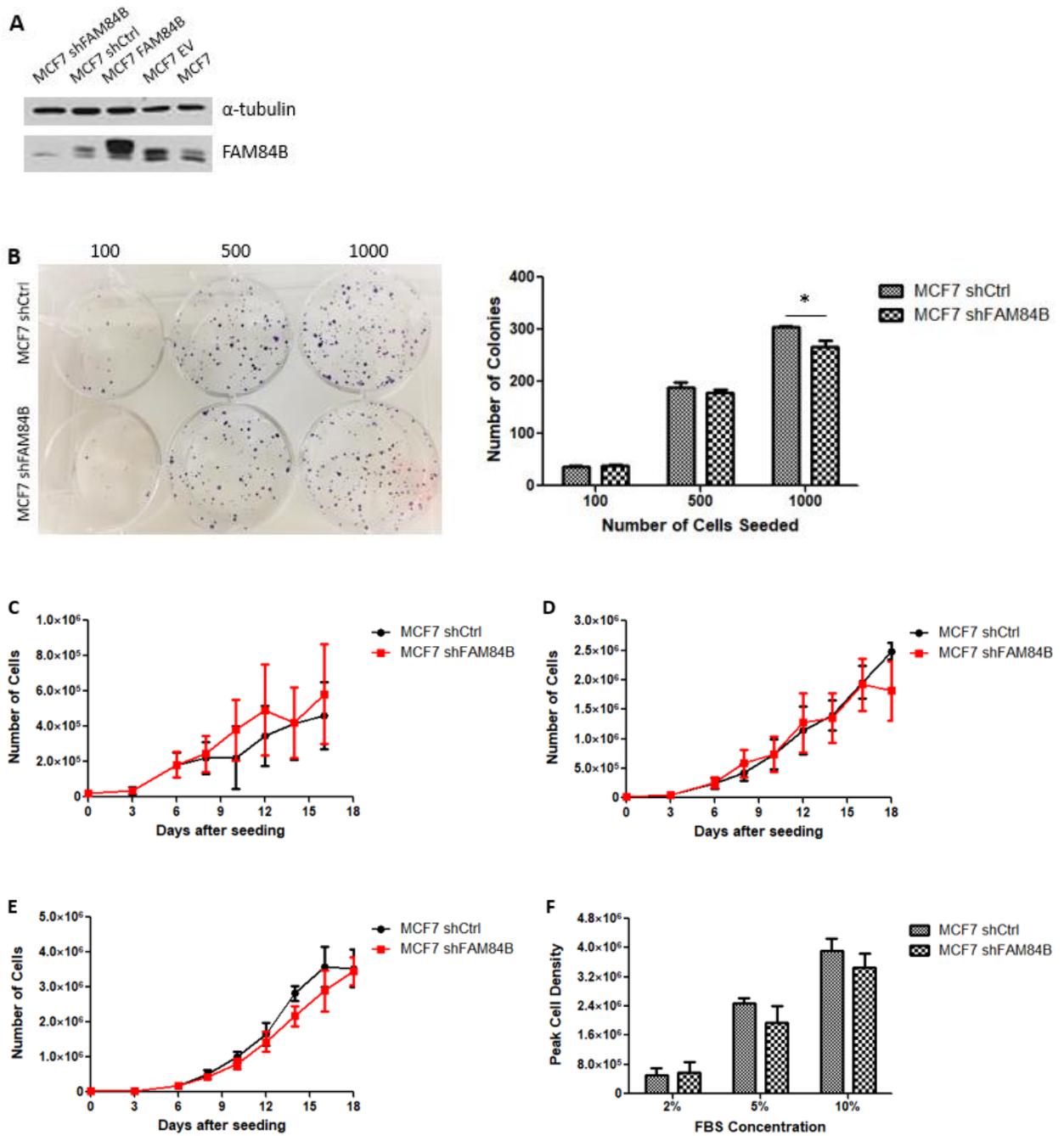


Figure 3. Knockdown of *FAM84B* reduces colony formation and has little impact on cell proliferation for MCF7 cells. (A) Stable transfection of MCF7 cells with the shFAM84B plasmid was examined via western blot analysis using antibodies for FAM84B and α -

tubulin. (B) 100, 500, and 1000 MCF7 shCtrl and shFAM84B stable cell lines were seeded and cultured for 2 weeks to allow for colony formation. The experiments were repeated 3 times and a typical image from a single repeat is shown. Quantification of the number of colonies for each seeding are displayed with means and standard error graphed; * indicates $p < 0.05$ (Two-way ANOVA with Bonferroni's post-hoc test). (C-E) 2×10^4 MCF7 shCtrl and shFAM84B cells were cultured for 18 days and counted every 2-3 days. The cells were grown in DMEM media supplemented with either 2% (C), 5% (D), or 10% (E) FBS. The x-axes represent the number days after initial seeding of cells and the y-axes represent the number of cells that were counted. The experiments were repeated 3 times and means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between shCtrl and shFAM84B cells. (F) Average peak cell densities reached by MCF7 shCtrl and shFAM84B cells were compared for each cell proliferation assay representing a different concentration of FBS-supplemented DMEM media; means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between shCtrl and shFAM84B cells.

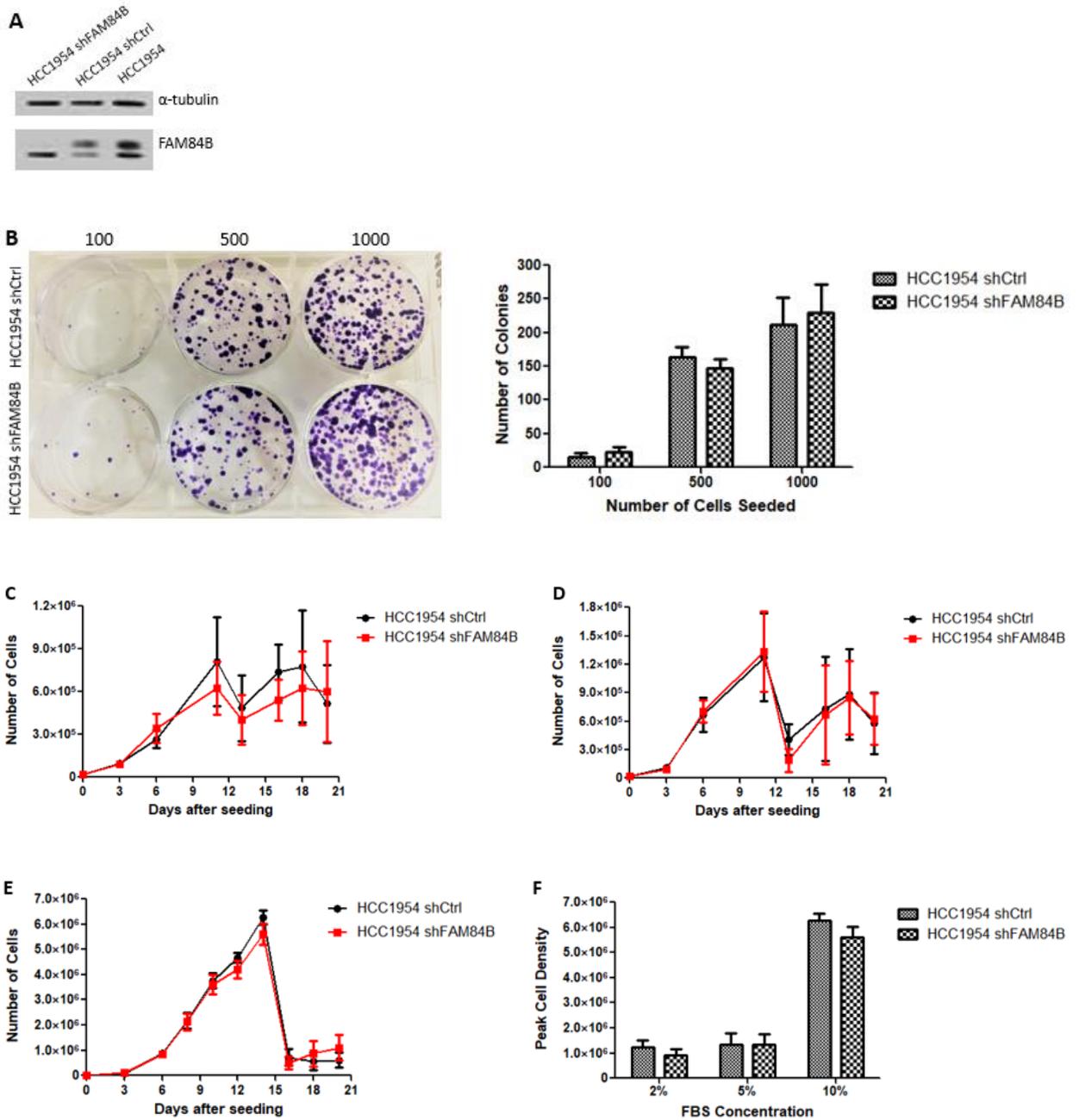
3.3 Knockdown of FAM84B has little impact on oncogenic-related events of HCC1954 cells

To compliment the analysis of the impact of FAM84B knockdown on the *in vitro* oncogenic properties of ER+ MCF7 cells, we performed a similar analysis using HER2+ HCC1954 cells. We stably knocked down FAM84B expression in these cells and verified the results of the transfection through western blot analysis (Fig. 4A). We noticed a significant reduction in the expression of FAM84B for the cells transfected with the shFAM84B plasmid (HCC1954 shFAM84B) in comparison to cells transfected with a knockdown control plasmid (HCC1954 shCtrl) and parental cells with baseline expression of FAM84B (HCC1954). Based on this observation, the transfection was a success.

In our analysis of the impact of FAM84B knockdown on HCC1954 colony formation *in vitro*, we observed similar numbers of colonies for both the control and knockdown cells in each of the three seedings (Fig. 4B). In the context of HCC1954 cell proliferation in different concentrations of FBS-supplemented RPMI 1640 media, we observed very similar growth patterns between the control and knockdown cells when cultured in 2%, 5%, and 10% media (Fig. 4 C-E). The peak cell densities for each of these proliferation assays were measured and the results showed that the numbers were very similar for both cell lines when cultured in 2% and 5% RPMI 1640 media (Fig. 4F). The control cells showed a trend towards a higher peak cell density in comparison to the

knockdown cells when cultured in 10% media; however, this difference was not statistically significant.

We also examined the impact of FAM84B knockdown on HCC1954 cell invasion. Figure 4G shows typical images of invasive HCC1954 shCtrl and shFAM84B cells that migrated through either control or matrigel inserts. While the knockdown cells were observed to be slightly less invasive than the control cells, the difference was not statistically significant (Fig. 4H).



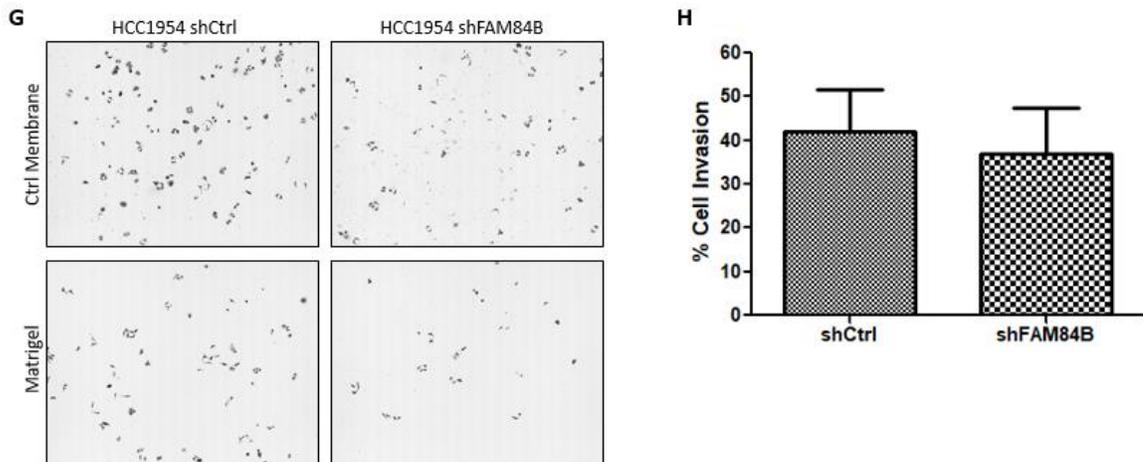


Figure 4. Knockdown of *FAM84B* has little impact on oncogenic-related events of *HCC1954* cells. (A) Stable transfection of *HCC1954* cells with the sh*FAM84B* plasmid was examined via western blot analysis using antibodies for *FAM84B* and α -tubulin. (B) 100, 500, and 1000 *HCC1954* shCtrl and sh*FAM84B* stable cell lines were seeded and cultured for 2 weeks to allow for colony formation. The experiment was repeated 3 times and a typical image from a single repeat is shown. Quantification of the number of colonies for each seeding are displayed with means and standard error graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between shCtrl and sh*FAM84B* cells. (C-E) 2×10^4 *HCC1954* shCtrl and sh*FAM84B* cells were cultured for 20 days and counted every 2-3 days. The cells were grown in RPMI 1640 media supplemented with either 2% (C), 5% (D), or 10% (E) FBS. The x-axes represent the number days after initial seeding of cells and the y-axes represent the number of cells that were counted. The experiments were repeated 3 times and means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between shCtrl and sh*FAM84B* cells. (F) Average peak cell densities reached

by HCC1954 shCtrl and shFAM84B cells were compared for each cell proliferation assay representing a different concentration of FBS-supplemented DMEM media; means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between shCtrl and shFAM84B cells. (G) 5×10^4 HCC1954 shCtrl and shFAM84B were seeded into control and matrigel inserts, followed by incubation for 48 hours to allow for cell invasion. The experiment was repeated 3 times and typical images from a single repeat are shown. (H) Cell invasion through matrigel was quantified and normalized to the respective cell invasion through the control insert; means with standard error are displayed. 2-tailed Student's t-test was used to analyze differences between shCtrl and shFAM84B cells.

3.4 FAM84B expression is associated with the early developmental stages of ductal BC

A tissue microarray (TMA) was performed to analyze the expression of FAM84B in patient-derived BC tissue representing various types and stages of progression for the disease. Typical images of positive and negative IHC staining of FAM84B are displayed in Figure 5A. In comparison to normal breast tissue, both ductal and lobular BC tissue express significantly higher levels of FAM84B (Fig. 5B). When compared to each other, FAM84B expression is significantly higher in ductal carcinoma than lobular carcinoma (Fig. 5B).

We also separated the ductal and lobular TMA slides into different groups according to the classification system that assigns tumours to one of five stages. We created two additional categories to represent early tumour development (ETD), which combines stages 0-2, and late tumour development (LTD), which combines stages 3-4. For ductal carcinoma, no significant difference in FAM84B expression was observed between the five stages of BC progression; however, FAM84B expression was significantly higher in early developed tumours than late developed tumours (Fig. 5C). For lobular carcinoma, no significant difference in FAM84B expression was observed between the individual stages nor between early and late developed tumours (Fig. 5D).

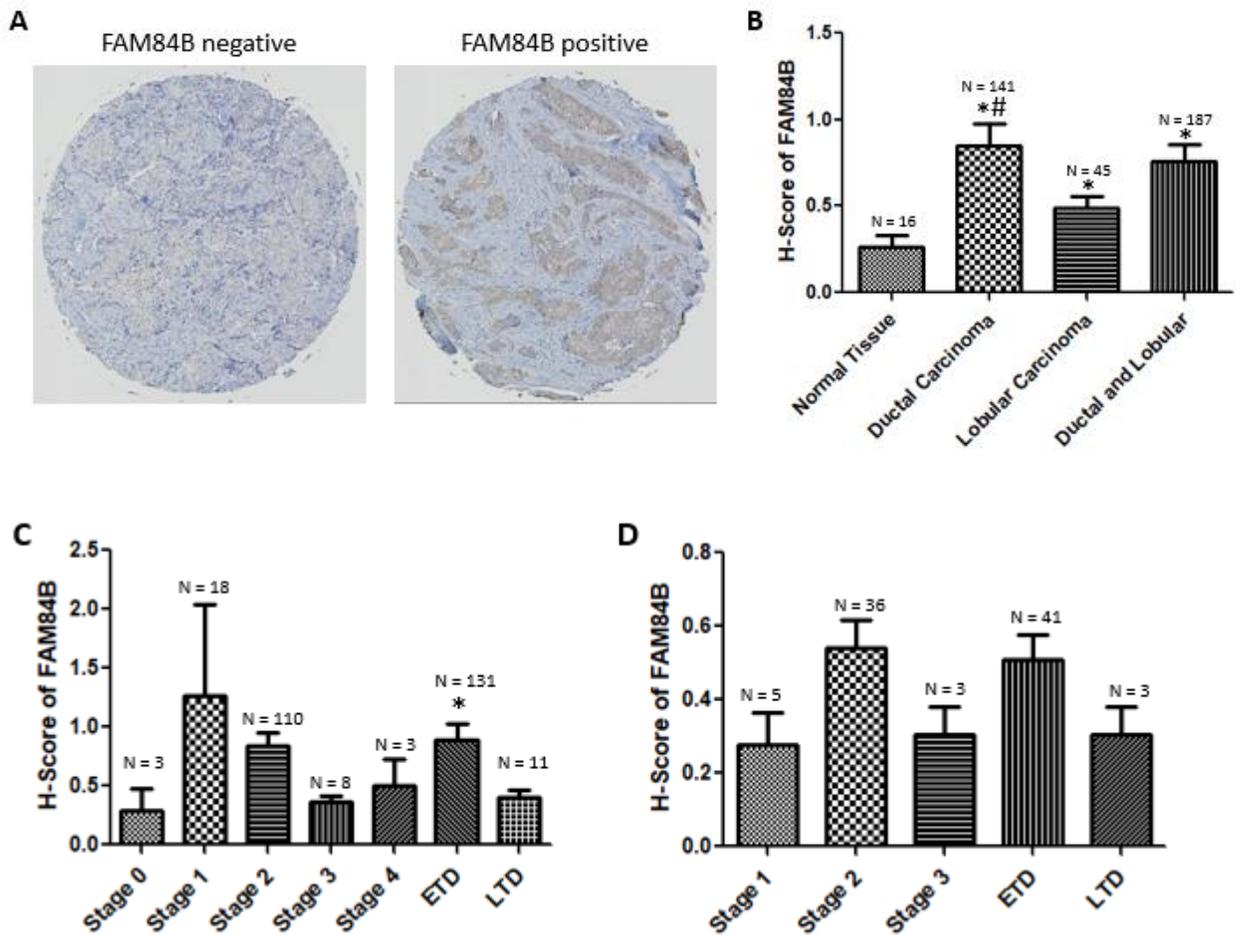


Figure 5. *FAM84B* expression is associated with the early developmental stages of ductal BC. (A) TMA slides were IHC stained and typical images of positive and negatively stained slides are displayed. (B) *FAM84B* expression was quantified as an H-Score and graphed for normal breast tissue, ductal carcinoma, lobular carcinoma, and combined ductal and lobular carcinoma. Mean and standard error are displayed; * and # indicate $p < 0.05$ in comparison to normal tissue and lobular carcinoma respectively (2-tailed Student's t-test with Welch's correction). (C-D) *FAM84B* expression was quantified as an H-Score and graphed for stages 0-4, early tumour development (ETD), and late tumour

development (LTD) for ductal (C) and lobular carcinoma (D). Mean and standard error are displayed; * indicates $p < 0.05$ in comparison to LTD (2-tailed Student's t-test with Welch's correction).

3.5 Deletion of FAM84B's HRASLS domain enhances MCF7 cell proliferation and colony formation

To examine the impact of deleting FAM84B's HRASLS domain on BC tumorigenesis, we stably expressed this mutant form of the protein in MCF7 cells. The result of the transfection was verified through western blot analysis (Fig. 6A). Expression of M2-Flag was used as an indicator of expression of transfected FAM84B and FAM84B(Δ HRASLS) since it was tagged to these proteins. We observed that M2-Flag expression was greater for cells transfected with FAM84B or FAM84B(Δ HRASLS) in comparison to cells transfected with an empty vector (EV). More importantly, the success of the transfection was confirmed based on the molecular weight of M2-Flag being lower in the FAM84B(Δ HRASLS) cells than in the FAM84B cells.

Our analysis of the impact of deleting FAM84B's HRASLS domain on the ability of MCF7 cells to form isolated colonies *in vitro* showed that there were similar numbers of colonies formed by EV and FAM84B(Δ HRASLS) cells when 100 and 500 cells were seeded (Fig. 6B). However, when 1000 cells were seeded we noticed that the FAM84B(Δ HRASLS) cells formed significantly more colonies than the EV cells.

Figures 6 C-E show the results of our analysis of the impact of deleting FAM84B's HRASLS domain on MCF7 cell proliferation in different concentrations of FBS-supplemented DMEM media. The EV and FAM84B(Δ HRASLS) cells showed similar patterns of growth throughout all 20 days when cultured in 2% and 5% DMEM. However, this was only the case for the first 14 days when the cells were cultured in 10%

DMEM. After this day the two growth curves began to diverge with the FAM84B(Δ HRASLS) cells proliferating faster than the EV cells. By day 20 the FAM84B(Δ HRASLS) cell number was measured to be significantly higher than the number of EV cells. As shown in Figure 6F, the peak cell densities of the EV and FAM84B(Δ HRASLS) cells throughout the proliferation assays were not significantly different for any of the three FBS concentrations.

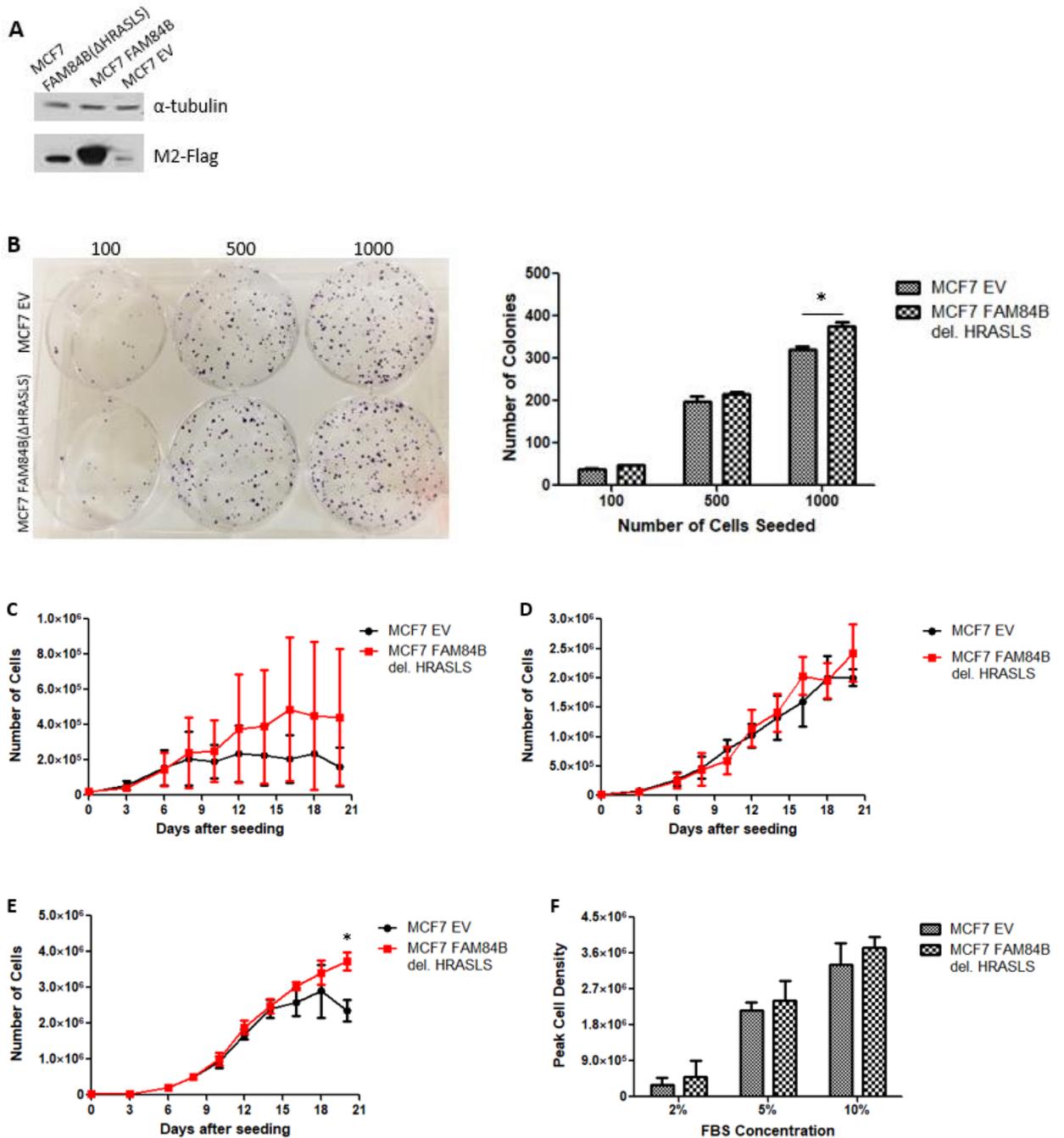


Figure 6. Deletion of *FAM84B*'s *HRASLS* domain enhances MCF7 cell proliferation and colony formation. (A) Stable transfection of MCF7 cells with FAM84B or FAM84B(ΔHRASLS) was examined via western blot analysis using antibodies for M2-

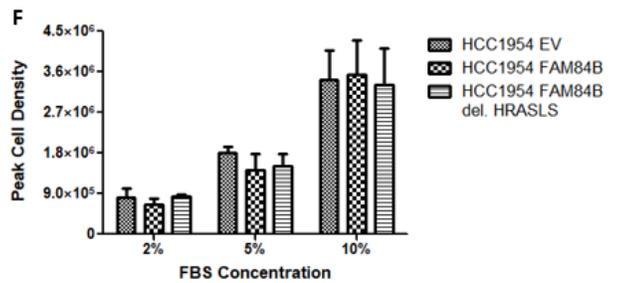
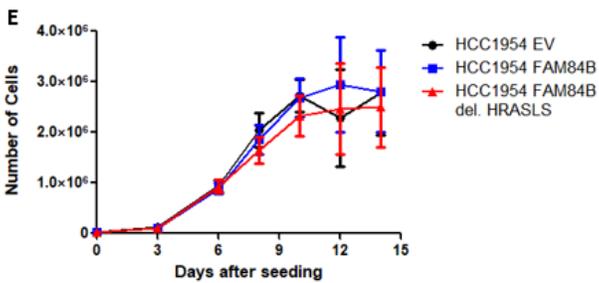
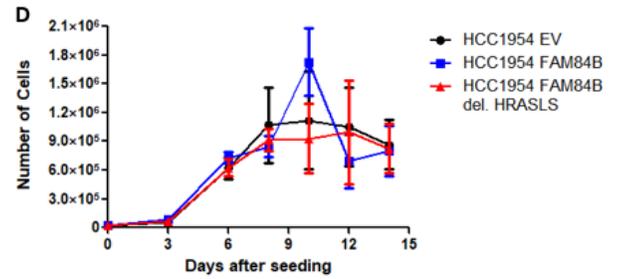
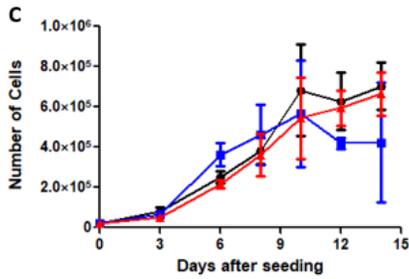
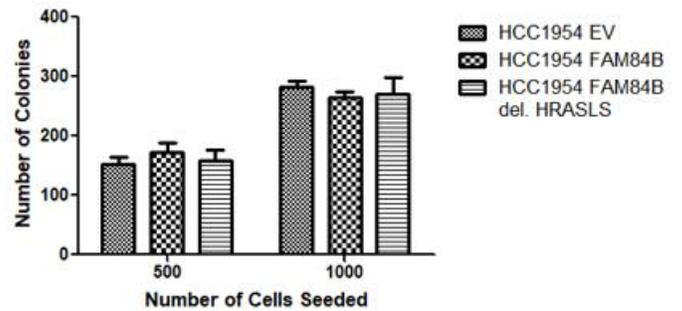
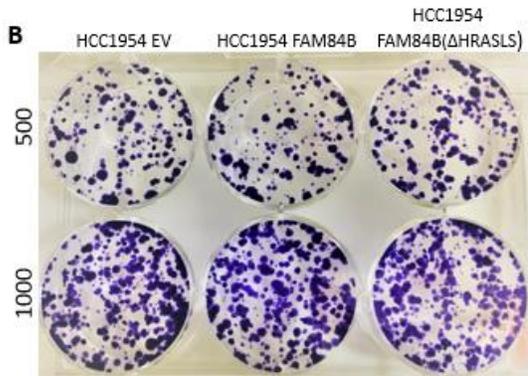
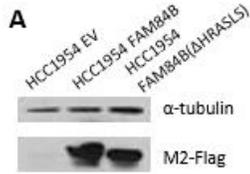
Flag and α -tubulin. (B) 100, 500, and 1000 MCF7 EV and FAM84B(Δ HRASLS) stable cell lines were seeded and cultured for 2 weeks to allow for colony formation. The experiment was repeated 3 times and a typical image from a single repeat is shown. Quantification of the number of colonies for each seeding are displayed with means and standard error graphed; * indicates $p < 0.05$ (Two-way ANOVA with Bonferroni's post-hoc test). (C-E) 2×10^4 MCF7 EV and FAM84B(Δ HRASLS) cells were cultured for 20 days and counted every 2-3 days. The cells were grown in DMEM media supplemented with either 2% (C), 5% (D), or 10% (E) FBS. The x-axes represent the number days after initial seeding of cells and the y-axes represent the number of cells that were counted. The experiments were repeated 3 times and means with standard error are graphed; * indicates $p < 0.05$ for the difference between the MCF7 EV and FAM84B(Δ HRASLS) cell numbers (Two-way ANOVA with Bonferroni's post-hoc test). (F) Average peak cell densities reached by MCF7 EV and FAM84B(Δ HRASLS) cells were compared for each cell proliferation assay representing a different concentration of FBS-supplemented DMEM media; means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV and FAM84B(Δ HRASLS) cells.

3.6 Deletion of FAM84B's HRASLS domain has little impact on oncogenic-related events of HCC1954 cells

To compliment the analysis of the impact on MCF7's *in vitro* oncogenic properties from deletion of FAM84B's HRASLS domain, we performed a similar analysis using HCC1954 cells. We stably expressed the FAM84B(Δ HRASLS) plasmid in these cells and verified the results of the transfection through western blot analysis (Fig. 7A). Once again we used expression of the M2-Flag as an indicator of transfected FAM84B and FAM84B(Δ HRASLS) and we observed that its expression was greater for cells transfected with FAM84B or FAM84B(Δ HRASLS) in comparison to cells transfected with an empty vector (EV). Again, success of the transfection was confirmed based on the molecular weight of M2-Flag being lower in the FAM84B(Δ HRASLS) cells than in the FAM84B cells.

Our examination of the effect of deleting of FAM84B's HRASLS domain on the ability of HCC1954 cells to form isolated colonies *in vitro* showed that there were no significant differences in the number of colonies formed by EV, FAM84B, and FAM84B(Δ HRASLS) cells for both seedings (Fig. 7B). In the context of HCC1954 cell proliferation in different concentrations of FBS-supplemented RPMI 1640, we observed no significant differences in the growth patterns of EV, FAM84B, and FAM84B(Δ HRASLS) cells when cultured in 2%, 5%, or 10% media (Fig. 7 C-E). The peak cell densities of the three cell lines were compared for all three cell proliferation assays and we did not observe any significant differences (Fig. 7F).

The cell invasion assay was used to measure the effect of deleting FAM84B's HRASLS domain on HCC1954 cell invasion. Figure 7G shows typical images of invasive HCC1954 EV, FAM84B, and FAM84B(Δ HRASLS) cells that migrated through either control or matrigel inserts. The EV and FAM84B cells showed very similar levels of invasion, which were both greater than the level of invasion observed for the FAM84B(Δ HRASLS) cells; however, these differences were not statistically significant (Fig. 7H).



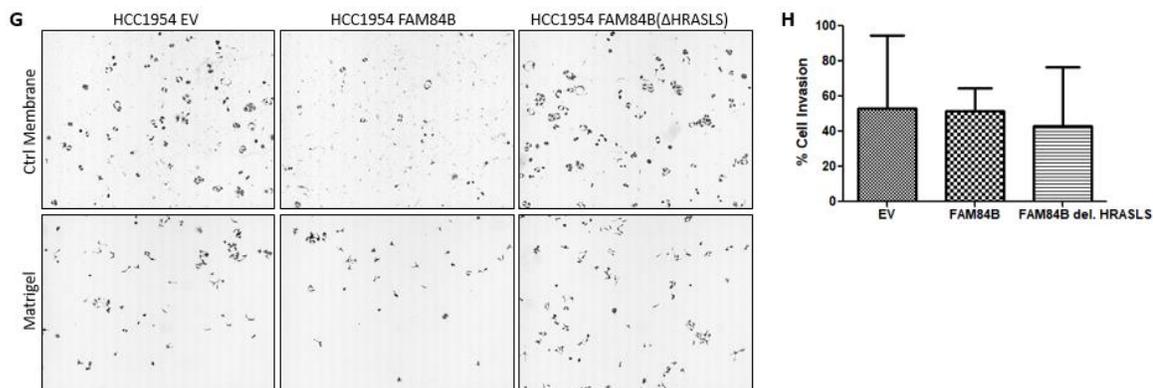


Figure 7. Deletion of *FAM84B*'s *HRASLS* domain has little impact on oncogenic-related events of *HCC1954* cells. (A) Stable transfection *HCC1954* cells with *FAM84B* or *FAM84B(ΔHRASLS)* was examined via western blot analysis using antibodies for M2-Flag and α -tubulin. (B) 500 and 1000 *HCC1954* EV, *FAM84B*, and *FAM84B(ΔHRASLS)* stable cell lines were seeded and cultured for 2 weeks to allow for colony formation. The experiment was repeated 3 times and a typical image from a single repeat is shown. Quantification of the number of colonies for each seeding are displayed with means and standard error graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV, *FAM84B*, and *FAM84B(ΔHRASLS)* cells. (C-E) 2×10^4 *HCC1954* EV, *FAM84B*, and *FAM84B(ΔHRASLS)* cells were seeded and cultured for 2 weeks and counted every 2-3 days. The cells were grown in RPMI 1640 media supplemented with either 2% (C), 5% (D), or 10% (E) FBS. The x-axes represent the number days after initial seeding of cells and the y-axes represent the number of cells that were counted. The experiments were repeated 3 times and means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV, *FAM84B*, and *FAM84B(ΔHRASLS)* cells. (F)

Average peak cell densities reached by HCC1954 EV, FAM84B, and FAM84B(Δ HRASLS) cells were compared for each cell proliferation assay representing a different concentration of FBS-supplemented DMEM media; means with standard error are graphed. Two-way ANOVA with Bonferroni's post-hoc test was used to analyze differences between EV, FAM84B, and FAM84B(Δ HRASLS) cells. (G) 5×10^4 HCC1954 EV, FAM84B, and FAM84B(Δ HRASLS) cells were seeded into control and matrigel inserts, followed by incubation for 48 hours to allow for cell invasion. The experiment was repeated 3 times and typical images from a single repeat are shown. (H) Cell invasion through matrigel was quantified and normalized to the respective cell invasion through the control insert; means and standard error are displayed. One-way ANOVA with Tukey's Multiple Comparison post-hoc test was used to analyze differences between EV, FAM84B, and FAM84B(Δ HRASLS) cells.

3.7 FAM84B expression does not confer resistance to TAM in MCF7 cells

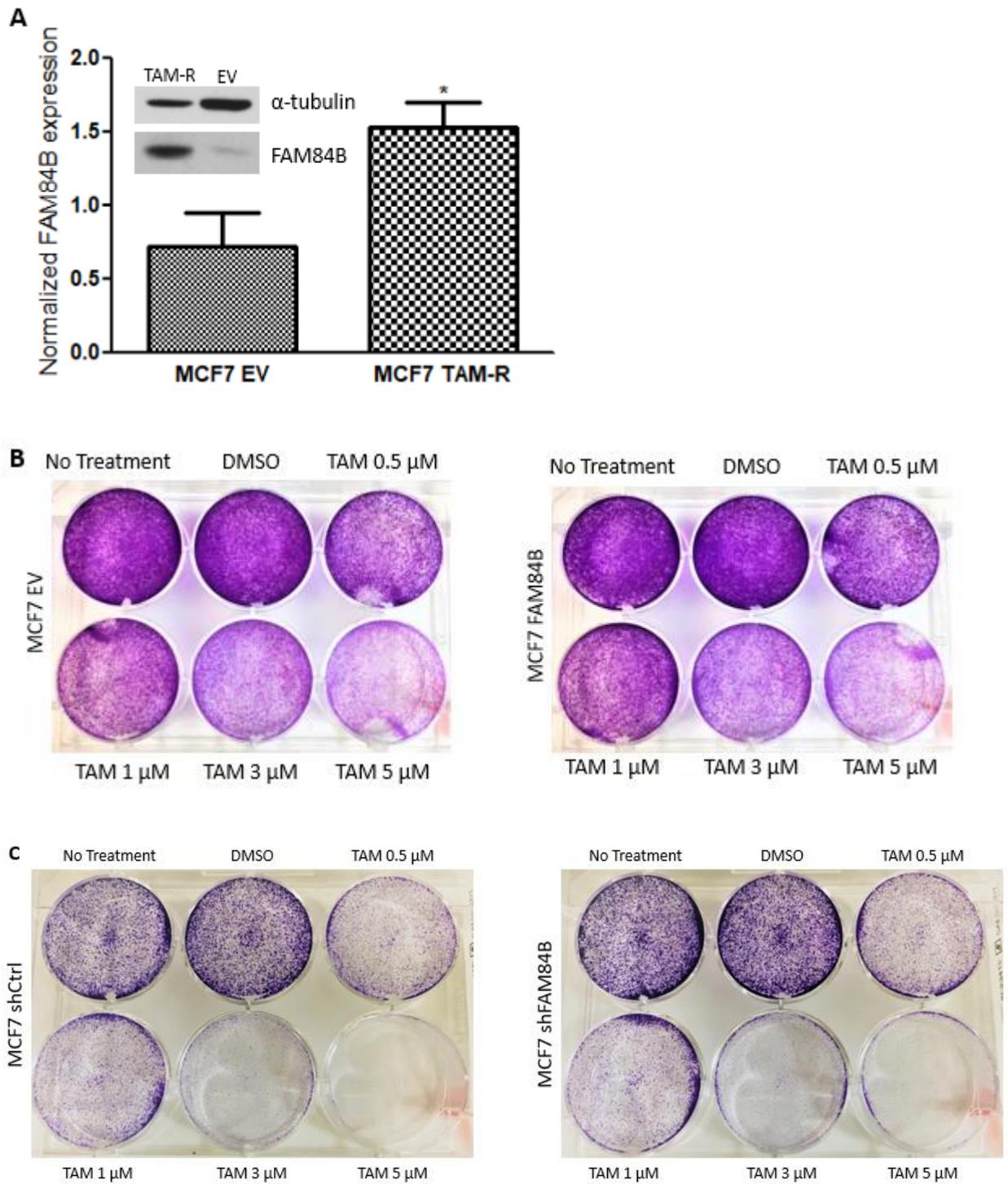
To help elucidate the mechanisms that contribute to TAM resistance in BC, our lab has established a TAM-resistant MCF7 cell line (MCF7 TAM-R). If FAM84B expression has a direct role in facilitating MCF7 TAM resistance, then its expression should be elevated in MCF7 TAM-R cells compared to normal MCF7 cells. We investigated this scenario through western blot analysis of MCF7 EV and TAM-R cell lysate with α -tubulin expression used as a loading control (Fig. 8A). The FAM84B band was more intense in the MCF7 TAM-R lane than the MCF7 EV lane; however, the α -tubulin band intensity was not equivalent for the two cells. Nonetheless, normalization of the FAM84B bands to their respective α -tubulin counterparts, followed by statistical analysis revealed that MCF7 TAM-R cells had significantly higher expression of FAM84B than MCF7 EV cells.

With this result in mind, we investigated the corresponding idea that FAM84B expression having a direct impact on TAM resistance implies that TAM-sensitive MCF7 FAM84B cells demonstrate stronger resistance to TAM treatment than TAM-sensitive MCF EV cells. We seeded MCF7 EV and FAM84B cells into 6-well plates and treated the cells with increasing concentrations of TAM (Fig. 8B). One well was a blank that received no treatment and another was a negative control that was treated with only DMSO, the reagent which TAM was dissolved in prior to treatment. The similar amounts of cell survival in the blank and DMSO wells indicate consistent seeding of the cells and that DMSO had little effect on cytotoxicity. For increasing concentrations of TAM, the

expected trend of decreasing cell survival was observed for both MCF7 EV and FAM84B cells; however, we observed no significant difference in cell survival between the two cell lines.

We repeated this analysis to investigate the complimentary idea that MCF7 cells with reduced expression of FAM84B should demonstrate weaker resistance to TAM than MCF7 cells with baseline expression of the protein. We seeded MCF7 shCtrl and shFAM84B cells into 6-well plates, followed by treatment with increasing concentrations of TAM (Fig. 8C). Again, consistent seeding of the cells was verified by similar amounts of cell survival in the blank and DMSO wells. For increasing concentrations of TAM, the expected trend of decreasing cell survival was observed for both MCF7 shCtrl and shFAM84B cells; however, we observed no significant difference in cell survival between the two cells.

This analysis was used one more time to examine the resulting impact of MCF7 TAM resistance due to deletion of the HRASLS domain from FAM84B's structure. In this case, MCF7 EV and FAM84B(Δ HRASLS) cells were seeded into individual wells of 6-well plates, followed by treatment with increasing concentrations of TAM (Fig. 8D). Consistent seeding of the cells was again verified as previously described. For increasing concentrations of TAM, we observed the same trend of decreasing cell survival for both MCF7 EV and MCF7 FAM84B(Δ HRASLS) cells; however there was no significant difference in cell survival between these two cell lines.



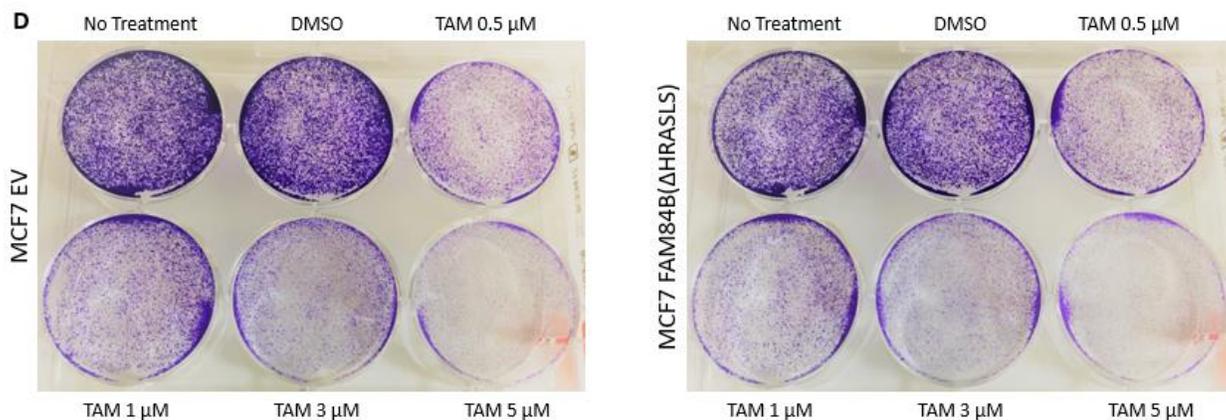


Figure 8. *FAM84B* expression does not confer resistance to TAM in MCF7 cells. (A)

Western blot analysis was used to examine the relative expression of FAM84B in MCF7 EV and TAM-R cells using antibodies for FAM84B and α -tubulin (inset). FAM84B expression was quantified, normalized to α -tubulin expression, and graphed for MCF7 EV and TAM-R cells. Mean and standard error are displayed; * indicates $p < 0.05$ in comparison to MCF7 EV (2-tailed Student's t-test). The experiment was repeated 4 times. (B-D) 10^5 MCF7 EV and FAM84B cells (B), 10^5 MCF7 shCtrl and shFAM84B cells (C), and 10^5 MCF7 EV and FAM84B(Δ HRASLS) cells (D) were seeded and treated with either DMSO, increasing concentrations of TAM, or nothing at all (see Materials and Methods section for details). The experiments were repeated 3 times and typical images from a single repeat are shown.

3.8 FAM84B expression is not associated with TAM resistance *in vivo*

To investigate the association of FAM84B expression with TAM resistance *in vivo*, we performed an IHC analysis of xenograft BC tumours that were derived from either MCF7 EV cells or MCF7 cells with overexpression of BMI1 (MCF7 BMI1). MCF7 BMI1 tumours were shown to be resistant to TAM treatment in comparison to MCF7 EV tumours based on a previous study from our lab (7). Figure 9A shows typical images of FAM84B staining for MCF7 EV tumours, MCF7 BMI1 tumours with no TAM treatment (MCF7 BMI1), and MCF7 BMI1 tumours with TAM treatment (MCF7 BMI1 + TAM). We observed that both types of MCF7 BMI1 tumours showed similar levels of FAM84B expression, which were both slightly higher than the expression of FAM84B in MCF7 EV tumours; however, these differences were not significant (Fig. 9B).

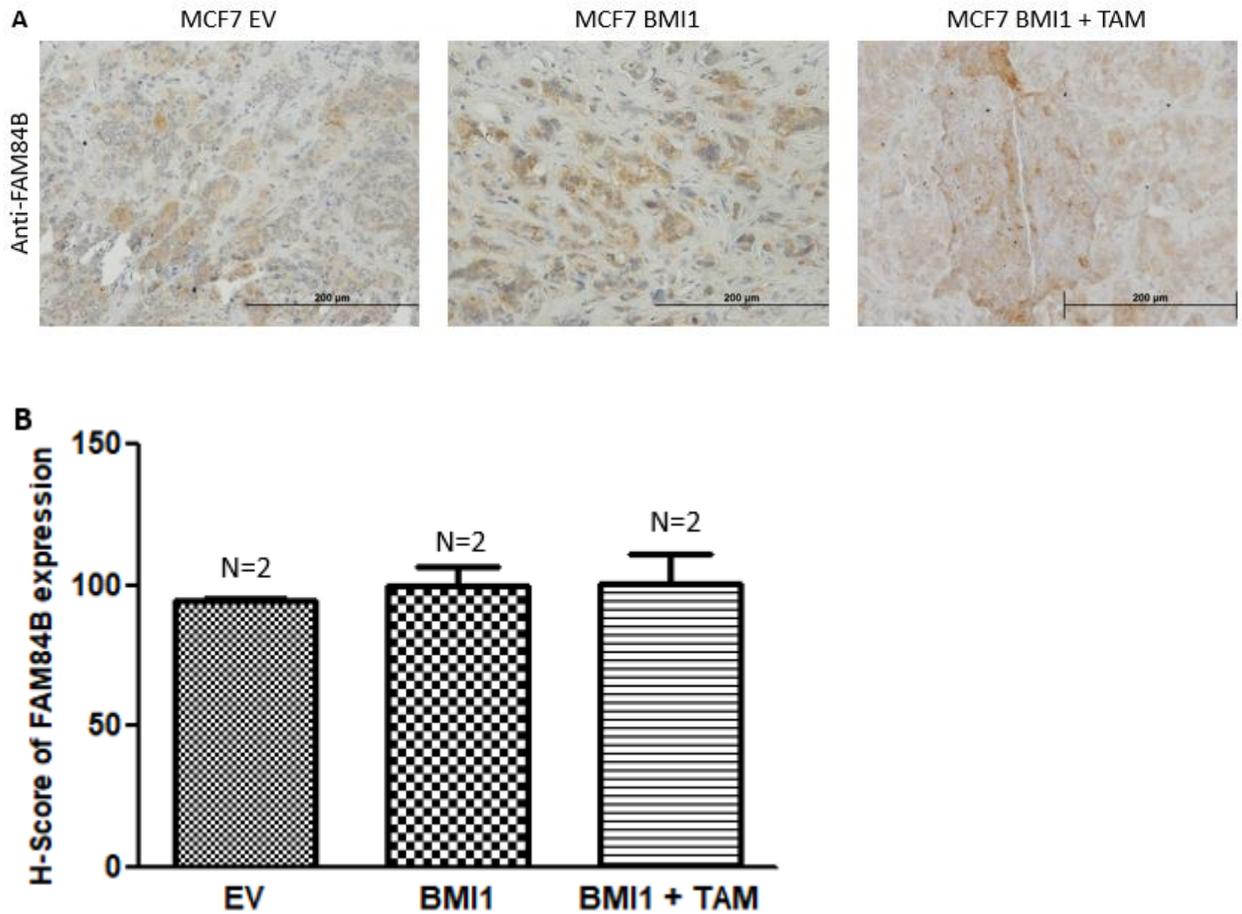


Figure 9. *FAM84B* expression is not associated with TAM resistance *in vivo*. (A) *FAM84B* expression in xenograft tumours produced by either MCF7 EV or BMI1 cells was confirmed by IHC analysis using an antibody for *FAM84B*. The tumours formed by MCF7 BMI1 cells were separated into those that were treated with TAM (MCF7 BMI1 + TAM) and those that had no drug treatment (MCF7 BMI1). The experiment was repeated twice and typical images from a single repeat are shown. (B) *FAM84B* expression was quantified as an H-Score and graphed for tumours formed by MCF7 EV cells (EV), MCF7 BMI1 cells with no TAM treatment (BMI1), and MCF7 BMI1 cells with TAM treatment (BMI1 + TAM); mean and standard error are displayed. One-way ANOVA

with Tukey's Multiple Comparison post-hoc test was used to analyze differences between EV, BMI1, and BMI1 + TAM tumours.

CHAPTER 4: DISCUSSION

The influence of FAM84B expression on tumour development and progression has been described for many cancers, such as ovarian (33, 34), colorectal (33, 37, 38), esophageal (45, 46), and prostate (33, 35, 36, 37); however, similar studies in BC have not been addressed. Due to the high rates of mortality in men and women due to BC (1, 2, 3), there is an increasing demand for research into the mechanisms of BC tumorigenesis and the discovery of new effective treatments. Our goal is to establish the role of FAM84B in BC tumorigenesis and the development of resistance to the SERM Tamoxifen (TAM). This could possibly lead to future research into this protein as a target for the development of new therapeutic drugs.

Since BC is a heterogeneous disease in which cells display varying expression combinations of the ER, PR, and HER2 (4, 5, 6, 7, 8), we were interested in using BC cells of different classifications as a basis for our analysis of FAM84B's role in BC development. MCF7 and T47D are both Luminal A cells due to their ER+, PR+, and HER2- expression profiles (17); as a result, these cells are typically less aggressive in comparison to other BC cell lines and their expression of ER renders them suitable for treatment with TAM (31, 32). The HCC1954 cell line, on the other hand, is classified as HER2+ (17), which means that these cells are comparably more aggressive than other BC cell lines and their absence of ER expression renders them unresponsive to TAM treatment (17, 18, 19, 31).

Our study demonstrates that FAM84B overexpression does not have an impact on oncogenic-related events of BC cells *in vitro*. We compared EV and FAM84B overexpression stable lines for MCF7, T47D, and HCC1954 cells and observed no significant differences when measured for colony formation, cell proliferation, and cell invasion. This is intriguing given the fact that our previous investigation of prostate cancer also showed that FAM84B overexpression had no effect on DU145 cell proliferation *in vitro* (42). However, that same study showed that overexpression of FAM84B significantly increased the invasiveness of DU145 cells in comparison to DU145 EV cells. This inconsistency with our results using HCC1954 cells may likely be attributed to the various differences between the tumour biology of prostate and breast cancer. However, we cannot exclude the possibility that FAM84B enhances *in vitro* oncogenic properties of triple negative BC cells, as my research did not cover this subtype of BC.

To get another perspective of the impact of altered FAM84B expression on MCF7 and HCC1954 oncogenic-related events *in vitro*, we stably knocked down expression of the protein in these cells. In the context of cell proliferation and invasion, reduced expression of FAM84B did not have any significant effects; however, we noticed that the ability of MCF7 cells to form isolated colonies was significantly impaired (Fig. 3B). Interestingly, this result was not observed for HCC1954 cells since those control and knockdown cells formed similar numbers of colonies for each seeding (Fig. 4B). This suggests that FAM84B may be a factor that mediates the viability of MCF7 BC cells and that this function becomes redundant when HER2 expression is positive. Since FAM84B

overexpression had no impact on MCF7 colony formation *in vitro*, this implies that regulation of the protein's function becomes saturated at its baseline expression level. In other words, the maximum rate of FAM84B's function occurs at its baseline level and that increased expression of the protein has no resulting effect. Future studies are needed to determine whether or not this idea is true.

The TMA provided important evidence about the impact of FAM84B expression on BC tumorigenesis *in vivo*. The results demonstrated that the protein's expression was significantly upregulated in cancerous tissue compared to normal breast tissue and that it was associated more with the development of tumours of the breast ducts than the lobules (Fig. 5B). Additionally, FAM84B expression was significantly higher in early developed tumours than late developed tumours for ductal carcinoma (Fig. 5C). This trend was also observed for lobular carcinoma, but the difference was not statistically significant (Fig. 5D); however, it should be noted that the number of cases for lobular carcinoma was limited, which may not support a meaningful statistical analysis. Overall, these results suggest a possible role of FAM84B in promoting early BC development and that its expression decreases as the tumour grows into a more aggressive, metastatic phenotype. This would explain why we did not observe any impact on HCC1954 cell invasion due to altered expression of FAM84B, since invasion and migration are typical properties of cells that have progressed to later stages of BC (48).

The similarities between FAM84B and the HRASLS subfamily of enzymes provide insight towards the structural regions of the former that may be crucial to its

tumorigenic function (42). Thus, we were interested in examining the impact of deleting FAM84B's HRASLS domain on various oncogenic properties of MCF7 and HCC1954 cells. We noticed that the deletion had no significant impact on the invasiveness of HCC1954 cells, which is in contrast to results we observed in prostate cancer where deletion of FAM84B's HRASLS domain significantly reduced the invasiveness of DU145 cells in comparison to cells with overexpression of the wild-type protein (42). As previously mentioned, this inconsistency is likely attributed to biological differences between prostate and breast cancer cells. In terms of its effect on BC colony formation *in vitro*, we noticed that deletion of FAM84B HRASLS domain significantly increased the ability of MCF7 cells to form isolated colonies in comparison to EV cells (Fig. 6B). This was followed by our observation that MCF7 cells expressing the mutated FAM84B protein showed enhanced rates of cell proliferation in 10% DMEM compared to EV cells following day 14, with the FAM84B(Δ HRASLS) cells growing to a significantly higher cell number than the EV cells upon day 20 (Fig. 6E). This provides further evidence towards a possible role of FAM84B as a factor that mediates the viability of BC cells. The HRASLS domain may function in BC to suppress molecular events related to the viability, growth, and division of these cells, which is consistent with the reported function of the HRASLS family as suppressors of H-Ras mediated oncogenic signalling (42, 47). Similar to the results from our analysis of FAM84B knockdown, we also observed that deletion of the protein's HRASLS domain did not have a significant effect on HCC1954 colony formation and cell proliferation. This further supports our notion that FAM84B's function is relevant to MCF7 cells and that it becomes redundant upon

positive expression of HER2. Further research is required to investigate the validity of these claims.

In our analysis of the impact of FAM84B expression on the development of TAM resistance in BC *in vitro*, we noticed that MCF7 TAM-R cells had significantly higher expression of FAM84B than MCF7 EV cells (Fig. 8A); however, we observed no difference in the abilities of TAM-sensitive MCF7 EV and FAM84B cells to resist treatment with increasing concentrations of the drug (Fig. 8B). Similarly, comparisons between MCF7 shCtrl and shFAM84B cells (Fig. 8C), as well as MCF7 EV and FAM84B(Δ HRASLS) cells (Fig. 8D), revealed that there were no differences in the abilities of these cells to resist TAM-derived cytotoxicity. Collectively, these observations suggest that FAM84B expression does not have an impact on the development of TAM resistance in BC; however, increased expression of the protein in BC cells seems to be an event that occurs following the acquisition of resistance.

We continued our analysis of FAM84B's impact on TAM resistance through an *in vivo* model. The xenograft tumours we analyzed were derived from MCF7 cells with overexpression of either an empty vector (EV) or BMI1. One of our previous studies confirmed that the BMI1 tumours were resistant to TAM in comparison to the EV tumours (7). Based on this result, our goal with this analysis was to determine whether or not these TAM-resistant tumours also showed differing levels of FAM84B expression when compared to TAM-sensitive EV tumours. The results showed that these two tumours had similar levels of FAM84B expression and that treatment with TAM did not

change the level of FAM84B expression in the BMI1 tumours (Fig. 9B). This is consistent with the results of our *in vitro* studies and it provides further evidence that FAM84B expression does not have an impact on the ability of BC cells to resist TAM. There were a few limitations to this analysis that should not be ignored; for example, each tumour group had a sample size of two mice which is very low. Ideally, at least five mice per group should be studied to obtain valid results. The differing levels of BMI1 expression between the TAM-sensitive and TAM-resistant tumours is another variable that could possibly affect our observed results. BMI1 is a component of the polycomb repressive complex 1 and it has reported oncogenic activity through repression of tumour suppressors of the INK4A-ARF locus (7, 49). There have not been any reports of a connection between BMI1 and FAM84B expression, although upregulation of the former correlates with increased expression of c-Myc (7, 49). Nonetheless, it would be ideal to perform this same analysis using TAM-resistant xenograft tumours without overexpression of a separate gene.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The results of this study provide evidence towards a possible role of FAM84B in promoting events that maintain the viability and stimulates the growth and division of MCF7 cells. This idea is supported by observations of these cells showing impaired capabilities of forming isolated colonies *in vitro* when FAM84B expression was knocked down. The enhancement of MCF7 colony formation and cell proliferation that resulted from deletion of FAM84B's HRASLS domain also support this notion and provides some insight towards an anti-tumorigenic function of this domain in BC. Since the oncogenic properties of HCC1954 cells were not affected by either FAM84B knockdown nor deletion of its HRASLS domain, it is possible that the presence of HER2 masks any manipulations to FAM84B.

The TMA results provide valuable evidence towards a possible role of FAM84B in directing events associated with the early developmental stages of BC. This idea is supported by our observations that FAM84B expression had no impact on HCC1954 cell invasion, which is a property that is typically associated with BC at later stages of progression.

We also demonstrate that FAM84B does not have an impact on the development of TAM resistance in BC based on the results of our *in vitro* and *in vivo* experiments. It seems more likely that increased FAM84B expression in BC cells is an event that occurs following the acquisition of TAM resistance.

Future studies will be conducted to improve our understanding of FAM84B's role in BC tumorigenesis and TAM resistance. To build on our idea of FAM84B promoting events associated with enhancing the viability of BC cells we will perform other experiments that measure this property such as the growth of isolated colonies in soft agar. We will also use Luminal B and triple negative BC cells to examine more closely the relationship between FAM84B and HER2 to determine whether the latter actually functions to mask any changes to the former. In addition, we are interested in examining the effect of FAM84B expression on the activity of different tumorigenic signalling pathways, such as AKT and MAPK. We are particularly interested in its interaction with members of the Ras family based on the possibility that FAM84B's HRASLAS domain has an anti-tumorigenic function that may suppress H-Ras mediated oncogenic signaling (42).

To build on our other idea of FAM84B directing events associated with early BC development, we will examine the impact of altered FAM84B expression on various multi-gene signatures representing different stages of BC progression. We will also improve the *in vivo* study of FAM84B's impact on TAM resistance by increasing the sample size of each tumour group to at least five mice. Instead of analyzing TAM-resistant tumours that were derived from BMI1 overexpression in MCF7 cells, we will use our culture of TAM-resistant MCF7 cells to form xenograft tumours and then perform an IHC analysis of FAM84B expression. Additional *in vivo* studies will investigate the ability of BC cells with either overexpression or knockdown of FAM84B to form xenograft tumours.

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