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# The Elov15 enzyme in breast cancer : regulation of cancer cell proliferation and metastasis

Trinh-Le-Vi Kieu

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**THESE DE DOCTORAT DE L'ETABLISSEMENT UNIVERSITE BOURGOGNE FRANCHE-COMTE  
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**Doctorat des Sciences de la Vie et de la Santé**

Par

**Mlle Trinh Le Vi KIEU**

# **L'enzyme Elovl5 dans le cancer du sein : régulation de la prolifération des cellules cancéreuses et du processus métastatique**

Thèse présentée et soutenue à Dijon le 15 décembre 2021

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## Résumé

Le cancer du sein est le cancer le plus diagnostiqué et la principale cause de décès par cancer chez les femmes. Le taux de survie à 5 ans est inférieur à 30 % pour le cancer du sein métastatique et les métastases représentent plus de 75 % des décès par cancer du sein. Le métabolisme des acides gras est altéré dans les cancers et contribue à la progression tumorale. Nous nous sommes intéressés au rôle de l'enzyme Elovl5 qui catalyse l'élongation des acides gras à longue chaîne par l'ajout de deux carbones. Nous avons observé que les tissus tumoraux de patientes atteintes d'un cancer du sein avaient une expression d'Elovl5 plus faible que le tissu du sein normal apparié. De plus, une faible expression d'Elovl5 est associée à un mauvais pronostic chez les patientes atteintes d'un cancer du sein de sous-type luminal (ER+) ou basal. Conformément à cette observation, nous avons montré que la diminution de l'expression d'Elovl5 est plus prononcée dans les tumeurs mammaires ER+ de patientes présentant des métastases dans les ganglions lymphatiques. Bien que la régulation négative d'Elovl5 limite la prolifération des cellules cancéreuses du sein et la progression tumorale, nous avons démontré que la répression de l'expression d'Elovl5 favorisait la transition épithélio-mésenchymateuse et le développement de métastases pulmonaires dans des modèles murins de cancer du sein. Une répression de l'expression d'Elovl5 dans les cellules cancéreuses du sein conduit à une augmentation de l'expression des récepteurs au TGF- $\beta$  induite par une accumulation de gouttelettes lipidiques. L'apparition de ces gouttelettes lipidiques est supprimée par un blocage de la synthèse des triglycérides avec des inhibiteurs pharmacologiques des enzymes DGAT1/2 et cette suppression a levé l'induction de l'expression des récepteurs au TGF- $\beta$ , de l'EMT et de l'invasion cellulaire activée par la répression de l'expression Elovl5. Au total, nous avons montré que l'expression de l'enzyme Elovl5 est un marqueur prédictif du risque de développement de métastases dans le cancer du sein et que cette enzyme régule le processus métastatique en modulant l'expression des récepteurs au TGF- $\beta$  induite par les gouttelettes lipidiques.

**Mots clés :** cancer du sein, élongation des acides gras, métastases, gouttelettes lipidiques, TGF- $\beta$ , Elovl5

## Abstract

Breast cancer is the most diagnosed cancer and the leading cause of cancer death in women. The 5-year relative survival rate is less than 30% for metastatic breast cancer and metastases account for more than 75% of breast cancer deaths. Fatty acid metabolism is altered in cancer and contributes to tumor progression and metastasis. Here, we were interested in Elongation of very long chain fatty acids protein 5 (Elovl5) which catalyses the elongation of long-chain fatty acids. We observed that the tumors from patients with a breast cancer had a lower expression of Elovl5 than paired normal breast tissue. However, low expression of Elovl5 is associated with a worse prognosis in breast cancer patients with a luminal (ER+) or basal-like subtype. In accordance with this finding, we showed that the decrease of Elovl5 expression is more pronounced in ER+ breast tumors from patients with metastases in lymph nodes. Although Elovl5 downregulation limits breast cancer cell proliferation and cancer progression, we demonstrated that repression of Elovl5 expression promoted EMT and lung metastases in murine breast cancer models. A repression of Elovl5 expression in breast cancer cells led to an upregulation of TGF- $\beta$  receptors mediated by an accumulation of lipid droplets which is suppressed by a blockade of triacylglycerol synthesis with pharmacological drugs inhibiting DGAT1/2. Interestingly, the abolition of Elovl5-regulated lipid droplet formation reversed the induction of TGF- $\beta$  receptors, EMT and cell invasion. Altogether, we showed that Elovl5 expression is a predictive marker for the risk of metastases development in breast cancer and that Elovl5 regulated the metastatic process through modulation of the expression of TGF- $\beta$  receptors mediated by lipid droplets.

**Keywords:** breast cancer, fatty acid elongation, metastasis, lipid droplets, TGF- $\beta$  Elovl5

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# Abbreviations

ABC	Advanced breast cancer
ABP	Albumin binding protein
ACAT	Acyl-CoA cholesterol acyltransferase
ACC	Acetyl-coenzyme A carboxylase
ACP	Acyl-binding site
ACS	Acyl-CoA synthase
ACSL	Acyl-CoA synthetase long-chain
AGPAT	Enzyme 1-acylglycerol-3-phosphate-O-acyltransferase
AI	Aromatase inhibitor
Akt	AKT Serine/threonine-protein kinases
ALA	Alpha-linolenic acid
ALK	Anaplastic lymphoma tyrosine kinase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AON	Antisense oligonucleotide
ARID1A	AT-rich interactive domain-containing protein 1A
ATF	Activating transcription factor
ATGL	Adipose triglyceride lipase
ATM	Ataxia-telangiectasia mutated Serine/Threonine Kinase
ATP	Adenosine Triphosphate
AURK	Aurora kinase activity
Bcl2	B-cell lymphoma-2
BLBC	Basal-like breast cancer
BMDC	Bone marrow dendritic cell
BRCA1	Breast cancer type 1
BRCA2	Breast cancer type 2
CAD	Acyl-CoA dehydrogenase/ Fatty acids dehydrogenase
CAF	Cancer-associated fibroblast
CBFB	Core-Binding Factor Subunit Beta
CCL	Chemokine ligands
CCNB1	Cyclin B1
CD36	Cluster of differentiation 36
CDH1	E-Cadherin
CDK	Cyclin-dependent kinases
CDKN1B	Cyclin Dependent Kinase Inhibitor 1B
CE	Cholesteryl esters
CHEK2	Checkpoint Serine/threonine kinase 2
CIN	Chromosomal instability
CoA	Coenzyme A
CPT	Carnitine-palmitoyltransferase
CSF-1	Colony stimulating factor 1

CTC	Circulating tumour cells
CTCF	CCCTC-binding factor
CTGF	Connective tissue growth factor
CXCL	C-X-C Motif Chemokine Ligand
DAP	Death-associated protein kinase
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
DFI	Disease-free interval
DGAT	Diacylglycerol acyltransferases
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DTC	Disseminated tumor cell
EBP $\beta$	Enhancer-binding protein $\beta$
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELOVL	Elongation of Very Long chain Fatty acid
EMT	Epithelial to mesenchymal transition
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ET	Endocrine therapy
ETF	Electron-transferring factor
EV	Extracellular vehicle
FA	Fatty acid
FABP	Fatty acid binding protein
FAO	Fatty Acid $\beta$ -oxidation
FASN	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transportation protein
FGFR	Fibroblast growth factor receptor
FIT	Fat storage-inducing transmembrane proteins
FOXA	Forkhead Box A
FOXO	Forkhead box protein O
G3P	Glycerol- 3-phosphate
GARP	Glycoprotein A repetitions predominant
GATA3	Globin transcription factor
GPAT	Sn-glycerol-3-phosphate acyltransferases
GSC	Glioma stem cells
HACD	3-HydroxyAcyl-CoA dehydratase
HER2	Human epidermal growth factor receptor 2
HEV	High endothelial venule
HIF	Hypoxia-inducible factor

HPC	Haematopoietic progenitor cell
HSL	Hormone sensitive lipase
Id	Inhibitor of differentiation
IDO	Indoleamine-2,3-dioxygenase
IFN $\gamma$	Interferon-gamma
IGF-1R	Insulin-like growth factor receptor 1
ILs	Interleukins
JAK2	Janus Kinase
JNK	c-Jun N-terminal kinases
KAR	3-KetoAcyl-CoA reductase
LCFA	Long-chain Fatty acid
LCIS	Lobular carcinoma in situ
LD	Lipid droplet
LEC	Lymphatic endothelial cells
LGL	Lethal giant larvae
LN	Lymph node
LOX	Lysyl oxidase
LPA	Lysophosphatidic
MAPK	Mitogen-activated protein kinase
MCFA	Medium-chain Fatty acid
MDSC	Myeloid-derived suppressor cell
MEK	Mitogen-activated protein kinase kinase
MET	Mesenchymal–epithelial transition
MGAT	Monoacylglycerol acyltransferase
MGL	Monoacylglycerol lipase
MH1	MAD homology 1 domain
MMP	Matrix metalloproteinases
MMTV	Mouse Mammary Tumor Virus
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
MTP	Mitochondrial trifunctional protein
MUFA	Monounsaturated Fatty acids
MYBL2	MYB proto-oncogene like 2
MYC	MYC Proto-Oncogene
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NBL1	Neuroblastoma suppressor of tumorigenicity 1
NCOR1	Nuclear Receptor Corepressor 1
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NF1	Neurofibromin 1
NK	Natural killer cell
NO	Nitric oxide
PAP	Phosphatidate phosphatase

PARP	Poly (ADP-ribose) polymerase
PATJ	Associated tight junction protein
PD-1	programmed cell death protein-1
PD-L1	programmed cell death ligand 1
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinases
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PKC	Protein kinase C
PLIN	Perilipin
PPAR	Peroxisome proliferator-activated receptor
PR	Progesteron receptor
PTEN	Phosphatase And Tensin Homolog
PUFA	Polyunsaturated Fatty acid
RAF	Rapidly Accelerated Fibrosarcoma
RAS	Rat sarcoma virus
RUNX1	Runt-related transcription factor 1
SCD	Stearoyl-CoA desaturase
SCFA	Short chain Fatty acid
SFA	Saturated Fatty acid
SKI	Small-molecule kinase inhibitor
SLUG	Snail C2H2-type zinc-finger protein
SNAIL	Zinc finger protein SNAI1
SREBP	Sterol regulatory element-binding transcription factor
STAT3	Signal transducer and activator of transcription 3
TAG	Triacylglycerols
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophils
TBX3	T-box transcription factor
TDSF	Tumor-derived suppressor factor
TER	2,3-Trans-enoyl-CoA reductase
TGF $\beta$	Transforming growth factor $\beta$
TGF $\beta$ R	Transforming growth factor $\beta$ receptor
TIE	TGF $\beta$ inhibitory element
TIEG	TGF- $\beta$ -inducible early response gene
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment
TNBC	Triple negative breast cancer
TNF $\alpha$	Tumor necrosis factor alpha
TP53	Tumor antigen p53
TWIST	Twist-related protein 1
UPC1	Mitochondrial uncoupling protein 1
VCAM-1	Vascular cell adhesion protein 1

VEGF	Vascular Endothelial Growth Factor A
VIM	Vimentin
VLCF	Very-long-chain Fatty acid
WNT	Wingless/Integrated
YAP	Yes1-associated transcriptional regulator
ZEB	Zinc finger E-box-binding homeobox

# **PART 1 Introduction**

# Chapter 1 Cancer

## 1 Cancer definition and history

The word “Cancer” has been originated from an ancient Greek word meaning “a crab” and is used to describe the resemblance of tumor and surrounding swollen veins as the crab limbs. Back to the history, Hippocrates (460-377 before JC) firstly used the term “carcinosis”, then Celsus (25 BC-50 AD) translated from “carcinosis” to “cancer”. In the period 131-201 after JC, Galien used the term “oncos” to indicate malignant tumor.

In present, the World Health Organization (WHO) defines cancer as “Cancer is a large group of diseases that can start in almost any organ or tissue of the body when abnormal cells grow uncontrollably, go beyond their usual boundaries to invade adjoining parts of the body and/or spread to other organs. The latter process is called metastasizing and is a major cause of death from cancer. A neoplasm and malignant tumour are other common names for cancer.”

## 2 Breast cancer

### 2.1 Breast cancer epidemiology

In 2020, Globocan reported that 2.26 million of women were diagnosed with breast cancer, representing 24.5% of total cancer cases. With almost 685 000 deaths worldwide, breast cancer is the first cause of women cancer mortality, accounted for 15.5 % cancer death (Figure 1) (Sung et al. 2021)

These data indicated that breast is one of the most common cancers in the world; women suffered from breast cancer have more lost in disability-adjusted life years (DALYs) than any other cancer types. The risk of breast cancer increases with patient age. From 1930s to 1970s, the mortality of breast cancer was slightly changed. The survival rate is beginning to improve in 1980s due to the early detection and invasive cancer treatment, however, the cancer death rate is still not significantly modified due to high number of cases (R. L. Siegel, Miller, and Jemal 2020) In worldwide, the socioeconomic inequalities and geographical distribution stably correlate with breast cancer incidence and mortality (Heer et al. 2020).

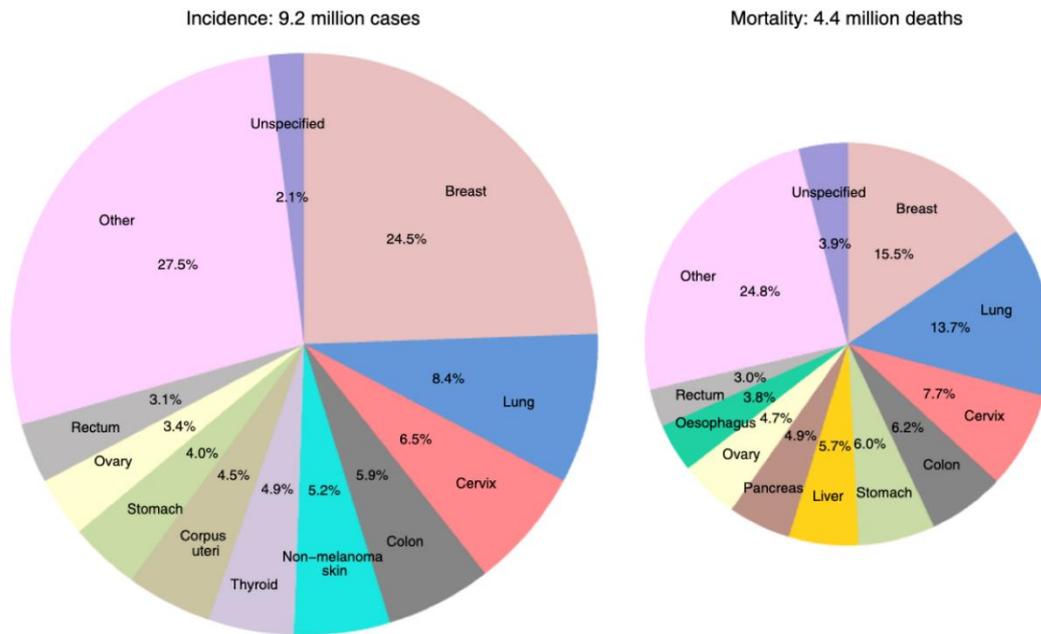


Figure 1: Percentage of most common cancers cases (left) and deaths (right) in female in 2020 (Sung et al. 2021)

## 2.2 Breast cancer definition, etiology and classification

### 2.2.1 Definition

Breast cancer is early established from breast tissue, mainly in the lobules or the ducts, due to the uncontrollable growth of breast cells, results in breast tissue characteristic modifications such as lump detection, skin irritation, bleeding, size and shape modification, swollen lymph nodes and painful situation (Harbeck et al. 2019).

At the beginning, the disease starts as non-invasive breast cancer like lobular carcinoma in situ (LCIS) or ductal carcinoma in situ (DCIS), then the breast cancer cells spread into neighboring breast stroma as primary invasive breast cancer and finally, the breast cancer can metastasize to draining lymph nodes and other distant organs (Quezado and Merino 2010). Unlike benign breast disorder, such as fibroadenoma or hyperplasia, advanced or metastatic breast cancer is considered as poor prognosis to patient due to the treatment resistance and unpredictable metastasis (Feng et al. 2018).

### 2.2.2 Etiology

Breast cancer etiology is one of the most complicated among different types of cancers and can be defined as sporadic or hereditary cancers.

Sporadic cancers represent approximately 90-95% of cancer cases; result from multiple spontaneous (non-inherited) genomic changes in somatic cells or hormone metabolism due to environmental factors.

Hereditary cancers represent almost 5-10% of breast cancer cases and result from germline mutations (Harbeck et al. 2019)

### 2.2.3 Sporadic cancers risk factors

More than 90% of breast cancer occur in women with no identified genetic predisposition factors. The evidence of environmental risk factor is determined by different studies, which show the movement of low-breast cancer migrates population from their countries to high-risk countries leading to the increase of their breast cancer risk (Hiatt and Brody 2018). The well-known environmental risks are including endocrine disruptors (contraceptives, diethylstilbestrol, polychlorinated biphenyls), obesity, physical activity, smoking and nutrition factors (diet and alcohol consumptions). The relationship between these risk factors and breast cancer shows the potential for disease prevention by a healthy lifestyle promotion (Feng et al. 2018)

#### *a) Endocrine disruptors*

Endocrine disruptors (synthetic hormones) are DES (diethylstilbestrol) or polychlorinated biphenyls (PCBs), oestrogen and progesterone in various pesticides, detergents, fuels, plastics, prescription drugs, contraceptives or other chemical synthetic solvents. For instance, the consumption of contraceptives is significant increase in breast cancer patients, even at low-dose formulation (Busund et al. 2018) Dangerously, these chemicals can be transferred into utero during pregnancy. For example, the daughter of women who consumed or exposed DES during gestation have more than two times the risk of developing breast cancer than other women in their age brackets who were not exposed to DES (Newbold, Padilla-Banks, and Jefferson 2006).

#### *b) Physical activity*

Physical activity is the external risk factor that is frequently evaluated in breast cancer prevention. The relationships between physical activity and premenopausal breast cancer risk can be due to the reduction of body-fat weight and estrogen levels (Ballard-Barbash et al. 2009). However, there is still no evidence that can specifically highlight the molecular mechanism related to physical activities and breast cancer risks development. Therefore, a precise understanding the interactions of physical activity and breast cancer would provide the opportunities for prevention.

*c) Smoking*

More than 20 carcinogens components are detected in the smoke of tobacco. Concerning the direct smoker, these substances are often detected in breast fluid and tissue of smoking-women (IARC 2004; Morabia 2002). From the database of International Agency for Research on Cancer (IARC), the molecular modifications of smoking-induced breast cancer are identified, which is due to the slow acetylation of N-acetyltransferase 2 slowing metabolism and detoxification of these carcinogens in active smokers (Secretan et al. 2009; Johnson et al. 2011). Concerning secondhand smokers, environmental tobacco smoke also increases breast cancer risk, which may also associate to N-acetyltransferase 2 slow acetylator or glutathione S-transferase Theta 1 low-expression genotypes (Ambrosone et al. 2008; Terry and Goodman 2006). All the studies are concerning only women. However, mechanisms by which environmental smoke might involve to early cancer development remain to be further determined.

*d) Nutrition factors (diet and alcohol consumption)*

Alcohol consumption is one of the most well-studies risk factors in breast cancer. The daily consumption of 10 g alcohol in both premenopausal and postmenopausal adult woman leads to a 7–10% increase in breast cancer risks (Secretan et al. 2009; Gladen, Ragan, and Rogan 2000; Gore et al. 2015). In fact, alcohol consumption has higher effect on risk of ER+/PR+ than ER-/PR- malignancies (R. Suzuki et al. 2008). These researches are confirmed on invivo experiments, in which, high exposure to alcohol corresponds to increase of tumorigenesis (Oyesanmi et al. 2010). However, the mechanism of action is remaining unclear, at this moment, the possible explanation is high alcohol consumption leads to genotoxin formation (i.e acetaldehyde) or regulates hormones and its receptors (Oyesanmi et al. 2010; Ambrosone et al. 2008).

Nutrition inducing breast cancer would account for 35% of cancer cases (Kotepui 2016). In 2008, a study of European Prospective Investigation into Cancer and Nutrition on 319 826 women indicated the positive correlation between saturated fatty acid consumption (SFA) and breast cancer risk (Sieri et al. 2008). In addition, diets enriched in high consumption of myristoleic, erucic acids, palmitic, margaric, linoleic acid, and stearic acids lead to the increase of breast cancer risk (Thiébaud et al. 2009). In contrary, polyunsaturated fatty acids (PUFA) n-3 from fish oil including eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) has been described to prevent breast cancer risk (Sczaniecka et al. 2012). Furthermore, low consumption of n-3 PUFA and high consumption of n-6 PUFA increase the risk of breast cancer (Murff et al. 2011). Another important point is the origin of fatty acids, which also have effects on breast cancer risk. For instance, alpha-linolenic acid (ALA) originated from fruit and vegetable oil decreases breast cancer risk while ALA from nut mixes and salty foods increases

this risk (Thiébaud et al. 2009; Gerber 2012). There are some explanations for this observation, the first reason is the nut mixes are as aperitifs items, therefore, the study population cannot exclude the residual confounding data by alcohol consumption. However, the molecular mechanism showing direct biological effect on ALA and nut in breast cancer occurrence has not been clarified.

#### *e) Obesity*

Obesity is determined by body mass index (BMI), which is associated with an elevated risk of postmenopausal breast cancer. The obesity patient has a BMI > 30 kg / m<sup>2</sup>. The alteration of lipid metabolism is widely observed in these patients since obesity is related to the accumulation of adipose tissue. In general, adipose tissue belongs to endocrine system, which is the energy pool and releases adipokines such as leptin, adiponectin, visfatin, resistin, apelin, etc. Therefore, abnormal levels of adipose tissue negatively impact on the body metabolism, lead to the development of metabolism related diseases (Singla, Bardoloi, and Parkash 2010).

The evidence suggests the ethnicity have strong impact on obesity-induced breast cancer. The Asian women responses stronger to the effects of obesity on breast cancer aggressiveness than African, American and non-white Hispanic women, especially when comparing in subtypes and menopausal status (Graham et al. 2001; Hart et al. 2016). In addition, 18% of premenopausal women (African-American) in the United States with high BMI and breast density can benefit from lifestyle modified-weight loss; however, this benefit is not applied to premenopausal women, especially in Asian population (Hiatt 2008).

In breast cancer patients with obesity, the disease-free and overall survival is much worse than these indexes in non-obese women with breast cancer. These patients also have less response to local and systemic therapies, especially endocrine therapy (K. Lee et al. 2019). The explanation for this problem is the chronic inflammation, which is widely observed together with the secretion of growth factors, cytokines, chemokines (i.e IL-6, IL-8, TNF $\alpha$ , CSF-1 and leptin). For example, pro-inflammatory interleukins IL-6 and IL-8 are detected in triple negative breast cancer (TNBC) patients (with obesity), suggest the highly aggressive phenotype (Nickel et al. 2018). Other releasing pro-inflammatory cytokines and chemokines such as TNF $\alpha$  or CSF-1 involved in inflammation also associate with cancer and tumor progression (Esquivel-Velázquez et al. 2015). Furthermore, leptin adipokine that is not only known for satiety factor roles but also involved in the activation of pro-tumor signaling pathways such as the PI3K / Akt pathway, the MAPK pathway and JAK2 / STAT3 due to the binding of leptin onto its receptor in breast cancer (Schäffler, Schölmerich, and Buechler 2007).

#### **2.2.4 Genetic predisposition in hereditary cancers**

The most common hereditary modifications in breast cancer patients are breast cancer type 1 (BRCA1-17q21) and type 2 (BRCA2-13q13) gene mutations. Women with individual or combination of genes mutations have higher risk (more than 72%) to be suffered with breast cancer at younger age compared to other women. By studying in a series of Jewish ancestry families, two BRCA1 mutation 185delAG and 5382insC, and one BRCA2 mutation 6174delT were found and called as founder mutation. If a Jewish woman genomic does not contain one of these mutations, a different mutation will be identified instead. The founder mutations have also been determined in Icelandic (BRCA2 999del5) and Polish population (Simard et al. 1994). However, penetrance of the BRCA2-6174delT mutation is identified less often than that of BRCA1-185delAG in breast cancer among all population (Warner et al. 1999). BRCA1 and BRCA2 involve in DNA repair, cell-cycle checkpoint control, protein ubiquitylation and chromatin remodeling, therefore, the treatment of breast cancer issues related to BRCA dysfunction have been discussed in several studies. Both genes are involved in DNA repair by homologous recombination with RAD51 in subnuclear clusters (Scully et al. 1997), thus in BRCA deficient cells, double-strand DNA breaks are remodeled by an error-prone mechanism, result in chromosomal rearrangements. Due to these characteristics, these cells are hypersensitive to the reagents that crosslink DNA strands or breaking double-stranded DNA like cisplatin or mitomycin C (Moynahan, Cui, and Jasin 2001; Tassone et al. 2003). Over the last decades, poly (ADP-ribose) polymerase (PARP) inhibitor is a class of drugs that have elucidated the effectiveness against BRCA1 and BRCA2 positive breast cancer cells in different preclinical models and clinical trials (McCabe et al. 2006). PARP involves in single-strand DNA repair, inhibition of this enzyme leads to the failure of DNA repair and enhancement of double-strand DNA impairment. This phenomenon is particularly damaging to cells with no intact BRCA protein family. For example, breast cancer cells with low heterozygosity contains mutation carriers, lead to cell death. Therefore, PARP inhibitor olaparib exhibits a great potential in metastatic hereditary breast cancer treatment and other agents in the same class can be applied to induce effectiveness of chemotherapies like cisplatin (Fong et al. 2009; Tutt et al. 2010).

Although BRCA demonstrated a high risk of breast cancer development, other gene mutations can be also involved in inherited breast cancers, such as CDH1 gene mutations coding E-cadherin protein. E-cadherin is a cell–cell adhesion protein, which is involved in epithelial differentiation. E-cadherin is a strong tumor suppressor in breast cancer; therefore, partial or complete loss of E-cadherin expression is associated to aggressiveness of breast cancer (poor prognosis, low-response to therapy) (Yoshida et al. 2001). The explanation for this problem is the location of CDH1 on human chromosome 16q22.1. This region is frequently suffered (loss of heterozygosity) in sporadic breast cancer (Cleton-Jansen

2002). In addition, hypermethylation of CDH1 promoter and the overlapping 5' CpG island associate with loss of E-cadherin expression, especially in primary ductal breast cancers (Graff et al. 1995). CDH1 dys-regulation is often detected at early non-invasive stages, results in cellular over-proliferation and promotes epithelial to mesenchymal transition (EMT), which can be a prognosis marker for breast cancer classification (Singhai et al. 2011).

### 3 Molecular classification

Due to key genomic alterations, breast cancer is classified into four main subtypes, including Luminal A, Luminal B, Basal-like, and HER2-enriched which were determined by using genomic-profiling and immuno-staining assay (Perou et al. 2000; Sørlie et al. 2001). The common biomarkers have been used to characterize breast cancer including human epidermal growth factor receptor 2 (HER2), progesterone receptor (PR) and estrogen receptor (ER).

Subtypes	ER	PR	HER2	Metastatic status	Prognosis
<b>Luminal A</b>	+	+/-	-	Low	Good
<b>Luminal B</b>	+	+/-	-	Average	Intermediate
<b>HER2+</b>	-	-	+	High	Poor
<b>Basal like-TNBC</b>	-	-	-	High	Poor

*Table 1: Breast cancer subtypes classification*

The intrinsic subtypes of breast cancer can be used for prognosis (Perou et al. 2000; Therese Sorlie et al. 2003). In detail, luminal A seems to consider as the most favourable outcomes in breast cancer categories, while the worse prognosis belong to luminal B, HER2-enriched, and basal-like phenotype. In clinic, the correlation between these intrinsic subtypes and three biomarkers ER, PR, and HER2 are observed. Luminal A tumor are likely to be ER+, PR+, HER2-. Luminal B tumors tend to be ER+, PR+/- and HER2- (Prat et al. 2013). The HER2-enriched tumors have HER2+/ER- while the basal-like subtype have triple negative characteristic (ER-, PR-, HER2-). Regardless of this correlation, the subtypes cannot be exactly identified based on these markers (Prat et al. 2013). Nevertheless, most of luminal A and B tumor are ER+ and/or PR+ and hence suitable models for endocrine therapy, most HER2-enriched cancers are HER2+ and hence suitable models for anti-HER2 therapies and finally most basal-like tumors are ER-, PR-, and HER2- and therefore possible candidates for chemotherapy regimens (Hoadley et al. 2014). The favorite metastatic sites for breast cancer are the bones, lymph nodes, liver, and lungs (Gote et al. 2021). Breast cancer can promote distant metastatic sites depending on the molecular subtype as presented in Figure 2.

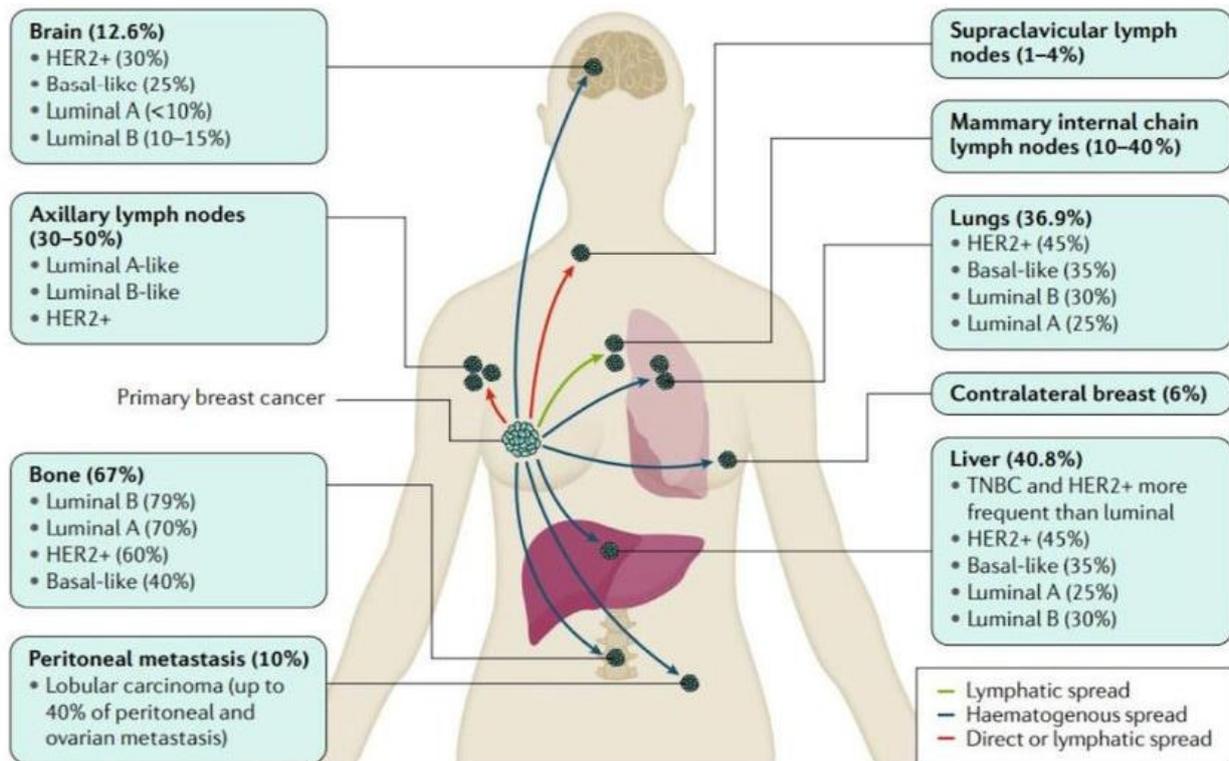


Figure 2: Common metastatic sites of different breast cancer subtypes (Gote et al. 2021)

### 3.1 Luminal A subtype

Among different breast cancer subtypes, luminal A accounts for 50% of total breast cancer cases (Voduc et al. 2010). Luminal A tumor is clinically defined by negative HER2, ER positive with 90% and/ or PR positive with 89% in corresponding patients (Perou et al. 2000). Luminal A tumor further contains low levels of Ki-67, results in moderately tumor growth and represents as an optimistic prognosis (Paik et al. 2006). The common metastatic sites of luminal A patients are brain, lymph nodes, bone, lungs and livers (Figure 2) (Gote et al. 2021).

As the most heterogeneous subtype, luminal A has high genomic instabilities. For example, mutations in TP53 gene up-regulate Aurora kinase activity (AURK). AURK consists of highly conserved serine/threonine protein kinase, which is strongly involved in mitosis and control the cell division (Willems et al. 2018). Additionally, chromosomes 1, 8, and 16 aberrations, PIK3CA, GATA3, AKT1, and MAP3K1 modulations also arise tumor abnormalities (Ciriello et al. 2013). Other genes such as CDH1, MAP3K1, MAP2K4, FOXA1, RUNX1, CBFβ, NBL1, CTCF, NCOR1, PTEN, CDKN1B, TBX3, ARID1A, and NF1 are also mutated with more than 5% per gene among total genomic profile (Hoadley et al. 2014). The highest number of repeatedly mutated genes but the lowest total mutations

number and copy number changes were observed in luminal A tumor, propounding that these alterations tend to be driver mutations (Ciriello et al. 2013).

The treatment with Tamoxifen reduces mortality rate by 31% in ER+ breast cancer, however, more than half of advanced ER+ breast cancers are resistant to this treatment and approximately 40% of the case develop the resistance during treatment (Hultsch et al. 2018). The explanation for this observation is due to genomic alterations, and lipid metabolism. In tamoxifen resistant breast cancer cells, luminal A or luminal B, neutral lipidic droplets and lysosomal free cholesterol are upregulated, together with lysosomal membrane permeabilization (LMP) induction (Hultsch et al. 2018). In addition, tamoxifen induces stemness and promotes metastasis into draining lymph nodes, lung and brain of ER $\alpha$ 36+ (estrogen receptor alpha) breast cancer cells (Q. Wang et al. 2018). Thus, improving current treatments and developing novel therapies associated with this breast cancer phenotype resistance are extremely crucial. The detail treatment plan of this subtype in early and advanced breast cancer will be discussed in the next part.

### **3.2 Luminal B subtype**

Associated with 10-20% of breast cancer cases, luminal B is important in breast cancer (Voduc et al. 2010). Until now, luminal B is determined by highly positive ER (98%) and/or positive PR (82%), together with high expression of proliferative control genes (CCNB1, MYBL2 and Ki-67). Among different luminal B patients, 24% of the case is HER2+ (Tran and Bedard 2011; Cheang et al. 2009). Luminal B tumor are mostly aneuploid with high-level of focal amplifications such as 11q13 cyclin D1 (56%) and 8p11-12 FGFR1 (23%) (Giltane et al. 2017). The common metastatic sites of luminal B patients are brain, lymph nodes, bone, lungs and livers (Figure 2) (Gote et al. 2021).

In 2012, based on genome-wide DNA methylation patterns of Cancer Genome Atlas Network, there is a subset of luminal B with hypermethylated phenotype. Other recurrently mutated genes are also important in luminal B cancer progression such as PIK3CA, GATA3, PTEN, and TP53 (Hoadley et al. 2014). Current studies have highlighted the importance of signaling modification in luminal B, correlated with the treatment efficacy. For example, the activation of insulin-like growth factor receptor 1 (IGF-1R), and loss of PR expression associate with cellular over proliferation and tamoxifen-induced apoptosis resistance (Osborne and Schiff 2011). In addition, fibroblast growth factor receptor 1 (FGFR1), which is involved in cellular capacities such as proliferation and angiogenesis, is highly amplified in PR-negative luminal-B breast cancer. Especially, FGFR1 overexpressing cells is resistant to endocrine therapy, while knockdown FGFR1 or using FGFR tyrosine kinase inhibitor could reverse these negative effects (Turner et al. 2010; Shiang et al. 2010).

Above examples must reflect the mandatory of focusing on the mechanisms, that responsible for luminal B breast cancer development, to constrain inappropriate treatment responses.

Ki-67 proliferation marker protein analysis, and genomic profiling are used to distinguish between Luminal A and Luminal B subtype (Loibl et al. 2021) (Loibl S, 2021). Luminal B tumors show higher proliferation grade with a higher Ki67 index therefore it has poorer outcome compared with luminal A tumors (Correa Geyer and Reis-Filho 2009; Wirapati et al. 2008).

### **3.3 HER2-enriched subtype**

Identifications of different breast cancer groups through genomic profiling highly elucidate the attention to HER2 positive phenotype, which associates with 10-15% of total breast cancers (Voduc et al. 2010). Immuno-staining has defined this hormone-receptor negative phenotypes containing prominent levels of HER2/ERBB2 gene amplification and protein overexpression whereas ER and PR are poorly express (Nielsen et al. 2004). The common metastatic sites of HER-enriched patients are brain, axillary lymph node, bone, lung and liver (Figure 2) (Gote et al. 2021).

HER2-positive tumor is mostly aneuploid with elevated levels of chromosomal destabilization. This type of tumor also has the highest single nucleotide mutation ratio and the common mutated genes are TP53 (71%) and PIK3CA (35%) (Cancer Genome Atlas Network 2012). Diagnosis with HER2+ cancer phenotype results in rapid tumor growth and metastatic driver signal (Moasser 2007). Approximately 62% of ER- patient have high pathological grade and high brain metastatic rate (Shen et al. 2015) .

### **3.4 Triple negative breast cancer (TNBC) subtype**

Triple negative breast cancer (TNBC) is characterized by lack of ER and PR, HER2 expression (only 8% ER+, 7% PR+ and 7% HER2+), with significantly aggressive properties (Badve et al. 2011). Most TNBC tumors have basal-like phenotype, which is defined by the similar appearance of tumor cells to the outer basal surrounding cells in mammary ducts. At this moment, there is still no precise diagnostic assay has been established yet for TNBC classification in routine practice. About 15-20% of breast cancers are TNBC, in which, basal-like (BL) accounted for the major rate of TNBC with 70–80% and corresponds to high metastatic degree (Harrell et al. 2012; Prat et al. 2013). The common metastatic sites of TNBC patients are lung, liver, bone, breast or chest wall (Gote et al. 2021).

Basal-like breast cancer (BLBC) frequently associated with women at early age, especially in African descent (Huo et al. 2009) and related to BRCA1 germline mutation (Therese Sorlie et al. 2003). Nevertheless, BLBC cells highly express myoepithelial genes and protein such as cytokeratins (5/6,

14 and 17), TP53, P-cadherin, caveolins 1 and 2, EGFR, p16, p53 and pRB alteration (Badve et al. 2011; Nielsen et al. 2004). More than 10% patient of this subtype has TP53 mutation, including amplification of 3q, loss of 4q and 5q. In the 12 tumor-type analysis, BLBC creates an identical group, which is highly distinct with another breast cancer (Hoadley et al. 2014). Remarkably, the Ki-67 labeling or mitotic counting of basal-like tumors illustrates great cellular proliferative potential in this phenotype (Badve et al. 2011).

In overall TNBC, BRCA1/2, PI3K/mTOR or RAS/RAF/MEK mutations, and cell-cycle checkpoint signaling alterations are often detected, thus, using the inhibitors of these pathways are the most targeting clinical treatments at this moment. Interestingly, TNBC is tumor micro-environmental heterogeneous, which highly contains tumor-infiltrating lymphocytes (TILs) and programmed cell death ligand 1 (PD-L1) protein expression, suggesting the positive possibilities in chemotherapy and immunotherapy response (Bianchini et al. 2016). Patients, who seriously suffer from BLBC and TNBC, have lower survival rate following faster metastatic probabilities, compared to other subtypes of breast cancer (Dent et al. 2007; Fulford et al. 2007).

#### **4 Breast cancer treatment**

The primary step for breast cancer treatment is subtype identification, which are estrogen receptor positive (ER+), human epidermal growth factor receptor 2 positive (HER2+), and triple negative breast cancer (TNBC, ER-, PR- and HER2-). After that, the evaluation of metastasis is important for the outcome of the treatment. Non-metastatic breast cancer is a good prognosis because the 5-year relative survival rate is almost 90% in women. Metastatic breast cancer is considered as poor outcome because the 5-year relative survival rate reduces to less than 30% for metastatic breast cancer and the median overall survival reaches only 3 years (Redig and McAllister 2013). Thus, evaluating the local or distant metastases of tumor is critical step to establish the possible combination treatments.

In general, the common treatment methods of cancer are surgery, irradiation, chemotherapy, immunotherapy, and hormone therapy. In detail, surgery is a local therapy to remove the tumor and the surrounding tissue including the draining lymph nodes. Irradiation therapy is the loco-regional treatment using ionizing radiation such as X-rays, Gamma rays, electron beams, protons... passing over the skin and superficial tissues to target the tumors and limit cancer cells growth. Chemotherapy is the therapeutic treatment of cancer using medicines or drugs against cancer cells progression through intravenous or oral route; the pharmacology reagents in chemotherapy can be either chemical substances or antibodies. Immunotherapy is the treatment method base on artificial regulation of immune system. Hormone therapy is the treatment that modulates the hormones activities to reduce or

block the growth of cancer cells. The common side effects of breast cancer treatment is widely observed in patients as presented in Table 2. The treatments of chemotherapy, immune therapy and hormone therapy are categorized into the same class on the table because they are all pharmacological molecules delivery into patient bodies. The listed side effects can be observed and visualized by the patient, however the complicated (molecular metabolism side effects) should be predicted by the medical doctors before, during and after treatment. Therefore, it is extremely important to determine the risks and select the best treatment methods.

<b>Treatments</b>	<b>Common side effects</b>
<b>Surgery</b>	Thrombosis, neighbor tissue damage, bleeding, pain, infection, movement impairments, lymphedema...
<b>Radiotherapy</b>	Nerve injury, pain, headache, cachexia, convulsion, numbness, exhausted, vomit, sore throat, hair loss, lymphedema, increase breast density, skin color changes, cardio-respiratory, movement impairments...
<b>Chemotherapy, Immunotherapy, Hormone therapy</b>	Cognitive dysfunction, anxiety, depression, pain, headache, cachexia, hair loss, vomit, anorexia, cardio-respiratory, osteoporosis, thrombosis, hematuria, low blood cell count, oliguria, brittle nails ...

*Table 2: Side-effects of breast cancer treatments*

In breast cancer, the common treatment procedure is combining different therapies. Surgery and irradiation therapies are often applied in all subtypes while chemotherapy treatment strategies are specific for each subtype characteristics.

The locoregional therapy is commonly used in early stage of breast cancer, including breast-preserving surgery and mastectomy and postoperative radiotherapy, followed by the reconstruction of breast structure. In addition, systemic therapy also involves in the decrease of locoregional recurrence (Mannino and Yarnold 2009). For instance, 10 years after the combination of breast-conserving operation and radiotherapy, the locoregional recurrence reach up to 2–3% with ER+, HER2+ breast cancer and 5% with TNBC without any significantly differences from mastectomy (van Maaren et al. 2019; Zumsteg et al. 2013). However, the axillary lymph node surgery is no longer the priority option

in the prevention of lymph node metastasis due to the efficacy of postmastectomy radiotherapy (Donker et al. 2014).

In clinic, postoperative radiotherapy is used to eliminate non detected-tumors during operation including breast-conserving surgery or mastectomy. The treatment locations are the breast, chest wall, drain lymph node with several risk factors in tumor microenvironment. In patients with high risk conditions and low ratio of nodal metastasis, the compensation of axillary surgery via nodal radiotherapy is highly recommended to avoid high-risk side effects (Donker et al. 2014; M. S. Katz et al. 2020). However, current difficulties of radiotherapy are its assimilation and optimization with breast rehabilitate, therefore it is important to identify patients' backgrounds (life quality, historical drug resistance, risk-associated radiotherapy) and select the appropriate treatment program.

#### **4.1 Chemotherapy strategies of ER positive and HER2 negative breast cancer**

Due to the expression of hormone receptor in this subtype, the endocrine therapy is a common standard 5 years treatment program in ER-positive early breast cancer patients. The continuation up to 10 years on the treatment has been demonstrated in many clinical trials, with reduction of the aggressiveness in high risk patients (node-positive, high genomic score) (Burststein et al. 2019). In the premenopause patient with ER+, HER2- and lymph node metastasis breast cancer, the combination of endocrine therapy (ET) and chemotherapy improves 5-year invasive disease-free survival (Kalinsky et al. 2021). The general principles of this treatment depend on ETs types, which can be divided into two main strategies (estrogen depletion and estrogen receptor (ER) targeting).

In cancer cells, estrogen is released from adrenal precursors, testosterone, and androstenedione, which are then converted to estradiol and estrone by aromatase activity (H. Zhao et al. 2016). Therefore, using aromatase inhibitors including anastrozole and letrozole (azole compounds), and exemestane (17-hydroxy steroid) are widely applied. The promising tolerance of these aromatase inhibitors is observed in randomized clinical trials in both the adjuvant and metastasis. The activity, side effects, and toxicity of three compounds are highly identical; therefore, the any one of them can be choose for treatment (Howell et al. 2005; Finn et al. 2016; Y. H. Park et al. 2019). In terms of directly target the ER, two strategies are used to dysregulate ER signaling, including ER modulators (tamoxifen or raloxifene), and selective ER down-regulators (Fulvestrant) with promising clinical trial outcome (Hamilton et al. 2020; Martín et al. 2015). The uses of these molecules are alone or in combination with other targeted agents. The detail about ER+ breast cancer treatment can be views in Figure 3 (Loibl et al. 2021).

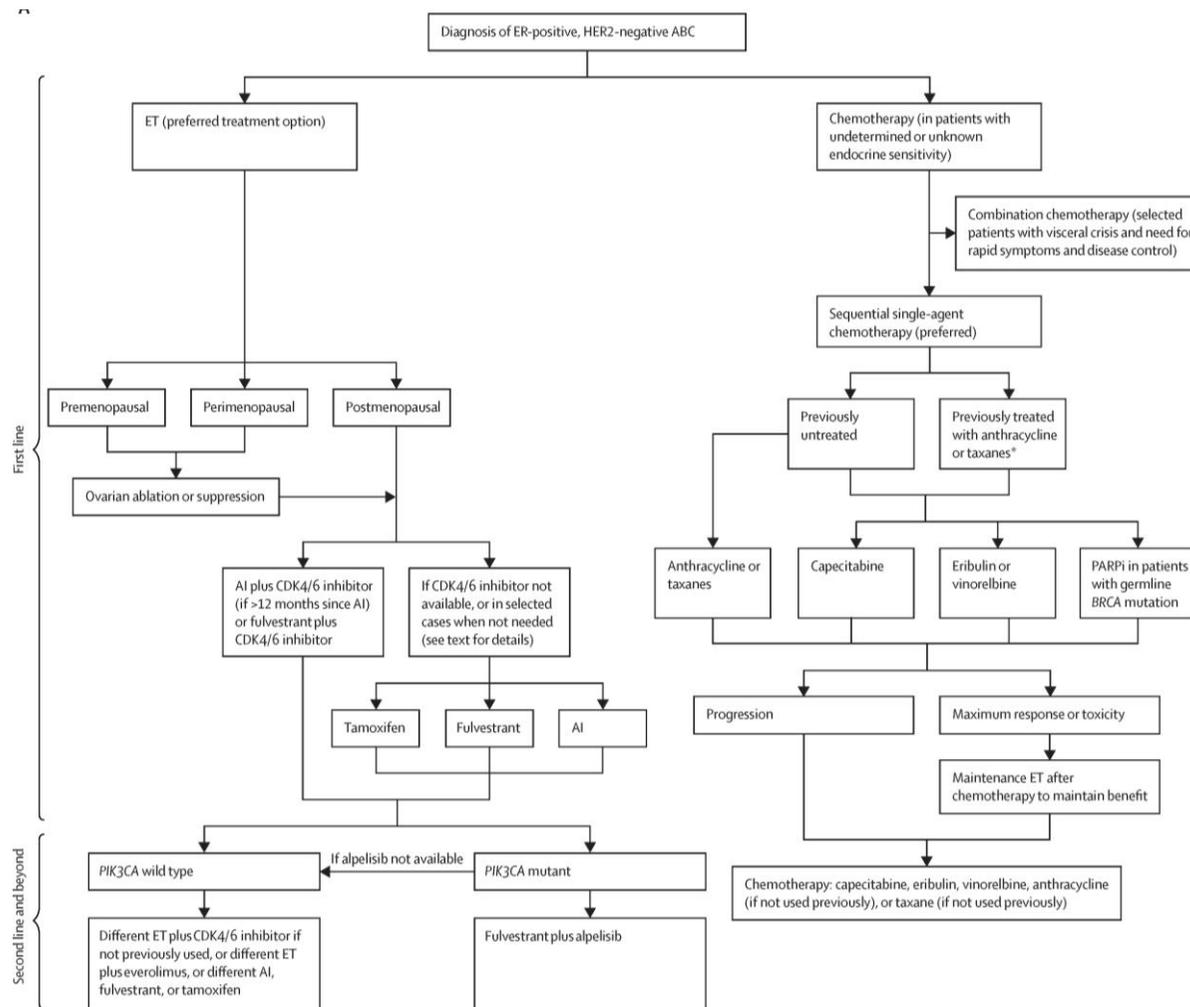


Figure 3 : Schematic represents ER-positive and HER2-negative metastatic breast cancer (endocrine-responsive) treatment (Loibl et al. 2021).

Abbreviations: ABC=advanced breast cancer. AI=aromatase inhibitor. DFI=disease-free interval. ET=endocrine therapy. PARPi=poly(ADP-ribose) polymerase inhibitor

## 4.2 Treatment of HER2-positive breast cancer

In HER2-positive metastatic breast cancer, the combination of anti-HER2 therapy, chemotherapy or endocrine therapy improves patient survival (Figure 4). For example, in first-line therapy, trastuzumab and pertuzumab is used to block HER2 in the presence of chemotherapy (i.e taxanes) is commonly applied. In second-line therapy, trastuzumab emtansine or trastuzumab and chemotherapy agent is recommended for the treatment. Another treatment option is the combination of Trastuzumab and lapatinib, the addition of endocrine therapy is widely used in HER-2 positive, hormone receptor-positive metastatic breast cancer (J. Wang and Xu 2019). Recently, a new antibody–drug conjugate trastuzumab deruxtecan against HER2 is initially studying in phase ½ clinic, which indicates the promising overall response rate at 61% in HER2-high patients, and 37% in HER2-low pretreated patients. However, the common side effects are nausea, neutrophil-count reduction, and anaemia (Modi et al. 2020). Therefore, the new development of anti-HER2 strategies will be the valuable direction for HER2-positive breast cancer cells treatment. The detail about HER2+ breast cancer treatment can be views in Figure 4 (Loibl et al. 2021).

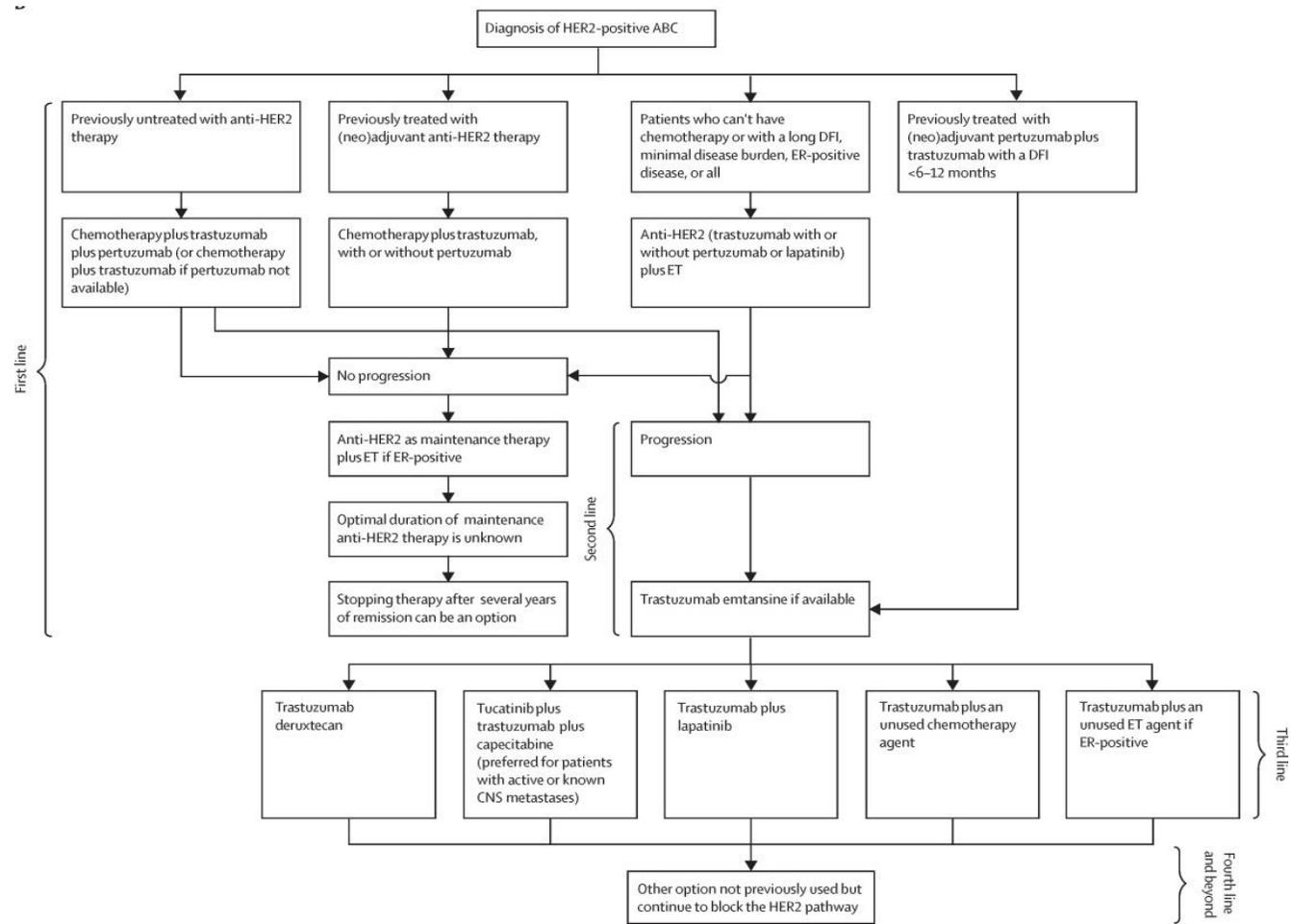


Figure 4: Schematic represents HER2-positive metastatic breast cancer treatment (Loibl et al. 2021).

Abbreviations: ABC=advanced breast cancer. AI=aromatase inhibitor. DFI=disease-free interval. ET=endocrine therapy. PARPi=poly(ADP-ribose) polymerase inhibitor

### 4.3 Treatment of Triple Negative Breast Cancer

Figure 5 indicates the representation diagram of TNBC breast cancer treatment. TNBC treatment is challenging because targeted therapy is still missing, together with aggressive phenotype and highly poor prognosis. Therefore, until now, there are no specific treatment guidelines for TNBCs, the combinations of chemotherapy, immunotherapy are now applied in the treatment methods depending on testing germ-line mutation or PD L-1 expression (Loibl et al. 2021). In TNBC cancer, progression-free survival and overall survival are improved after immune checkpoint inhibitor atezolizumab (PD-1 blockage) and nab-paclitaxel co-treatment in PD-L1-positive population (Schmid et al. 2020). In addition, treatment of inhibitor of PD-1 receptor pembrolizumab together with one of three following chemotherapy nab-paclitaxel, paclitaxel, and carboplatin–gemcitabine has desirable results on progression-free survival improvement in PD-L1-positive TNBC with high degree of metastasis (Schmid et al. 2020). The idea protocol for treatment of patients with PD-L1-positive and germline BRCA1 or BRCA2 mutants is firstly applied checkpoint inhibitor molecules and secondly provided PARP inhibitor, in this clinical study, the rate of PD-L1-positive is independent to BRCA status (Emens et al. 2019). However, chemotherapy is still remaining the optimal systemic therapy for TNBC patients. The detail about TNBC breast cancer treatment can be views in Figure 5 (Loibl et al. 2021).

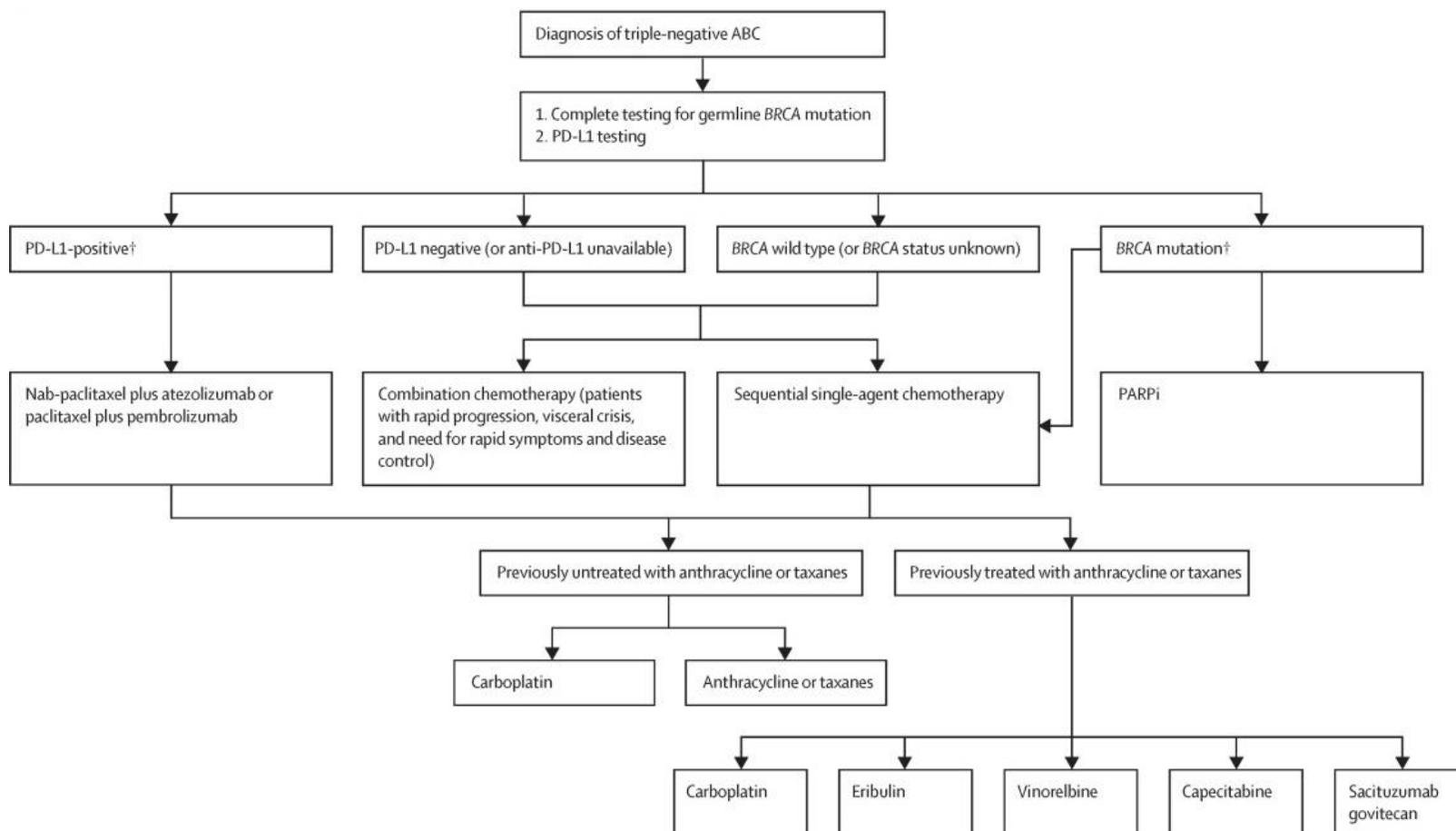


Figure 5 : Triple-negative metastatic breast cancer (Loibl et al. 2021)

Abbreviations: ABC=advanced breast cancer. AI=aromatase inhibitor. DFI=disease-free interval. ET=endocrine therapy. PARPi=poly(ADP-ribose) polymerase inhibitor

## Chapter 2 Breast cancer progression and metastasis

### 1 Molecular alterations in breast cancer

Breast cancer is a heterogeneous disease characterized by complex and distinct molecular alterations. By using next-generation sequencing, the genomic profiling of breast tumor tissue, circulating tumour cells (CTCs) and circulating tumor DNA (ctDNA) identifies two classes of genomic alterations, including germline mutations and somatic mutations (Shatsky et al. 2019).

The heritable germline alterations (mostly in BRCA1 and BRCA2-inducing breast cancer phenotype) are divided into high, moderate and low penetrance genes. The higher penetrance of the genes correlates to higher development of breast cancer induced by these genes (Godet and Gilkes 2017). The high-penetrance genes (*i.e.* TP53, PTEN, BRCA1, BRCA2, MLH1, MSH2) have more than 4-fold increase, moderate-penetrance genes (*i.e.* ATM, CHEK2, BRIP1, PALB2) have from 2 to 4-fold increase and low-penetrance genes (*i.e.* MAPK3K1, FGFR2, LSP1) have less than 2-fold increase in developing breast cancer risks compared to healthy situation (Shiovitz and Korde 2015; Sud, Kinnersley, and Houlston 2017).

Somatic mutations (*i.e.* AKT1, PIK3CA PTEN and TP53 genes) have been used to classify primary breast cancer tumor subtypes (Y. Chen et al. 2015). Somatic mutations are inherited and associated with clinical symptoms of breast cancer patient (Hofree et al. 2013; He et al. 2017; Ronen, Hayat, and Akalin 2019; Arslanturk, Draghici, and Nguyen 2020). However, the studies of genomic alteration composition in these classes should be taken into account together due to the complex relationships of mutated gene systems in breast cancer.

In early of all breast cancers, the common genomic mutations in the tumor cells are TP53 (41%), PIK3CA (30%), MYC (20%), PTEN (16%) and CCND1 (16%), ERBB2 (13%), FGFR1 (11%) and GATA3 (10%) (Nik-Zainal et al. 2016). These genes are responsible for cell-cycle inactivation (*e.g.* p53) or activation (*e.g.* cyclin D1), proliferation induction, apoptosis inhibition, and oncogenic pathways activation (MYC, HER2, FGFR1, PTEN). Most of breast cancers are developed due to the accumulation of low-penetrant mutations, the representative genomic alteration pathways as presented in Figure 6. The molecular drivers are depended on the subtypes. Therefore, the most common mutations are as following: luminal A tumors have PIK3CA (49%) mutations, basal-like tumors have prevalent mutation of TP53 (84%) while triple-negative breast cancer (TNBC) have PI3KCA (55%),

AKT1 (13%) and CDH1 (13%) gene mutations, and finally, HER2 positive has TP53 (74.6%), CDK12 (64.6%) and PIK3CA (46.4%) mutations (Hoadley et al. 2014).

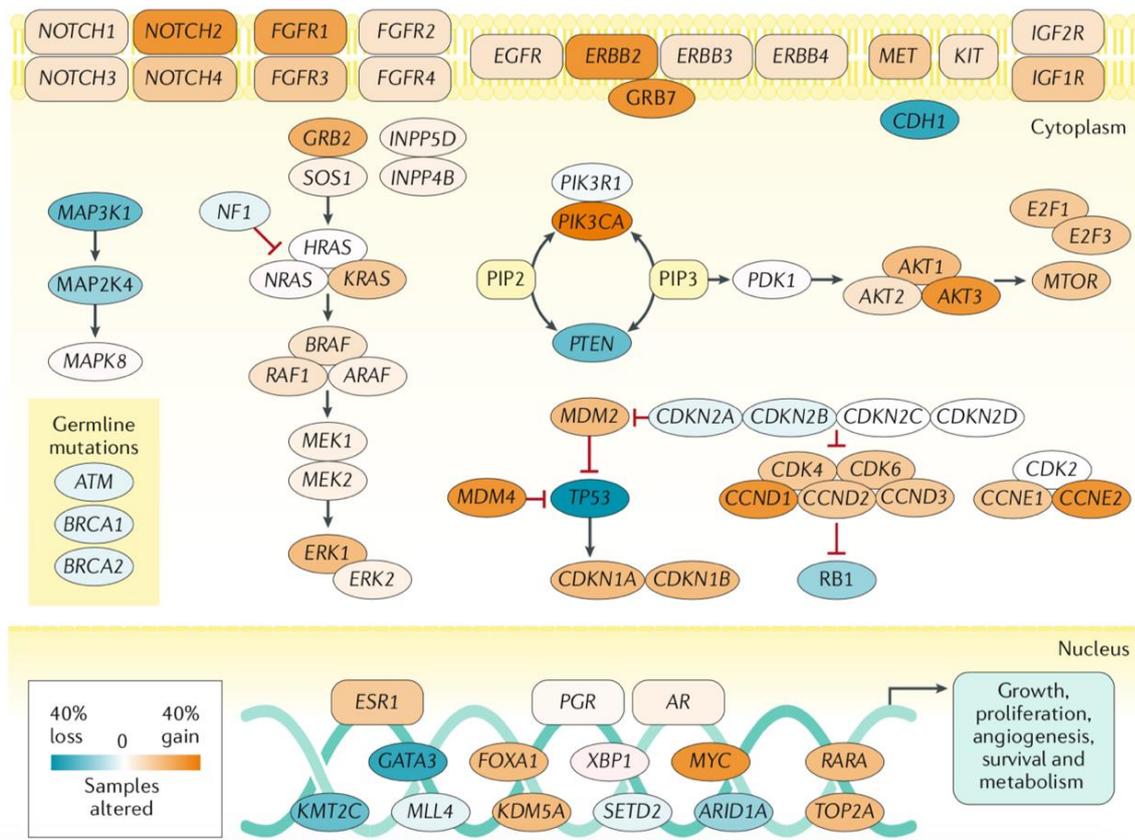


Figure 6: Common genetic alterations in breast cancer (Hoadley et al. 2014)

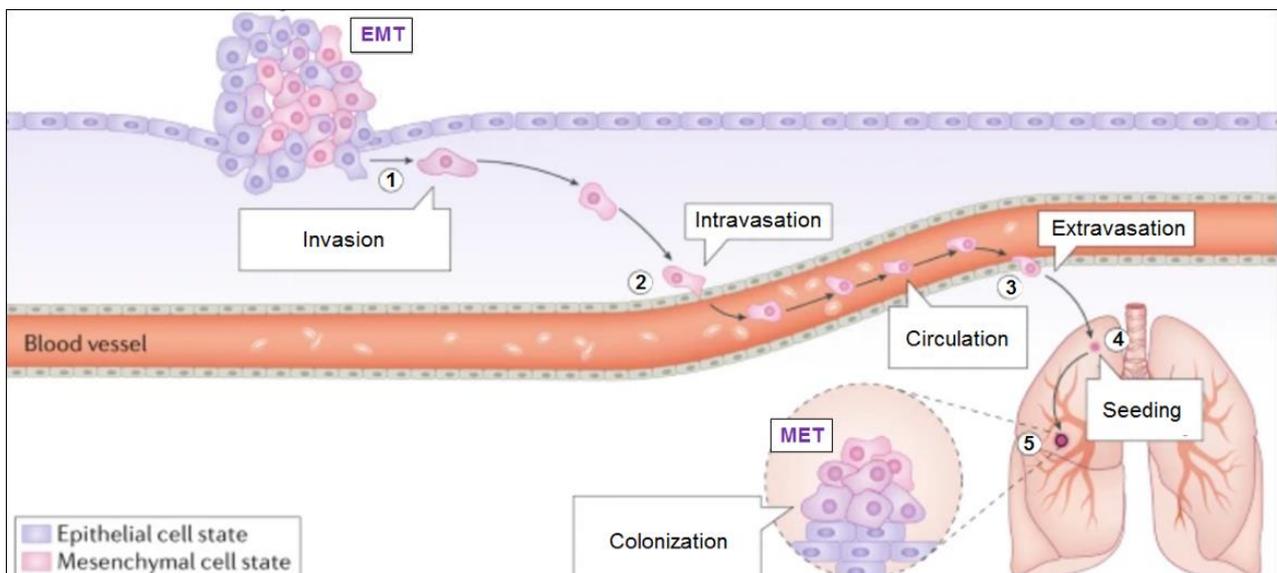
Shading color of each gene reflects the degree of mutation. Orange: amplification induction-gain-of-function; blue: homozygous reduction-loss-of-function.

Epigenetic alterations can contribute to the changes of gene expression. The histone tail modifications such as the induction of chromatin structure changes can cause these gene expressions regulation and remodel nucleosome (Z. Zhao and Shilatifard 2019). CpG sites modifications including global hypo-methylation may lead to chromosome instability and regulates gene expressions/activation; local hyper-methylation on specific locus may lead to DNA repair gene silencing, genetic imbalance and regulates gene repressions/ inactivation. These epigenetic modifications are reversible and mainly based on enzyme mediation, therefore it can be a potential target for breast cancer treatments (Ediriweera, Tennekoon, and Samarakoon 2019).

## 2 Metastatic stages

Metastasis is defined as the secondary development at distant of the initial primary tumor and considered as a hallmark of cancer (Hanahan and Weinberg 2011). The metastatic cascade combines

of six main processes, including firstly the invasion of carcinoma cells to the neighbor healthy tissue, secondly, these cells intravasate into the micro-vessels and macro-vessels draining the tumor-surrounding tissues. The third step is the dissemination and circulation of these carcinoma cells into the vessels (blood and lymph). The fourth and fifth steps are extravasation and continuously seeded of these cells into target tissues respectively, which are also considered as micro-metastases. Finally, the outgrowth of macro-metastases is the consequence of previous micro-metastases, which is called secondary tumoral colonization (Figure 7) (Valastyan and Weinberg 2011; J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020).



*Figure 7 : Five main steps of metastasis: invasion-intravasation, circulation, extravasation, pre-metastatic niche seeding and colonization (J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020)*

## 2.1 Invasion and intravasation

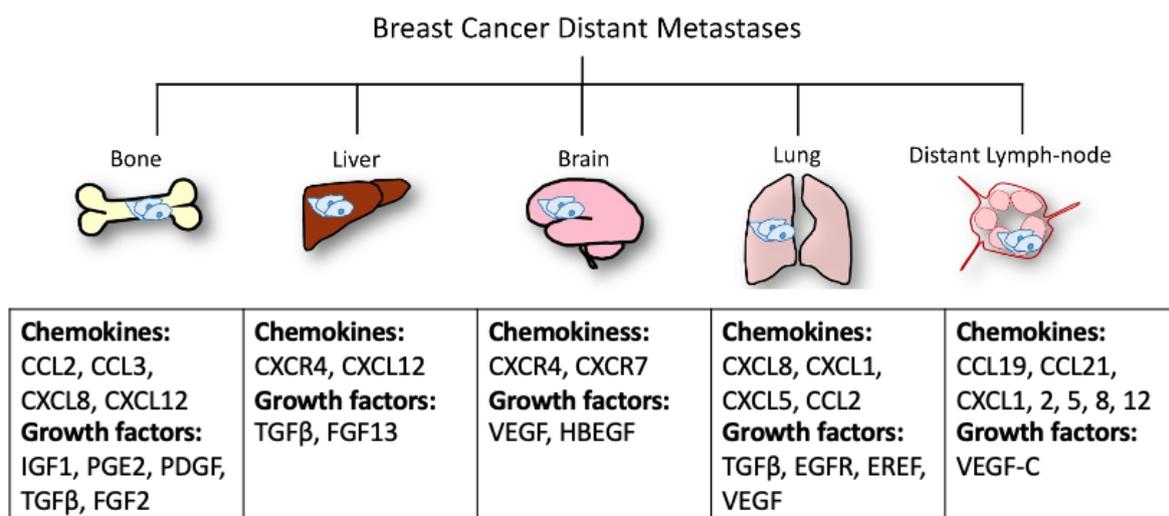
Cancer cells dissemination and invasion are the first step of metastasis cascade (Lambert, Pattabiraman, and Weinberg 2017). The initial step is the degradation of the extracellular matrix (ECM) to allow the tumor cells invade the neighbor tissue and disseminate in blood and lymph vessels (Yilmaz and Christofori 2009) (Figure 7). This process is affected by proteinases such as matrix metalloproteinases (MMPs) which are secreted by dynamic actin-rich membrane protrusions (invadopodia and podosomes) of the tumor cells (Linder 2007; Kessenbrock, Plaks, and Werb 2010). High expressions of MMPs family proteins in breast cancer associate to high tumor grade and poor prognosis (Bachmeier et al. 2001; Ren et al. 2015; Köhrmann et al. 2009).

An important cellular process correlated to the invasive or metastatic initiation program in many cancers is the epithelial to mesenchymal transition (EMT) of cancer cells from epithelial state toward a mesenchymal state. Actin-dependent protrusion of cell pseudopodia is a common character of mesenchymal cell migration and cancer metastasis (Shankar et al. 2010). In breast cancer, the activation of HER2 is associated with E-cadherin down-regulation (epithelial marker) and Vimentin up-regulation (mesenchymal marker) provides a huge advantage during tumor initiation and booster detachment of breast tumor cells from primary tumors (Dua, Gui, and Isacke 2005; Hanahan and Weinberg 2011). However, the detail discussion on cellular EMT phenotype will be described in the next part of the chapter. Beside EMT, another example of cancer cells modification to induce metastatic phenotype is epigenetic mutations. Indeed, the chromosomal instability (CIN) of cancer cells associates with chromosome segregation during kinesin-13-regulated mitosis through microtubule depolymerization, leads to the disruption of micronuclei and genomic DNA release into cytosol (Ems-McClung and Walczak 2010). This defect consequently activates cytosolic DNA-relating pathways such as cyclic GMP-AMP synthase modulating interferon (IFN), or downstream NF- $\kappa$ B signaling (Bakhoun et al. 2018) and activate cellular dissociation.

There are several pro-metastatic inducers in TME such as TGF $\beta$ , WNT, cancer associated fibroblasts cells (CAF), immune cells (macrophages, neutrophils) or hypoxia (Olsen et al. 2010; Hanahan and Weinberg 2011). These regulators involve in the activation of tumor cell transformation, which firstly over-proliferate from primary tumor, then induce metastatic phenotypes as previously described (Kessenbrock, Plaks, and Werb 2010; H. Liu et al. 2010; Thiery et al. 2009; Cano et al. 2000). In breast cancer, common metastatic preference sites are different between subtypes (Figure 2). The metastatic destinations depend on primary tumor locations, for instance, breast cancer cells are primarily metastases to the lung *via* intracardiac and pulmonary vein (Minn et al. 2005). In addition, the cancer cells have to be attracted by chemoattractant cytokines and growth factors, which were released by the secondary metastatic site, to identify which distant organs is the correct destination.

In fact, chemokines are chemoattractant cytokines, which act as signaling molecules in cellular communication through the binding to their corresponding receptors (Mackay 2001). The classification of chemokines depends on their composition of amino acid, especially the first two cysteine residues on the conserved tetra-cysteine motif. Two common groups of chemokine are CC and CXC. There are 27 different members of CC subgroup in mammalian, called CC chemokine ligands (CCL). The CXCL (CXC ligand) subgroup contains ELR amino acid motif or (ELR+CXCL) such as CXCL1-3, 5-8, 14 and 15 and non-ELR motif (ELR-CXCL) such as CXCL13 (Hughes CE, 2018). In breast cancer, chemokines and its receptors are important during metastasis, for example,

high-expression of chemokine receptors (CXCR4 and CCR7) were found in human breast cancer cells, malignant breast tumours and metastases while their corresponding ligands (CXCL12/SDF-1alpha and CCL21/6Ckine) are up-regulated in organs representing the first sites of breast cancer metastasis (e.g. lymph node) (Müller et al. 2001). Therefore, chemoattraction cytokines and growth factors at secondary tumor sites are remaining as the main impacts to direct metastatic cells toward the corresponding organs (i.e. bone, liver, brain, lung and lymph node) (Figure 8) (Razmkhah et al. 2014; Coniglio 2018; C. Zhang et al. 2018; L. Ji et al. 2021; Skobe et al. 2001; Wenjing Chen et al. 2018a).



*Figure 8: Representative of chemokines and growth factors at metastatic sites of breast cancer*

Abbreviations: CCL=chemokine ligands. CXCL= C-X-C motif chemokine ligand. CXCR= C-X-C motif chemokine receptor. IGF= insulin-like growth factor. PGE2= Prostaglandin E2. PDGR= Platelet-derived growth factor. FGF= Fibroblast Growth Factor. TGFb= Transforming growth factor beta. VEGF= Vascular endothelial growth factor. EGFR= estimated glomerular filtration rate. HBEGF= Heparin Binding EGF Like Growth Factor.

## 2.2 Pre-metastatic niche seeding

Before entering to the circulation, tumor cells prepare the premetastatic niche to create an appropriate environment (nutrients, extracellular matrix and immune cells) for supporting the disseminated cancer cells seeding at metastatic sites. Metastatic niche is composed of tumor-derived suppressor factors (TDSFs), bone marrow dendritic cells (BMDCs), regulatory/suppressive immune cells, and stromal components at the distant metastatic sites (Chin and Wang 2016; Sceneay, Smyth, and Möller 2013; Quail and Joyce 2013). The pre-metastatic niche support metastatic cells seeding by inducing immunosuppression, inflammation, permeabilize vascular to support angiogenesis, lymphangiogenesis, organotropism, and reprogramming of metabolic environment (Y. Liu and Cao 2016).

### 2.2.1 At distant organs

The preparation of pre-metastatic niche at distant organs are including two main phases: priming and licensing as presented in Figure 9.

In priming phase (Figure 9A), primary tumors are highly proliferating, create hypoxic and inflammatory environments. These events support the production of different tumor-derived suppressor factors (TDSFs, *i.e.* VEGF, TNF $\alpha$ , TGF $\beta$ ), extracellular vehicles (EVs, *i.e.* exosomes, microvesicles, large oncosomes), and other molecular components, which increase bone marrow dendritic cells (BMDCs) mobilization and regulatory/suppressive immune cells to the future distant site. These soluble factors are transported along blood vessels to reach the metastatic niche and initiate the reprogramming of host stromal environment of the metastatic organs (Y. Liu and Cao 2016). Therefore, at this stage, primary tumor cells mainly trigger the establishment of an immature pre-metastatic niche in the secondary organs or in the same organ but distinct from the primary tumor.

Licensing phase can be the linkage of the pre-metastatic niche maturation and tumor metastasis initiation (Figure 9B and Figure 9C). During this process, BMDCs and other regulatory/suppressive immune cells are continuing their movement and recruitment into the metastatic sites in response to previous TDSFs and EVs secreted by primary tumor. TDSFs and EVs are transported from primary tumor to metastatic niche through blood vessels. Interestingly, the high content and average activation duration of these TDSFs are appropriate to convert the pre-mature niche to matured-niche with the enriched tumor-promoting substances and abundant environment for the incoming metastatic cancer cells (Y. Liu and Cao 2016). These soluble molecules promote the mobilization and recruitment of different cell populations to secondary organ sites, including BMDCs (*i.e.* haematopoietic progenitor cells (HPCs) expressing VEGFR1) and CD11b<sup>+</sup> myeloid cells, and other regulatory/suppressive immune cells such as MDSCs, Treg cells, TAMs, and tumor-associated neutrophils (TANs). For example, the recruitment of immune cells (MDSCs) and cytokine (IL1 $\beta$ ) induce EMT of tumor cells and promote tumor cell metastasis (Tazzari et al. 2014; Cui et al. 2013). The associations of TDSFs, tumor-recruited cells, and local stroma may establish an appropriate niche microenvironment for metastatic tumor cell colonization. For instance, increase of extracellular matrix (ECM) remodeling and activation of integrins and chemokines involve in the pre-metastatic niche formation at distant sites (Winkler et al. 2020). Hypoxia and ECM remodeling also induce pre-metastatic niche formation. The interaction between TDSFs, BMDCs, and host stroma (*i.e.* fibroblasts) at secondary sites are important. This relationship regulates the local microenvironment and forms a mature pre-metastatic niche for the further seeding and colonization of tumor cells.

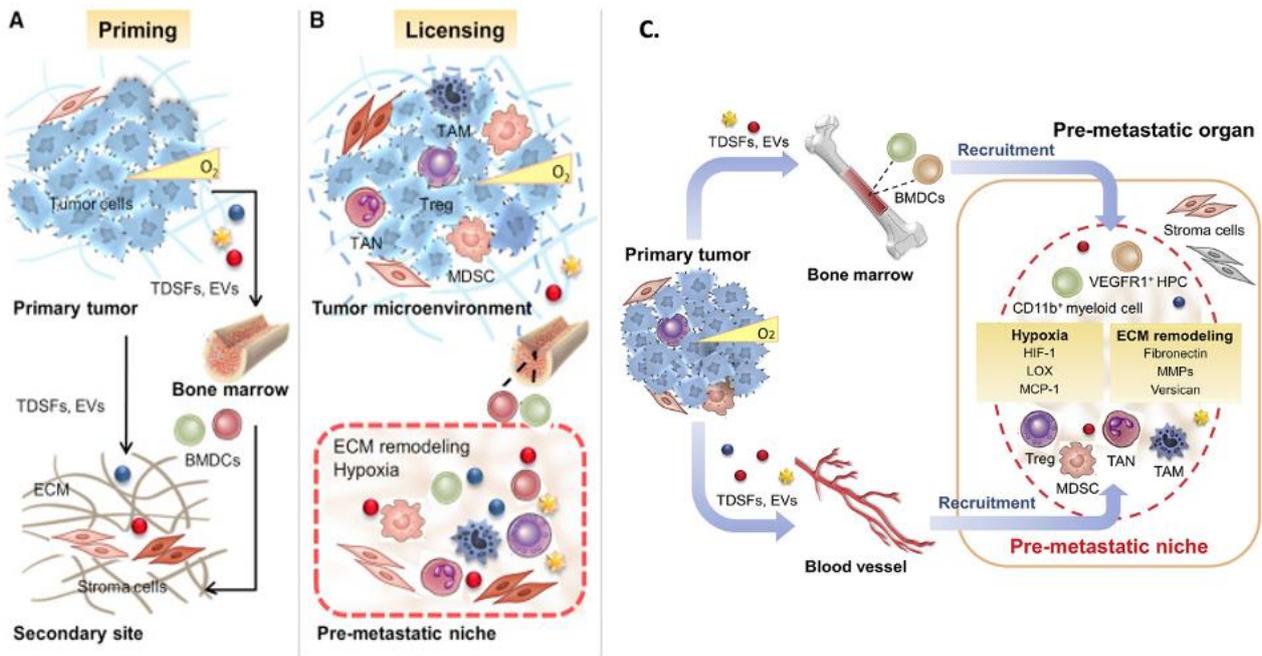


Figure 9: Pre-metastatic niche preparation at distant organs ((Y. Liu and Cao 2016).

(A) During priming stage, primary cancer cells release numerous soluble factors such as TDSFs, EVs, and other molecular molecules, to induce the immature pre-metastatic niche establishment in the distant organs or in the same organ but different from primary tumor.

(B) During the licensing stage, BMDCs and regulatory/suppressive immune cells are activated and recruited into the metastatic sites in response to tumor-derived molecular signals. The interactions between TDSFs, recruited cells and host stroma replace and produce the microenvironment, finally create a mature pre-metastatic niche

(C) TDSFs and EVs enhance the movement and recruitment of different cell types to metastatic site such as BMDCs (VEGFR1 + HPCs and CD11b + myeloid cells), and regulatory/suppressive immune cells (MDSCs, Treg cells, TAMs, and tumor-associated neutrophils (TANs)). The interaction among these factors contribute to the microenvironment creation of the niche for cancer cell colonization. Hypoxia and ECM remodeling also induce the niche formation.

### 2.2.2 At lymph node

Prior to metastatic dissemination, the primary tumor remodels the draining (sentinel) lymph node by secreting soluble factors or extracellular vesicles that are transported by lymphatic vessels (Figure 10). The lymph node pre-metastatic niche preparation is not completely similar to this process in other distant organs. The establishment of pre-metastatic niche in lymph node includes the following steps: Firstly, tumor derived endothelial growth factors VEGF-A, VEGF-C and VEGF-D; extracellular vesicles, TGF- $\beta$  and lysyl oxidase (LOX), these signaling molecules recruit macrophages, myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) to create immunosuppressive microenvironment in lymph node. Secondly, tumor-deriving factors also induce the proliferation of lymphatic endothelial cells (LECs) and fibroblastic reticular cells (FRCs). Thirdly, these effects support the releasing of LN-derived factors including chemokines (CCL19; CCL21; CXCL1, 2, 5, 8, and 12); TGF $\beta$ ; matrix metalloproteinases (MMPs); indoleamine- 2,3-dioxygenase (IDO); and nitric

oxide (NO), which increase the remodeling of high endothelial venule (HEV). Finally, these molecules stimulate lymph-angiogenesis and create the chemoattraction to support cellular metastasis into these lymph node (Gillot et al. 2021) .

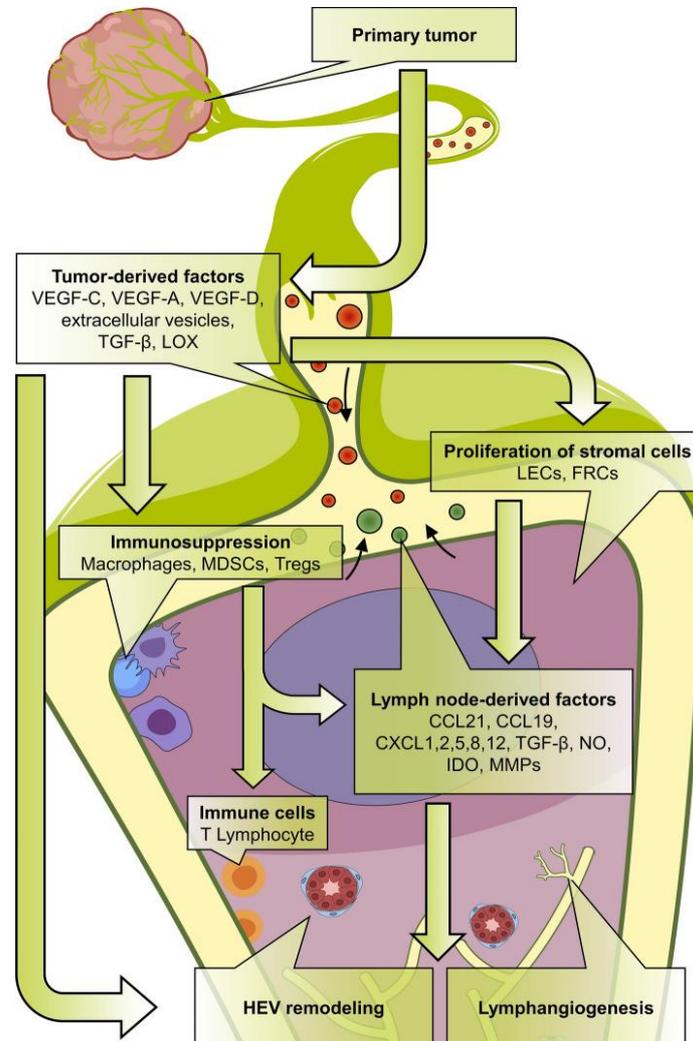


Figure 10: Pre-metastatic niche preparation at draining lymph node (Gillot et al. 2021)

Abbreviation: VEGF=vascular endothelial growth factor VEGF-A, VEGF-C and VEGF-D. LOX=lysyl oxidase. MDSCs= myeloid derived suppressor cells. Tregs= regulatory T cells. LECs=lymphatic endothelial cells. FRCs=fibroblastic reticular cells. Chemokines=CCL19; CCL21; CXCL1, 2, 5, 8, and 12. MMPs=matrix metalloproteinases. IDO=indoleamine 2,3-dioxygenase. NO=nitric oxide (NO). HEV=high endothelial venule.

### 2.3 Intravasation

After the preparation of pre-metastatic niche, tumor cells enter into circulation (intravasation) *via* two main entrances, which are hematogenous vessels and lymphatic vessels (Chiang, Cabrera, and Segall 2016). The tumor cells disseminate to intravasate into either blood or lymphatic vessels that is not only depended on internal mechanisms of metastatic tumor cells but also tightly dependent on physical

environment established in primary tumors (Wong and Hynes 2006). Blood or lymphatic vasculature access of tumor cells can also affect to metastatic initiation pathway.

The hematogenous intravasation is the major entrance of tumor cells into the circulatory system. This entrance is observed by microfluidic technique, which provides the insight how metastatic cells enter to the vessels (Wenjing Chen et al. 2018b; Blaha et al. 2017). The breast transformed cancer cells (MDA-MB-231, MCF-10A) with thin filaments (pseudopodia) can push against the vessel wall, create the space in endothelial lining and allow for transmigration (M. B. Chen et al. 2013). Due to the thickness of vessel walls, veins are the preferences invasive vessels than arteries; the tumor cells often follow the venous blood flow to reach secondary metastatic sites (Sandoo et al. 2010). Hematogenous intravasation can be an active or inactive process, depending on characteristics of the tumor, structure of vessels or TME conditions (Chiang, Cabrera, and Segall 2016; Quail and Joyce 2013).

Recently, different studies show that lymph node metastasis is a prognostic parameter in many cancers' types and an entrance for further dissemination to distant organs (Brown et al. 2018; Pereira et al. 2018). Concerning lymphatic intravasation, due to the fact that lymph vessels assemble the main thoracic duct, tumor cells invading-lymph vessels allow these cells to enter to the ductal capillaries and therefore indirectly drain into the blood (Wong and Hynes 2006). However, along the metastatic way in this mechanism, tumor cells have to face with series of lymph nodes, especially sentinel (draining) lymph nodes (LN) as the closest site of primary tumor (Nathanson 2003). Therefore, the primary tumors have to remodel draining node before the appearance of metastatic cell and create LN pre-metastatic niche to support the survival and growth of these cells (Figure 10). As one of the primary metastatic sites, sentinel lymph nodes are frequently used to check early appearance of metastasis.

## 2.4 Circulation

Once the transformed cancer cells have detached from the primary solid tumor or lymph node and started to circulate the bloodstream, these metastatic cells are described by a general term: circulating tumor cells (CTCs) (Micalizzi, Maheswaran, and Haber 2017). CTCs are the results of adaptive mechanisms that resist again constrictive conditions to enhance their survival during migration (H.-A. Park, Brown, and Kim 2020). For example, metastatic tumor cells, especially breast cancer cells, can activate and maintain cell fate inducing pathways over the long term *via* inducing cell pro-survival mechanisms (Furlow et al. 2015) or by promoting epithelial to mesenchymal transition (EMT), which result in a high invasive phenotype (Cognart, Viovy, and Villard 2020; Irianto et al. 2017). Therefore, CTCs are heterogeneous populations of cells, including epithelial tumor cells, epithelial-to-mesenchymal cells, hybrid epithelial/EMT cells, and cancer stem cells (Barriere et al. 2014).

Starting from the primary tumor, single CTCs or CTCs-clusters have to pass through different types of flow including arterial flow, lymphatic flow and venous flow to interact with other CTCs or blood elements, which affect their metastatic potential (Marrella et al. 2021). To survive during the circulation, CTCs have to escape anoikis, which is an apoptotic cell-death type induced by loss of attachment to the extracellular matrix, blood flow pressure (mechanical stress) and immune intervention (Szczerba et al. 2019; Wculek and Malanchi 2015; Aceto et al. 2014b; Douma et al. 2004) (Figure 11). The three processes act together to inhibit intravascular survival and metastatic effects of tumor cells. Follain G and colleagues divide the three main processes above into seven independent stages based on movement of CTCs in different size of vessels and velocities of flow.

The stages 1, 2 and 3 take place in arterial vessels, high velocity of the flow and shear stress cause either CTCs fragmentation, cell cycle arrest and cell death respectively. Until reaching capillary beds of distant organ, CTCs reach lower blood flow velocities. This circulation promotes CTC arrest and cancer cell adhesion to endothelial walls of either the main vein activation of cell adhesion molecules and increase of adhesion energy (stage 4) or small capillaries via occlusion-mediated arrest (stage 5).

In addition, a venous blood flow velocity is lower than arterial blood flow, which leads to the interaction of CTCs with other blood cells including neutrophils, platelets or cancer-associated fibroblasts (CAFs) and travels as clusters (stage 6). The interaction between CTCs and its cluster can prevent the destruction of CTCs by other immune cells such as natural killer (NK) cells and escape immune surveillance. For example, platelets drive various bioactive soluble factors that induce EMT through NF- $\kappa$ B pathway and prevent NK cells effects through TFG $\beta$  and PDGF signaling, these metastatic CTCs- platelets clusters also have the protective cover (fibrinogen) (Labelle and Hynes 2012; Palumbo et al. 2007). After that, within clusters, CTCs with partial epithelial markers are observed, which will induce secondary organ colonization (Stage 7). For instance, once associated with CTCs, platelets secrete ATP, which further induce vascular permeabilization, these platelet-CTCs clusters then strongly attach to vasculature endothelial walls and contribute to the beginning of extravasation (Schumacher, 2013). Finally, in lymphatic system, the low velocity of blood flow and less shear stress induce CTC survival, arrest, lymph node colonization and haematogenous dissemination (stage 8), which also contribute to the starting of extravasation (Follain et al. 2020).

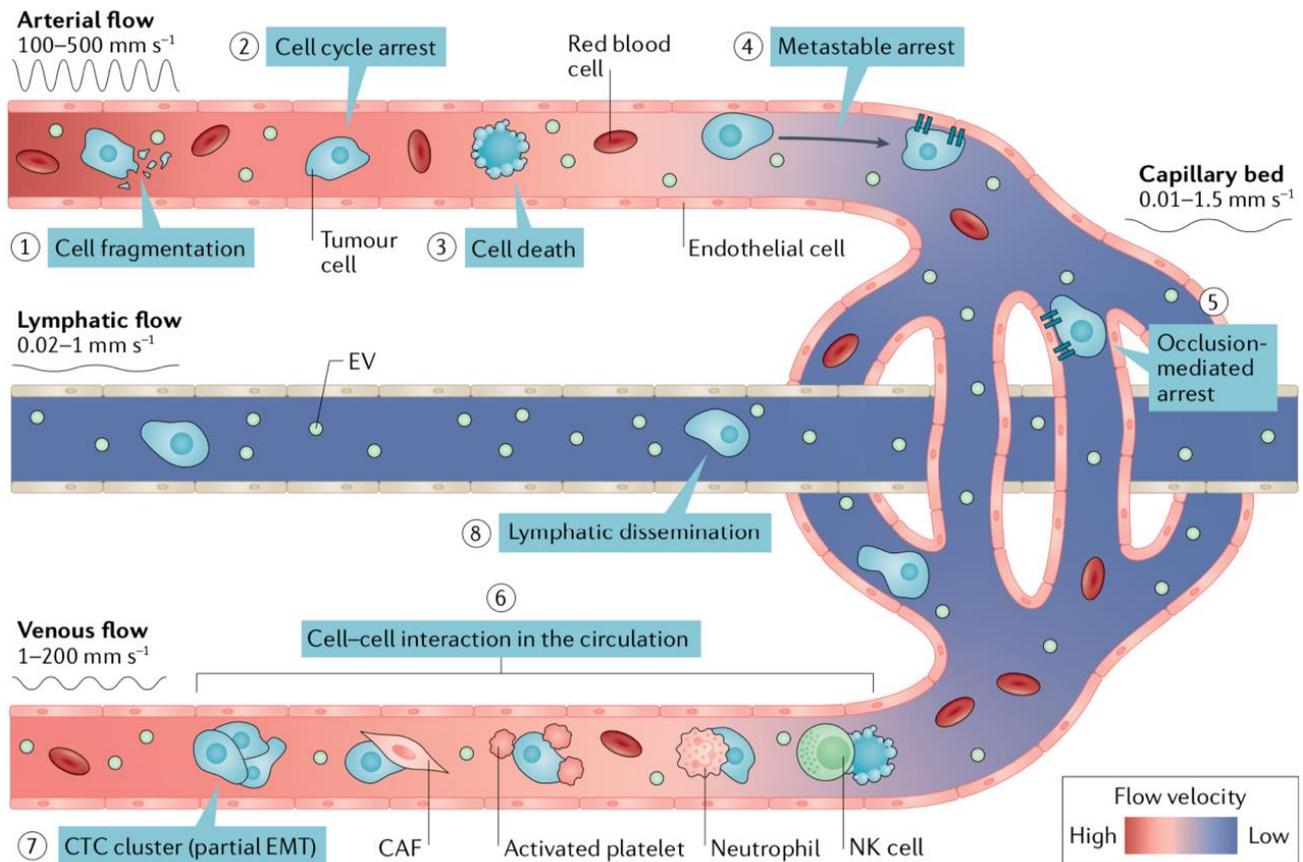


Figure 11: Circulation of CTCs in the blood and lymphatic vessels (Follain et al. 2020)

Abbreviations: CTCs=Circulating tumor cells (CTCs). NK=natural killer cells. CAFs=cancer-associated fibroblasts (CAFs). EMT=epithelial–mesenchymal transition. EV=extracellular vesicle.

Interestingly, CTCs–neutrophil clusters also metastasize in EMT independent manner. For examples, neutrophils are highly contained tumorigenic potential, support distant colonization to other organs such as lung. In the blood samples from clinical patient and mouse models, CTCs are mostly associated with neutrophils–derived leukotrienes, Ki67 up-regulation is also detected in these CTCs–neutrophil clusters, which indicates higher proliferation rate of this CTCs population than EMT and platelet clusters (Szczerba et al. 2019; Wculek and Malanchi 2015). Therefore, the stories of CTCs–neutrophil could be the alternative explanations for distant metastasis without EMT transition. The evaluations of breast metastatic cells compositions and CTCs from tumor liquid biopsies are not only promising for prognosis but also useful to specify the tumoral response during therapies.

## 2.5 Extravasation

Extravasation indicates the abilities of CTCs to partially or completely leave the blood vessels and distantly penetrate another organ. Once rested predominantly in appropriate capillaries at specific metastatic sites (Kienast et al. 2010), CTCs either start to migrate within blood vessels and then

extravasate the endothelium as a group of cells (Leong et al. 2015; Tayoun et al. 2019), or directly transmigrate as single cells (Gassmann et al. 2009; Kienast et al. 2010; Schumacher et al. 2013; M. D. Martin et al. 2010).

There are three main events that implicate tumor cells extravasate. Firstly, cancer cells express specific proteins that are responsible to initiate the process. In breast cancer, breast cancer cells secrete angiopoietin-like 4 (ANGPTL4) or its C-terminal fibrinogen-like domain (cANGPTL4) to antagonize vascular endothelial-adherent junctions and promote extravasation (R.-L. Huang et al. 2011; Padua et al. 2008). ANGPTL4C-terminal domain is then directly interacted with integrin  $\alpha 5\beta 1$ , VE-cadherin, and claudin-5 in a temporally manner to induce metastasis (R.-L. Huang et al. 2011). Extravasation can be considered as intravasation-mirroring process; hence breast cancer cells extravasation is also promoted by intravasation-involving proteins like epidermal growth factor (EGF) receptor ligands (*i.e.* epiregulin (EREG), heparin-binding EGF-like growth factor (HBEGF), prostaglandin-synthesizing enzyme cyclooxygenase 2 (COX2), matrix-remodeling metalloproteinases MMP1 and MMP2) (Bos et al. 2009; Gupta et al. 2007). The second step of tumor cell extravasation is its interaction with endothelial layer in the blood vessels to open the cellular barrier. Before invading the secondary organ site, the CTCs includes its adhesion to the endothelium and regulates the endothelial barrier to create the physical escaping spaces on the vessels wall and then reach the invade tissue (Strilic and Offermanns 2017). Thirdly, tumor cells migrate between two endothelial cells, which require the rearrangements of inter-endothelial and loss of cell-cell junctions (Leong et al. 2015; Schumacher et al. 2013). In some cases, single tumor cell has been individually penetrated through the endothelium, which is called transcellular migration (Khuon et al. 2010; Tremblay, Huot, and Auger 2008).

The success of tumor cell extravasation is not only depended on tumor cells and endothelial cells interactions but also tumor cell and other blood cells association. For example, platelets and myeloid cells recruitment into the neighbor of tumor cells not only support CTCs survival but also promote CTCs extravasation (Coupland, Chong, and Parish 2012; Kitamura, Qian, and Pollard 2015; Labelle and Hynes 2012; Reymond, d'Água, and Ridley 2013; Stegner, Dütting, and Nieswandt 2014). Platelets are also an extravasation inducer by the activation of EMT phenotype in CTCs and maintain this characteristic as previously described in circulation process (Labelle, Begum, and Hynes 2011; Takemoto et al. 2017).

## **2.6 Dormancy state and Metastatic niche seeding**

### **2.6.1 Dormancy state**

After extravasation from circulation, tumor cells are rapidly entered to distant organ to form secondary tumor at dormancy state. During this period, tumor cells can firstly establish micro-metastatic clusters or remain as single disseminated tumor cells (DTCs) (Luzzi et al. 1998). However, these cells often stay in dormancy and low-metabolism state, which results in low-proliferation. The explanation of this problem is due to the adaptation of these cells to pre-metastatic niche to create a supportive and receptive TME, because the new TME does not share the similar regulatory networks to their primary tumor such as stromal cells, growth factors, or immune cells (Aguirre-Ghiso 2007). During seeding, the CTCs have to self-regulate its characters to consequently adapt the new TME. For example, circulating breast cancer cells with EMT-phenotype supposed to revert EMT progress into MET after dissemination in order to reestablish the similar TME conditions to the origin tumors, and also restore the loss of epithelial cellular features during circulation (Brabletz 2012). Besides that, EMT also drives CSCs characters, which are essential demands for metastatic colonies success and dormancy escape, because disseminated tumor cells with CSCs-phenotypes are important to be metastatic colonies precursor (Mani et al. 2008).

### 2.6.2 Metastatic niche seeding

One reaching the target organs, the metastatic niche seeding is extremely important in contributing to the metastasis process, which is categorized into two distinct phases (initiation and progression) as presented in Figure 12.

In the initiation phase (Figure 12A), under the support of primary tumor-support and the maturation of pre-metastatic niche, the initiation of metastasis is promoted by facilitating the extravasation of CTCs from vessels and attracting cancer cell colonization into these niches (Y. Liu and Cao 2016). For example, the induction of adhesion molecule (fibronectin) in the pre-metastatic lung promote metastatic seeding through attracting and enhancing attachment of CTCs to the niche (Yamamura et al. 2015). Hypoxia-inducible factor 1 (HIF1) is induced in the pre-metastatic niche, which favorites cancer cells extravasation and chemotaxis of by regulating hypoxia-induced proangiogenic factors such as VEGF and platelet-derived growth factor (PDGF) (Unwith et al. 2015). In addition, exosomal transfer of microRNA (*i.e.* miR-23b) from bone marrow mesenchymal stem cells (MSCs) improves breast cancer cell dormancy in the pre-metastatic niche by suppressing myristoylated alanine-rich C-kinase (MARCK), which is the cellular substrate for protein kinase C (Ono et al. 2014). In addition, many pro-metastatic factors (*i.e.* immunosuppression and ECM remodeling) in the pre-metastatic niche support tumor cells escape from dormancy (Eyles et al. 2010; Kienast et al. 2010). Therefore, well-prepared pre-metastatic niche is necessary to promote the seeding, colonization, survival, and

proliferation of metastatic tumor cells, resulting in the success of metastasis and formation of micrometastases.

In the progression phase (Figure 12B), there are an increase in the number of tumor cells which continue to disseminate the metastatic primary site and colonize the mature pre-metastatic niche of the secondary organ. At this moment, pre-metastatic niche components directly induce tumor cell growth and increase secondary tumor size in the metastatic organ, resulting in the progression of secondary tumor from micrometastases to macrometastases (Y. Liu and Cao 2016). For example, in breast cancer, abnormal expression of VCAM-1 in the bone microenvironment induces the progression from micrometastases to bone macrometastasis by regulating  $\alpha 4\beta 1$ -positive osteoclast progenitors (X. Lu et al. 2011, 1). In addition, the tumors that contain pre-metastatic niche or establish metastatic lesions can effectively increase tumor dissemination and improve the formation of macrometastases (Y. Liu and Cao 2016).

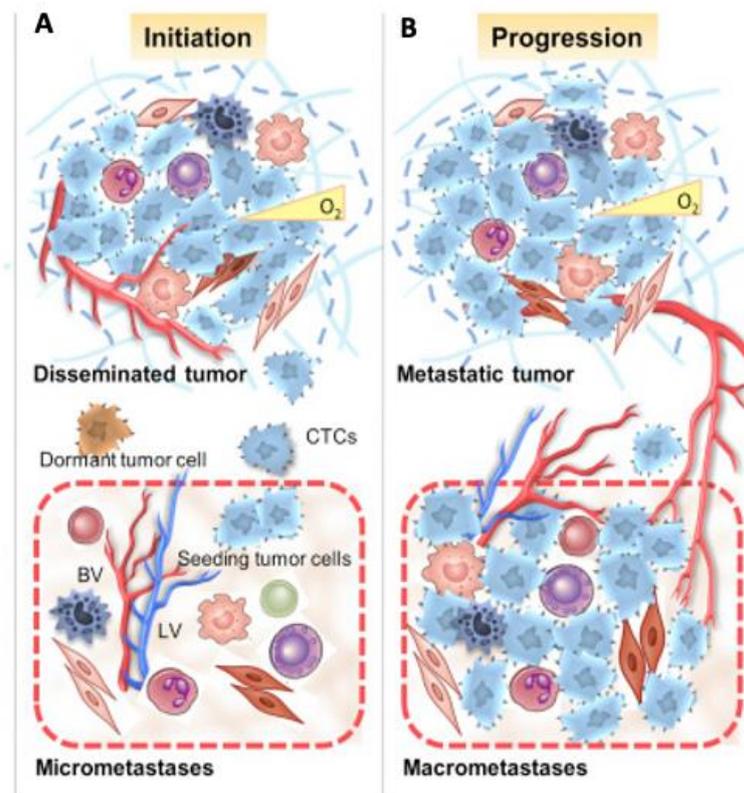


Figure 12: Metastatic niche seeding and tumor progression (Y. Liu and Cao 2016)

(A) In the initiation stage, circulating tumor cells (CTCs) mobilize and colonize at the pre-metastatic niche, survival cells enter to dormancy until the niche environment are well-prepared. The mature pre-metastatic niche favorites cellular seeding, colonization, and development (micro-metastasis).

(B) In the progression stage, the pre-metastatic niche accepts high number of metastatic tumor cells and directly increases metastatic tumor progression at the niche (macro-metastasis).

## 2.7 Colonization

In general, multiple breast cancer metastases potentially appear in different organs such as brain, lung, bone, liver and lymph node (Hanahan and Weinberg 2011; Taverna et al. 2020). At these metastatic sites, various reversible transitions could be able to process after metastatic initiation such as CTCs cluster tight junctions, disseminated tumor cells DTCs-ECM interactions and hence definitively certify colonization formation (Aceto et al. 2014a; Shibue et al. 2012). By subsequent xenotransplantation delivery to promote tumor formation, the DTCs subclones, which particularly inaugurate primary tumor growth, are extremely necessary for metastasis in multi-organ and support metastasis. The explanation for this observation is due to enrichment of LAMA4, FOXQ1 and NAP1L3 genes expression, which induce the yield of metastatic process. In fact, FOXQ1 up-regulate LAMA4 a key proliferative signaling player of DTCs, LAMA4 then promotes its substratum disengagement due to  $\beta$ 1-integrin molecular pathway, leads to clonal proliferation, enlargement, and tumor re-initiation (Ross et al. 2015). Consistently, the over-growing capacity of CSCs is associated with the adaptation of TM4SF1 protein and collagen receptor DDR1 and facilitates cellular dominance to multi-organelle metastases (Gao et al. 2016).

After the mature-metastatic niche seeding, the tissue-specific adaptive regulations of carcinoma cells are activated, for example, breast cancer cells associate with pulmonary abundant macrophages through VCAM-1-intergrin connection, and up-regulate PI3K–Akt survival signaling to enter to organ microenvironment, supporting lung metastasis (Q. Chen, Zhang, and Massagué 2011). Another survival regulation of breast cancer cell was studied in cerebral metastasis, which depend on serpins secretion to prevent plasminogen-associated cell death from responsive astrocytes (Valiente et al. 2014). Additionally, the cooperation between breast cancer and neuronal cells is another explanation for brain invasive tumor growth, in which, the activation of glutamate ligands of N-methyl-D-aspartate receptors (NMDARs), a crucial cerebral metastatic handler, through glutamate secretion, lead to pseudo-tripartite synapses formation between glutamatergic neurons and DTCs, and for the first time represent synaptic-associated breast carcinomas models (Zeng et al. 2019). These breast DTCs survival opportunities may also connect with the capability of consuming extracellular energy sources from microenvironment to generate and obtain the additional metabolic benefits. In the other studies, the enhancements of breast cellular proliferation are also diverse; for instance, based on osteolytic metastasis, a quantity of RANKL stimulators from tumor cells such as IL-11 and/or MMPs release bone matrix growth factors, lead to the induction of carcinoma cells proliferation together with the increase of osteoblast activity as a positive feedback loop (Mundy 2002). However, it seems likely that in certain cases, colonization enhancement can be inversely proportional to survival signals. For example, under the activation of stress hormones mediator-glucocorticoid receptor, ROR1 kinase and

other anti-survival signaling pathways are activated, while this activation promotes tumor heterogeneity and metastasis, implicate the attention with glucocorticoids treatment protocol (Obradović et al. 2019). These findings demonstrate the diversity of multi-metastatic mechanisms in different organs and turn to the advance diagnosis and treatment in breast cancer patient.

### **3 Epithelial to mesenchymal transition**

#### **3.1 General concept**

Epithelial–mesenchymal transition (EMT) is a cellular program that temporally turns cells from epithelial to full or partial mesenchymal status (Dongre and Weinberg 2019). During this reversible process, epithelial cells continuously lose their epithelial morphology in the cellular monolayer to endorse a mesenchymal phenotype with spindle-shaped structure. In contrast, the mesenchymal cells can also revert into their initial epithelial state, which is also called mesenchymal–epithelial transition (MET).

The EMT results in decrease of epithelial markers such as E-cadherin and Occludin (Dongre and Weinberg 2019). E-cadherin (CDH1) is a homophilic protein that regulates the cellular adherence junctions to its surrounding cells via extracellular domains interaction. The downregulation and dysfunction of CDH1 is widely observed during EMT (Gheldof and Berx 2013). In breast cancer, CDH1 plays as a tumor suppressor, therefore, the loss of E-cadherin expression is associated with poor prognosis in breast cancer patients. Down regulation of CDH1 is due to internal mutation including the loss of chromosome heterozygosity or external microenvironment impact on the inhibition of transcription (Berox and Van Roy 2001). Occludin is a major component of the tight junction cellular barriers, and act as a tumor suppressor. Occludin is highly expressed in low-metastatic breast tumor tissues (ER+) and slightly expressed in high metastasis tumor tissues (TNBC), loss of Occludin associates with breast cancer progression and metastasis (T. A. Martin, Mansel, and Jiang 2010; T. A. Martin et al. 2016). In addition, inducing of EMT phenotypes leads to the increase of mesenchymal markers such as Vimentin. Vimentin (VIM) is an intermediate filament (type 3), which involve in cytoskeleton formation and maintain cellular integrity (Korsching et al. 2005). In breast cancer, VIM supports tumor cells migration, therefore, expression of VIM is correlated to tumor invasiveness and chemoresistance. The effect of VIM on the aggressiveness of breast cancer is often explained by the mesenchymal phenotypes of these cells indicated the initiation of metastatic process (Korsching et al. 2005; Z. Chen, Fang, and Ma 2021).

The high-grade malignant progression depends on the activation of pleiotropic EMT in neoplastic cell populations and association with carcinoma cells traits mutations (Mani et al. 2008; Dongre and

Weinberg 2019). Dongre A and Weinberg RA described a typical EMT process as presented in Figure 13. In healthy condition, the apical–basal epithelial cells are strongly connected together by different junctions (tight and adherences), desmosomes and are attached to basement membrane layer by hemidesmosomes. These cells explicit the proteins that are responsible for maintain epithelial-like state and cell polarity (Figure 13 yellow and light orange boxes). The activation of EMT causes the expression of EMT-inducing transcription factors (EMT-TFs) (Figure 13 grey box), which restrict the epithelial-associated genes (Figure 13 yellow box) and accordingly promote the mesenchymal-associated genes (Figure 13 light orange box). These genomic expression modifications lead to the cellular reprogramming including epithelial cell–cell junctions' dissolution and cell polarity destruction via repression of junction-regulated and apical–basal polarity-regulated genes (crumbs, PALS1-associated tight junction protein (PATJ) and lethal giant larvae (LGL)). The acquisition of mesenchymal phenotypes together with continuous loss of epithelial features result in cellular front-to-back polarity acquisition and cytoskeleton reorganization, these cells transiently express the specific molecules and EMT-TFs that induce and preserve mesenchymal status (Figure 13 orange box). The mesenchymal cells have high capacity of motility and invade to distant organs. As a reversible process, mesenchymal-like cells can revert to epithelial-like state at the metastatic site to establish secondary tumor by following mesenchymal–epithelial transition (MET). However, most of the carcinoma cells that spontaneously grow as tumors are often transform partially into mesenchymal status in order to balance between EMT and MET in physiology or pathology conditions.

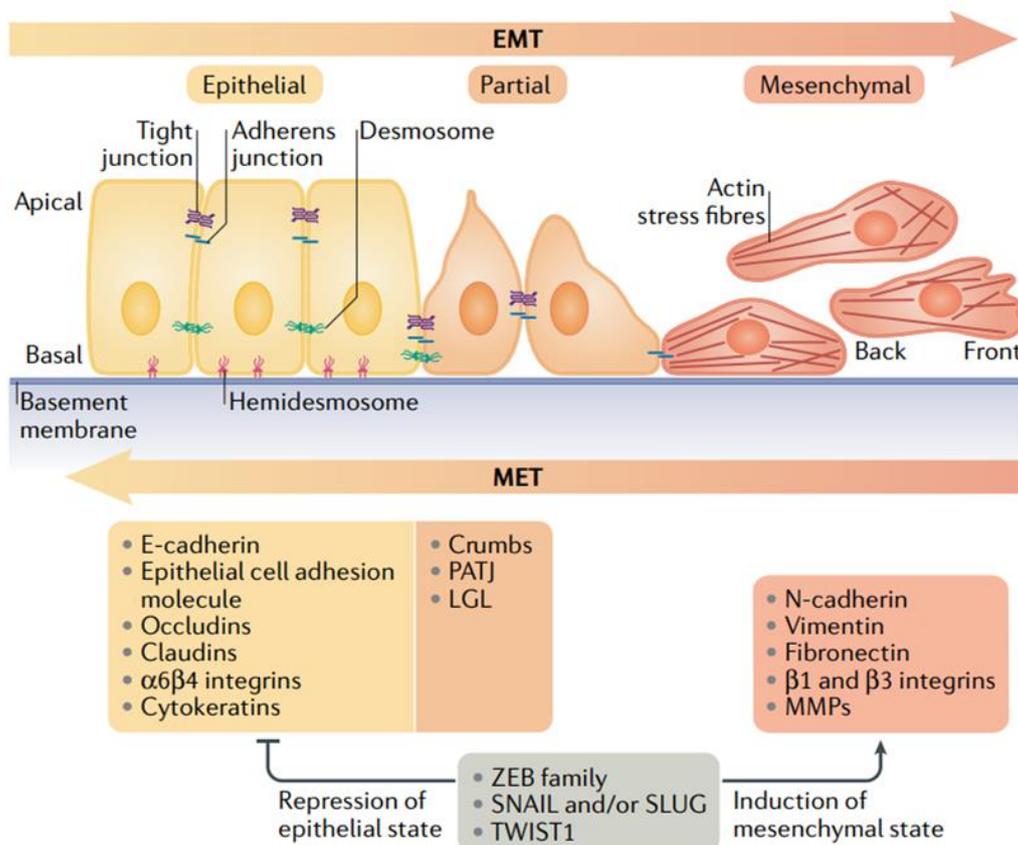


Figure 13 : Representation of a standard EMT program (Dongre and Weinberg 2019)

Yellow-box: epithelial markers, light orange box: polarity markers, dark orange box: mesenchymal markers, grey box: EMT-inducing transcription factors (EMT-TFs).

Abbreviations: EMT=epithelial–mesenchymal transition. EMT-TFs=EMT-inducing transcription factors. ZEB=Zinc Finger E-Box Binding Homeobox. SNAIL=Snail Family Transcriptional Repressor. TWIST=Twist-related protein. PATJ=PALS1-Associated tight junction protein. LGL=Lethal Giant Larvae. MET=mesenchymal–epithelial transition. E-cadherin=epithelial cadherin. MMP=matrix metalloproteinase. N-cadherin=neural cadherin.

### 3.2 Signaling pathways regulate EMT

The activation of EMT is due to the contribution together of different intrinsic pathways, results in the up-regulation of EMT-regulated transcriptional factors such as Zinc Finger proteins (ZEB, SNAIL, SLUG) or basic helix–loop–helix protein (TWIST) (Figure 13 box grey). During carcinoma progression, the cancer cells activate transforming growth factor- $\beta$  (TGF $\beta$ ), WNT and NOTCH representative pathways and the downstream signaling of these regulations induces EMT as presented in Figure 14 (Dongre and Weinberg 2019).

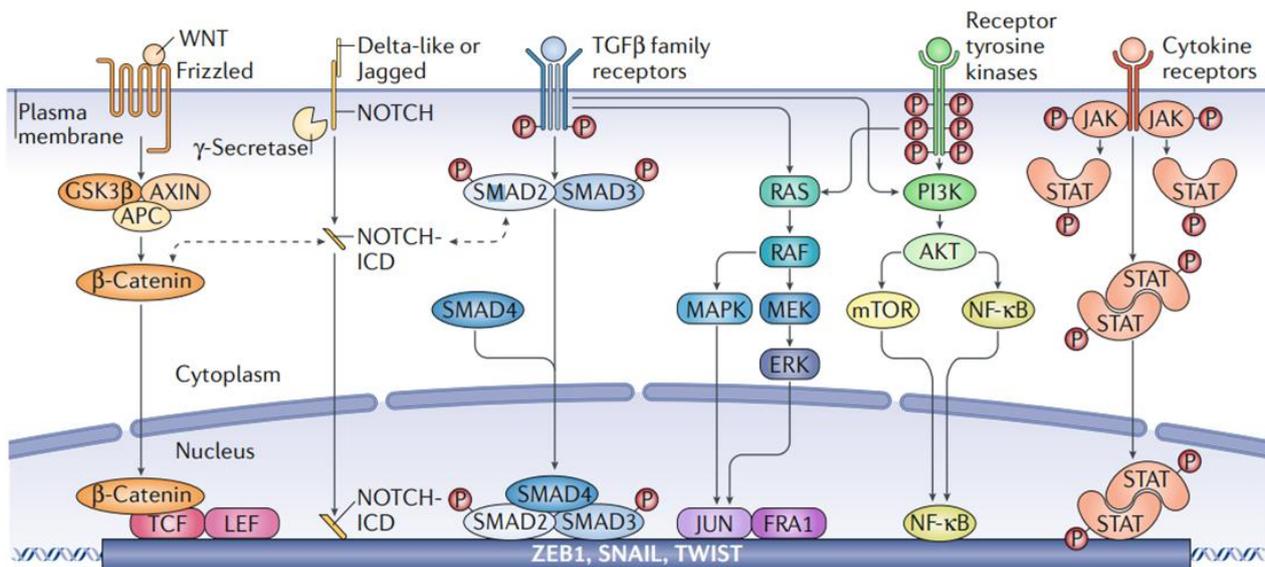


Figure 14: Signaling pathways involve in EMT activation (Dongre and Weinberg 2019)

Abbreviations: ZEB, SNAIL and TWIST=epithelial–mesenchymal transition (EMT)-inducing transcription factors. TGF $\beta$ =transforming growth factor- $\beta$ . GSK3 $\beta$ =glycogen synthase kinase-3 $\beta$ . AXIN=axis inhibition protein. APC=adenomatous polyposis coli protein. TCF=T cell factor. LEF=lymphoid enhancer-binding factor. NOTCH-ICD=intracellular domain of the NOTCH receptor. NF- $\kappa$ B=nuclear factor- $\kappa$ B. JAKs=Janus kinases. STAT= Signal transducers and activators of transcription

In detail, the activation of canonical WNT pathway is due to the binding of WNT ligand to membrane receptors (Frizzled family proteins), leads to  $\beta$ -catenin releasing from GSK3 $\beta$  (glycogen synthase kinase-3 $\beta$ )–AXIN (axis inhibition protein)–APC (adenomatous polyposis coli protein) complex into cytosol and translocate into the nucleus (Komiya and Habas 2008). Inside the nucleus, the  $\beta$ -catenin then bind to transcription factors TCF (T cell factor) and LEF (lymphoid enhancer-binding factor), repress cellular proliferation, activate EMT-regulated genes and promote EMT (Komiya and Habas 2008; Santiago et al. 2017).

Another example is the activation of NOTCH pathway after the binding of ligands (Delta-like or Jagged family proteins) to NOTCH receptor (Schroeter, Kisslinger, and Kopan 1998). These bindings generate different proteolytic cleavage reactions that terminate the intracellular domain of NOTCH receptor (NOTCH-ICD) release. This active domain then arrives to cell nuclei and acts as co-activator for transcription of EMT-inducing TFs genes (Z. Wang et al. 2009).

The other representative signaling is TGF $\beta$  pathway, which can be activated after the binding of TGF $\beta$  ligands to the corresponding receptors. Briefly, family of TGF $\beta$  receptors is then phosphorylated and activates SMAD proteins (via phosphorylation), which can translocate into nucleus and promote EMT program. In addition, these SMAD complexes also co-interact with  $\beta$ -catenin and NOTCH-ICD to play as a central crosstalk between the three mentioned pathways (TGF $\beta$ , WNT and NOTCH) (X. Guo

and Wang 2009; Hata and Chen 2016; C. Chen et al. 2015). The TGF $\beta$  pathway also participates with other signaling pathway such as the RAS–RAF–MEK–ERK pathway, MAPK (p38) pathway and PI3K–AKT pathway. The PI3K–AKT signaling axis leads to mTOR complex and  $\kappa$ -light-chain-enhancer nuclear factor activating B (NF- $\kappa$ B) activation and activate EMT-inducer genes (Dongre and Weinberg 2019). However, the detail literature concerning TGF $\beta$  signaling is discussed in following parts of the thesis.

The last representative EMT-initiated pathways are activation of downstream signaling after the binding of cytokines to their associated receptors; result in the activation of Janus kinases (JAKs) via phosphorylation and activator of transcription (STAT) via phosphorylation and/ or dimerization. These stable STAT dimers are able to promote the EMT-TFs gene transcription via activation of EMT-inducing transcription factors (ZEB, SNAIL, TWIST) (Thomas et al. 2015).

## Chapter 3 TGF $\beta$ signaling in cancer

In order to metastasize effectively, the tumor cells should be able to adapt multiple microenvironments, be flexible and profit the regulatory network with sufficient signaling molecules to maintain survival state and reestablish tumor growth.

For example, cancer cell transition from dormant status to active state can be induced *via* local environmental modification, such as dormancy-escaping and proliferation-inducing activities *via* neo-vascularization after TGF $\beta$ 1 and periostin (POSTN) secretion (Ghajar et al. 2013), angiogenesis (Kienast et al. 2010) or inflammation under the mediation of pro-inflammatory cells in pulmonary metastasis (De Cock et al. 2016).

Among different metabolic pathways and TME factors supporting the tumor progression, TGF $\beta$  signaling is a wide range of reactions that results in a variety of gene responses leading to TME element modification (S. Liu, Ren, and Ten Dijke 2021). TGF $\beta$ -signaling network varies from early tumor formation to advanced stage of cancer development (Figure 15).

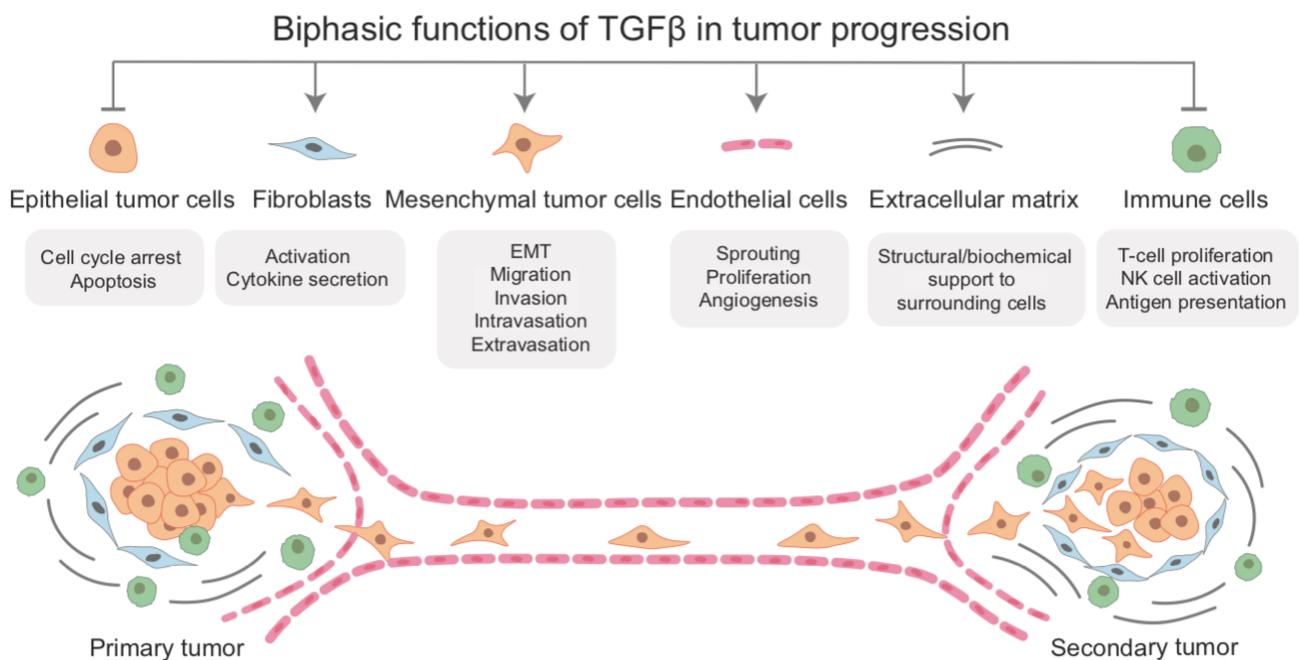


Figure 15: TGF $\beta$  functions in tumor development and metastasis (S. Liu, Ren, and Ten Dijke 2021)

### 1 TGF $\beta$ pathway members and activation signaling

The transforming growth factor  $\beta$  (TGF $\beta$ ) is a cytokine family with ambivalent action in a tumor context. It restricts neoplastic development at an early stage but promotes tumor progression and the metastatic process at the advanced stage. TGF $\beta$  cytokine belongs to transforming growth factor superfamily, which has three isoforms TGF $\beta$ 1, TGF $\beta$ 2 and TGF $\beta$ 3 (Flanders et al. 2016). These TGF $\beta$  isoforms share similar biological activity but tissue expression specificity. TGF $\beta$  cytokines are

frequently secreted by cancer cells, white blood cells (macrophage, B-cells), T-cells (Regulatory T cells) and cancer associated fibroblast (CAF) (Bjarnadóttir et al. 2016; S. Liu, Ren, and Ten Dijke 2021). The receptors of this cytokine include TGF $\beta$  type I receptor (TGF $\beta$ RI) family, which contains activin-like receptor ALK-5, TGF $\beta$  type II receptor (TGF $\beta$ RII) (Joan Seoane and Gomis 2017a). Seven types of TGF $\beta$ RI and five types of TGF $\beta$ RII have been clarified in human (Wrzesinski, Wan, and Flavell 2007). There is also TGF $\beta$ R3 ( $\beta$ -glycan) considered as a co-receptor presenting TGF $\beta$  to TGF $\beta$ RII, which recruits and then activates TGF $\beta$ RI by transphosphorylation (Tzavlaki and Moustakas 2020).

The activation of TGF $\beta$  signaling is due to the secretion of the cytokine into the extracellular matrix as a latent complex consisting of the cytokine and a latent associated peptide (LAP). By activation, TGF $\beta$  detaches from LAP and binds to TGF $\beta$ RII at docking site. After that, TGF $\beta$ RI can auto-activate, however, it is mainly recruited by TGF $\beta$ RII to form TGF $\beta$  bidimeric-structure receptors complex (heterotetrameric structure with 2 TGF $\beta$ RI and 2 TGF $\beta$ RII molecules) (Heldin and Moustakas 2016; Ikushima and Miyazono 2010). The TGF $\beta$ RII then phosphorylates both serine and threonine residues in TGF $\beta$ RI, which leads to the activation of this receptor complex and continuously transmits the signal *via* the activation of down-stream signaling (Figure 16). The TGF $\beta$  pathway is associated with pro- and anti-tumor properties present at different stages of the disease. The downstream pathways of TGF $\beta$  signaling combine of the canonical Smad pathway and the non-canonical (Smad-independent) pathways.

In canonical pathway, activation of TGF $\beta$  receptors leads to the phosphorylation and activation of Smad2 and Smad3 proteins (R-Smad; Smad receptor-associated) by TGF $\beta$ RI to form a complex with Smad4 (Co-Smads; common-partner Smads). The Smad complex translocates into the nucleus where it will play a role as transcriptional co-activator or co-repressor depending on the context (Figure 16). This pathway is also regulated by I-Smad (Smad inhibitors) like Smad7, which inhibits the activation of R-Smad (Tzavlaki and Moustakas 2020). The termination of Smads signaling is followed by the polyubiquitination of active-Smads complex (Joan Seoane and Gomis 2017a). The degradation of active R-Smads is due to E3 ubiquitin ligase (e.g. smurf2, Nedd4L) activities, which is recruited by I-Smad (Smad7) (Yan et al. 2016)

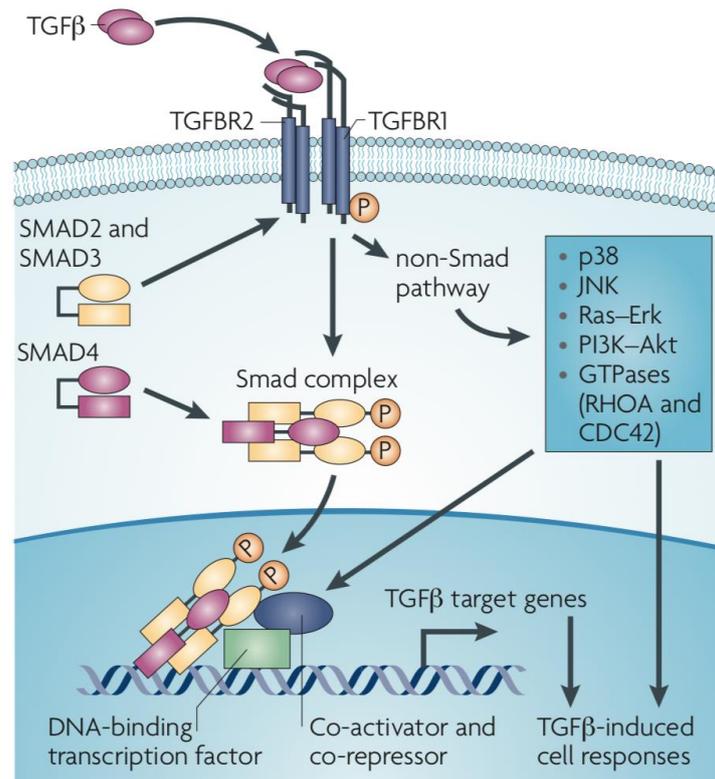


Figure 16: Intracellular downstream signaling of TGFβ (Ikushima and Miyazono 2010).

Transforming growth factor-β (TGFβ) signaling is transformed through Smad (canonical) and non-Smad (non-canonical) pathways. TGFβ cytokine binds to its receptors (TGFBR2 and TGFBR1). Phosphorylation of TGFBR2 phosphorylates (P) TGFBR1, lead to the phosphorylation and activation of SMAD2 and SMAD3 to create a complex with SMAD4 and translocate into the nucleus. This complex then co-interacts with other activator or repressor and induce TGFβ responsive genes at transcriptional level. TGFβ stimulation also activates other signaling cascades such as p38, JNK, Ras-Erk, PI3K-Akt, and small GTPases (RHOA and CDC4).

The main mediator of canonical pathway is Smad protein family. Smad proteins are originally described in *Drosophila* (Mad) and *C. elegans* (Sma) and well-known in humans as Smad, a contraction of Sma and Mad (Attisano and Lee-Hoeflich 2001). Depending on the functions in response to TGFβ signaling, Smad protein family is categorized into three subclasses including receptor-regulated Smads (R-Smads: Smad 2, Smad 3), common-partner Smads (Co-Smads: Smad 4) and inhibitory Smads (I-Smads: Smad 7) as presented in Figure 17. The structure of Smad proteins contains different domains, which are dependent on the subclass. From the left to the right of structure schemes in Figure 17, only R-Smads (Smad 3) and Co-Smad (Smad 4) possess an N-terminal MAD homology 1 domain (MH1) to support DNA binding via a β-hairpin structure, which then inserts into the major groove of DNA. However, full-length Smad 2 does not bind to DNA because the insertion of amino-acid sequence coded by exon 3 in Smad 2 mRNA near the β-hairpin can prevent DNA binding. In addition, the linker domain in all Smad subtypes can be phosphorylated by different kinases (including ERK, AKT, JNK, and PI3K) during signaling regulation. The MH2 domain also presents in all Smads, which contains

the L3 loop for protein-protein interaction such as the connection of R-Smad and activated-TGF $\beta$ RI or Co-Smad trimeric complexes. Finally, the R-Smads have Ser-X-Ser (SxS) motif at C-terminus, which is a TGF $\beta$ RI phosphorylation site. Because of the inhibitory activities, I-Smad contains only MH2 domain with L3 loop, not MH1 and linker domains like other Smads (Tzavlaki and Moustakas 2020).

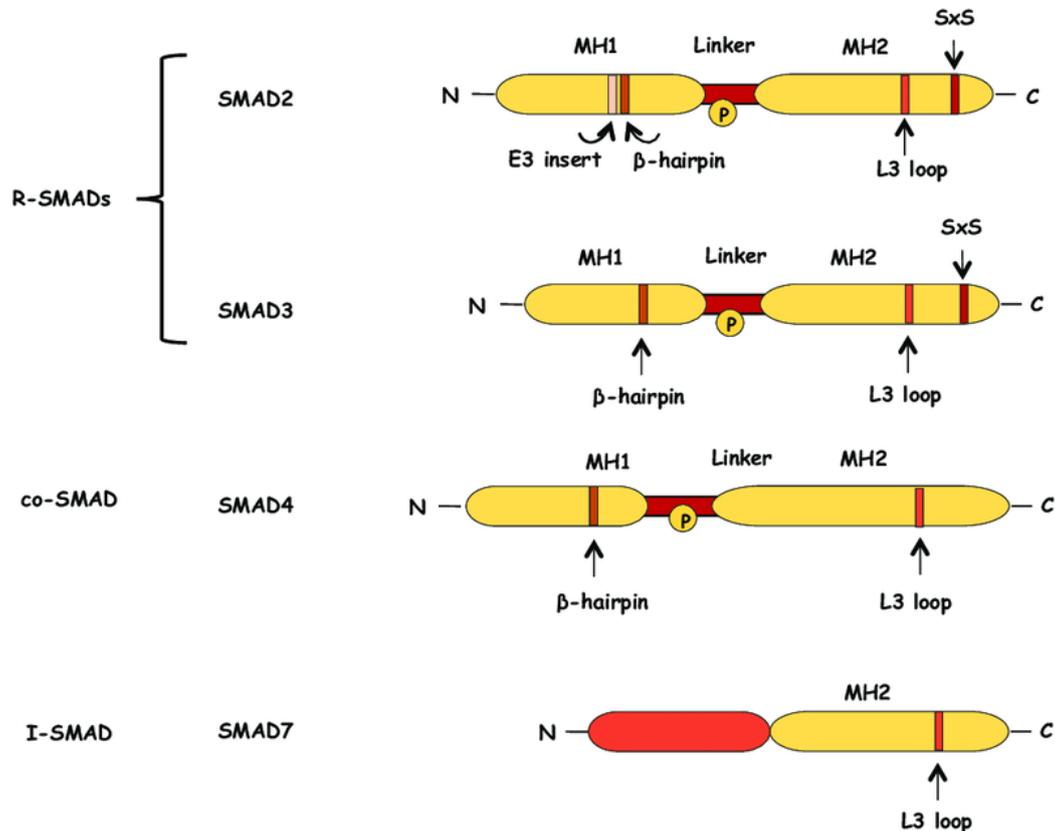


Figure 17: The SMAD proteins structure (Tzavlaki and Moustakas 2020)

Abbreviations: R-Smads=receptor-regulated Smads (Smad 2, Smad 3). Co-Smads=Common-partner Smads (Smad 4). I-Smads=inhibitory Smads (Smad 7)

In addition to canonical Smad signaling pathways, TGF $\beta$  also regulates non-canonical Smad-independent pathways including several kinase cascades (Figure 16). The representative kinases pathways are Erk-Ras, p38 MAPK kinase (TAK-MKK4-p38), Jun N-terminal kinase-JNK (Tak-MKK3/6-c-Jun NH2-terminal kinase), PI3K–Akt and small GTPase (Rho-Rac-cdc-42). These non-canonical pathways involve in a large number of cellular processes such as proliferation, differentiation, apoptosis, inflammation and migration (Joan Seoane and Gomis 2017a).

## 2 Tumor suppression effect of TGF $\beta$

### 2.1 Effects on cell proliferation and cell cycle

TGF $\beta$  signaling is able to suppress proliferation by blocking the cell cycle and inducing apoptosis (Joan Seoane and Gomis 2017a). TGF $\beta$  inhibits cell-cycle arrest at the R-point in the phase G1 during

cell-cycle progression via two main processes including repression growth-promoting transcription factors expression and the induction of specific CDK inhibitors expression (Y. Zhang, Alexander, and Wang 2017) (Figure 18).

Concerning the first mechanism, TGF $\beta$  represses transcription factors controlling cell growth and differentiation such as the growth inducer c-Myc (Figure 18A) (Alexandrow and Moses 1995; Y. Kang, Chen, and Massagué 2003). For the first example, in c-Myc-low cells response to TGF $\beta$  signal, Smad3 binds to TGF $\beta$  inhibitory element (TIE) region of MYC promoter, which have repressive Smad-binding element (RSBE) and a consensus E2F-binding site. At this region, the repressor complex containing Smad3, E2F4/5, co-repressor p107 and DP1 preassembles in the cytoplasm then translocates into the nucleus to response the signaling of TGF- $\beta$ . This complex thereby binds to MYC promoter and represses MYC transcription (C.-R. Chen et al. 2002; Yagi et al. 2002; Frederick et al. 2004). The low-expression of c-Myc not only inhibits cell proliferation but also initiates the activation of different CDK inhibitor genes in response to TGF $\beta$  signals at the same time (Eisenman 2001)

Concerning the second mechanism, induction of cell cycle arrest response to TGF $\beta$  signals is also regulated by CDK inhibitors (Figure 18b). The activation of CDKs (CDK2, CDK4, and CDK6) is required during  $G_1 \rightarrow S$  transition, in which CDK2 binds to cyclin E, CDK4 or CDK6 bind to cyclin D and phosphorylate its substrates. However, inhibitor of cyclin-dependent kinase 4 (INK4) protein family containing p15INK4B can specifically binds to CDK4 and CDK6 to prevent their binding to cyclins, leads to the inhibition of cell-cycle progression. In addition, other CDK inhibitors such as p21CIP1, p27KIP1 and p57KIP2, which belong to CIP/KIP family, can associate with cyclin-CDK complexes and prevent kinase activities (Murray 2004). Interestingly, the regulation of these inhibitors is controlled by c-Myc, in fact, c-Myc creates a complex with the zinc-finger protein (Miz1) and binds to the promoters of p15INK4B and p21CIP1, leads to the inhibition of these genes' expressions in proliferating cells. Therefore, in the response to TGF $\beta$  signaling, suppression of c-myc expression promotes transcriptional induction of CDK inhibitors, results in cell cycle-arrest (J. Seoane et al. 2001; Joan Seoane, Le, and Massagué 2002; Staller et al. 2001)

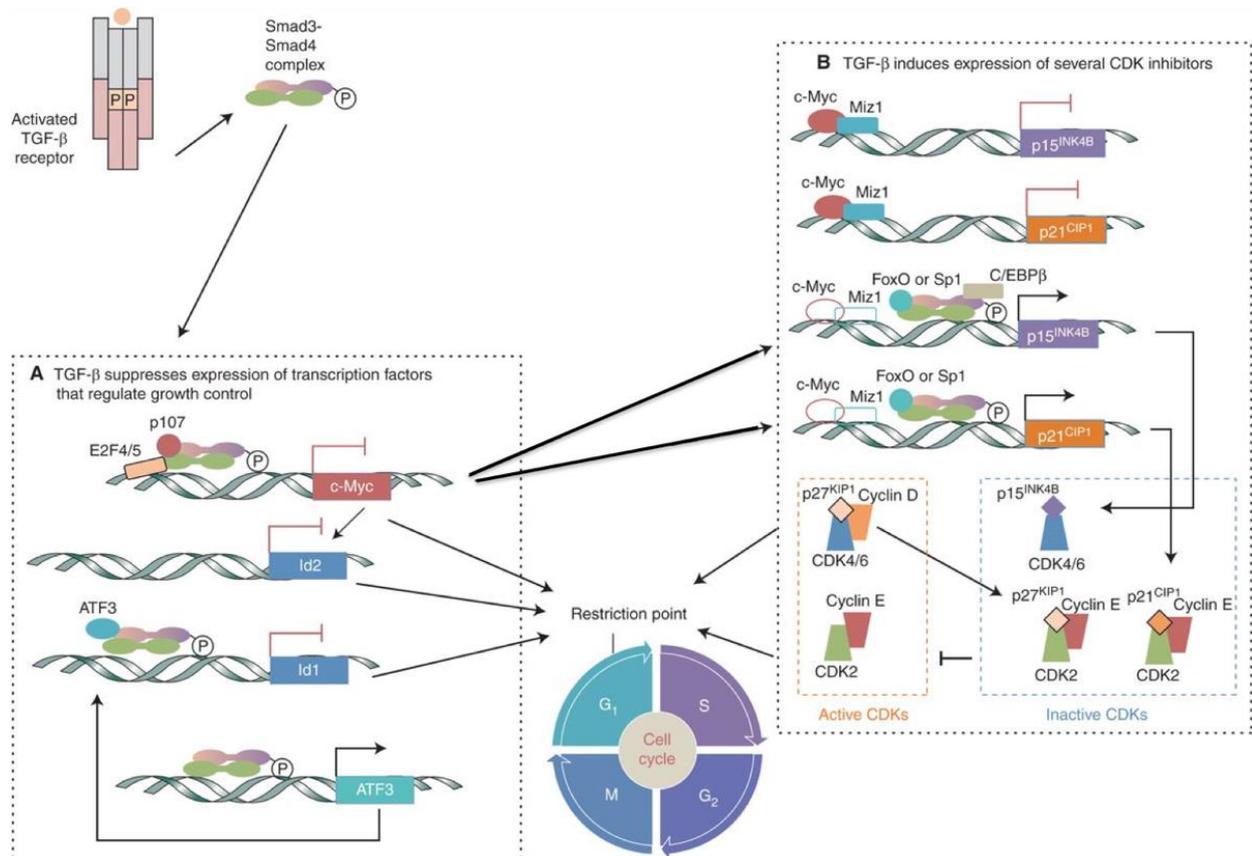


Figure 18 : Tumor suppression effects of TGF $\beta$  signaling in epithelial cells cell-cycle (Y. Zhang, Alexander, and Wang 2017)

(A) TGF- $\beta$  reduces the transcription factors expression that control cellular growth including c-Myc and Id encoded proteins.

(B) TGF- $\beta$  increase cyclin-dependent kinase (CDK) inhibitors expression (p15<sup>INK4B</sup> and p21<sup>CIP1</sup>). TGF- $\beta$  also improves p27<sup>KIP1</sup> function without modifying *CDKN1B* gene transcription, due to p27<sup>KIP1</sup>-CDK4/6-cyclin D complexes disruption.

## 2.2 Effects on cells apoptosis

The effect of TGF $\beta$  on apoptosis is strongly dependent on the stage of cancer development. Although TGF $\beta$  can either induce or suppress apoptosis, pro-apoptotic effects of TGF $\beta$  have been observed in premalignant state of cancer. However, the studies on tumor suppression effects of TGF $\beta$  signaling in breast cancer are mainly focused on growth inhibition induced by cell cycle arrest, not regulation of apoptosis.

In canonical pathway responding to TGF $\beta$  signaling, Smad transcriptional complexes, especially Smad 2/3 and Smad 4, regulate many apoptotic target genes in different types of cancer (Figure 19). For example, this Smad complex induces TGF- $\beta$ -inducible early response gene 1 (TIEG1)-a zinc-finger transcription factor in pancreatic epithelial cells. TIEG1 can reduce Bcl2 expression, which is a main inhibitor of cellular apoptotic intrinsic pathway. Therefore, TIEG1 induction in response to TGF $\beta$  contributes to its proapoptotic effects (Y. Zhang, Alexander, and Wang 2017). In addition, connective tissue growth factor protein (CTGF) is transcriptionally induced by TGF $\beta$  in breast cancer, leads to the

low-expression of Bcl2 protein, results in the induction of apoptosis (Hishikawa et al. 1999) . Another mechanism in hepatocytes showed that death-associated protein kinase (DAP-kinase) expression is increased by TGF $\beta$  mediated Smad2/3 and Smad4 complex, which then activates caspase activities inducing cell apoptosis (Jang et al. 2002) .

In non-canonical pathway, a human septin-like protein, which is also called apoptosis-related protein in the TGF $\beta$  signalling pathway (ARTS), is important for TGF $\beta$ -regulating apoptosis. During TGF $\beta$  activation, ARTS is released from mitochondria, leads to up-regulation of caspase 3 (a pro-apoptotic protein) activity (Larisch et al. 2000). Furthermore, adaptor protein Daxx can bind to either Fas receptor or interact with TGF $\beta$ RII to activate JNK pathway-enhanced apoptosis and promote Fas-mediated apoptosis in both cancer cells (human cervix-carcinoma) and immune cells (lymphocytes) (Perlman et al. 2001; X. Yang et al. 1997). Finally, Id family, which includes three isoforms Id1, Id2, and Id3, can inhibit the cell differentiation and induce proliferation. In general, Id1 induces Ras and causes the mammary tumorigenesis. In the presence of TGF $\beta$ , the cytokine down-regulates Id1 expression, lead to the modification of Ras in human breast epithelial cells, leads to the decrease of their proliferative abilities, results in the suppression of tumor formation (Grusch et al. 2010). Therefore, these findings illustrate the importance of TGF $\beta$  in controlling cellular growth and development.

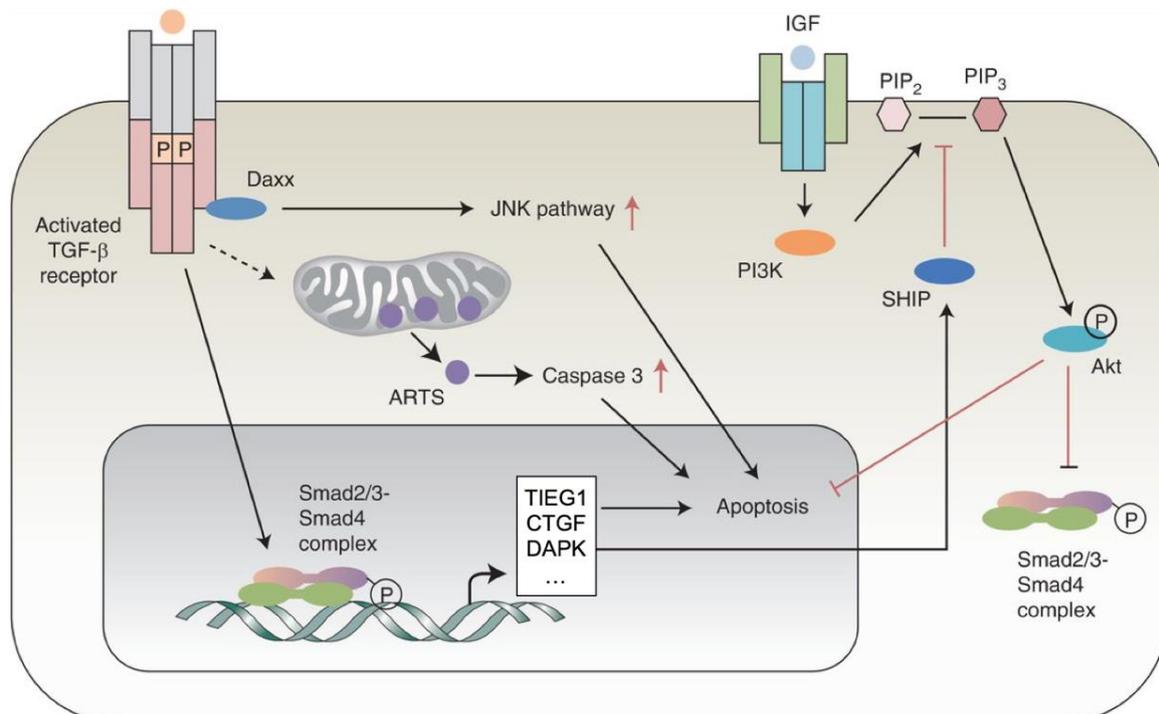


Figure 19 : TGF- $\beta$ -induced cellular apoptosis mechanisms (Y. Zhang, Alexander, and Wang 2017)

Abbreviations: TIEG1=TGFbeta Inducible Early Gene-1. CTGF=Connective tissue growth factor. DAPK= Death-Associated Protein Kinase . Daxx= The death-associated protein 6. IGF= Insulin-like growth factors. PIP= Prolactin Induced Protein. SHIP= inositol polyphosphate-5-phosphatase.

### 2.3 Suppressive effects on tumor growth in cancer

TGF $\beta$  signaling acts as a tumor suppressor through three different signaling aspects including cell cycle/ cell proliferation inhibition, induction of apoptosis promotion, and prevention of cell immortalization.

In breast cancer, many studies on transgenic mice (MMTV-PyMT and MMTV-Neu model) demonstrated the effect of TGF $\beta$  on inhibiting breast cancer tumor growth. These transgenic models contain the murine mammary tumor virus (MMTV) promoter, which drives the expression of breast carcinogenesis gene in mice such as ErbB2/Neu (called MMTV-Neu), polyoma middle T antigen (called MMTV-PyMT).

The MMTV-Neu and MMTV-PyMT transgenic mice has the lack of lactation during their pregnancy, which activates estrogen breast cancer responsive transgene and results in tumorigenesis (lung and lymph node metastasis) (Fantozzi and Christofori 2006; Attalla et al. 2021). MMTV-PyMT mice is similar to human luminal B breast cancer. The chemical carcinogen induction of TGF $\beta$ 1 cytokine prevents the formation of breast tumors at early stage of tumor formation (Attalla et al. 2021; Maglione et al. 2001). In addition, Py811 is the cancer cell line derived from MMTV-PyMT mammary tumor (Py8119), by knock down of TGF $\beta$ R2 expression in this cell line, there is the increase of cell proliferation and tumor growth in a primary tumor site, which suggests the role of TGF $\beta$ R2 and TGF $\beta$  signaling in delay tumor growth (Biswas et al. 2014). Conditional knockout of TGF $\beta$ R2 in the mammary epithelium of MMTV-PyMT mice also leads to the induction of tumor growth (Forrester et al. 2005). In addition, conditional knockout of TGF $\beta$ R2 (Tgfr2-KO) in MMTV-PyMT can downregulates TGF $\beta$  signaling, which leads to the increase of IL17 and promotes tumor growth. Treatment with anti-IL-17 Ab can reverse these effects on this model (Novitskiy et al. 2011). Moreover, MMTV-Neu model is established based on MMTV promoter driving the ErbB2/Neu expression, which is mimicking human HER2+ breast cancer due to the high expression of ErbB2 (Neu). The crossing of MMTV-Neu mice with MMTV- DNIIR (dominant-negative gene of TGF $\beta$ R2) show an increase of tumor growth, which shows the role of TGF $\beta$ R2 high-expression in suppressing breast cancer tumor growth (P. M. Siegel et al. 2003).

TGF $\beta$  signaling and its composition can also inhibit tumor growth in different cancers (L. H. Katz et al. 2013). In melanoma, TGF $\beta$  inhibited tumor growth of B16F1 bearing mice due to the reduction of plasmin activity (Ramont et al. 2003). Interestingly targeting TGF $\beta$  isoforms by T $\beta$ R1-T $\beta$ R2-Fc, a chimeric protein including the extracellular domains of TGF $\beta$ R1 and/or TGF $\beta$ R2 fused with the Fc

region of human immunoglobulin (IgG), support both inhibition of tumor growth in subcutaneous mouse melanoma and melanoma metastasis (Kodama et al. 2021). In addition, TGF $\beta$ 2 cytokine reduces the growth of mesothelioma cancer tumor in vivo model by inducing host antitumor immunity (E. Suzuki et al. 2004).

### 3 Tumor promoting effect of TGF $\beta$ on cell death and cell metastasis

#### 3.1 Effects on cell proliferation

In the advanced cancers, TGF $\beta$  pathway promotes tumor progression and the development of metastases, which is called pro-oncogenic activity. In tumor development, the carcinoma cells escape the tumor-suppression effect of TGF $\beta$ . In certain cases, tumor cells have genomic mutations or silencing of TGF $\beta$  signal transduction pathway components genes (*i.e.* Smads and TGF $\beta$  receptors), therefore they escape the antitumoral function of TGF $\beta$  cytokine.

In other cases, the compositions of TGF $\beta$  signaling pathway sometimes remain unchanged, however, cellular resistances against anti-proliferation effect of TGF $\beta$ , are highly observed (Joan Seoane and Gomis 2017b). For examples, in breast cancer, the TGF $\beta$ -mediated cytostatic effect is selectively damaged. In 2010s, the isolated cells from pleural fluids of metastatic breast cancer patients showed a loss of cytostatic effect of TGF $\beta$  in different clinical studies. In this population, half of the patients' samples has a down-regulation of p15INK4b and up-regulation of c-Myc in response to TGF $\beta$  signaling while other TGF $\beta$ -signaling regulated genes are remaining unchanged. These modifications lead to the increase of breast cancer cells proliferation (Gomis et al. 2006; Arnal-Estap   et al. 2010). The explanation for this observation is associated to CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ). C/EBP $\beta$  is tumor suppressor with anti-proliferation effects via forming with the complex with RB/E2F repressor to negatively modulate E2F target genes including c-Myc and cyclin A2 (Sebastian and Johnson 2006). The C/EBP $\beta$  mRNA is translated into two major isoforms, LAP (liver-enriched activating protein) and LIP (liver-enriched inhibitory protein). LAP expression correlates with forkhead box protein FOXO and Smads to activate CDK inhibitor p15INK4b and repress c-Myc expression, LIP expression blocks LAP activity and induces proliferation by turning the TGF $\beta$  responses toward p15INK4b repression and c-Myc activation. Therefore, the increase of LIP:LAP ratio is observed in both malignant tumors associated with poor prognosis (Milde-Langosch, L  ning, and Bamberger 2003; Zahnow et al. 1997) and TGF $\beta$ -modified cytostatic response in metastatic breast cancer (Gomis et al. 2006). In additions, ER negative breast cancer patients with metastatic phenotype have high expression of heterogenous Id1 in response to TGF $\beta$ . As mentioned before, Id1 can promote the cancer cell proliferation (B. Tang et al. 2007). Therefore, in breast cancer cells responding to

TGF $\beta$ 1, Smad 3 and Smad 4 bind to the upstream locus on the Id1 gene, lead to the induction of Id1, resulting in breast cancer cell over-proliferation (Y.-Y. Liang, Brunicardi, and Lin 2009, 1).

### 3.2 Effects on cell death

Under certain conditions, TGF $\beta$  signaling can act as a survival signal and prevent apoptosis. For example, TGF $\beta$  promotes Dec1 expression, which is a transcription factor known to prevent apoptosis, and induce cell survival in mouse mammary carcinoma cells, including breast cancer cells (Ehata et al. 2007). In breast cancer epithelial cells (NMuMG and 4T1), TGF $\beta$  activates PI3K-Akt signaling and thus improves cell survival (Y. E. Zhang 2009). Interestingly, the cross talk between PI3K-Akt and TGF $\beta$  is used to determine the proliferation, cell-cycle arrest, and apoptosis of different cells (Figure 19). For example, activated Akt directly interacts with Smad 3 to form a Akt-Smad3 complex, which can decrease the phosphorylation and nucleus translocation of Smad 3, leads to the inhibition of cell apoptosis (Conery et al. 2004; Remy, Montmarquette, and Michnick 2004). Akt and its downstream target-rapamycin (mTOR) are also able to inhibit Smad3 activities (Das et al. 2013). In addition, PI3K-Akt pathway can inhibit FoxO transcription factors and hence reduce the TGF $\beta$ -induced cell apoptosis (Joan Seoane et al. 2004). However, there is a contradictory study which indicates the activation of PI3K by TGF- $\beta$  only observed in fibroblasts but not in epithelial cells (Wilkes et al. 2005). This observation is interesting because 4T1 cells have both mesenchymal and epithelial markers while NMuMG cells can modify from epithelial status to mesenchymal status (EMT) in response to TGF $\beta$  effects (Gal et al. 2008; Drasin, Robin, and Ford 2011). Therefore, there is a possibility that EMT can significantly regulate cellular response to TGF $\beta$ .

### 3.3 Effects on breast cancer tumor growth and metastasis

Elevated TGF $\beta$  expression or increased activity of its receptors correlates with breast cancer aggressiveness by promoting malignant phenotypes including EMT, cancer stemness, angiogenesis, tissue invasion, and metastasis (Padua et al. 2008). For example, the switch from anti-tumoral effect to pro-tumoral effects of TGF $\beta$  can be explained by the metastatic process, which depends on the HER2 / EGFR signaling pathway inducing the epithelial to mesenchymal transition (EMT) under the control of Smad3 (F. Huang et al. 2018). EMT-induced TGF- $\beta$  is considered to be an essential event in the metastatic process (Dongre and Weinberg 2019). Many studies on *in vivo* mouse models also create the link between TGF $\beta$  and metastasis, tumor growth as well as indicate the possible treatment targeting TGF $\beta$  signaling.

Firstly, studies on transgenic model such as MMTV-Neu and MMTV-PyMT also show the modulation of TGF $\beta$  signaling on increasing metastasis. For example, high TGF $\beta$ 1 content in double-transgenic

MMTV-Neu-TGF $\beta$ 1 mice promotes cancer cells circulating in the vessels and lung metastases, this mutation has not shown any impact on primary tumor development (Muraoka et al. 2002). In addition, the crossing of MMTV-Neu mice with MMTV-TGF $\beta$ RI shows an increase of tumor metastasis while crossing of these mice with MMTV- DNIIR show a decrease of tumor metastasis and increase of tumor growth. DNIIR is the dominant-negative gene of TGF $\beta$ RII (P. M. Siegel et al. 2003). Interestingly, MMTV-Neu mice in the presence of DNIIR in mammary epithelium were used to study the relationship between TGF $\beta$  signaling and HER2. Low activation of TGF $\beta$  signaling decreases tumor growth and increases metastases in MMTVNeu<sup>DNIIR+</sup> mice in a TGF $\beta$ RII-VEGF signaling dependent manner (Novitskiy et al. 2014). In MMTV-PyMT model, the cancer cell line that is derived from MMTV-PyMT mamary tumor (Py8119) has mesenchymal-like phenotype together with high TGF $\beta$ RII expression, suggesting the high metastatic possibility of this models (Biswas et al. 2014). Conditional knockout of TGF $\beta$ RII in the mammary epithelium of MMTV-PyMT mice also induces lung metastasis and reduce tumor growth (Forrester et al. 2005).

Secondly, in cancer cell transplantation model, the introduction of Ki26894 molecule (TGF $\beta$ RI inhibitor), decrease *in vivo* bone metastasis of MDA-MB-231 bearing nude mice (TNBC mimicking model) (Ehata et al. 2007). Another molecule is IN-1130 (TGF $\beta$ RI inhibitor) which decreases matrix metalloproteinase (MMP2 and 9), blocks Smad2 activities (phosphorylation and nucleus translocation), and inhibits 4T1 with EMT phenotype in 4T1-xenografted model. This treatment reduces lung metastasis, increases survival rate of these mice and implicates the importance of TGF $\beta$  in aggressiveness of breast cancer (C.-Y. Park et al. 2014). In addition, 1D11 (TGF $\beta$  cytokines inhibition) treatment also suppresses lung metastasis in 4T1-bearing mice due to CD8+ T cells activation (Nam et al. 2008). These results suggest the role of TGF $\beta$  cytokines and its composition in breast cancer metastasis. Interestingly, these above molecules are now investigating in clinical study, which highlight the important of TGF $\beta$ 1 and efficacy of these *in vivo* models in breast cancer treatment. In fact, due to the paradox effects of TGF $\beta$  signaling (antiproliferation and metastatic inducer), the main target of these treatments is to select the appropriate treatment dose to reduce the metastasis without inducing breast tumor growth.

Moreover, many TGF $\beta$  /Smad signaling-dependent pathways were discovered in correlation with EMT-inducing phenotype. For instance, Smad-driven EMT induces both stemness characteristics and metastatic seeding in breast cancer (Labelle, Begum, and Hynes 2011; Scheel et al. 2011). TGF $\beta$  signaling in breast cancer is also tightly correlated to lung metastasis and to the expression of SMAD-dependent protein: angiopoietin-like 4 (Angptl4), which causes the disruption of vascular endothelial cell–cell junctions and supports extravasation of circulating tumor cells (Padua et al. 2008).

Upregulation of other metastatic-supported genes such as connective tissue growth factor (CTGF), interleukin-11 (IL-11), and Jagged 1 (JAG1) is also dependent on TGF $\beta$ /Smad signaling to improve bone metastasis of breast carcinoma cells (Maroni et al. 2021; Sethi et al. 2011). In addition, targeting TGF $\beta$  signaling via its downstream pathways compositions in breast cancer cells is widely applied *in vivo*. For example, Casitas B-lineage lymphoma b (CBL-b) is the E3 ubiquitin ligases that can directly bind to Smad3, prevent Smad3/Smad4 complex formation and inhibit Smad3 nucleus translocation. By using MDA-MB-231<sup>low</sup> CBL-b xenografted athymic nude mice, the study shows the activation of TGF $\beta$  signaling, result in low-tumor growth and high metastasis. In fact, the data suggests the role of TGF $\beta$  as tumor inducer at early stage of breast cancer progression in CBL-b dependent manner (J. M. Kang et al. 2012). In addition, down-regulation of TGF $\beta$ 1 in MDA-MB-435 xenografted immunodeficient mice shows the decrease in breast tumor metastasis but not tumor development. Interestingly, both TGF $\beta$  receptor I and II expression suggest the positive feedback loop of TGF $\beta$  signaling. The study shows the correlations between TGF $\beta$  signaling compositions and promising target (blocking TGF $\beta$ 1), together with other treatments (for primary tumor) to treat breast cancer metastatic induced by TGF $\beta$  (Moore et al. 2008)

Furthermore, TGF $\beta$  also regulates EMT-correlated transcription factors such as SNAIL, SLUG, TWIST, and ZEB1/2 under the supports of inflammatory machineries and tumoral associated genes as listed in Figure 20

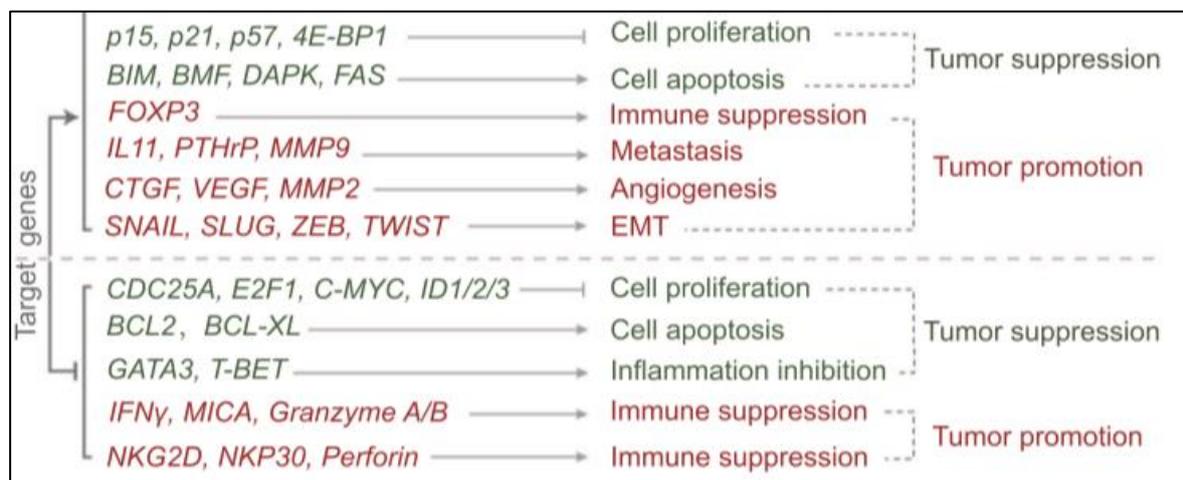
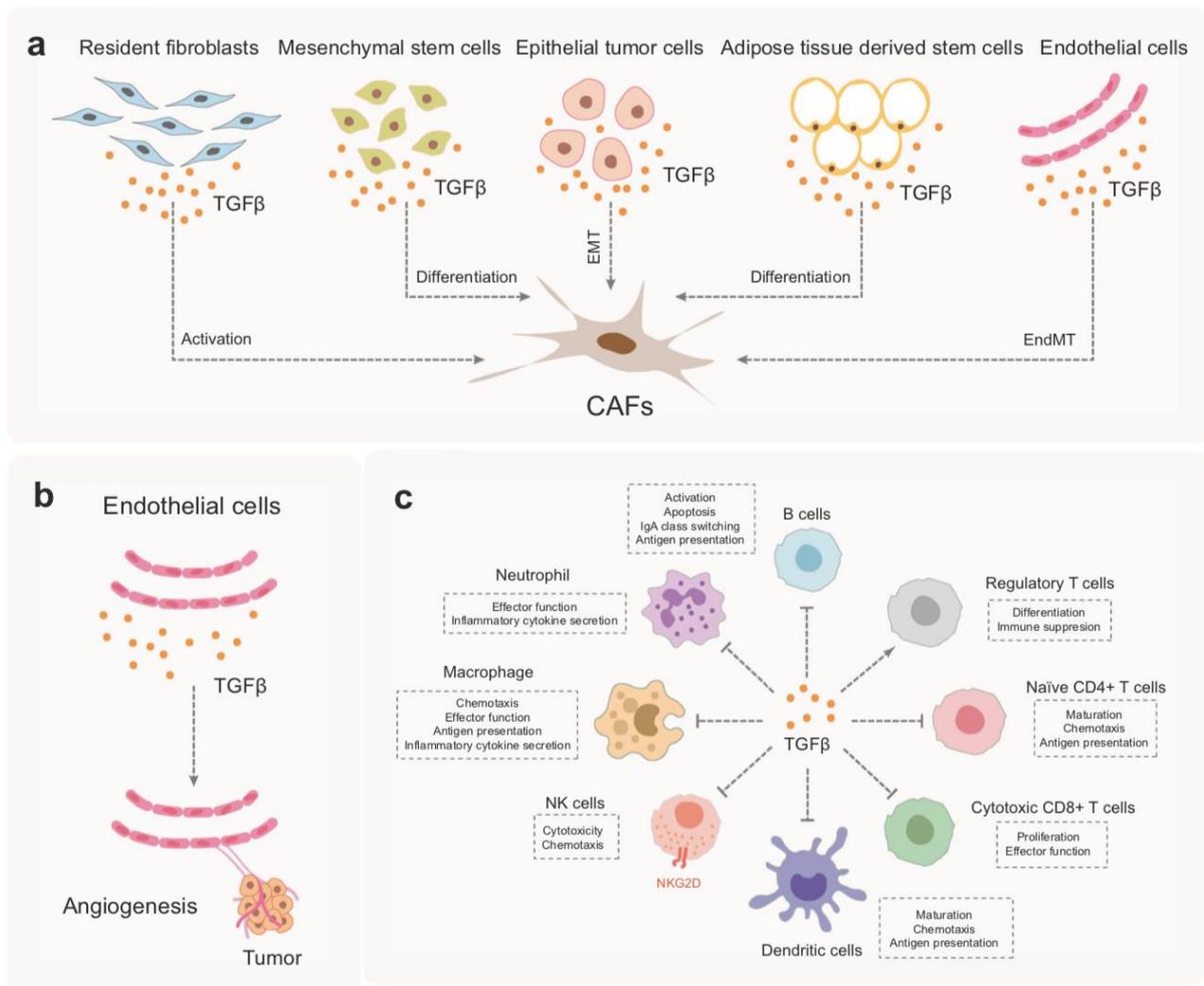


Figure 20 : TGF $\beta$  regulated genes. Green: tumor suppression associated-genes, Red: tumor promotion modulated-genes (S. Liu, Ren, and Ten Dijke 2021)

#### 4 Role of TGF $\beta$ and tumor microenvironment

The TME includes extracellular matrix (ECM), signaling cytokines, and many classes of cells, such as local and infiltrating cancer-associated fibroblasts (CAFs), immune-associated cells, endothelial cells,

and adipocytes that enclose the tumor cells in the surrounding environment. The TME is constructed from multiple effects of TGF $\beta$  on tumor stroma cells such as ECM production abilities, CAFs activations, immune system promotion, and angiogenesis initiation. Releasing from tumor stroma cells, TGF $\beta$  cytokine drives associated TME to exhibit absolute effects on tumor development as described in Figure 21.



*Figure 21 : Roles of TGF $\beta$  in tumor microenvironment (TME). A. TGF $\beta$  and naïve stroma cells differentiation. B. TGF $\beta$  and angiogenesis progression. C. TGF $\beta$  and immune cells regulations (S. Liu, Ren, and ten Dijke 2021).*

The first TGF $\beta$ -associated cellular populations in TME are tumor-inducing CAFs and tumor-reducing CAFs, which act as the main resources of the TME and plays as tumoral-supporting environment due to their roles in the production of ECM and cytokines, the invasion of immune cells, and angiogenesis (Kalluri 2016). Normally, CAFs stay as heterogeneous population such as four defined-CAF subpopulations in breast tumor, including vascular CAFs, matrix CAFs, cycling CAFs, and developmental CAFs (dCAFs) (Bartoschek et al. 2018). In CAFs formation, TGF $\beta$  activates local

quiescent fibroblasts, derives mesenchymal stem cells from bone marrow differentiation and stem cells from adipose tissue to form CAFs ( Figure 21) (Ping et al. 2021). TGF $\beta$  pathway activation also induces the transformation of epithelial and endothelial cancer cells, including transition into myofibroblasts cells (*via* epithelial to mesenchymal transition-EMT), and fibroblast-like cells (*via* endothelial-mesenchymal transition- EMT) respectively ( Figure 21a) (Petersen et al. 2003; Zeisberg et al. 2007). CAFs produced autocrine TGF $\beta$  acts as reagent attractant to promote fibroblasts recruitment into the TME surrounding primary tumor to favorite the metastatic initiation. In hypoxia condition, CAFs secrete TGF $\beta$ 2 and associate with hypoxia-inducible factor to promote cellular stemness phenotype and therefore induce chemoresistance in cancer treatment (Y.-A. Tang et al. 2018). In addition, the density of CAFs surrounding tumoral environment causes tight TME matrix and decreases the blood vessels density, which supports the formation of physical barrier covering the tumor cells, leads to the inaccessibility of anti-cancer drugs. Therefore, molecular characterization and mechanistic highlights of CAFs and its subtypes will be the opportunities to precise the cancer therapy target (Kanzaki and Pietras 2020) .

The second TGF $\beta$ -associated cells in TME are endothelial cells, which reside on blood and lymphatic vessels the surface and support tumor nourishment by supporting vessels functions including transferring blood, oxygen, nutrients, limiting waste, and regulating the in/out of immune cells and other elements ( Figure 21b). The relationship between TGF $\beta$  level and micro-vessel density is determined in different cancers, in which, endothelial cells contain two members of activin receptor-like kinase ALK1 and ALK5 in association with TGF $\beta$ RI. The TGF $\beta$ -ALK5 pathway can either directly inhibit endothelial cell proliferation and migration or indirectly regulates these cellular functions via VEGF, CTGF, and fibroblast growth factor 2 (FGF2). In the condition of endoglin positive, the TGF $\beta$ -ALK1 signaling directly induces cell proliferation and migration. In angiogenesis, the JNK pathway regulates the TGF $\beta$  proangiogenic effects, TGF $\beta$  also induce VEGF-C production to promote lymph-angiogenesis. However, the role of TGF $\beta$  in cancer-associated lymphatic formation is not fully discovered (S. Liu, Ren, and ten Dijke 2021).

The third TGF $\beta$ -associated cells in TME are immune-associated cells, there are numerous innate and adaptive immune cells involves in TME regulations ( Figure 21C). In tumorigenesis, myeloid cells such as MDSCs, macrophages, and neutrophils promote early-tumor development to reduce the response of T cells and maintain immunosuppressive conditions (Ginefra, Lorusso, and Vannini 2020). Among these immune cells, dendritic cells (DCs) distribute tumor antigens to T cells and natural killer (NK) cells, which exhibit the antitumor cytotoxic influence in physiological conditions, however, these reactions are usually inhibited during tumor progression. In TME, TGF $\beta$  shows central

immunosuppressive properties on DCs and NK cells antitumor effectiveness (Gonzalez, Hagerling, and Werb 2018). In early cancer development, TGF $\beta$  reduces proliferation and differentiation of myeloid cells by down-regulation of IFN $\gamma$  while in advanced-stage of tumor progression, myeloid cells release TGF $\beta$  and MMPs that again reduce the antitumoral effects of immune systems and therefore promote metastasis (L. Yang, Pang, and Moses 2010). TGF $\beta$  pathway inactivation in myeloid cells results as the improvement of antitumor activity. TGF $\beta$  suppresses NK cell activation and inhibits their cytotoxic capability by blocking the expression of C-type lectin receptor NKG2D (Lazarova and Steinle 2019). Macrophages mostly express IL1 receptor-associated kinase M (IRAK- M), which negatively regulates Toll-like receptor (TLR) signaling. Induction of IRAK-M antagonize TLR signaling under the activities of TGF $\beta$ , tumors are secured against TLR-regulated antitumor effects of macrophages (Standiford et al. 2011). In adaptive immune response, TGF $\beta$  supports cancer progression by blocking T cell activation, proliferation, differentiation, and migration. TGF $\beta$  can also inhibit naive CD4<sup>+</sup> helper T cells conversion into specific effector sub-populations, however, TGF $\beta$  promotes the differentiation naive T cells into Treg - also called suppressor T cells to restrain immune response (Jiao et al. 2019). Recently, a study indicated that removal of TGF $\beta$ RII in CD4<sup>+</sup> T cells results in tissue healing and remodeling of the blood vasculature, leads to hypoxia associated cancer and difficult tumor survival in distant tissues, therefore exhibits cancer suppression characteristics (M. Liu et al. 2020). In addition, TGF $\beta$  prevents cytotoxic CD8<sup>+</sup> T cell activation and maturation by reducing the tumor antigen presentation by dendritic cells, inhibits these cell proliferations through IFN $\gamma$  and IL2 down-regulation (McKarns and Schwartz 2005). In CD8<sup>+</sup> T cells, TGF $\beta$  induces antigen-induced programmed cell death protein-1 (PD-1) expression, leads to the dysfunction of T cells (B. V. Park et al. 2016). Recently, Mishra S and colleagues indicates the TGF $\beta$  pathway preserves the immune-suppressive identity of a CD8<sup>+</sup> Treg cell population, which is regulated by Eomesodermin (Eomes) transcription factor, hence, TGF $\beta$  and Eomes associate together to induce CD8<sup>+</sup> Treg cells homeostasis. TGF $\beta$  modulates lymphocyte B cells activation, proliferation, apoptosis and its antibody switching, however, TGF $\beta$  regulating B cell-mediated antitumor properties remains unclear (Tamayo, Alvarez, and Merino 2018).

## **5 TGF $\beta$ members and expression in breast cancer**

A study of 623 invasive breast carcinoma patients shows that most of tumors express extracellular-TGF $\beta$ 1 (78%), TGF $\beta$ 2 (91%), TGF $\beta$ 3 (93%), TGF $\beta$ RII (72%), and phospho-Smad2 (61%) proteins. Intracellular TGF $\beta$ 1 was expressed in 32% of tumors. In this study, three TGF $\beta$  ligands are correlated to a good prognosis such as small tumor size, and low grade in both ER<sup>+</sup> and ER<sup>-</sup> subtype. TGF $\beta$ RII

expression is associated with small tumor size only in primary ER- tumors while expression phospho-Smad 2 is observed in ER- patients with positive lymph node metastasis (Figuroa et al. 2010).

Another study observes higher protein levels of TGF $\beta$ 1 and TGF $\beta$ 3 in breast tissues of cancer patients (n=101) compared to normal tissues, however, no significant differences in TGF $\beta$ 2 expression are detected at all the stages. Only TGF $\beta$ 1 and TGF $\beta$ 3 indicate a higher protein expression in advanced lymph node with nodal positive and metastatic breast tumors. In this study, luminal A subtype has the correlation between the expression of three TGF $\beta$  isoforms and TGF $\beta$  receptors (RI and RII), basal-like subtype has the correlation between the expression of TGF $\beta$ 1, TGF $\beta$ 3 and TGF $\beta$ RII while HER2+ subtype has only the correlation between the expression of TGF $\beta$ 2 and TGF $\beta$ RI (Hachim et al. 2018). Concerning mRNA levels (n = 520) of TGF $\beta$ 2, TGF $\beta$ 3 and TGF $\beta$ RII are less expressed in TNBC and HER2+ breast cancer patients, suggesting a poor prognosis. In contrast, low TGF $\beta$ RI expression in patients with small (diameter  $\leq$  2 cm) tumors detection can have better clinical outcomes (C. Chen et al. 2015).

These results suggest the roles of these isoforms in tumor progression and the metastatic process as prognosis markers. Again, the transforming growth factor beta (TGF $\beta$ ) pathway can be tumor suppressor or a tumor-promoter in human breast carcinogenesis. However, the analyses of TGF $\beta$  members in breast cancer are still limited. Up to date, there are no certain classification methods to correlate and recognize TGF $\beta$  expression and aggressiveness of different breast cancer subtypes in clinic. Therefore, understand the expression of TGF $\beta$  members is important for breast cancer diagnosis and treatment.

## **6 Therapeutic targeting TGF $\beta$ pathway in cancers**

At this moment, there are many anti-cancer pharmacological strategies that focus on specific regulators or activators of TGF $\beta$  signaling mechanisms (Figure 22). These interventions show the promising outcomes in pre-clinical animal tests and therefore have been examined in human clinical trials. The main anti-TGF $\beta$  strategies under the current researches are antisense oligonucleotide (AON), neutralizing antibody (antibody), cyclic RGD pentapeptide, TGF $\beta$  ligand trap (trap), and small-molecule kinase inhibitor (SKI). These therapies are classified based on the targeting of different TGF $\beta$  signaling components including TGF $\beta$  mRNA, GARP/integrins that are responsible for the promotion of latent TGF $\beta$ , and ligands that correlates to TGF $\beta$  receptors and TGF $\beta$ RI kinase activities.

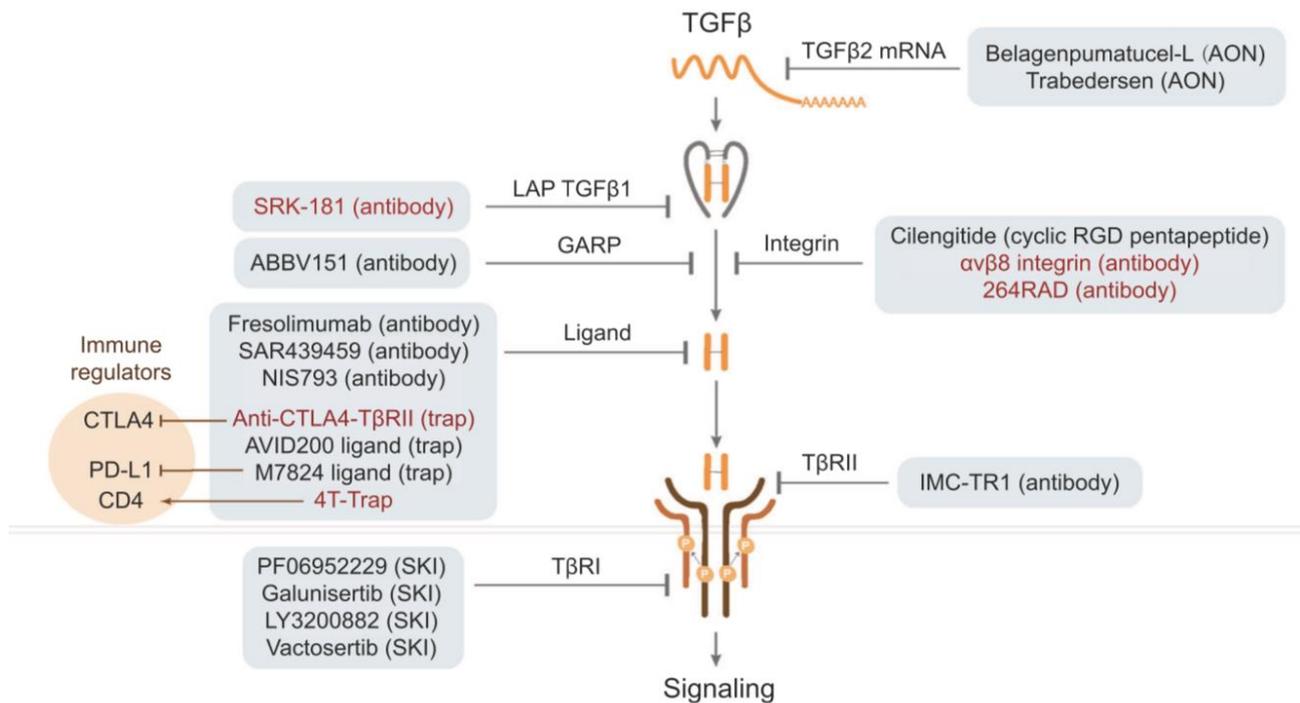


Figure 22: The representative treatment molecules targeting TGFβ signaling. Red: strategies under pre-clinical trials. Orange circle: immune regulatory targets (S. Liu, Ren, and ten Dijke 2021)

There are different anti-cancer pharmacological molecules that target specific regulators of TGFβ signaling pathway or TGFβ signaling compositions, which have been evaluated in human clinical trials or pre-clinical animal models in breast cancer treatment with promising outcome. The pharmacological treatments can be performed alone or in combination with other therapies (i.e radiotherapy, other chemotherapy...) as presented in Table 3. However, treatment targeting TGFβ signaling (i.e TGFβRI kinase inhibitors) also increase several side effects on patient including cardiac (hemorrhagic, degenerative, and inflammatory heart valves lesions) and skin problems (eruptive keratoacanthomas, hyperkeratosis, cutaneous squamous cell carcinomas, and basal cell carcinoma) (S. Liu, Ren, and ten Dijke 2021). For example, human monoclonal antibody Fresolimumab (GC1008, Genzyme), which neutralizes TGFβ1 and TGFβ2 cytokines, have caused reversible cutaneous keratoacanthomas, squamous cell carcinoma (SSCs) and hyperkeratosis effects in clinical studies with malignant melanoma and metastatic breast cancer (Morris et al. 2014; Formenti et al. 2018). Therefore, it is important to personalize the dose of treatment based on patient profil

Stragery	Compound	Drug	Breast cancer	Project	Phase	Combined-Treatment	Situation/Results
<b>Neutralizing antibody</b>	Fresolimumab	TGF $\beta$ 1/2/3	Metastatic breast cancer	NCT01401062	2	Radioztherapy	Finished/Promising
<b>Ligand trap</b>	M7824	L1	TNBC	NCT03579472	1	Eribulin mesylate	Recruiting
			HER2+	NCT03620201	1		Recruiting
			HER2-	NCT03524170	1	Radiotheray	Active
			Metastatic breast cancer	NCT04296942	1	Ado-trastuzumab emtansine, entinostat, and BN-brachyury vaccine	Recruiting
<b>Small-molecule inhibitors</b>	Galunisertib	TGF $\beta$ RI	Metastatic breast cancer	NCT02538471	2	Radiotheray	Finished/Promising
			TNBC	NCT02672475	1	Paclitaxel	Recruiting

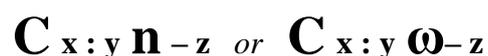
Table 3 : Representative pharmacological strategies focusing TGF $\beta$  for breast- cancer therapy in clinical trials (S. Liu, Ren, and ten Dijke 2021)

## Chapter 4 Fatty acid metabolism and cancer

### 1 Definition and structure of fatty acid

Fatty acids (FAs) are building unit of lipids; contain carbon chains with a carboxylic acid at one end and a methyl group at the other end. There are two different elements for FA characterization, including chain length and FA saturation status. Concerning the FA chain, the short chain fatty acids (SCFAs) have fewer than 6 carbon (C) atoms in their chain, which are often generated in the colon by bacterial fermentation of fibers and starch. Medium-chain fatty acids (MCFAs) have a 6-12 carbon atoms chain. Long-chain fatty acids (LCFAs) have 13 to 21 carbon atoms in the tail, while very-long-chain fatty acid (VLCFA) contain more than 22 carbons in their structure. The FAs without double bonds between carbon atoms in the chain are called saturated fatty acids (SFAs). In the presence of one or more double bond (s), the FAs are specified as monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) respectively.

The FAs are either represented by their systematic names based on the International Union of Pure and Applied Chemistry (IUPAC) nomenclature or by nonsystematic (trivial) names. However, over decades, the most common abbreviation way of naming FAs in lipid analysis is using the shorthand nomenclature according to the number of carbon atoms and number of double bonds as the following notation (Berg, Tymoczko, and Stryer 2002):



*x* represents the number of carbon atoms.

*y* represents the number of carbon-carbon double bonds.

*z* represents the location of double bonds that is the closest to methyl end (*n* or  $\omega$ ) site.

The omega ( $\omega$ ) position of the first double bond in shorthand nomenclature would be useful in certain cases to avoid any confusion with other FAs, which are only different from each other in first double bond position but with similar number of carbons. For example, the PUFAs including eicosapentaenoic acid (EPA; C20:5) of the n-3 family (omega-3 or  $\omega$ -3), and arachidonic acid (AA; C20:4) of n-6 family (omega-6 or  $\omega$ -6) contain similar carbon number, however there are the differences in the number of the double bond and their first position, which are located 3 carbons and 6 carbons respectively from the methyl end Figure 23 (Cockbain, Toogood, and Hull 2012).

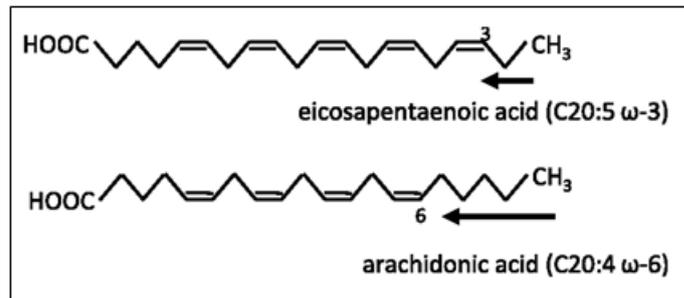


Figure 23: Structure and nomenclature of PUFAs in cis position (Cockbain, Toogood, and Hull 2012)

Fatty acids are the constituents of different classes of complex lipids (i.e. triglycerides, phospholipids, cholesterol esters, ceramide, sphingolipid) and are supplied by foods or *de novo* synthesis (lipogenesis), which will be described in the next part.

## 2 Fatty acid metabolism and breast cancer

In cancer, the transformation from a normal cell to a cancer cell is due to the accumulation the series of mutations which provide the cellular proliferative and survival advantages, leading to the appearance of a cancerous clone, followed by various subclones to form a tumor. In 2000, Hanahan and Weinberg summarized the particular characteristics of cancer cell, including five keys' factors to explain tumor development, as presented in Figure 24. In 2011, these authors subsequently updated these characterizations by taken into account four other criteria, which are different from genetic mutations, to further explore tumor progression (Hanahan and Weinberg 2011).

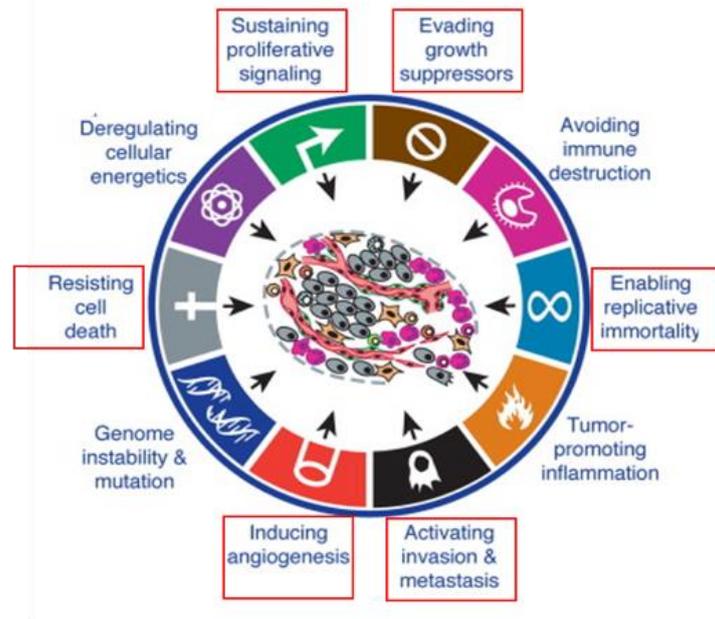


Figure 24: Hallmarks of cancers representative schematics (Hanahan and Weinberg 2011)

Among these new concepts, cellular energetic dysfunctions contribute to define a cancer hallmark. Back to the history, in 1924, Otto Warburg found one of the main changes in tumor cells is the low production of ATP (Adenosine Triphosphate) via oxidative phosphorylation, which are then compensated by ATP production via lactate-associated glucose fermentation. This modification is now called the Warburg effect (Warburg 1956). However, the transformation of a glucose molecule into lactate releases only two ATP molecules whereas via oxidative phosphorylation, the catabolism of glucose produces 36 molecules of ATP. In cancer cell proliferation, this metabolic reprogramming is observed despite normal oxygen-level conditions, leads to the first hypothesis of Warburg about the defect of the mitochondrial respiratory chain. However, different researches in the period have indicated that most of cancer cells do not exhibit the damage of mitochondrial function (Weinhouse 1976). The glycolysis pathway supports to speedily produce ATP, however, the glucose production per mole remains at lower levels compared to oxidative phosphorylation. Therefore, the current hypothesis concerning the activation of this pathway is not only due to ATP production but also due to the adaptation of cancer cells in aerobic glycolysis to induce lactate and pyruvate concentration. After these observations in cancer cells glycolysis deregulation, different studies have also indicated other metabolic pathways alterations in cancer cell. It is now widely accepted that cancer cell metabolisms are altered including lipid metabolism (Cruz et al. 2020). Thus, the metabolic reprogramming inducing by cancer cells will provide cellular benefits on different aspects such as: cancer cells develop metabolite influx mechanisms, supporting nutrients and energy requirements for proliferation, these synthesized nutrients contribute to the inducing-tumorigenic metabolic pathways,

these substances also support the energy storage to maintain cellular physiology in stressed condition including hypoxia or tumor metastases. Metabolic modification also consequently impacts on the cancer cells differentiation and tumor microenvironment (TME) components (Pavlova and Thompson 2016).

Fatty acid metabolism alteration is a part of malignant properties in breast cancer. Different fatty acid synthesis and oxidation enzymes also involve in breast tumor growth and metastasis. The mRNA expression analysis shows that the luminal subtypes contain the imbalance between *de novo* fatty acid synthesis and  $\beta$ -oxidation, which is important for storage and energy supplements. In basal-like and hormone receptor negative subtypes, the upregulation of genes played in exogenous fatty acids consumption are commonly detected (Monaco 2017). Therefore, with these information, the treatments for each subtype could be addressed.

### 3 Fatty acid metabolism process and breast cancer

The involvement of lipid metabolism is extremely important in cellular function, including energy production, plasma and organelle membrane synthesis and signal transduction cascades. In order to conform these requirements, the cells need to tightly control fatty acid uptake, synthesis (lipogenesis), storage and fatty acid oxidation as described in Figure 25.

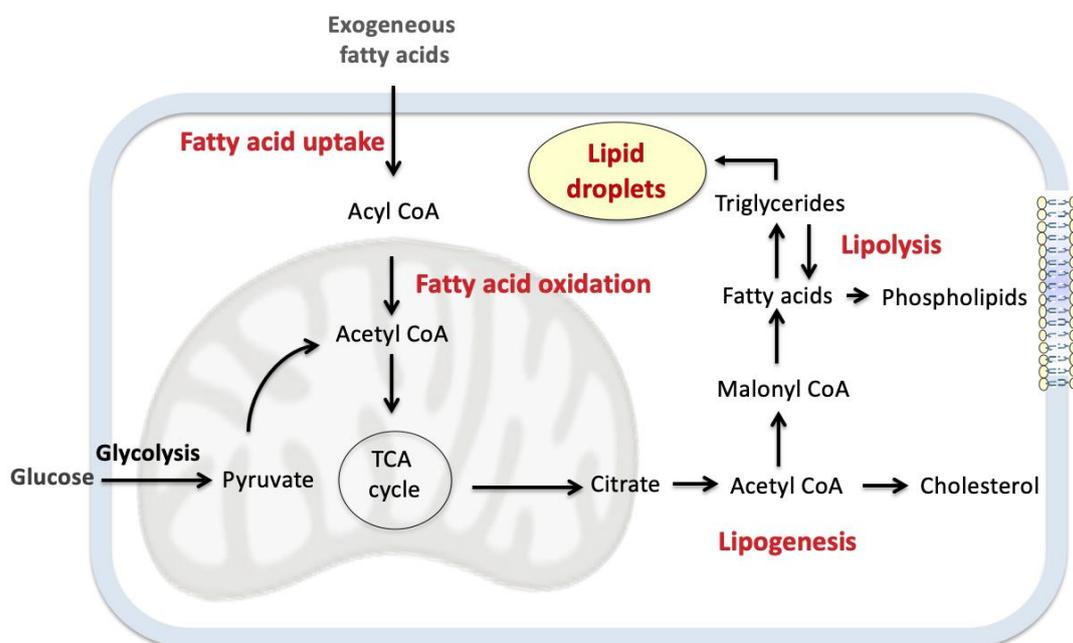


Figure 25 : The main lipid-metabolism pathways in mammalian cells.

#### 3.1 Fatty acid uptake, cellular trafficking and breast cancer

### 3.1.1 Fatty acid uptake

Over the decades, many researchers have been elucidated the transportation mechanisms especially FAs uptake and flux control in various mammalian tissues. In most of tissues, excepted in liver and adipose tissue, the ability to activate lipogenesis is dependent on the FAs uptake and FAs transportation. Concerning the transportation, short-chain fatty acid (SFA) can permeate through cellular membrane by non-ionic diffusion and apical membrane SCFA-/HCO<sub>3</sub><sup>-</sup> exchange (Charney, Micic, and Egnor 1998). Medium and long chain FAs can diffuse simply through phospholipid bilayers or cross through integral membrane protein (Ehehalt et al. 2006).

The first process in cellular nutrient homeostasis is FAs uptake at plasma membrane levels; these FAs can be released by neighbor adipocytes in the surrounding environment (Tucci et al. 2021). This uptake process is highly modulated by certain receptors located on the membrane. The binding proteins can act independently or in combination with albumin binding protein (ABP) to improve the efficiency of FA delivery through cellular membranes (van der Vusse et al. 2002). The rates of FAs uptake depend on the rates of FAs consumption, which were determined by numerous instant-factors such as acetylCoA/CoA conversion rate, malonyl CoA, substrate competitors (glucose, lactate...) or by long-term factors including genes expression of FAs-regulating proteins like peroxisome proliferator-activated receptors (PPARs) (van der Vusse GJ , 2002). Different types of proteins participate in lipid transportation: fatty acid translocase (FAT/CD36), caveolin-1, fatty acid binding proteins (FABPs), long chain acyl-coA synthase (ACS) and fatty acid transportation proteins (FATPs) (W. Zhang et al. 2018) (Figure 26). After the entrance of FAs, these membrane receptors can co-interact with other intracellular proteins to promote downstream transduction signaling or induce the utilization of other intracellular nutrition for intracellular trafficking (W. Zhang et al. 2018).

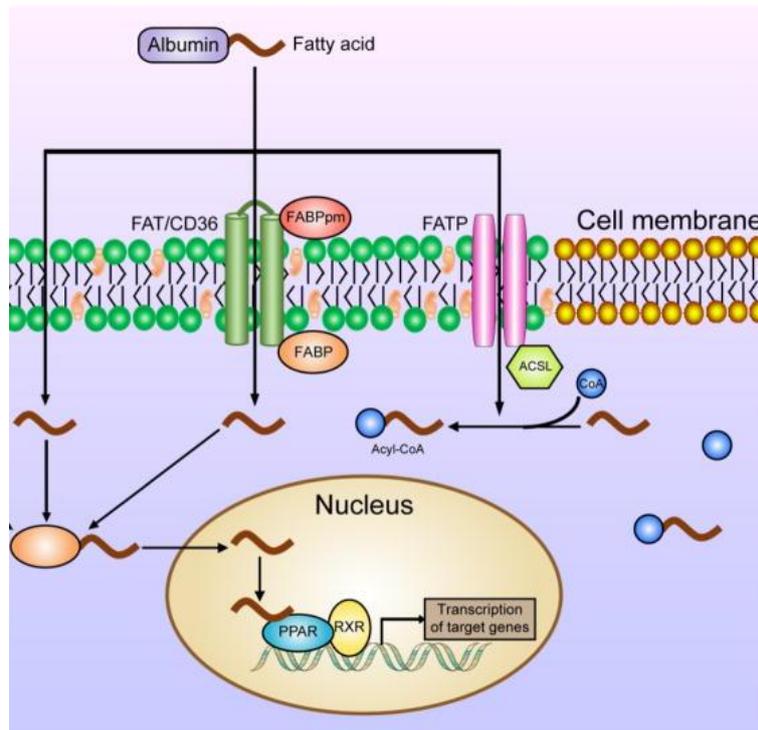


Figure 26: General scheme of PUFAs uptake (W. Zhang et al. 2018)

PUFAs are diffused through the plasma by albumin and lipoproteins. After release from these proteins, free-PUFAs (uncharged molecules) bind to the luminal surface of the cells, incorporate into the external phospholipid membranes, and translocate to the inner leaflet of this membranes by auto-flip-flop or transporter-dependent manner. Abbreviations: FAT/CD36=fatty acid translocase. FABPs=fatty acid binding proteins. ACS=acyl-coA synthase. FATPs=fatty acid transportation proteins. Mfsd2a=major facilitator superfamily domain-containing protein. PPARs=Peroxisome proliferator-activated receptors

### 3.1.2 Fatty acid uptake in breast cancer

In cancer progression, fatty acid transport protein family (FATPs), plasma membrane fatty acid-binding proteins (FABPpm), the fatty acid translocase protein (FAT/CD36) display an increase in tumoral gene and protein expression (Gyamfi et al. 2021). This phenomenon facilitates the efficient of FAs movement across the plasma membrane to adapt metabolic alteration in cancer.

#### FATPs

Fatty acid transportation proteins (FATPs) are membranes protein (both in plasma membrane and intracellular organelle membranes) that supports the movement of long-chain fatty acids. The protein contains six isoforms (FATP1–6), which share similar structure (two highly conserved domains), and favorite FA transportation, hydrolysis and ATP-binding (Stuhlsatz-Krouper, Bennett, and Schaffer 1998). After the enter of PUFAs into the cell, FATPs co-interact with fatty acyl-CoA synthase (ACS) to activate intracellular fatty acids and inhibit the additional PUFAs efflux (Coe et al. 1999). FATPs also act as Acyl-CoA enzyme ligase and responsible for the coupling of FAs uptake to support first FA-utilization in the reaction chain (Ehehalt et al. 2006). In breast cancer development, the increase

of FATP1 expression support tumor growth due to lipids transfer between cancer-associated fibroblast (CAFs) and breast cancer cells (Lopes-Coelho et al. 2018). Unfortunately, the effect of FATP protein family has not been widely studied in breast cancer context.

In other cancer types progression including lymphoma, lung and colon and pancreatic cancer, FATP2 is highly expressed in polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), which activates neutrophils, results in tumoral escape, failure of treatment and poor prognosis (Veglia et al. 2019; Wellenstein and de Visser 2019). Interestingly, a study has also showed the importance of adipocyte-derived FAs in the melanoma progression. Melanoma aggressiveness is positively regulated by adipocyte–melanoma cross talk of adipose-derived fatty acid through FATP1 lipid transporters (W. Zhang et al. 2018). However, the roles of other FATP family members in cancer remain an opportunity for study.

### **FABPs**

Fatty acid binding proteins (FABPs) are classified into two groups based on their localization, including plasma membrane proteins (FABP<sub>pm</sub>) and cytoplasmic proteins (FABP<sub>c</sub>). There are nine FABP<sub>c</sub> proteins (FABP<sub>c</sub> 1-9) that can be found in different tissues (i.e: brain, liver, heart) (Zhang W , 2018). On the one hand, FABP<sub>pm</sub> is a single polypeptide that presents at phospholipid bilayer outer surface and interacts with long-chain fatty acids (Berk et al. 1990; Stremmel et al. 1992). FABP<sub>pm</sub> induces the release of fatty acids from albumin complex which causes fatty acids accumulation at outer leaflet of cell membrane, these fatty acids then switch into the inner leaflet for its functions. On the other hand, FABP<sub>c</sub> is responsible for albumin-free long-chain fatty acid transfortation from outside to inside the cells (Berk et al. 1990; Stremmel, Kochwa, and Berk 1983; Stremmel et al. 1985). In general, FABPs protein family activities are important during metabolism by regulating fatty acid transportation across the plasma membrane and nucleus receptors (PPARs). FABPs can also limit unsaturated fatty acids oxidation, modulate fatty acids cellular uptake and induce intracellular fatty acid storage (Wolfrum 2007). The upregulation of FABP protein families (i.e FABP<sub>c</sub>4 and FABP<sub>c</sub>5) is observed in breast, prostate, colorectal and ovarian cancers, suggests the lipid-related metabolic signaling exchange between tumors cells and TME during tumorigenesis (S. Guaita-Esteruelas et al. 2018).

In the development of cancer, the high expression of FABP<sub>c</sub>7 regulate S/G2 phase cell cycle arrest and cell death death in TNBC via PPAR- $\alpha$  signaling (Kwong et al. 2019). Therefore, the expression of FABP and its isoform can regulate tumor development (cell growth and cell death) in varoious TME conditions. In addition, adipocyte-associated transportation process can also affect cancer cell

proliferation, niche seeding and distant metastasis. For example, adipose releasing-exogenous FABP4 induces breast cancer cell proliferation via the activation and overexpression of intracellular FABPc5 (Sandra Guaita-Esteruelas et al. 2017). Adipocyte-derived FAs molecules can be also transferred from adipocyte to breast cancer cells, results in FABPc5 high expression and leads to induction of breast tumor development (D. Yang et al. 2018).

In breast cancer progression, the decrease of FABPc1 and FABPc2 expression induces the communication between cancer-associated fibroblast (CAFs) and breast cancer cells via lipid transferring molecules, leads to breast tumor growth and metastasis (Lopes-Coelho et al. 2018). During hypoxia, the induction of FABPc7 reduces adipocyte differentiation and thermogenesis in ER negative breast cancer cells, leads to tumor aggressiveness and therapy resistance (Kawashima et al. 2020). This observation is also detected in glioblastoma hypoxic cells proliferation and metastasis, which have high level of FA uptake, accumulation of lipid droplets due to the effects of hypoxia-inducible factor (HIF)-1 $\alpha$  and the overexpression of FABPc3 and FABPc7 (Bensaad et al. 2014).

### **FAT/CD36**

In rat and human, the fatty acid translocase protein (FAT) shares 85% similarities in the structure with Thrombospondin receptor CD36 (leukocyte differentiation antigen), therefore FAT was also identified as CD36. This protein contains two trans-membrane domains that anchor in lipid rafts of plasma membrane. FAT/CD36 has high affinities with long-chain fatty acids binding and acts as main inducers of albumin-releasing free fatty acid. The uptake of FAT/CD36 regulated-fatty acids into tissue is also correlated to caveolin-1 and lipid rafts (Ehehalt et al. 2006). Apart from free fatty acids, FAT also connect with other lipid-related molecules such as collagen, thrombospondin, anionic phospholipids and oxidized low-density lipoprotein depending on cellular demands (Greenwalt et al. 1992). High expression of CD36 associates to poor prognosis of different tumour types such as including breast, ovarian, gastric and prostate (Calvo et al. 1998; Ladanyi et al. 2018).

In breast cancer development, over-expression of CD36 plays as pro-tumorigenic factor which enhance ER-positive breast cancer cells proliferation due to the induction of proliferative and anti-apoptotic genes (Y. Liang et al. 2018). In the TME, exogenous lipids induce breast cancer cells growth via CD36 channel transportation (J. Zhao et al. 2017). However, downregulation of CD36 in TME fibroblasts lead to the releasing of fibroblasts activin A (a transcriptional factor belonging to TGF $\beta$  superfamily which acts through SMAD2/3-dependent pathway), leads to the inhibition of solid tumors formation in TNBC and luminal cell line models (Cheng et al. 2020). CD36 can also co-interact with other protein such as FABP4 and UPC1 (mitochondrial uncoupling protein 1, a mitochondrial carrier protein located

in brown adipose tissue) to regulate adipocyte-induced fatty acid uptake and metabolism in breast cancer, hence this collaboration reduces breast tumor cells apoptosis (Gyamfi et al. 2021).

In breast cancer progression, the expression of CD36 in tumorigenesis have controversial results, for instance, high expression of CD36 induce ER-positive breast cancer migration (Y. Liang et al. 2018), however, low expression of CD36 also induce tumor progression and metastasis in neoplastic human breast (Cleazardin et al. 1993). In addition, CD36<sup>+</sup> cancer cells have highly metastatic abilities in nutrient-rich environment in several cancer types including oral, breast cancer and melanoma (Pascual et al. 2017). Therefore, the precise roles of CD36 in breast cancer tumorigenesis, needs more exploration.

## ACS

Mammalian Acyl coenzyme A synthetase family (ACS) has five sub-members based on the linked acyl groups and fatty acid chain length, including acyl-CoA synthetase short-chain (ACSS): C2 to C4; medium-chain (ACSM): C4 to C12; long-chain (ACSL): C12 to C20; bubblegum (ACSBG): C14 to C24 and very long-chain (or also called solute carrier family 27A-SLC27A): C18 to C26. Concerning the ACSL, there are five sub-genes that identified from ACSL1 to ACSL6, which are corresponding to ASCL1, 2,3,4,5 and 6 protein (Soupene and Kuypers 2008). ACS enzyme is responsible for the conversion of free fatty acids (FAs) to CoA esters by two thioesterification reactions. In the first reaction, cytosolic adenosine monophosphate (acyl-AMP) intermediate is formed from adenosine triphosphate (ATP), this AMP molecule is then replaced with CoA to generate the activated acyl-CoA (Soupene and Kuypers 2008). After that, these acyl-CoA molecules are participated in various lipid-related mechanisms such as *de novo* lipid biosynthesis, FA  $\beta$ -oxidation, and membrane remodeling (W. Zhang et al. 2018).

In cancer, ACSLs are widely studied because it activates long-chain fatty acids, which are highly abundant and commonly interfered in cancer, leads to poor survival rate in cancer patients. The expression of ACSLs protein is different between each isoform, up regulation of ACSL1, ACSL2, ACSL3, ACSL4 are observed in breast cancer (Y. Wang et al. 2017; Belkaid, Ouellette, and Surette 2017) while down-regulation of ACSL5 is found in worse prognosis breast cancer (M.-C. Yen et al. 2017). Until now, no study demonstrated the expression of ASCL6 in breast cancer. The impacts of ACSL1, ACSL3 an ACSL4 proteins overexpression in breast cancer are described in the Table 4. Treatment with triacsin C (ACSL inhibitor) prevents enzyme activity of ACSL 1, 3, 4, and 5 and also supports breast cancer therapy (M.-C. Yen et al. 2017; X. Wu et al. 2013).

Isoforms	Subtype	Characteristics
ACSL1	TNBC	Induce cell proliferation, and viability, colony formation
ACSL3	TNBC	Induce cell proliferation and viability, reduce $\beta$ -oxidation, induce lipid droplets.
ACSL4	ER+	Induce cell proliferation, induce drug efflux and chemotherapy resistance.
	TNBC	Induce cell invasion and independent growth in vitro, induce tumor growth in vivo.

Table 4: Effect of acyl-CoA synthetase long-chain (ACSL) isoenzymes deregulation in breast cancer. (Orlando et al. 2019; Wright et al. 2017; X. Wu et al. 2013; W.-C. Chen et al. 2016)

### 3.2 Lipogenesis

The *de novo* synthesis of fatty acids occurs in the cytoplasm and depends on several enzymes-including acetyl-coenzyme A carboxylase (ACC) and fatty acid synthase (FASN). There are two isoforms of ACC (ACC1 and ACC2), ACC1 stays in the cytosol and ACC2 being correlates with the mitochondrial membrane (Wakil and Abu-Elheiga 2009). ACC is the rate-limiting enzyme of lipogenesis and is responsible for the production of malonyl-coenzyme A from acetyl-coenzyme A. The malonyl-CoA generated by ACC1 is used by FASN for the synthesis of fatty acids in the cytosol whereas malonyl-CoA generated by ACC2 acts as an inhibitor of carnitine-palmitoyltransferase-1 (CPT-1) involved in the entry of FAs into the mitochondria for  $\beta$ -oxidation (Wakil and Abu-Elheiga 2009). The regulation of ACC is mandatory to control the quantity of malonyl CoA during the reaction, in which, AMP-activated protein kinase (AMPK) induces the phosphorylation of ACC, leading to the enzyme inactivation. This phosphorylation of ACC can cause to the suppression of lipogenesis (Lally et al. 2019; Fediuc, Gaidhu, and Ceddia 2006).

The malonyl-CoA generated by ACC1 is taken up by FASN to extend the acetyl-CoA chain on the acyl-binding site (ACP) of FASN. FASN is a multienzymatic protein composed of 7 different catalytic sites including acetyl / malonyl-CoA transferase,  $\beta$ -ketoacyl synthase,  $\beta$ -ketoacyl reductase,  $\beta$ -hydroxylacyl dehydratase, enoyl reductase, thioesterase (Schweizer and Hofmann 2004). The fatty acid chain synthesis reactions catalyzed by FASN, which transforms 2-carbon acetyl-coenzyme A into

16-carbon palmitic acid (C16: 0) after 7 reaction cycles. During each round, 2 carbons from malonyl-CoA are added into acyl carbon chain attached to ACP site of FASN. ACC has a key role in the neo-synthesis of fatty acids due to the consumption of a malonyl-CoA molecule to form 2 carbons-extended fatty acid carbon chain in each cycle. The last step occurs after the carbon chain linked to the ACP has reached a length of 16 carbons: the fatty acyl-ACP thioesterase breaks the bond connecting between this carbon chain to the ACP, releasing mainly palmitic acid (C16: 0) and other intermediaries (Figure 27). The activity of ACC and FASN enzymes requires the supplement of ATP (Adenosine TriPhosphate) and the reduction of NADPH, H + (Nicotinamide Adenine Dinucleotide Phosphate).

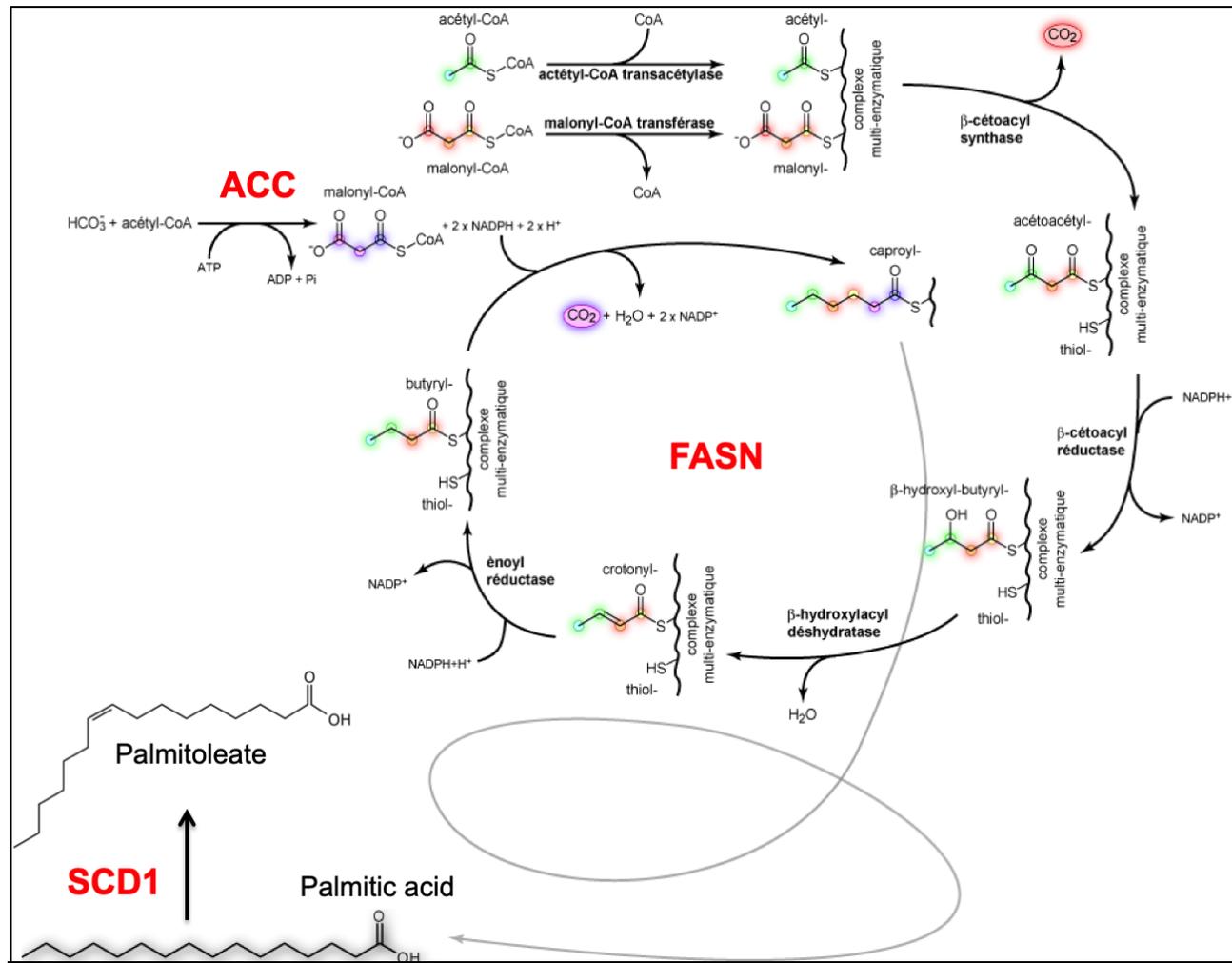


Figure 27 : De novo lipogenesis of saturated fatty acids and synthesis of unsaturated fatty acids

Abbreviations: ACC=acetyl-coenzyme A carboxylase. FASN=fatty acid synthase. SCD=Stearoyl-CoA desaturase

Unsaturated fatty acids are obtained by the desaturation of saturated fatty acids, which occurs in the endoplasmic reticulum (ER) membrane. The desaturation of C16:0 and C18:0 fatty acids is carried out by Stearoyl-CoA desaturase (SCD). SCD enzyme belongs to  $\Delta$ -9 fatty acid desaturase and contains four SCD isoforms (SCD1–4 in mouse) and 2 SCD isoforms (hSCD1 and 5 in human) (X. Liu, Strable, and Ntambi 2011). The SCD isoforms have similar enzymatic functions and different tissue distributions. Among these isoforms, SCD1 is predominance and highly expressed in different tissues such as liver, adipose and glands (meibomian, harderian and preputial). SCD isoforms locate in endoplasmic reticulum and introduces a double bond in position  $\Delta$ 9 from the terminal carboxyl group of saturated fatty acids C16:0 and C18:0, resulting in the formation of palmitoleic acid (C16: 1 n-7) and oleic acid (C18: 1 n-9) respectively (Ntambi and Miyazaki 2004). Quantitatively, palmitoleic acid and oleic acid are the major building blocks of monounsaturated fatty acids, which are also actively synthesized inside cells. From these monounsaturated fatty acids, the carbon chains can be lengthened by elongases enzyme.

### 3.2.1 Lipogenesis in breast cancer

Lipogenesis dysfunction is frequently observed in breast cancer, which often correlates to high expression of ACC (mostly ACC1a) and SCD1 (Mounier, Bouraoui, and Rassart 2014). Similarly, FASN expression is low in normal cells; however, this enzyme is over-expressed in breast cancer (Yoon et al. 2007). Furthermore, fatty acid synthesis is necessary for breast cancer brain metastasis (Ferraro et al. 2021).

Lipogenesis is constantly supplied to promote the growth and proliferation of cancer cells, including breast cancer (Hilvo et al. 2011). In early stage of breast cancer, ACC is highly expressed therefore silencing of ACC lead to inhibition of growth and apoptosis in these cancer cells (Khan et al. 2014; Yoon et al. 2007). Induction of FASN promotes cancer cells proliferation and induces S-phase during cell cycle progression, the inhibition of FASN leads to breast cancer cell apoptosis (Javier A. Menendez and Lupu 2007; P. Li, Tian, and Ma 2014).

Enzymes associate with fatty acid metabolism also are proved to involve to tumor progression and metastasis. Many studies show the correlation between ACC 1/2 and FASN expression and activity with invasion and EMT of breast cancer tumors, especially pre-malignant lesions formation (Simeone et al. 2021). p-ACC1 de-phosphorylation prevention can maintain an inhibition of the lipogenesis and activate tumor suppressor function of breast cancer susceptibility gene 1 (BRCA1) to limit hereditary breast cancer aggressiveness (Brunet et al. 2008). TGF $\beta$ 1 induces EMT and also inhibits ACC activity in MCF7 cell lines and highlights role of TGF $\beta$ 1-inducing lipid alteration in breast cancer metastasis

via AMP-activated protein kinase pathway (Rios Garcia et al. 2017). In addition, ACC also involves in fatty acid oxidation (FAO), therefore, suppression of mitochondrial ACC2 promotes fatty acids oxidation via EMT transcriptional factor (snail). The inhibition of ACC2 by Snail induces CPT1-dependent FAO, releases ATP and reduces NADPH consumption and leads to the improvement of breast cancer cells pro-survival in a starved environment (J. H. Yang et al. 2020).

In breast cancer development, HER2 protein overexpression promote the FASN production while FASN protein can induce HER2+ breast cancer cell growth (Jin et al. 2010). In addition, hormone dependent breast cancer cells (i.e MCF7) is growth under the support of estradiol, however, FASN inhibition reduces the estradiol-stimulated breast cancer cell proliferation and anchorage-independent colony formation, leads to the improvement of breast cancer treatment (J A Menendez and Lupu 2017). In breast cancer progression, with HER2+ breast cancer isoform, FASN involves in cellular metastatic phenotype transformation via EGFR signaling (Javier A. Menendez et al. 2004). FASN also induces the breast cancer metastasis by modify the fatty acid metabolism, especially the accumulations of C16:0, C18:0 and C18:2 and total free fatty acids (S. Xu et al. 2021).

The role of SCD1 in breast cancer is closely depended on the regulation of its substrates and products in breast cancer. In fact, the induction of MUFAs content has been detected in metastatic breast cancer cells indicating SCD1 role in tumorigenesis (Bougnoux et al. 1992). In breast cancer development, high expression of SCD1 is correlated to the increase in proliferation and decrease in breast cancer cell death (Tracz-Gaszewska and Dobrzyn 2019). Low SCD1 expression is correlated with a reduction of breast cancer cells proliferation, cell cycle and tumor growth in ERK1/2 and b-catenin inactivation-manner (Mauvoisin et al. 2013). In addition, depletion of SCD1 on breast cancer cells leads to the inhibition of cells migration; however, treatment with oleic acid (main SCD1 product) rescues the cellular phenotypes. Interestingly, in the same study, cancer-associated fibroblasts (CAF) induces SCD5 levels, lead to the stabilization in ER+ and TNBC breast cancer cells (EMT) survival (Angelucci et al. 2018). Finally, co-activation of SCD1 and FABP4 in TME induce breast tumor resistance to ferroptosis because of the accumulation of LDs to protect the cells from oxidative stress, therefore and leads to tumor recurrence (Luis et al. 2021). These findings suggest the role of SCD proteins; especially SCD1 in breast cancer development and it is promising to target SCD for breast cancer treatments.

### 3.2.2 Lipogenesis fatty acids products in breast cancer

Profiling fatty acid content and determining the balance between SFA and MUFA is one of breast cancer predictor (V. Chajès et al. 1999; Véronique Chajès et al. 2008). However, some specific polyunsaturated fatty acids (PUFA) have effects on cancer. There are two main groups of PUFA:

omega-6 polyunsaturated fatty acids (n-6 PUFA) and omega-3 polyunsaturated fatty acids (n-3 PUFA). In n-6 PUFA, Linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6) are the two common fatty acids, which have cancer-inducing effects (Pla et al. 2008; Horrocks and Yeo 1999). In n-3 PUFA,  $\alpha$ -Linolenic acid (ALA, 18:3n-3), Docosahexaenoic acid (DHA, 22:6n-3) is a common predominant form, which has cancer-inhibiting effects (Burdge 2004). The following table (Table 5) indicates the representative effects of SFA, MUFA, n-3 PUFA and n-6 PUFA in breast cancer.

<b>Fatty acid</b>	<b>Effect on breast cancer</b>
SFA	Induce risks and DNA damage resistance
MUFA	Induce risks, suppress HER2 expression (Oleic acid)
n-6 PUFA	Induce mammary tumor growth, cell proliferation; mammary protumorigenic. Promote tumor genesis via circulating estrogenic molecules and inflammation. Associate with HER2 expression.
n-3 PUFA (low-fat dose)	Reduce BC cell growth, tumor growth and tumor cell proliferation, HER2 expression and circulating estrogenic molecules. Induce apoptosis.

Table 5: The influence of fatty acids on breast cancer development (MacLennan and Ma 2010)

### 3.3 Fatty acid elongation

The elongation of FAs is the carbon chain length extension of FA. FAs elongation occurs in endoplasmic reticulum (ER) in the presence of different elongase enzymes (Table 6, Figure 28). The elongation process determines the chain length of intracellular FAs, including SFAs, MUFAs, and PUFAs with minimum 12-carbons chain. The substrates of process are endogenous-derived FAs and exogenous dietary FAs (Jump 2009).

#### 3.3.1 Elongation of very long chain fatty acids

The FAs elongation process takes places in ER, includes the enzymatic activities of four enzymes (Figure 28). The elongation cycle includes four steps: condensation, reduction, dehydration and reduction (Jakobsson, Westerberg, and Jacobsson 2006). In the step 1, the condensation of acyl-CoA with malonyl-CoA produces 3-ketoacyl-CoA. This condensation process is considered as rate-limiting step of the circle, under the catalysis of FA elongase enzyme (ELOVL). The second step includes the reduction of 3-ketoacyl-CoA to form 3-hydroxyacyl-CoA (3-keto intermediate). This reaction is catalyzed by 3-ketoacyl-CoA reductase and used NADPH as reducing substrate. Step 3 is the

dehydration of the 3-Hydroxyacyl-CoA (3-hydroxy species) by 3-hydroxyacyl-CoA dehydratase, following the release of 2,3-trans-enoyl-CoA. Finally, the step 4 includes the reduction of step 3 product in the presence of NADPH and trans-2,3-enoyl-CoA reductase enzymatic activities (Cinti et al. 1992; Moon and Horton 2003). The catalytic action under ELOVL activities allow the insertion of 2 carbons to the chain of long and very long-chain FAs (VCLFAs) after each cycle.

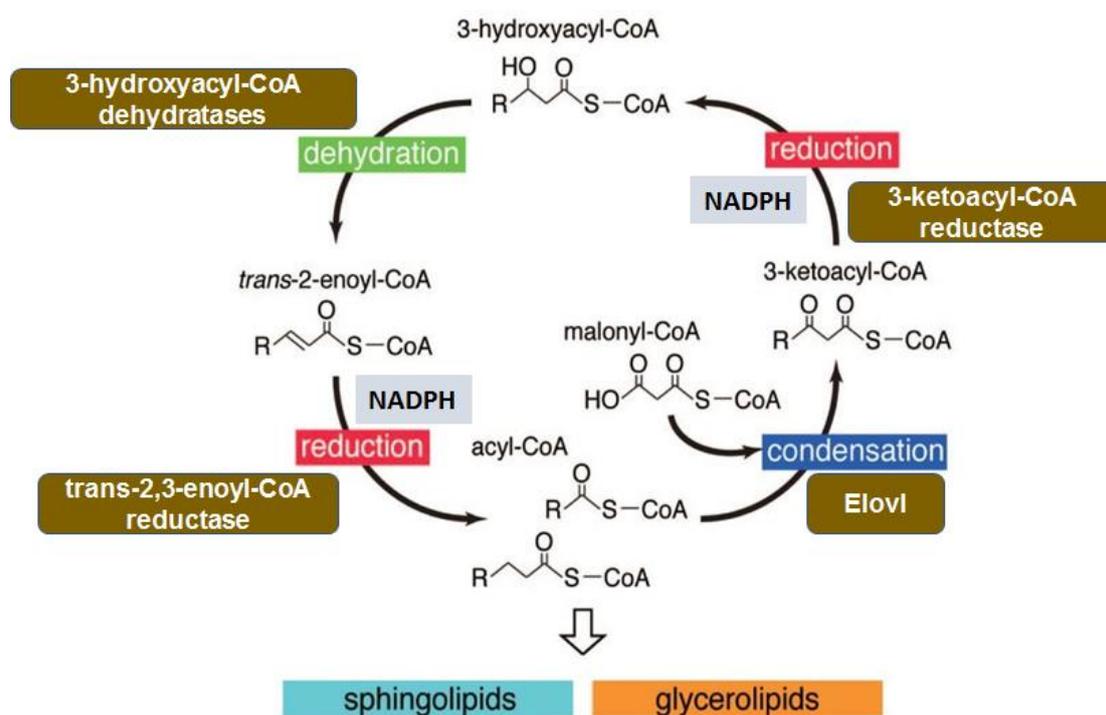


Figure 28: Four steps of fatty acid elongation cycle

Different human genomic analysis has defined the related genes concerning their function, the enzymatic abnormal functions can lead to several diseases Table 6

Classification	Genomic identification	Diseases
FA elongase	ELOVL1–7	STGD3, ichthyosis, neurological disorders (human <i>ELOVL4</i> ), prostate cancer (human <i>ELOVL5</i> )
3-Ketoacyl-CoA reductase	KAR	Breast cancer (human <i>17β-HSD12</i> )
3-Hydroxyacyl-CoA dehydratase	HACD1–4	Centronuclear myopathy (dog <i>HACD1</i> )
2,3-Trans-enoyl-CoA reductase	TER	Non-syndromic mental retardation (human <i>TER</i> )

Table 6: Four FA elongation enzymes and related diseases (Kihara 2012; Tsachaki et al. 2020; Centenera et al. 2021)

### 3.3.2 ELOVL family proteins properties

#### a) *Structure*

The elongation-of-very-long-chain-fatty acids protein family performs the first-important step (condensation) in the elongation process. These elongases consist of seven members in human and rodents (Elovl 1-7) (Jump 2009).

The enzymes locate on endoplasmic reticulum (ER) and are able to form a multimeric structure depending on their status (Okuda et al. 2010). The structure of each member is a multi-pass trans-membrane protein that contains an ELO domain, which is integral membrane protein (X.-M. Zhang et al. 2003). ELOVL proteins have N-linked glycosylation at the N-terminus histidine motif for elongase-catalytic function, a dilysine ER-retention motif (KXKXX) at the C-terminus for positioning the protein location on ER membrane (Deák et al. 2019). The detail structural identification of each protein member, except Elovl-4 and Elovl-7 are still unavailable to date. Several researches have been identified the ELOVL topological model base on Elovl-4 with either five membrane domains with SOUSI tool in Figure 29A-left (Molday RS , 2010) or seven trans-membrane domains with MEMSAT-SVM, MEM-SAT3; ENSEMBLE, Phobius, and TMHMM2 tools in Figure 29A-right (Ozaki et al. 2015). In 2021, Nie L research team also demonstrates the precise structure of Elovl-7 with seven transmembrane (TM) helices (1–7) via protein crystallization. In both protein models, N-terminus is localized on the ER lumen while C-terminus locates on the ER cytoplasmic side. Overall, the remarkable difference between 5 and 7 TM topologies is catalytic histidine motif location, in the 5-TM (Figure 29A), this motif stays at the ER lumen-site, close to TM3, while nucleophilic histidine in 7-TM places in TM4 (Elovl-4, Elovl-7) and TM5 (Elovl-7) on the ER cytoplasmic side as shown in Figure 29A-right and Figure 29B (Deák et al. 2019; L et al. 2021). These histidines in Elovl-7 can interact with 3-keto acyl-CoA thioester for the condensation or sometimes with metal ion in fatty acyl desaturases, however, the metal-associated catalytic function of Elovl-7 is still no clue (L et al. 2021)

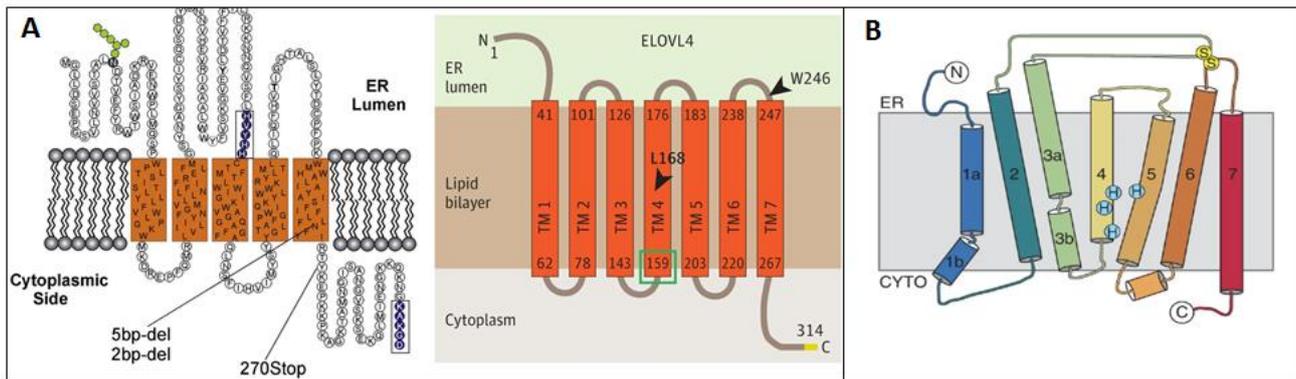


Figure 29: Schematic illustration of the Elov-4 (A) and Elov-7 (B) topology (Deák et al. 2019; L et al. 2021)

*b) Mechanism of action in mammalian species*

Some ELOVL proteins in the family contain substrate specificity and responsible for their corresponding elongation reactions. Therefore, the ELOVL family can catalyzes several elongation reactions, resulting in a wide range of PUFAs and SFA final products. In Figure 30A, Elov11-7 enzymes (excepted Elov15 and Elov12) are involved in VLC-SFA synthesis reactions. Some family enzyme members (Elov16 and Elov14) perform only specific steps while other elongases Elov11, Elov13 and Elov17 often involve together in different steps. In Figure 30B, the VLC-PUFA are desaturated by fatty acid desaturase-1 (FADS1 or  $\Delta 5$  desaturase), fatty acid desaturase-2 (FADS2 or  $\Delta 6$  desaturase) and elongated by Elov1-5 (Deák et al. 2019).

Among these members of Elov1 family, Elov1-5 is responsible for the elongation of long and very long chain-PUFA, long and very long chain-SFA with 18 and 22 carbons chain, especially the activity toward C18:3(n-6) and C20:3(n-6) acyl-CoA (Leonard et al. 2000; Ohno et al. 2010).

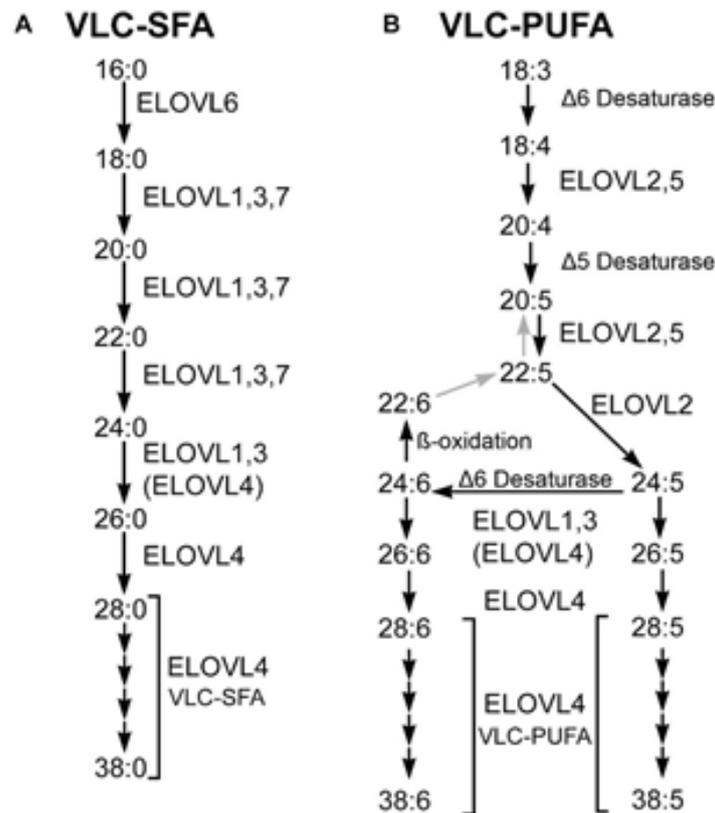


Figure 30 : Representation schematic of VLC elongation and desaturation from SFA (A) and PUFA (B) (Deák et al. 2019)

### c) *ELOVL family in cancer context*

A number of epidemiologic studies have indicated the association between ELOVL family enzymes and cancer development, contributing to suggest a role of lipid metabolism in carcinogenesis and tumor progression. For instance, upregulation of Elov11 in colorectal cancer tissues leads to the accumulation of VLCFA-TAG and nonesterified VLCFA, suggesting the development of colon cancer risks (Hama et al. 2021). In case of glioblastoma, Elov12 is highly expressed in glioma stem cells (GSC) causing cell growth and tumor initiation via polyunsaturated fatty-acid synthesis and Epidermal growth factor receptor (EGFR) signaling (Gimple et al. 2019). Elov17 involves in prostate cancer growth via Sterol regulatory element-binding transcription factor (SREBP1) regulation, and Elov15 drives prostate tumor progression and metastasis via the release of reactive oxygen species (ROS) (Centenera et al. 2021; Tamura et al. 2009).

To date, there are only limited research that elucidate the role of ELOVL family in breast cancer, especially Elov1-1 and Elov1-6. The metabolomics analysis of ER-positive, PgR-positive, HER2-neg (EP+H-) and TNBC patients (n=74) indicates high expression of Elov1-1 and Elov1-6, thus these proteins are important for breast cancer diagnosis (Y. Yamashita et al. 2017). In addition, in

postmenopausal breast cancer patient after curative mastectomy, high Elov1-6 expression negatively correlates to patient recurrence-free survival, however, Elov1-6 expression has no interaction with patient characteristics including primary tumor size, lymph node metastasis, stage, grade, estrogen receptor, progesterone receptor, HER2 and age. Therefore, high Elov1-6 expression can be only considered as a poor prognostic factor in post-surgery breast cancer patients (FENG et al. 2016).

### 3.4 Fatty acid $\beta$ -oxidation (FAO)

#### 3.4.1 Fatty acid oxidation in general

In general, FA  $\beta$ -oxidation is the metabolic process, by which the FAs are converted into acetyl-CoA. The efficacy of the oxidation pathway depends on intracellular concentration and structure of free-FAs, for example, incorrect double bonds positions in free MUFAs and PUFAs can prevent the oxidation. FAs  $\beta$ -oxidation processes appear first in peroxisome for very long chain fatty acids (VLCFA), branched-chain fatty acid (BRCFA) then finish the oxidation and degradation in mitochondria. The  $\beta$ -oxidation of short, medium and long chain fatty acids are fully taken place in mitochondria (Adeva-Andany et al. 2019).

In the mitochondria compartment, short-chain FAs can be delivered directly across mitochondria membranes while medium and long-chain FAs with more than 12 carbons must be transported through acyl-carnitine transporter. The Carnitine palmitoyltransferase (CPT) system composes of two complexes CPT1 and CPT2 in the outer and inner mitochondria membranes respectively. In addition, the CPT1 protein exists with three isoforms CPT1a, b and c, which are tissue specific and coded by three different genes (Virmani et al. 2015). In fact, CPT1, especially CPT1b plays a role as rate-limiting step during mitochondrial  $\beta$ -oxidation and CPT1b activities are inhibited by malonyl-CoA. Therefore, in the high-active malonyl-CoA concentration, FA  $\beta$ -oxidation is suppressed, leading to *de novo* fatty acid biosynthesis induction. In the low-active malonyl-CoA conditions (fasting, exercise or starvation), FA  $\beta$ -oxidation is induced leading- to the suppression of *de novo* fatty acid biosynthesis. The transportation through carnitine-dependent channel requires the co-working of CPT1-CPT2 proteins, acyl-CoA synthase (ACS) and carnitine-acylcarnitine translocase (CACT) (Figure 31).

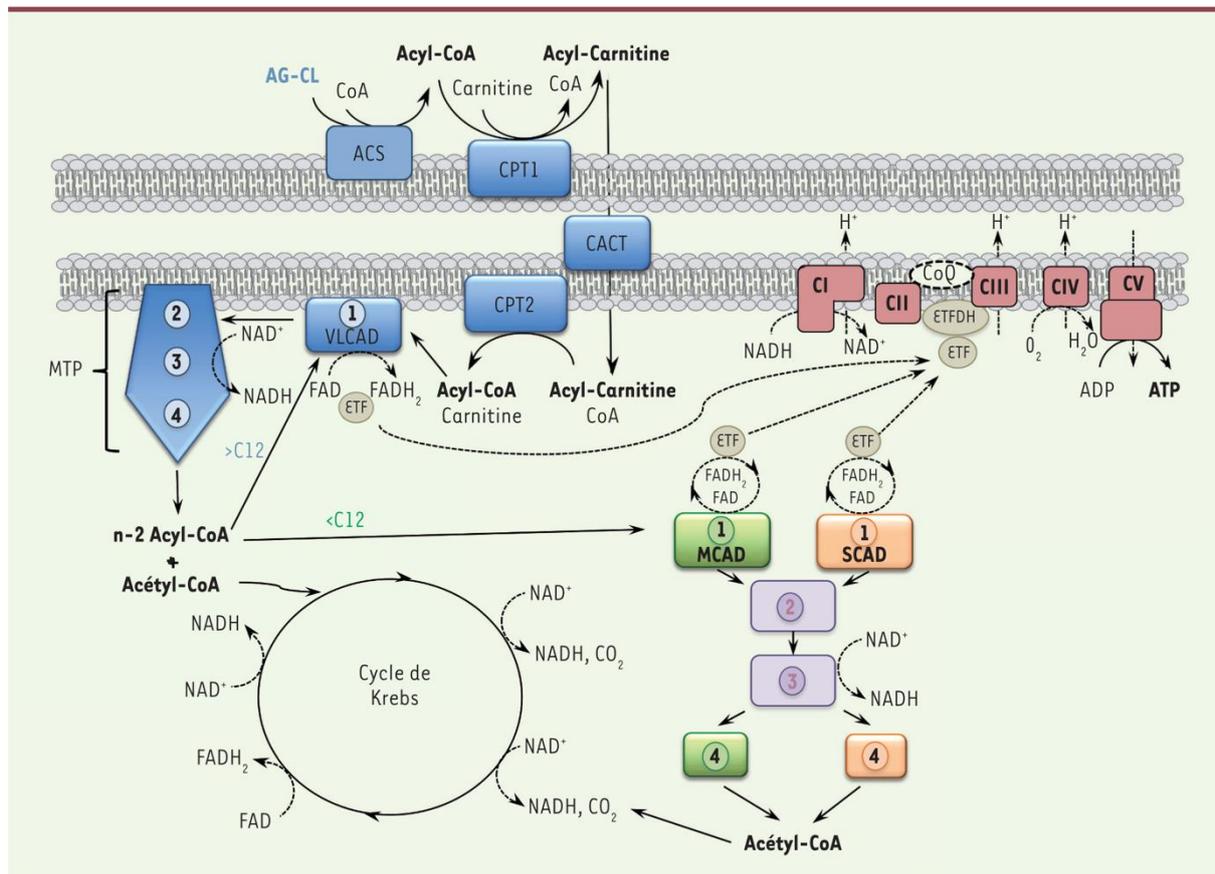


Figure 31: Transportation of fatty acids across mitochondria and mitochondria  $\beta$ -oxidation (Bastin and Djouadi 2019).

Abbreviations: MTP=mitochondrial trifunctional protein. VLCAD=very-long-chain acyl-CoA dehydrogenase. LCFA=free long-chain fatty acid. CPT=Carnitine palmitoyltransferase. MCAD=medium-chain fatty acids dehydrogenase. SCAD=short-chain fatty acid dehydrogenase. LCHAD=long-chain 3-hydroxyacyl-CoA dehydrogenase. ETF=electron-transferring factor. ETF-DH=electron-transferring factor dehydrogenase.

In the outer membrane of mitochondria, the free long-chain fatty acid (LCFA) is converted into acyl-CoA by ACS enzymatic activities. This acyl-CoA molecule is then transformed into acyl-carnitine by CPT1, which is also responsible for acyl-carnitine transportation to inter-membrane space. After mitochondria membrane crossing, CPT2 converts acyl-carnitine into acyl-CoA and release free carnitine for other FAs transportation cycle. Subsequently, the intra-mitochondrial acyl-CoA is followed Lynen helix process, which contains four steps as numbering from 1 to 4 in Figure 31 (dark blue illustration). In each oxidative cycle, acyl-CoA is oxidized by VLCAD (very-long-chain acyl-CoA dehydrogenase-step 1) then by MTP (mitochondrial trifunctional protein-step 2 to 4) to form the acyl-CoA with two-carbon shortened ( $n-2$  acyl-CoA), and release one acetyl-CoA, one NADH, one FADH<sub>2</sub> molecules (Bastin and Djouadi 2019).

In fact, Acyl-CoA dehydrogenase enzyme family has three enzymatic isoforms (long, medium and short chain). These isoforms are highly specific to fatty acid chain and identify as follow: very long-

chain fatty acids dehydrogenase (VLCAD) catalyzes C14 to C20 fatty acids, medium-chain fatty acids dehydrogenase (MCAD) correlates to the fatty acid with less than 12 carbon-length oxidation and short-chain fatty acid dehydrogenase (SCAD) associates to fatty acid less than 6 carbon-length oxidation (Swigonová, Mohsen, and Vockley 2009). Acyl-CoA dehydrogenase involves in the first step of all the mitochondrial  $\beta$ -oxidation reaction cycles. In addition, mitochondrial trifunctional protein (MTP) is a membrane-bound enzyme that composes of three long-chain sub-complexes including long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), long-chain enoyl-CoA hydratase, and long-chain thiolase. The protein MTP has four  $\alpha$ - and four  $\beta$  subunits, which is responsible for the catalysis of last three steps in mitochondrial  $\beta$ -oxidation (Jl et al. 1996). These steps are identically reproduced until reaching the medium chain acyl-CoA with less than 12 carbons length from initial long chain acyl-CoA.

Continuously, the medium chain acyl-CoA is then directed to medium chain acyl-CoA dehydrogenase (MCAD-step 1), to common isoforms for the step 2 and 3 and to specific isoform in step 4. Once acyl-CoA chain reduced to 6 carbons, this molecule is finally reacted with SCAD (short chain acyl-CoA dehydrogenase-step 1), with the common isoforms in steps 2 and 3, and with the specific isoform of the short chains in step 4. These acetyl-CoA continues to enter the Krebs cycle to produce the energy (GTP/ATP) for cellular metabolism and citrate as a substrate for lipogenesis.

The complete conversion of LCFA into acetyl-CoA successively mobilizes all the Acyl-CoA dehydrogenase enzymatic isoforms (long, medium and short chain) (Bastin and Djouadi 2019; Leslie et al. 1993).  $\beta$ -oxidation releasing-molecules are closely associated with respiratory chain activity, which allows the massive production of ATP. For example, the electrons of FADH<sub>2</sub> produced in step 1 are firstly donated to the ETF (electron-transferring factor). ETF is non-protein molecule that can interact to proteins and enzymes and drives electrons passage in electron transport chain (Munro and McLean 2013; 2013). ETF introduces FADH<sub>2</sub>-releasing electrons into the respiratory chain via a specific acceptor, ETF-DH (electron-transferring factor dehydrogenase). In addition, NADH produced during fatty acids  $\beta$ -oxidation also supplies the respiratory chain at complex I level (Bastin and Djouadi 2019).

### 3.4.2 Fatty acid oxidation (FAO) in breast cancer

The CPT protein family, especially CPT1 controls fatty acids movement into mitochondria as material supplement, therefore, it is considered the rate-limiting step in FAO (Figure 31) (Qu et al. 2016). The FAO dysfunction in cancer often associates with carnitine palmitoyltransferase (CPT) modifications and mitochondrial trifunctional (MTP) family. For example, down regulation of FAO is observed in

many tumor types especially breast tumor; this phenomenon is mainly associated to modulation of FAO related-proteins such as high-expression of CPT systems or low-expression of MTP family (Aiderus, Black, and Dunbier 2018; Qu et al. 2016). In cancer development, low-activated rate of FAO leads to high risk of breast cancer tumor growth and promote cancer cell proliferation. In accordance, an overexpression of CPT gene (CPT1a) in breast tumor cells significantly reduces their proliferation compared to non-tumor cells (Aiderus, Black, and Dunbier 2018). The MTP is not widely studied in breast cancer; however, a research on high oxidative lung carcinomas showed the inhibition of MTP expression reduces tumor growth (Amoedo et al. 2021).

In breast cancer progression, the up regulation of FAO in breast cancer development is also reported in differences study. For example; CPT1a overexpression also induces cell migration in TNBC cell line, which implicates roles of CPT protein family and FAO activation in metastasis and breast cancers recruitment (Han et al. 2019). Not only FAO enzymes but also signaling pathways associating FAO are important in breast cancer tumorigenesis. For example, JAK/STAT3 promote FAO in breast cancer stem cells (BCSCs) via CPT1b promoter binding, lead to the maintenance of cellular stemness properties, lipid metabolism alteration, chemotherapy resistance and induce metastasis (T. Wang et al. 2018). In MYC-high TNBC phenotype, suppression of FAO inhibits energy metabolism and reduces tumorigenesis (Camarda et al. 2016). Dysfunction of Hippo pathways activate Yes1-associated transcriptional regulator (YAP), which participates in FAO regulation and supports cancer cells including breast cancer cells metastasis through tumor-draining lymph nodes (LNs) (C.-K. Lee et al. 2019). The involvement of TME including adipocytes also contributes to breast cancer cells stemness properties and promotes tumor progression (Y. Y. Wang et al. 2017).

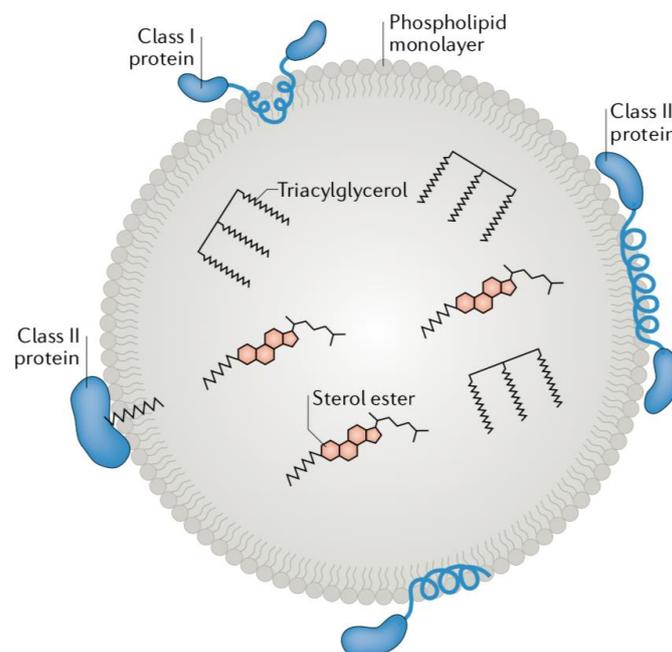
These studies strongly demonstrate the essential role of FAO in the lipid metabolic reprogramming of cancer cells, especially in breast cancer and help to develop therapy treatments. In conclusion, high activation of FAO promotes breast cancer metastasis.

### **3.5 Lipid droplet (LD)**

Lipid droplets (LDs) are cellular organelles that store neutral lipids, the diversity of LDs including their number, size or contents can be found in the same cell type or different types, depending on cellular metabolism status. Although variation of morphology does exist, the LDs share a similar structure that separates them from other organelles. From Figure 36, LDs are covered by phospholipid monolayer surrounding a neutral lipids-filled core, which mainly contains triacylglycerol, sterol esters and ceramides (Tauchi-Sato et al. 2002). Emerging from endoplasmic reticulum (ER), thus, the LD's monolayer contains similar composition to ER bilayer such as phospholipids molecule with polar head

groups contact with cytosol whereas the acyl chain stayed towards the hydrophobic-lipid core (Olzmann and Carvalho 2019)

The monolayer is also associated with numerous functional proteins on the LD surface including perilipin family member via specific binding structures (hydrophobic hairpins or amphipathic helices). Perilipin (PLIN) family has five isoforms (PLIN1-5), among these isoforms, PLIN2 and 3 are widely expressed whereas PLIN1, 4 and 5 have tissue specific expression. The PLIN1 and 2 are tightly associated with LDs while PLIN 3, 4 and 5 are cytoplasm exchangeable proteins that also attach with LDs. PLIN sequesters intracellular LDs by protecting them from lipase effects (Sztalryd and Brasaemle 2017). These associated proteins also drive the location of intracellular LDs to co-support other organelles. Therefore, the flexibilities in LDs modifications clearly demonstrate the LDs proteome alterations. Furthermore, the specificities of the LDs surface protein also indicate distinct LD populations, which are tightly regulated by organism species, cellular metabolism response and available nutrients.



*Figure 32: Lipid droplet structure (Olzmann and Carvalho 2019)*

### 3.5.1 Lipid droplet composition

The LD biogenesis contains multiple steps and mainly appears in ER; however, the mechanisms are poorly understood. In 2019, Olzmann JA and Carvalho P divided the process into three main steps,

including: the synthesis of neutral lipid and establishment of lens, initializing of LD, and finally its growth and maturation (Figure 37).

The first process of LD is synthesis of neutral lipids, including triacylglycerols (TAG) and cholesterol esters, synthesis due to the esterification of diacylglycerol or a sterol (cholesterol) respectively. TAG and cholesterol esters synthesis are described as bellow.

*a) Triacylglycerols (TAG) synthesis*

The TAG which mainly locate in adipose tissue, are produced by tissues in order to store lipids in cytoplasmic lipid droplets for energy supply and to manage lipotoxicity (Olzmann JA , 2019). TAG are composed of a glycerol molecule esterified by three fatty acids in position either sn1 (R), sn2 (R') or sn3 (R''). In detail, unsaturated fatty acids preferentially bind to the central sn2 position while the binding at peripheral positions (sn1 and sn3) (Figure 33)

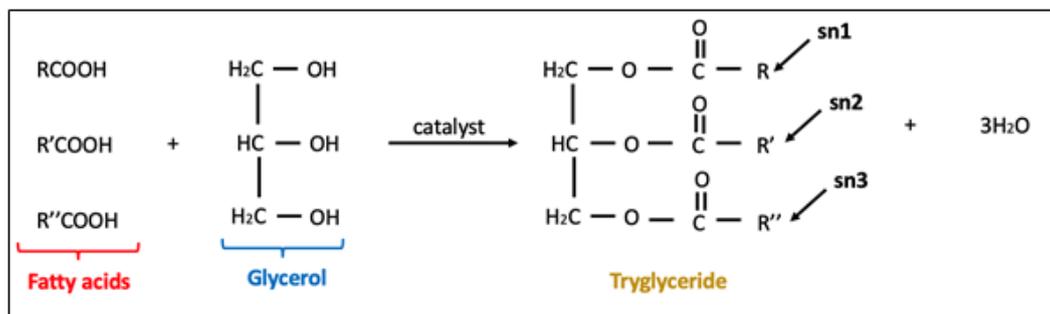


Figure 33 : Chemical reaction (esterification) of Glycerol and three FAs to form triglyceride (Karen 2018)

TAG synthesis appears onto the surface of ER or LD (Chitraju et al. 2017). There are three common synthesized mechanisms including glycerol-3-phosphate acylation, dihydroxyacetone phosphate acylation and of sn-2-monoacylglycerol acylation (Coleman and Mashek 2011). The Figure 34 shows two major pathways including glycerol phosphate (Kennedy pathway-GP) and the monoacylglycerol (MG) pathway. In which, glycerol phosphate pathway is taken place in most cells while the MG pathway appears in specific cell types (enterocytes, hepatocytes, and adipocytes) (Coleman et al. 2002; Xia et al. 1993). These pathways use fatty acyl-CoA as acyl donors. The substrate of GP pathway is Glycerol-3-Phosphate, which is then catalyzed into Diacylglycerol by sn-glycerol-3-phosphate acyltransferases (GPAT), enzyme 1-acylglycerol-3-phosphate-O-acyltransferase (AGPAT) and Phosphatidate phosphatase (PAP) enzymes. The substrate of MG pathway is monoacylglycerol, which is then catalyzed into Diacylglycerol by MGAT enzymes. In the final reaction of these two pathways, DGAT catalyzes the formation of an ester linkage between an activated fatty acid (FA CoA) and the free hydroxyl group of 1,2-diacylglycerol (DG) to form a complex (Figure 13B). One fatty acyl-CoA

and diacylglycerol (DG) molecule are covalently assembled to this complex to form TAG under the catalytic activities of acyl-CoA:diacylglycerol acyltransferase (DGAT) (C.-L. E. Yen et al. 2008a). Biosynthesis of TAG is mainly occurred in endoplasmic reticulum (ER) (Weiss, Kennedy, and Kiyasu 1960)

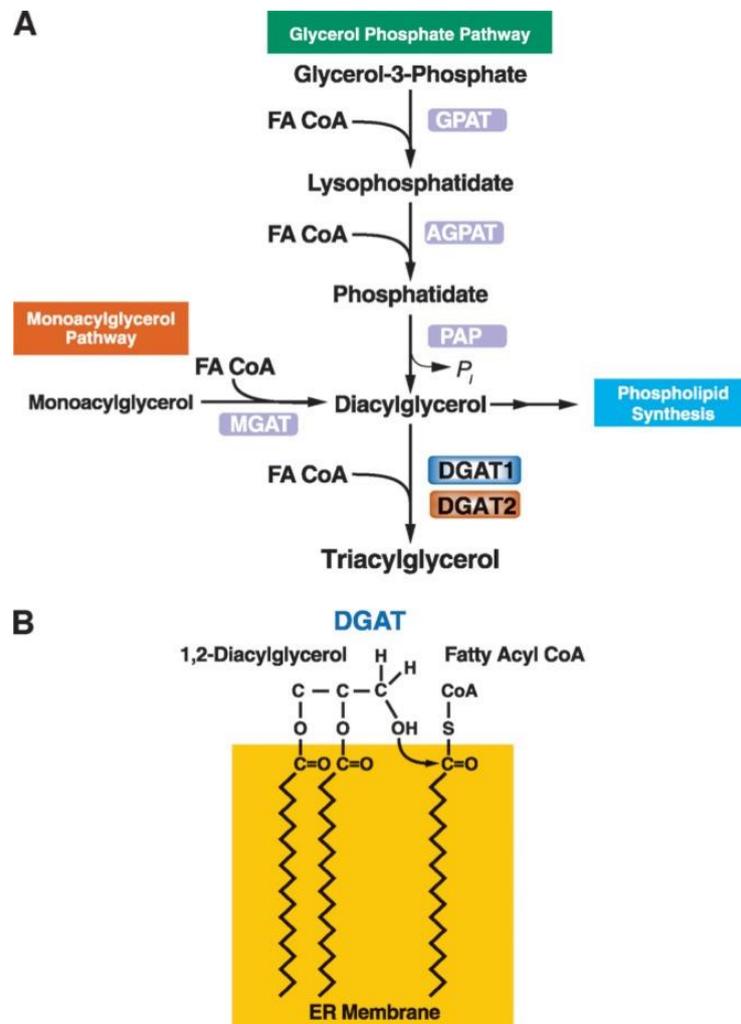


Figure 34 : Triglycerides (TAG) synthesis mechanism (C.-L. E. Yen et al. 2008a)

(A) TAGs are the final product of a catalytic chain under the enzymatic activities of DGAT1 or DGAT2  
 (B) DGAT is responsible for the creation of an ester connection between a fatty acyl CoA and the free hydroxyl group (OH) of diacylglycerol, which occurs at the endoplasmic reticulum (ER) membrane surface.  
 Abbreviations: GPAT=glycerol-phosphate acyltransferase. AGPAT=acylglycerol-phosphate acyltransferase. PAP=phosphatidic acid phosphohydrolase. MGAT=acyl CoA:monoacylglycerol acyltransferase.

Some key enzymes involve in the process are GPAT, PAP and DGAT. Firstly, the sn-glycerol-3-phosphate acyltransferases (GPAT) is the central enzyme of glycerolipid synthesis, including triglyceride regulation and phospholipid synthesis. GPAT is responsible for glycerol-3-phosphate and long-chain acyl-CoA transformation into lysophosphatidic acid (Takeuchi and Reue 2009). There are four GPATs isoforms in mammalian cells, however; only two groups were classified based on their

subcellular localization, favorite substrate, and N-ethylmaleimide (NEM) sensitivity. The first group is GPAT1 and GPAT2 in outer membrane of the mitochondria; the second group is GPAT3 and GPAT4 in the membrane of endoplasmic reticulum (ER) (B. Lu et al. 2010). The dysfunction of this family protein can lead to obesity, hepatic steatosis, and insulin resistance (J. Lu et al. 2018; Wendel et al. 2010).

Secondly, the PA phosphatases (PAP-lipin) is important for the hydrolysis of circulating lipid phosphates and signaling PA. The catalysis is conducted after the release of these lipid molecules from membrane phospholipids by phospholipase D (Sigal, McDERMOTT, and Morris 2005). The main function of PAP is producing the DAG, which acts as precursor for phospholipid and TAG synthesis (Coleman and Mashek 2011).

Finally, Diacylglycerol acyltransferases (DGAT) with DGAT1 and DGAT 2 isoforms are involved in the final esterification step of TAG synthesis (Stöveken et al. 2005). DGAT1 and DGAT2 proteins both locate in ER and lipid droplet membrane. The DGAT2 is also detected on mitochondrial-associated membranes in high FAs-conditions (Stone et al. 2009). Although DGAT enzymes are important in TG synthesis, they have different protein sequences and distinct biochemical, cellular, and physiological properties. In detail, DGAT1 belongs to acyl-CoA cholesterol acyltransferase (ACAT) protein family, which also contains retinol acyltransferase, monoacylglycerol acyltransferase (MGAT), and monoester wax synthase activities (C.-L. E. Yen et al. 2005; 2008b). Therefore, DGAT1 can catalyze TAG, retinyl esters or wax esters synthesis. DGAT1 can be found in several organs such as small intestine, adipose tissue, mammary gland, testis, thymus, skeletal muscle, spleen, heart, and skin. DGAT2 shares some similarities to DGAT1 in terms of protein structure but belongs to family of MGAT1, 2, and 3 (C.-L. E. Yen et al. 2008a). The DGAT2 is mainly expressed in liver, adipose, mammary gland, testis, peripheral leukocytes, and heart.

#### *b) Cholesteryl ester synthesis*

Cholesteryl esters (CE) consist of a cholesterol molecule bound to a long chain fatty acid at the hydroxyl group (Figure 35). They represent an intracellular fatty acid storage form in lipid droplets, which are mainly found in the liver, adrenals, and intravascular cholesterol transportation form. In addition, cholesterol can be unesterified to play a role as cellular membranes component (plasma membrane microdomains referred as lipid rafts) and acts as a precursor in bile acids and steroid hormones formation (Chimento et al. 2019).

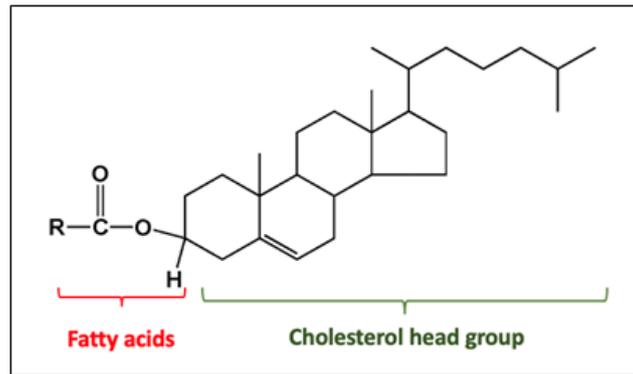


Figure 35: Cholesteryl ester molecule's structure (Proitsi et al. 2015)

The cholesteryl ester is formed by esterification of free cholesterol and acyl CoA under the catalytic activity of cholesterol acyltransferase (ACAT) enzyme (Figure 36) (Chang et al. 2009). Newly produced cholesteryl esters accumulate as cytoplasmic lipid droplets component (Olzmann and Carvalho 2019).

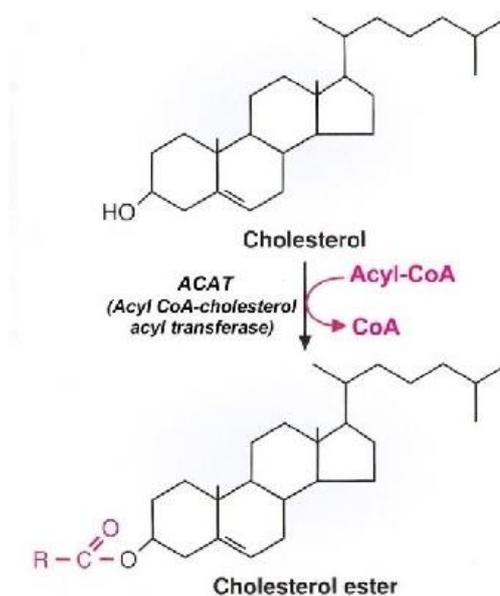


Figure 36 Cholesteryl ester formation from cholesterol (Chang et al. 2009)

The acyl coenzyme A cholesterol acyltransferase (ACAT) is important during this biosynthesis. In mammalian, the protein has two isoforms ACAT-1 and ACAT-2. ACAT-2 shares 44% identical amino acid sequence with ACAT-1 and both isoforms have tissue specific functions. Most of ACAT-1 locates in the tubular rough endoplasmic reticulum while ACAT 2 mainly localized in cytosol and partially in nucleoplasm (Sakashita et al. 2000).

### 3.5.2 Lipid droplet formation process

The first process of LD is biogenesis of neutral lipids, including triacylglycerols (TAG) and cholesterol esters, synthesis due to the esterification of diacylglycerol or a sterol (cholesterol) respectively as described before. These neutral lipids distribute between the leaflets of ER membranes at low concentrations, whereas they can coalesce to form the oil lens at proper concentrations, for examples 5–10% with TAG concentrations (Khandelia et al. 2010). The principle of the lens-formation process is related to physical chemistry parameters while direct involvement of proteins during this process have not been discovered yet (Olzmann and Carvalho 2019). At this stage, these LDs are intermediates and difficult to study due to micro-size and short-time survival.

The second step is the initiation of LDs budding from ER membrane after neutral lipid enlargement. In the cell, this developing mechanism is promoted by fat storage-inducing transmembrane proteins (FIT), especially FIT1 and FIT2 in ER membranes via its direct binding to neutral lipids and diacylglycerol signaling pathway, other mechanisms could be involved in this process, however these hypotheses have not yet fully proved despite many related recent studies (Olzmann and Carvalho 2019). Therefore, FIT depletion or dysfunction causes LD budding inhibition, resulting lipid lenses ingrained in ER membrane, following by the severe metabolic pathologies (Choudhary et al. 2015). The LD budding growth is started by the recruitment of Seipin to form oligomer in the lens structure and LD biogenesis proteins (perilipins) from cytosol to the lens monolayer membrane. These LD are then emerged into cytosol via luminal surface tension and ER-bilayer cytosolic leaflets. The important factors that drive the successful of LDs-intermediates releasing into cytosol are phospholipid composition of ER membrane and the surface tension (Skinner et al. 2009; Thiam and Forêt 2016). On the one hand, surface tension appears to maintain the round form, limits the contact between neutral lipids and intra-cellular aqueous environment, and busting budding effectiveness (Choudhary et al. 2018). On the other hand, phospholipid compositions and asymmetrical protein involve in geometrics (conical shape) acquisition to co-control budding effects, including diacylglycerol, phosphatidylethanolamine (negative regulation) or lysophospholipids (positive regulation) (Ben M'barek et al. 2017; Choudhary et al. 2018). Therefore, the phospholipids and masking proteins between membrane layers direct the tensions imbalances toward the efficacy of budding formation.

The final process is LDs growth due to fusion or lipid synthesis. After budding step, the LDs accumulation is observed by these droplet fusions via the delivery of TAG to LDs through ER membrane connecting proteins or the direct synthesis of local lipids on LD surface, resulting in expanding of LD hydrophobic core (Wilfling et al. 2013). Numerous enzymes such as DGAT1, DGAT2 and GPAT4 involved in this synthesis process are trans-located from ER to LD surfaces,

which support LD growth through phospholipid addition to the LD monolayer and maintain phospholipid homeostasis balance in intense LD expansion conditions (Olzmann and Carvalho 2019). LD formation process is described in Figure 37

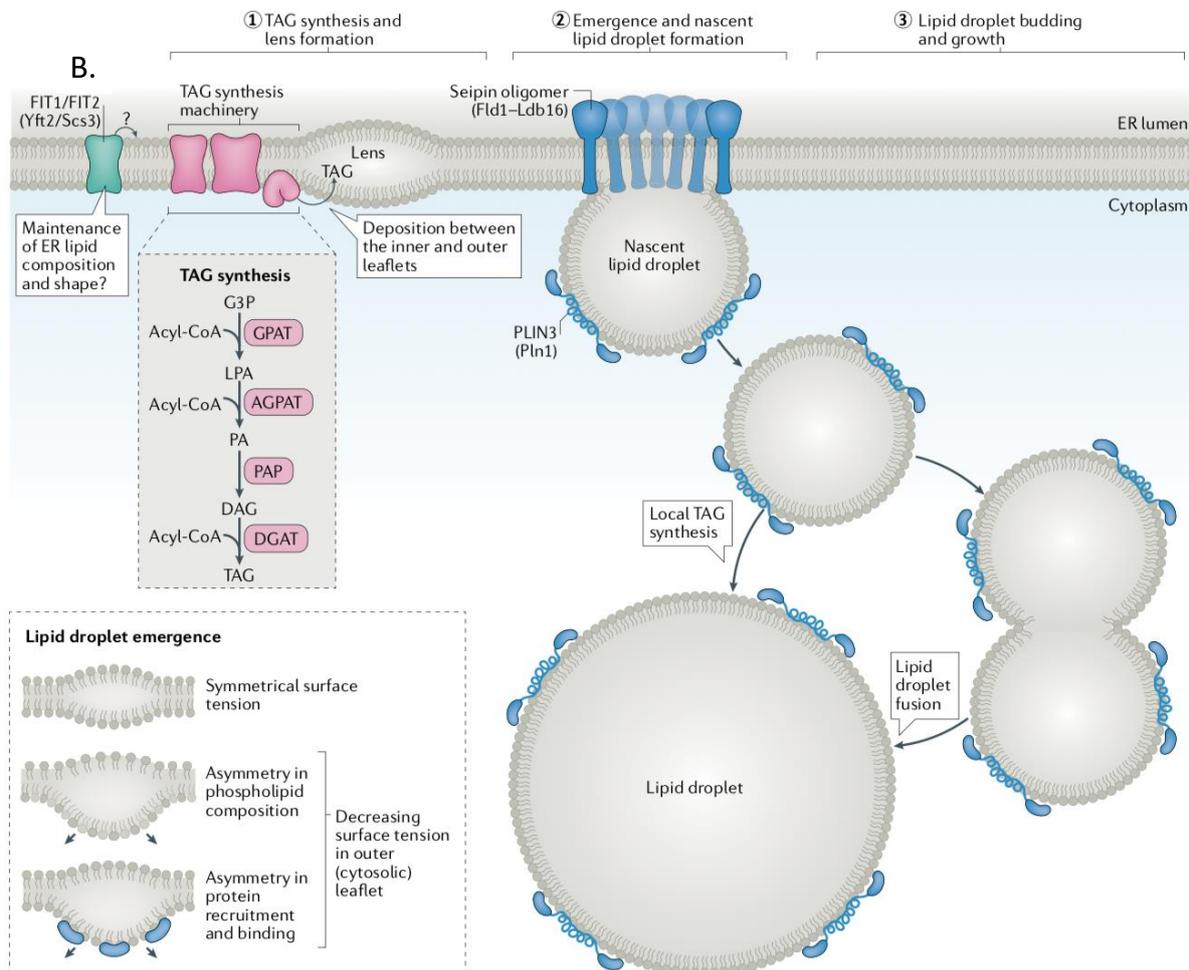


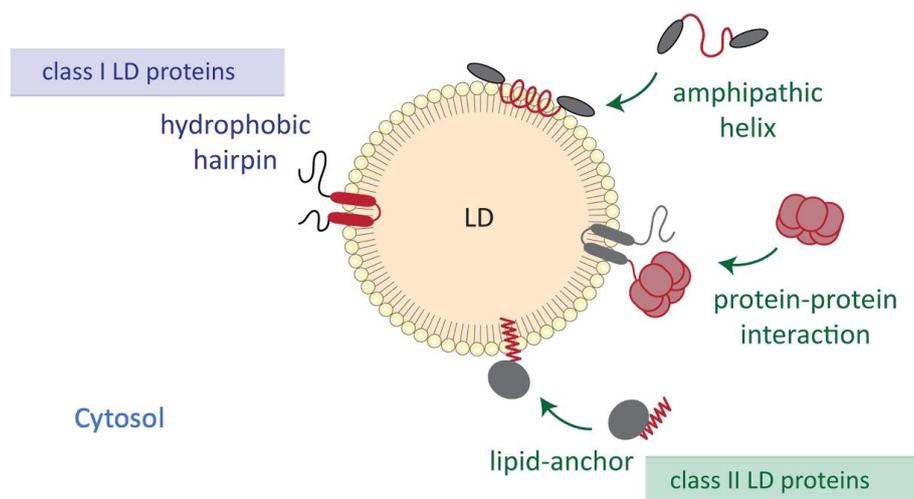
Figure 37 : Lipid droplet biogenesis processes (Olzmann and Carvalho 2019)

Abbreviations: GPAT=glycerol-phosphate acyltransferase. AGPAT=acylglycerol-phosphate acyltransferase. PAP=phosphatidic acid phosphohydrolase. MGAT=acyl CoA:monoacylglycerol acyltransferase.

### 3.5.3 Lipid droplet-associated proteins

Many proteomics studies have elucidated the complete collections of LDs connected proteins, which are mainly divided into two classes, including class I with LD and ER membrane associated-proteins, class II with cytosol recruited-proteins to LD surface. The class I proteins are enzymes that involve in lipid biosynthetic such as diacylglycerol acyltransferase (DGAT1 and DGAT2) or ubiquitination including ubiquitous protein 1 (AUP1), UBX domain containing protein 8 (UBXD8/ FAF2) as presented in Figure 38. The association of these proteins and phospholipid membranes is hydrophobic hairpins insertion with the midway into the bilayers and amino-carboxyl terminal directing the cytosol,

the simple structure containing special polytopic domains without luminal loops is the advantages of the proteins to localize on both ER bilayer and LD monolayer membrane, however, the distribution of class I proteins in ER and the LD is asymmetrical (Ingelmo-Torres et al. 2009). The class II proteins (such as CCT $\alpha$ , perilipin family member PLIN4) are recruited from cytosol to LD surface and directly interact with LD surface either by amphipathic  $\alpha$  helices or lipid anchor, they can also associate with LD surface through protein-protein interactions as describe in *Figure 38* (Dhiman et al. 2020). These structures are classified by the separation of polar and hydrophobic residues to another sides the helix in the membrane plane. The class II protein insertion depends on LDs formation, membrane packing defects or FAs modification, lack of LDs can lead to these proteins degradation via ubiquitin-proteasome due to the system heat shock cognate 71 kDa protein (HSC70) bearing as an example. The surface proteins allow LDs to connect with different organelles including ER, nuclear envelope, mitochondria, peroxisome, lysosome and other LDs, therefore implicate the function of LDs in cellular metabolism.



*Figure 38 : Lipid droplet associated proteins and binding strategies (Dhiman et al. 2020)*

### 3.5.4 Roles of lipid droplet in cells

Low-nutrition or cellular growth and division conditions highly require the expansion and biosynthesis of cellular membranes, which are fueled by the mobilized TAG from the LDs core after lipolysis or lipophagy (Zechner, Madeo, and Kratky 2017). For example, LDs can protect cells against ER stress, which occurs with incorrect ER protein folding, calcium uptake and lipid composition imbalances, and results in several mechanisms involvement and the cellular adaptive response (unfolded protein response-UPR) initiation to control the ER stress and reconstruct homeostasis (Walter and Ron 2011). In detail, UPR transducers including inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR)-

like ER kinase (EIF2AK3) and activating transcription factor 6 (ATF6) are activated by unfolded proteins, lead to the delay in protein translation and promotion of genes-controlled ER protein folding, degradation and lipid synthesis. The UPR regulations are also triggered by LDs-related molecular mechanisms such as TAG synthesis-LDs biogenesis perturbation, LDs over-storage or LDs-dependent proteins functions like RAB GTPase RAB18 (part of ER-lipid droplet tethering complex in adipocyte) after oleate treatment (D. Xu et al. 2018). Therefore, the increases of LDs are observed to response again in UPR inducers small molecules (tunicamycin and dithio-threitol) and ER-related genetic imbalances, suggest the possibility that LDs are up regulated to prevent ER stress (Rutkowski et al. 2008).

LDs are also important for the preventions of mitochondria damage in autophagy after long-term nutrient deprivation. During autophagy, various amino acids and lipids release from proteins and organelles membrane respectively, under the tightly control of mTOR complex 1 (mTORC1) (Efeyan, Comb, and Sabatini 2015), however, numbers of these lipids are instantly re-esterified into triacylglycerol to be packaged into new source of LDs. Constant finding demonstrated that autophagy related protein 5 (ATG5) knock out or autophagy inhibitor treatments can inhibit LDs formation in the similar nutrient deprivation conditions or direct blockage of mTORC1 (Hale et al. 2013). These studies indicate the relationship between LDs and autophagy induced by starvation, which is regulated by mTORC1 together with induction of LDs biosynthesis, suggesting that LDs can act as a conditioned buffer against stress under autophagic flux environments.

Another function of LDs is to prevent lipotoxicity in both cell-autonomous and non-cell autonomous behaviors. For example, in adipose tissue, which contains excess fat, causes in ectopic fat impeachment, results in high concentration of circulating FAs in blood stream, leads to the development of several metabolic diseases. In order to limit this negative effect, these FAs are converted and package into LDs for other functions. In addition, the cells that have high amounts of LDs in cytoplasm also contain high concentration of reactive oxygen species (ROS), elucidating their protection the nearby cells from lipotoxicity (Herms et al. 2013). Interestingly, the peroxidation of PUFAs that contain phospholipids is necessary to identify ferroptosis programmed-cell death, figures out the relationship between lipid metabolism and various cell death programs (J.-Y. Lee et al. 2021). The studies on LDs function implicate its roles in metabolism, and dysfunction of LDs biogenesis can cause different diseases such as obesity, fatty-liver disease, cardiovascular disease, neutral lipid storage disease (NLS), lipodystrophy or Hereditary spastic paraplegia (HSP) (Olzmann and Carvalho 2019)

### 3.5.5 Role of lipid droplet in cancers

Breast cancers are correlated to high LD content, which also associates with the presence of estrogen/progesterone receptors (Koundouros and Poulogiannis 2020). However, the role of LDs in breast cancer proliferation and apoptosis are not widely demonstrated. In different non-transformed cancer cells, the synthesis of LD is increased under the modulation of cell cycle during S phase (Cruz et al. 2019). During mitosis, LDs is polarized while late G1 check point depends on lipid associated PTEN regulations (Patel et al. 2017). Another interesting point is the oncogenic protein such as PI3K, ERK1, ERK2, p38, PKC and caveolin-2 also locate on LDs in different cell lines such as immune cells, MDSC and leukocytes and liver cancer cell line (Fujimoto et al. 2001; Olzmann and Carvalho 2019), indicating the possibilities of LDs involve in kinases signaling driving cell proliferation. The study is promising to understand the role of LDs in TME, especially in breast cancer context, however, no study has demonstrated the role of kinases-associated LD in this pathway up to date. During apoptosis, LD formation delays the toxic FAs productions and maintains cellular homeostasis. Tumor cell apoptosis activates p53, inhibits mTOR inhibitor Myc, and FA  $\beta$ -oxidation, which again turn the FAs into *de novo* lipogenesis circle and results in LD accumulation (Henique et al. 2010; Sanders and Griffin 2016). The up regulation of LD is considered as markers for tumor cell apoptosis *in vivo* after cancer treatment via proton nuclear magnetic resonance (1H) (Blankenberg 2008). The studies on various cancer cases can support the future demonstration of LDs in breast cancer contexts including proliferation, cell death or tumor growth. In breast tumor progression, LD accumulation was typical characteristics of mesenchymal breast cancer cells due to the differences in lipid profile between epithelial-like and mesenchymal-like cells. In order to initiate metastatic events in breast cancer *in vitro*, the primary tumors show an up regulation of LDs (Giudetti et al. 2019). During metastasis, low-accumulation of LDs is observed to support the process, in which, the triple negative breast cancer cells with high expression of CUB-domain containing protein 1 (CDCP-1) decrease the lipid amount and increase cell invasion (Wright et al. 2017). Therefore, the LDs are probably used for the cellular protection at the initiation of metastasis and energy supplement during metastasis. Interestingly, the intracellular LD-associated protein perilipin-1 (PLIN1) expression is significantly decreased in human breast cancer cells while the extracellular expression of PLIN1 is increased in ER+ and TNBC subtypes, blocking PLIN1 leads to the prevention of breast cancer cell proliferation, migration, invasion *in vitro* and tumorigenesis *in vivo* (Zhou et al. 2016). In addition, conditioned media of adipocyte-derived stem cells positively improves proliferation, migration and invasiveness of malignant breast cells (MCF-7) due to the induction of CD36 receptor, promotion of fatty acid uptake and accumulation of lipid droplets in cellular cytoplasm. Inhibition of CD36 can reduce the malignant phenotypes of breast cancer cells. Thus, high lipid droplet storage correlate poor prognosis in breast cancer due to the transfer of FAs from adipocytes to breast cancer cells via CD36 (Zaoui et al. 2019).

These findings on LDs and cancer contribute a new vision into the complexities of LD biogenesis and support the involvement of TME in cancer aggressiveness, therefore encourage the adaptation of cancer treatment.

### 3.6 Lipolysis and breast cancer

#### 3.6.1 Lipolysis in general

Lipolysis is a metabolic pathway through which triacylglycerol (TAG) hydrolysis led to the cleavage of their constitution components including glycerol and free fatty acids (FFAs) from lipid storage organelles (i.e LDs). The FFAs molecules are non-esterified and eventually functioned as substrates for energy generation, precursors for lipid synthesis and cellular membrane formation, or mediators for different molecular signaling (i.e post-translational modifications) in cells. Lipolysis involved enzymes are being studied, including glyceride hydrolase enzymes adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Figure 39).

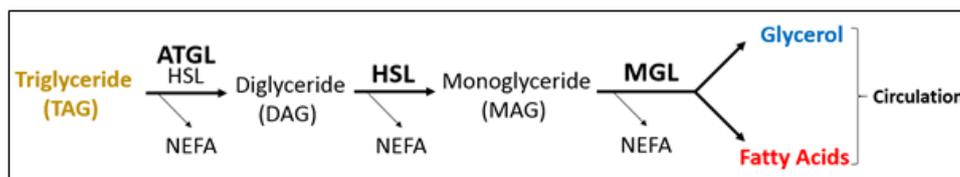


Figure 39: Triacylglycerol hydrolysis into fatty acids and glycerol (Lass et al. 2011)

Abbreviations: ATGL= adipose triglyceride lipase. HSL= hormone sensitive lipase. MGL= monoacylglycerol lipase. NEFA=Non-esterified fatty acids.

#### 3.6.2 Lipolysis in cancer

Lipolysis is important for cancer progression; for example, lipolysis of adipocytes indicates the impact of obesity on breast cancer growth. Fatty acids releasing from adipocytes induce breast cancer cell proliferation and promote migration via ATGL and HSL (Balaban et al. 2017)

#### ATGL

ATGL is annotated as patatin-like phospholipase domain-containing protein A2 (Fischer et al. 2015), which can be found at the surface of lipid droplets. ATGL catalyzes the first step in the lipolysis of adipose triglyceride, releasing DAGs and non-esterified FAs. The stable transition state of ATGL and its substrate is maintained by the glycine-rich oxyanion hole in patatin domain of the protein. In addition, the ATGL protein also contains hydrophobic expanse region (amino acid 315-360) in C-terminal, which is located next to the patatin domain, functions as a lipid-binding site and determines partially enzyme activity (Schweiger et al. 2008).

Among lipolysis enzymes, ATGL is up regulated in breast cancer with adipocytes-enriched TME, supporting to aggressiveness of high-grade tumors (Vegliante et al. 2018). In addition, fatty acids releasing from adipocytes induce breast cancer cell proliferation and promote migration via ATGL and HSL enzymatic activities (Balaban et al. 2017). Despite of widely study on ATGL and lipid metabolism, the roles of this enzyme in breast cancer have not yet fully understood. In another type of cancer, reduction of ATGL leads to the inhibition in the proliferation of colorectal cancer cells and non-small-cell lung carcinoma cell lines, thereby reduces the aggressiveness (Ou et al. 2014; Zagani et al. 2015). In prostate cancer cells, ATGL low-expression reduce the development of this cancer (Wen Chen et al. 2017; Mitra et al. 2017). Therefore, it is important to elucidate the role of tumor in cancer progression.

### **HSL**

Concerning hormone-sensitive lipase (HSL), this enzyme can hydrolyze diacylglycerol molecule to form monoglyceride and NEFA. HSL is located in both the cytoplasm and LDs surface with a to1A domain in the N-terminal region, an HSL\_N domain and alpha/beta hydrolase domain in the central region, and a C-terminal end (J. Huang et al. 2016). The HSL\_N domain is responsible for lipid binding mediation, enzyme dimerization and protein interactions (i.e fatty acid binding protein 4) whereas the catalytic  $\alpha/\beta$  hydrolase domain with folding structure hydrolyses lipid substrates. The catalytic domain also contains enzymatic regulatory module with five phosphorylated sites of the HSL protein.

HSL protein highly expressed only HER2+ subtype, not luminal and TNBC suggest the relationship of HSL activities and HER2 expression (S. Kim, Lee, and Koo 2015). In addition, HSL also associates with ATGL enzymatic activities to regulate the release of fatty acids from adipocytes to induce breast cancer cell proliferation and promote migration (Balaban et al. 2017). However, there are not many studies that indicate the role of HSL in breast cancer. In pancreatic cancer, mutation of RAS genes (KRAS) regulates the storage and utilization of LD via of hormone-sensitive lipase (HSL). Therefore, disruption of the KRAS–HSL signaling pathway reduces the accumulation of LDs, leads to the inhibition of cellular invasion migration *in vitro* and metastasis *in vivo* (Rozeveld et al. 2020). The relationship of HSL and cancer should be further studied.

### **MGL**

Monoacylglycerol lipase (MGL) hydrolyzes monoglycerides into glycerol and free fatty acid (Deng H , 2020). MGL belongs to  $\alpha/\beta$  hydrolase folding hydrolase enzyme family and contains two independent

groups (Bertrand et al. 2010). The enzyme is highly expressed in cell membrane and has tissues specificity (Karlsson et al. 1997).

MGL strongly regulates the aggressiveness of human cancer cells and primary tumors, including breast cancer, leading to the increase of *in vitro* cellular migration, invasion, survival, and tumor growth (Nomura et al. 2010). In endometrial cancer, inhibition of MGL significantly reduces body weight, myometrial invasion, and metastasis (X. Li et al. 2019). In addition MGL knockdown also blocks colorectal tumor cells growth (Ye et al. 2011). In lung cancer, MGL inhibition also reduces the invasion and metastasis of human lung cancer cells via CB1 Cannabinoid Receptor-related pathway (Prüser et al. 2021). Therefore, it is important to target MGL in cancer treatment.

## **PART 2 Study hypothesis and objectives**

The effects of lipid alterations in tumoral context are starting to be described and definitely support the evidence of metabolism driving cancer aggressiveness, especially in breast cancer. In breast cancer, the involvement of TGF $\beta$  signaling-inducing-distant metastasis and treatment therapies targeting-TGF $\beta$  are continuing to be further examined in *in vitro*, *in vivo* models and clinic. However, the role of fatty acid elongation in regulating the proliferation and invasive properties of cancer cells in metastatic breast cancer context has not been addressed yet. This limitation raises the question on these cancer cell modification status for example epithelial to mesenchymal transition (EMT) and its effects on tumoral microenvironment after the dysfunction of long and very long chain fatty acid synthesis.

Therefore, my thesis focuses on identifying the role of elongation of very long chain fatty acid protein 5 (Elovl5) on breast cancer cells proliferation and metastasis. Elovl5 is an enzyme belonging to fatty acid elongase family, which modifies the length of fatty acid chain by the addition of two carbons. The study also elucidates the involvement of this enzyme in TGF $\beta$ -regulating metastatic initiation process through lipid droplet formation.

## **PART 3 Results**

## Chapter 1 An introductory reminder

Breast cancer is the most frequent and common pathology in women worldwide and the second common cancer in general. In 2020, this disease that has a contradictory effect on women with 2.26 million of new cases and one of the leading causes of cancer-related deaths with approximately 685,000 deaths worldwide (Ferlay et al. 2021). Aggressiveness of breast cancer is determined by the high degree of metastasis and considered untreatable with actual single-therapy procedures. In general, the increase of breast cancer incidence and mortality is globally recorded, which indicates the population aging and development, as well as modifications in the predominance and placement of breast cancer risk factors, especially socioeconomic-associated development and geographical location (Heer et al. 2020; P. Ji et al. 2020; Ferlay et al. 2021)

Breast cancer is a heterogeneous disease with various mutations of histological and biological properties due to instabilities of genetic, epigenetic and transcriptomic factors, lead to the discovery of different clinical studies and therapy treatments. At molecular levels, there are different subtypes of breast cancer based on genomic expression of estrogen receptors, progesterone receptor and human epidermal growth factor receptor 2. These subtypes are Luminal A and B, HER-2 positive, Basal like (triple negative TNBC) breast cancers (Perou et al. 2000; T. Sorlie et al. 2001). This phenotypic classification is important in breast cancer diagnosis, prognosis and treatment

A good prognosis is considered as non-metastatic breast cancer, which has high percentage of 5-year relative survival rate (75%) (Dillekås, Rogers, and Straume 2019; P. M. Siegel et al. 2003; Frank et al. 2020; J. Wang and Xu 2019). In contrast, the metastatic breast cancer is clarified as poor-prognosis due to only 30% of patients have approximately 3 years-overall survival (R. L. Siegel, Miller, and Jemal 2020; Cardoso et al. 2018).

Metastasis is the primary criteria to determine cancer aggressiveness. The metastatic process is a multistep mechanism, which contains the local breast cancer cell invasion from primary tumor to distant metastatic sites including lymph nodes, bones, lungs, liver and brain (Gong et al. 2017). Due to the activation of several pathways such as epithelial-to-mesenchymal transition (EMT), cancer cells initiate the dissemination, migration and invasion steps to promote the cell metastasis. After that, circulating cancer cells intravasate the blood vessels and enter to the circulations. The cells then extravasate at desirable distant metastatic sites, start to colonize the new organs as well as proceed mesenchymal to epithelial transition (MET) mechanism for secondary tumor progression (Riggio, Varley, and Welm 2021). EMT play a critical role in metastatic effectiveness of breast cancer cells, which is defined by the diminution of epithelial apical-basal polarity, decrease of cell-cell contacts and

cell-extracellular matrix (ECM) connections. The common modifications of EMT molecular markers are low expression of epithelial markers such as E-cadherin or occluding together with high expression of mesenchymal markers such as Vimentin or N-cadherin (J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020). The mesenchymal phenotype is widely observed in circulating tumor cells with high migrative and invasive potential (Chaffer et al. 2016).

Deregulating cellular energetics in hallmark of cancers is described the metabolic adaptations of cancer cells to tumor microenvironment (TME) (Hanahan and Weinberg 2011). Cellular lipid modulations and fatty acid (FA) reprogramming is detected in the progression of many cancer types. Breast cancer is reported to be associated with quantitative and qualitative alterations in FA composition at cell membrane and TME (S. Guo et al. 2014; Hilvo et al. 2011). Endogenous fatty acid composition remodeling of breast cancer cells is corresponding to up-regulation of *de novo* fatty acid synthesis pathway due to the high expression of the key enzymes in this process such as lipogenic enzymes ATP Citrate Lyase (ACLY), Acetyl-CoA Carboxylase (ACC) and Fatty Acid Synthase (FASN). Reduction of lipogenesis reaction through the blockage of lipogenic enzyme activities (i.e ACC, FASN) limits cancer cell proliferation and metastatic process (Hilvo et al. 2011; Stoiber et al. 2018; Rios Garcia et al. 2017; J. Li et al. 2014). Exogeneous fatty acid is also important in breast cancer progression due to its involvement in cancer cells proliferation and metastasis (J. Zhao et al. 2017; Pascual et al. 2017). In addition, remodeling of FA in cancer cells are highly regulated by desaturation and elongation processes and these expression modifications are also widely described in breast cancer context (Hilvo et al. 2011; Y. Yamashita et al. 2017). The rate limiting step of fatty elongation is the condensation under fatty acid elongases enzymatic activities. Seven very long-chain fatty acid elongases (Elovl 1–7) in the protein family have been identified in the different species including mouse, rat, and human by genomic analysis. The specific catalytic activities of these enzymes are associated with the high selectivity on substrate carbon chain length, among these elongases, Elovl5 is mainly responsible for C18 and C20 unsaturated FA elongation (Leonard et al. 2000). Therefore, we focus on the role of Elovl5 in breast cancer progression and introduce different study models to indicate Elovl5 function in tumor growth and metastasis. In the current data point of view, breast tumor growth correlates to the expression of Elovl5. The analysis of Elovl5 effect on breast cancer metastasis is considered as a key element in the diagnosis of the disease. Interestingly, we observed that loss of Elovl5 expression reduces the proliferation of breast cancer cells and results in a delay of tumoral progression; however, the low overall survival of breast cancer patient is observed in the case of low Elovl5 expression. Loss of this enzyme leads to the activation of EMT in breast cancer cells and then initiates the metastatic

properties of these cells via invasion and migration. The transition of these cells is correlated to the increase of lipid droplet accumulation and demonstrated in shElov15-MCF7 xenografts in nude mice, PyMT-MMTV Elov15 knockout mice and overexpression of 4T1 xenografts in Balb/c mice. Finally, we have explained the molecular mechanism of Elov15 regulation in breast cancer by demonstrating the involvement of the lipid droplet formation for fat storage and TGF $\beta$  receptors signaling pathway.

## **Chapter 2    Publication**

**Downregulation of Elov15 expression initiates breast cancer metastasis through a lipid droplet-mediated increase in expression of TGF- $\beta$  receptors.**

Kieu TLV, Pierre L, Derangère V, Perrey S, Truntzer C, Jalil A, Causse S, Groetz E, Dumont A, Guyard L, Arnould L, Pais de Barros JP, Apetoh L, Limagne E, Jourdan T, Demizieux L, Masson D, Thomas C, Ghiringhelli F and Rialland M.

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Downregulation of Elov15 expression initiates breast cancer metastasis through a lipid droplet-mediated increase in expression of TGF- $\beta$  receptors.

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## Competing interests

The authors declare no competing interests.

**Contributions**

K.TLV. designed and conducted all experiments, analyzed the data and wrote the manuscript; P.L. conducted in vitro experiments; D.V. performed IHC experiments; P.S. conducted in vivo experiments; T.C. performed METABRIC analysis; J.A, G.E, D.A., L.E., J.T., D.L. helped for methodology; P.de B. JP. supervised the lipidomic experiments; C.S. helped for writing; G.L, A.L. and A.L. provided human samples and MMTV-PyMT mouse model; T.C. performed metabolic analysis and discussed the data; M.D. and G.F. discussed the data; R.M. conceived the study, analyzed the data, wrote the manuscript and supervised the project.

**Abstract**

Breast cancer is the most diagnosed cancer and the leading cause of cancer death in women. The 5-year relative survival rate is less than 30% for metastatic breast cancer and metastases account for more than 75% of breast cancer deaths. Fatty acid metabolism is altered in cancer and contributes to tumor progression and metastasis. Here, we were interested in Elongation of very long chain fatty acids protein 5 (Elovl5) which catalyzes the elongation of long-chain fatty acids. We observed that the tumors from patients with a breast cancer had a lower expression of Elovl5 than paired normal breast tissue. Furthermore, low expression of Elovl5 is associated with a worse prognosis in breast cancer patients with a luminal (ER<sup>+</sup>) or basal-like subtype. In accordance with this finding, we showed that the decrease of Elovl5 expression is more pronounced in ER<sup>+</sup> breast tumors from patients with metastases in lymph nodes. Although Elovl5 downregulation limits breast cancer cell proliferation and cancer progression, we demonstrated that repression of Elovl5 expression promoted EMT and lung metastases in murine breast cancer models. A repression of Elovl5 expression in breast cancer cells led to an upregulation of TGF- $\beta$  receptors mediated by an accumulation of lipid droplets which is suppressed by a blockade of triacylglycerol synthesis with pharmacological drugs inhibiting DGAT1/2. Interestingly, the abolition of Elovl5-regulated lipid droplet formation reversed the induction of TGF- $\beta$  receptors, EMT and cell invasion. Altogether, we showed that Elovl5 expression is a predictive marker for the risk of metastases development in breast cancer and that Elovl5 regulated the metastatic process through modulation of the expression of TGF- $\beta$  receptors mediated by lipid droplets.

## Introduction

Breast cancer is the most frequent cancer in women with 2.26 million of new cases and the leading cause of cancer death in women with almost 685,000 deaths worldwide in 2020<sup>1</sup>. The incidence and mortality of breast cancer steadily increase in the world with geographical and socioeconomic inequalities<sup>2,3</sup>. Breast cancer is a heterogeneous disease with different subtypes defined by a molecular classification: Luminal A and B (estrogen receptor positive ER<sup>+</sup>), Her2 positive (HER-2<sup>+</sup> and ER<sup>-</sup>), Basal-like (triple negative breast cancer -TNBC-; ER<sup>-</sup>, PR<sup>-</sup> and Her-2<sup>-</sup>) breast cancers<sup>4-6</sup>. A non-metastatic breast cancer has a good prognosis and the 5-year relative survival rate is approximately 90% in women<sup>7-9</sup>. On the other hand, the 5-year relative survival rate decreases to less than 30% for metastatic breast cancer with a median overall survival of approximately 3 years<sup>7,10</sup>. Metastasis involves a multistep process resulting in the dissemination of cancer cells from the primary tumor to secondary sites (lymph nodes, bones, lungs, liver and brain in breast cancer)<sup>11</sup>. The generation of metastases requires the activation of migration and invasion properties in cancer cells in order to leave the primary tumor site. Cancer cells then enter the circulatory system by intravasation and metastatic seeding occurs after extravasation at distant sites<sup>12</sup>. The epithelial-to-mesenchymal transition (EMT) might be a critical mechanism for metastasis and is characterized by loss of epithelial apical-basal polarity, decrease of cell-cell or cell-extracellular matrix interactions, downregulation of epithelial marker expression (i.e. E-cadherin or occludin) and up-regulation of mesenchymal markers (i.e. vimentin or N-cadherin)<sup>13</sup>. Cancer cells with mesenchymal traits acquire migrative and invasive abilities essential to metastasis<sup>14</sup>.

Metabolic adaptation is a hallmark of cancers and reprogramming of fatty acid (FA) metabolism is observed in many cancer cells. Breast cancer is associated with quantitative and qualitative changes in FA composition<sup>15</sup>. The remodeling of fatty acid content in breast cancer cells is correlated to high expression of the lipogenic enzymes ATP Citrate Lyase (ACLY), AcetylCoA Carboxylase (ACC) and Fatty Acid Synthase (FASN) suggesting high rates of *de novo* fatty acid synthesis. Abolition of

lipogenesis through inhibition of lipogenic enzyme activity decreases cancer cell proliferation and metastatic processes<sup>15-18</sup>. Exogenous fatty acids also contribute to proliferation of breast cancer cells and development of metastases<sup>19,20</sup>. Modifications of cellular FA by desaturation and elongation are common in cancer cells and alterations in the expression of these involved enzymes is described in breast cancer<sup>15,21</sup>. Seven very long-chain fatty acid elongases (Elovl 1–7) have been identified in the mouse, rat, and human genomes. These enzymes determine the rate of overall fatty acid elongation with substrate selectivity depending on carbon chain length and unsaturation degree; Elovl5 preferentially elongates C18 and C20 unsaturated FA<sup>22</sup>.

In the present study, we were interested in the role of Elovl5 in breast cancer progression. We developed different murine cancer models and showed that Elovl5 controlled tumor growth and metastasis process. We provided evidence of the implication of lipid droplets and TGF- $\beta$  receptors in the modulation of proliferation and invasion by Elovl5 expression in breast cancer cells.

## Results

### **Elovl5 is downregulated in breast cancer and low expression of Elovl5 is associated with poor clinical outcome.**

Analysis of Elovl5 mRNA levels in the METABRIC dataset showed that expression of Elovl5 mRNA is downregulated in breast cancer tissues (n= 957 samples) compared to non-matched normal breast tissues (n= 144 samples) (Fig. 1a). In addition, we analyzed Elovl5 mRNA expression in samples from 30 women with breast cancer for which we had breast tumor tissues and paired normal breast tissues (Suppl. Table 1). Overall the analysis showed that the content of Elovl5 mRNA was significantly reduced in breast cancer tissues compared to matched normal tissues (Fig. 1b). We found also that the expression of Elovl5 mRNA was differently expressed between molecular subtypes of breast cancer (PAM50). Indeed, breast cancer of the luminal subtypes expressed higher Elovl5 mRNA levels than Her2<sup>+</sup> and Basal-like subtypes in the METABRIC dataset (Fig. 1c). We established a H-score for

immunohistochemistry (IHC) staining of Elov15 and we confirmed that the average expression of Elov15 was higher in ER<sup>+</sup> breast cancer samples than in ER<sup>-</sup> breast cancer tissues (Her2<sup>+</sup> and TNBC) and was downregulated in breast cancer (T) tissue compared to adjacent normal (NT) breast tissue (Fig. 1d and Suppl. Table 2). From the METABRIC dataset, we determined the overall survival (OS) of breast cancer patients according to Elov15 mRNA expression. We found that a lower expression of Elov15 mRNA is associated with a shorter OS time for breast cancer patients without distinction of subtypes (Fig. 1e). In patients with Luminal A and B cancers, low Elov15 mRNA expression was associated with a worse OS prognosis compared to patients with high Elov15 expression. A similar albeit not significant trend was observed for Basal-like cancers. Finally, no such association was observed for Her2<sup>+</sup> cancer patients (Fig. 1f-i).

#### **Silencing of Elov15 expression inhibits breast cancer cell proliferation and tumor growth.**

To precise the role of Elov15 in breast cancer cell proliferation, we used mammary cell lines with different basal Elov15 expression levels, in which we further downregulated or overexpressed Elov15 expression (Suppl. Fig. S1a). We validated the reduction of Elov15 mRNA and protein levels at 48 hours using siRNA and shRNA targeting Elov15 compared to control siRNA and shRNA in murine 4T1 or human MCF-7 breast cancer cells (Suppl. Fig. S1b-e). In the same way, we generated a stable overexpression of Elov15 in 4T1 cells (Elov15-0 and Elov15-3) compared to control (ctrl1 and ctrl3) 4T1 cells (Suppl. Fig. S1f-g). Lipidomic analysis of mono- and polyunsaturated FA composition by Gas Chromatography-Mass Spectrometry (GC-MS) between Elov15-silenced breast cancer cells and their respective control breast cancer cells resulted in accumulation of C18:3 n-6 FA (Suppl. Fig. S1h-j) whereas C18:3 n-6 content decreased in 4T1 cells stably overexpressing Elov15 (Suppl. Fig. S1k). We then showed that stable or transient reduction of Elov15 expression with an shRNA or an siRNA, respectively decreased MCF-7 cell proliferation analyzed by crystal violet staining (Fig. 2a-b). Likewise, transient downregulation of Elov15 expression with an siRNA in 4T1 cells inhibited the

proliferation (Fig. 2c). Conversely, stable overexpression of Elov15 in 4T1 cells (Elov15-0 and Elov15-3) resulted in an increase of *in vitro* proliferation compared to control 4T1 (ctrl1 and ctrl3) (Fig. 2d).

Next, we assessed the effect of Elov15 overexpression in tumor growth by grafting 4T1 cells stably overexpressing Elov15 (Elov15-0 and Elov15-3) or control (ctrl1 and 3) 4T1 cells in the fourth mammary fat pad of female Balb-c mice. Our results showed that Elov15 expression increased 4T1 tumor growth in mice (Fig. 2e). We also used the MMTV-PyMT mammary cancer mouse model which mimics human luminal B breast cancer<sup>23</sup>. To evaluate the role of Elov15 invalidation in breast cancer progression, we crossed *Elov15* full knockout female C57BL/6 mice with MMTV-PyMT male C57BL/6 mice. After validation of Elov15 gene invalidation (Suppl. Fig. S2a-c), we found as expected that mammary tumor tissues from MMTV-PyMT;*Elov15*<sup>-/-</sup> (hereafter termed *Elov15*<sup>-/-</sup>) mice had a significantly increased percentage of C18:3 n-6 FA compared to mammary tumor tissues of MMTV-PyMT;*Elov15*<sup>+/+</sup> (hereafter termed *Elov15*<sup>+/+</sup>) (Suppl. Fig. S2d). We then monitored tumor growth until the sacrifice of the mice at 180 days of age; all female mice developed at least one mammary tumor at this time point. The surface of the aggregated tumor lesions at 180 days was lower in *Elov15*<sup>-/-</sup> than in *Elov15*<sup>+/+</sup> mice (Fig. 2f). However, we observed a slight delay in tumor onset in *Elov15*<sup>-/-</sup> compared to *Elov15*<sup>+/+</sup> mice which could explain difference in tumor surface (Fig. 2g). Therefore, we determined the tumor growth rate in the 22 days starting from the the detection of the first tumor. In these 22 days, the mean aggregated tumor surface for 22 days increased 11.6 fold in *Elov15*<sup>+/+</sup> mice and only 7.4 fold in *Elov15*<sup>-/-</sup> mice (Fig. 2h and 2i). Altogether these data show that Elov15 controlled proliferation and tumor growth in breast cancer.

### **Loss of Elov15 promotes development of lung metastases**

A weak expression of Elov15 is associated with a worse prognosis in breast cancer. However, the breast cancer aggressiveness is not dependent on cancer cell proliferation since low expression of Elov15 inhibited proliferation (Fig. 2). Therefore, we investigated the role of Elov15 in metastasis which remains the main risk of death in breast cancer patients<sup>24</sup>. First, we analyzed the presence of metastases

in lungs of Elov15<sup>-/-</sup> and Elov15<sup>+/+</sup> mice at an age of 180 days. We observed that Elov15<sup>-/-</sup> mice developed more metastases on their lung surface than Elov15<sup>+/+</sup> mice (Suppl. Fig. S3a and S3b). The quantification of metastases area in H&E-stained sections from lungs using QuPath analysis confirmed that the area occupied by metastases relative to the total lung area was greater in Elov15<sup>-/-</sup> mice than in Elov15<sup>+/+</sup> mice (Fig. 3a). Moreover, mice transplanted with Elov15-overexpressing (Elov-0 and Elov-3) 4T1 cells in their mammary fat pad showed lower metastases counts on their lung surface and less lung area was invaded by metastases in comparison to control (ctrl1 and 3) 4T1 tumor-bearing mice (Suppl. Fig. S3c and Fig. 3b). To further investigate the role of Elov15 in metastatic process, we decided to inject MCF-7 or 4T1 cells with different Elov15 expression levels in the tail vein, in order partially to mimic metastasis. As expected, we observed that stable downregulation of Elov1-5 in MCF-7 (shRNA Elov15) resulted in more lung surface with metastases (Fig. 3c) and higher numbers of metastases compared to shRNA ctrl MCF-7 cells (Suppl. Fig. S3d) in female NMRI-nude mice. In contrast, tail vein injection of 4T1 cells stably overexpressing Elov15 (Elov15-0 and Elov15-3) in female Balb-c mice led to a decrease in metastases numbers and lung surface with metastases in comparison to control (ctrl1 and ctrl3) 4T1 cells analyzed by QuPath (Suppl. Fig. S3e and Fig. 3d). To confirm the association of Elov15 expression and lymph node metastasis in breast cancer, we conducted an IHC analysis of patients with or without lymph node invasion (Suppl. Table 3). The Elov15 H-score defined with QuPath analysis on breast cancer tissues was significantly higher in ER<sup>+</sup> patients without metastases in lymph nodes (N0) than with metastases in lymph nodes (N1). We didn't observe any differences in the Elov15 H-score between N0 and N1 status in patients with Her2<sup>+</sup> or TNBC cancers (Fig. 3e). Moreover, we confirmed that the Elov15 H-score is higher in ER<sup>+</sup> patients (both N0 and N1) than in Her2<sup>+</sup> or TNBC patients (Fig. 3e). These results demonstrate that a decrease in Elov15 expression correlates to lymph node invasion in ER<sup>+</sup> breast cancers and promotes the formation of lung metastases in mouse breast cancer models.

**Downregulation of Elov15 increases invasiveness and expression of EMT markers.**

The metastatic process at the cellular level is associated with the acquisition of invasive properties and a mesenchymal phenotype. Thus, we investigated the effect of Elov15 expression in cell invasion and the expression of EMT markers. To perform invasion assays, we seeded cancer cells in a Boyden chamber with a layer of Matrigel and determined cell invasion by crystal violet staining after 48 hours. These experiments were carried out in low invasive MCF-7 cells in which Elov15 expression was silenced. Stable or transient Elov15 downregulation significantly promoted invasiveness in MCF-7 cells (Fig. 3f and 3g). In 4T1 cells with high basal invasive properties, knockdown of Elov15 expression (siRNA Elov15) also increased cell invasion compared to control (siRNA ctrl) 4T1 cells (Fig. 3h) whereas Elov15 overexpression (Elov-0 and Elov-3) limited invasiveness of 4T1 cells in comparison to control (ctrl1 and ctrl3) 4T1 cells (Fig. 3i). Changes in cell morphology through actin cytoskeleton remodeling is critical for invasion (ref). Therefore, we visualized actin fibers (F-actin) with FITC-conjugated phalloidin staining and found that suppression of Elov15 with an siRNA for 48 hours in 4T1 and MCF-7 cells induced a mesenchymal-like morphology (Fig. 3j). Then, we analyzed by western-blotting the expression of EMT markers which showed a decrease in the expression of epithelial markers (E-cadherin and occludin) and an increased expression of mesenchymal markers (vimentin and N-cadherin) in Elov15-silenced breast cancer cells (Fig. 3k and 3l). In contrast, overexpression of Elov15 in 4T1 cells (E0 and E3) reduced the expression of mesenchymal markers (vimentin and N-cadherin) and slightly increased expression of epithelial markers (E-cadherin and occludin) (Fig. 3m). These data highlight that repression of Elov15 expression sustains invasiveness and EMT in breast cancer cells.

**Elov15-regulated expression of TGF- $\beta$  receptors controls cell proliferation and invasion**

The TGF- $\beta$  pathway is a major contributor of EMT and metastasis <sup>25</sup>. Thus, we aimed to investigate the expression of TGF- $\beta$  receptors in breast cancer cells upon modulation of Elov15 expression. Silencing of Elov15 expression in both 4T1 and MCF-7 cells induced the expression of TGFBR1 and

TGFBR2 mRNA, as shown by RT-qPCR (Fig. 4a-c). Conversely, the stable overexpression of Elov15 in 4T1 cells (Elov15-0 and Elov15-3) decreased the expression of TGFBR1 and TGFBR2 mRNA compared to control 4T1 cells (ctrl1 and ctrl3) (Fig. 4d). We confirmed at the protein level that expression of TGF- $\beta$  receptor 1 (TGF $\beta$ -R1) by immunofluorescence staining (Fig. 4e) and expression of TGF- $\beta$  receptor 2 (TGF $\beta$ -R2) by western-blotting (Fig. 4f) is enhanced in Elov15-depleted breast cancer cells. Furthermore, we demonstrated an increase in TGF $\beta$ -R1 and TGF $\beta$ -R2 expression at the plasma membrane by flow cytometry analysis of breast cancer cells with suppressed Elov15 expression (Fig. 4g-i) while stable overexpression of Elov15 reduced their exposure at the plasma membrane (Fig. 4j). Given that TGF $\beta$ -R1 and TGF $\beta$ -R2 were more expressed in Elov15-silenced breast cancer cells, such cancer cells should present a higher activation of downstream pathways after TGF- $\beta$  treatment. In order to evaluate the activation of the TGF- $\beta$  pathway, we analyzed phosphorylation of smad2/3 (p-smad2/3) by western-blotting. We showed that TGF- $\beta$ 1 treatment (5 ng/ml) for 30 and 60 minutes in Elov15-silenced MCF-7 cells induced higher p-smad2/3 than in control MCF-7 cells suggesting that the TGF- $\beta$  pathway is more activatable (Fig. 4k). A similar result was obtained in Elov15-depleted 4T1 cells treated for 30 minutes with TGF- $\beta$ 1 (Fig. 4l). It is noteworthy that there is an induction of p-smad2/3 in MCF-7 and 4T1 cells with downregulation of Elov15 compared to control cells in untreated conditions (Fig. 4k and 4l) suggesting the presence of TGF- $\beta$  in the extracellular microenvironment. We evaluated the mRNA expression of the different TGF- $\beta$  isoforms and we found an increase in TGF- $\beta$ 3 transcripts in Elov15-depleted MCF-7 and 4T1 cells (Suppl. Fig. S4a and S4b). In order to demonstrate the role of TGF- $\beta$  receptors in Elov15-dependent cell invasion and proliferation, we inhibited TGF- $\beta$  receptor activity with pharmacological drugs (LY2157299 and LY2109761). We confirmed that repression of Elov15 expression enhanced invasiveness (Fig. 5a-c). However, the treatment with LY2157299 and LY2109761 prevented the increased cell invasiveness of Elov15-depleted MCF-7 and 4T1 cells compared to control cells (Fig. 5a-c). Moreover, treatment with the inhibitors of TGF- $\beta$  receptors (LY2157299 and LY2109761) alleviated repression of proliferation

induced by Elov15 silencing in 4T1 and MCF-7 breast cancer cells (Fig. 5d-f). Collectively, these data suggest a regulation of TGF- $\beta$  receptor expression by Elov15 and confirm the role of TGF- $\beta$  receptors in proliferation and invasion.

### **Suppression of Elov15 expression drives DGAT1/2-dependent accumulation of lipid droplets.**

In order to address the modification of lipid metabolism under Elov15 regulation, we analyzed the expression of lipogenic and triacylglycerols (TAG) synthesis enzymes by western-blotting. Elov15-depletion in MCF-7 cells induced a lipogenic program characterized by decreased inhibitory phosphorylation of ACC and increased Scd1 expression (Fig. 6a). Furthermore, we evaluated the expression of Diacylglycerol Acyltransferases (DGAT) 1 and 2 which participate in TAG synthesis, and found an increase in DGAT1 and 2 expression in Elov15-silenced cancer (Fig. 6b and Suppl. Fig. S5a). We then determined the total FA content by GC-MS and TAG content by enzymatic assay in breast cancer cells. We observed that transient depletion of Elov15 in breast cancer cells using an siRNA for 48 hours or its stable depletion by an shRNA in MCF-7 cells led to an intracellular accumulation of total FA (Fig. 6d and Suppl. Fig. S5b) and TAG (Fig. 6d and Suppl. Fig. S5c) compared to control cancer cells. Furthermore, mammary tumor tissues from MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>) mice also harbored higher FA and TAG content compared to mammary tumor tissues from MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>) mice (Fig. 6e and 6f). In addition, we analyzed TAG content in breast tumor tissues in which we showed that Elov15 expression was lower compared to their paired non-tumoral counterparts from patients with different cancer subtypes (Fig. 6g). The TAG content was significantly higher in breast tumor tissues than in paired non-tumoral tissues from patients with ER<sup>+</sup> and Her2<sup>+</sup> breast cancer; a non-significant increase in TAG concentration was also observed in tumor tissues of the TNBC subtype compared to paired normal tissues (Fig. 6g). Moreover, the average TAG concentration was different between subtypes with the highest concentration in the ER<sup>+</sup> subtype (Fig. 6g). TAG are a major neutral lipid constituent of lipid droplets (LD), storing excess of FA. LD are a reservoir of substrates for fatty acid oxidation contributing to acetyl-CoA production and their

formation depends on DGAT1/2 activity that catalyzes the final step of TAG synthesis<sup>26</sup>. Therefore, we analyzed the effect of inhibiting Elov15 expression on LD content. Breast cancer cells with siRNA-mediated depletion of Elov15 expression using siRNA transfection increased the content of intracellular LD (red dots in cells) detected by fluorescence microscopy using lipophilic Nile red staining (Fig. 6h). The quantification of LD after Nile red staining in MCF-7 and 4T1 cells 24 and 48 hours after siRNA transfection was carried out by flow cytometry and showed an accumulation of LD in Elov15 expression silenced-breast cancer cells compared to control cancer cells (Fig. 6i). We confirmed that LD content was increased in MCF-7 cells with stable downregulation of Elov15 expression (Suppl. Fig. 5d). Moreover, as expected, stable overexpression of Elov15 in Elov150 and Elov15-3 4T1 cells reduced LD formation (Fig. 6j). In order to explore the role of DGAT isoforms in LD formation in a Elov15-dependent manner, we inhibited DGAT1 and DGAT2 activity with pharmacological agents A922500 (DGAT1i) and PF-06424439 (DGAT2i) respectively and observed a decrease in LD accumulation in Elov15-depleted breast cancer cells treated with the DGAT inhibitors (Fig. 6k and Suppl. Fig. S5e-f). Furthermore, we showed that silencing of Elov15 expression in MCF-7 cells led to increased acetyl-CoA levels which is reversed by inhibition of DGAT1 and DGAT2 activity (Fig. 6l and 6m). Altogether, the data highlight the role of Elov15 in DGAT1/2 dependent-LD formation.

### **Elov15 extinction-induced lipid droplets regulate expression of TGF- $\beta$ receptors, EMT markers and metastasis.**

To further investigate the functions of LD, we first analyzed levels of TGF- $\beta$  receptors at the plasma membrane of breast cancer cells lacking Elov15 expression and exposed to DGAT inhibitors. We confirmed by flow cytometry that TGF- $\beta$  receptors 1 (TGF $\beta$ -R1) and 2 (TGF $\beta$ -R2) was present at higher levels at the plasma membrane of Elov15-silenced MCF-7 and 4T1 breast cancer cells than in control cells (Fig. 7a-b and suppl. Fig S6a). However, treatment with inhibitors of DGAT1 (A922500, DGAT1i) and DGAT2 (PF-06424439, DGAT2i) activity for 48 hours was able to counteract the

increase of plasma membrane TGF $\beta$ -R1 and TGF $\beta$ -R2 levels in Elov15-depleted cancer cells (Fig. 7a-b and Suppl. Fig. S6a). Similarly, the analysis by RT-qPCR demonstrated that preventing LD accumulation by A922500 and PF-06424439 for 48 hours abrogated the induction of TGFBR1 and TGFBR2 mRNA expression in MCF-7 and 4T1 cells without Elov15 expression (Fig. 7c-d and Suppl. Fig. S6b). Next, we evaluated the impact of LD reduction on expression of EMT markers in breast cancer cells. A mesenchymal phenotype with upregulation of vimentin and downregulation of epithelial markers (E-cadherin and occludin) was obtained in breast cancer cells with stable or transient silencing of Elov15 expression as assessed at 48 hours by RT-qPCR (Fig. 7e-f and Suppl. Fig. S6c) and western-blotting (Fig. 7g-h). However, treatment with DGAT inhibitors (DGAT1i and DGAT2i) reversed the EMT triggered by Elov15 extinction in breast cancer cells (Fig. 7e-h and Suppl. Fig. S6c). The effect of LD repression on TGF- $\beta$  receptor expression and the mesenchymal phenotype led us to assess the effect of repressing LD formation on cell proliferation and invasion. We clearly confirmed that ablation of Elov15 expression with siRNA or shRNA in breast cancer cells inhibited cell proliferation (Suppl. Fig. S6d and Fig. 7i-j) and promoted cell invasion (Suppl. Fig. S6e and Fig. 7k-l). Nevertheless, the prevention of LD accumulation using DGAT inhibitors (DGAT1i and DGAT2i) in Elov15-depleted 4T1 and MCF-7 cells restored cell proliferation and decreased cell invasion (Suppl. Fig. S6d-e and Fig. 7i-l). Collectively, the data demonstrate that LD accumulation induced by Elov15 downregulation controls proliferation and invasion through TGF- $\beta$  receptor induction.

## Discussion

In this study, we demonstrated a role of Elov15 in breast cancer growth and metastasis through the expression of TGF- $\beta$  receptors, mediated by the storage of fat in lipid droplets. Our results showed that downregulation of Elov15 expression inhibited proliferation of breast cancer cells and mammary tumor growth in murine models, which is consistent with a recent publication on prostate cancer<sup>27</sup>. Indeed, depletion of Elov15 expression in prostate cancer cells led to inhibition of cell proliferation and

metastasis<sup>27</sup>. Interestingly, we showed that formation of metastases and metastasis-associated features (cell invasion, TGF- $\beta$  receptor expression and EMT) in breast cancer models were promoted by dampening Elov15 expression. In contrast, overexpression of Elov15 in murine 4T1 cells decreased the development of lung metastases. The data obtained from breast cancer patients confirmed a relationship between Elov15 expression and metastasis. In our case, a significant downregulation of Elov15 expression was observed in patients with metastatic ER<sup>+</sup> breast cancers (N1) compared to non-metastatic ER<sup>+</sup> breast cancers (N0). We did not find any differences in Elov15 expression between patients with metastatic Her2<sup>+</sup>/TNBC breast cancers (N1) compared to non-metastatic Her2<sup>+</sup>/TNBC breast cancers (N0). Of note, Elov15 expression in patients with an N0 status is already much lower in Her2<sup>+</sup> and TNBC breast cancer tissues compared to ER<sup>+</sup> breast cancer tissues, and is closer to the downregulated Elov15 expression in ER<sup>+</sup> breast cancer tissues from patients with N1 status. It is reported that more than 75% of breast cancer death are caused by metastases<sup>24</sup>. Therefore, the correlation between a low expression of Elov15 and a worse prognosis in patients with luminal breast cancers could be explained by the fact that metastasis is promoted by Elov15 silencing.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling exerts ambivalent properties in cancer with tumor suppressive effects at early stages and pro-metastatic action at a later stage<sup>28</sup>. TGF- $\beta$  signaling has the ability to induce cell cycle arrest and/or apoptosis as well as to promote EMT and cell invasion in cancer cells including breast cancer cells<sup>29,30</sup>. Active TGF- $\beta$  elicits its biological effects by binding to serine/threonine kinase TGF- $\beta$  receptors (TGF $\beta$ -R) 1 and 2 directly or with the help of the accessory TGF $\beta$ -R3. Binding of TGF- $\beta$  on the receptor complex triggers TGF $\beta$ -R2-induced transphosphorylation and activation of TGF $\beta$ -R1. Then, canonical TGF- $\beta$  transduction signal depends on TGF $\beta$ -R1-mediated phosphorylation of Smad2 and Smad3. The phospho-Smad2/3 heterodimer complex interacts with Smad4 for nuclear translocation of the heterotrimer complex which runs a transcriptional program<sup>28</sup>. We demonstrated an increase in expression of TGF- $\beta$  isoforms and receptors in breast cancer cells lacking Elov15 expression. Furthermore, Elov15 depletion induced an

increase in phosphoSmad2/3 content suggesting activation of the TGF- $\beta$  pathway and anti-proliferative and pro-metastatic effects of TGF- $\beta$  signaling which were reversed by inhibitors of TGF $\beta$ -R1 and TGF $\beta$ -R2. Drugs against TGF- $\beta$  signaling might offer a therapeutic response to cancer cell dissemination and several inhibitors of the TGF- $\beta$  pathway are tested for clinical evaluation <sup>31</sup>. However, the ambivalent role of TGF- $\beta$  requires a precise selection of eligible patients with advanced breast cancer. Furthermore, dosing of anti-TGF- $\beta$  signaling drugs has to be personalized due to possible cardiac and cutaneous side effects <sup>31,32</sup>. Elov15 expression might be a predictive biomarker and help to determine eligible recipients of anti-TGF- $\beta$  signaling therapy.

Alteration of lipid metabolism contributes to cancer progression and pre-clinical studies demonstrated the rationale of targeting lipid metabolism. It has been shown that inhibition of exogenous uptake or endogenous synthesis of FA repressed proliferation of breast cancer cells <sup>33-35</sup>. Elongation of fatty acid is under the control of seven fatty acid elongases (Elovl) and expression of Elovl1 and Elovl6 is increased in breast cancer tissues <sup>21</sup>. Yamashita *et al.* also described increase of Elovl5 mRNA expression in breast cancer tissues while they did not find any changes in protein levels of Elovl5 in IHC staining comparing breast tumors to normal paired tissues <sup>21</sup>. In our study, we did not confirm an increase of Elovl5 mRNA expression in breast tumors but we were able to show a decrease of Elovl5 expression by RT-qPCR as well as by IHC staining in breast cancer tissues. As a consequence of Elovl5 downregulation, there was accumulation of LD in breast cancer cells which controlled their proliferation and invasion. The increase in total FA and TAG content in breast cancer cells upon depletion of Elovl5 was consistent with an activation of lipogenesis and LD formation. LD abundance is correlated with cancer aggressiveness (i.e. in glioblastoma) <sup>36</sup> and breast cancer cell lines show an increasing trend in LD content when going from the non-malignant MCF10A cells, to the intermediately aggressive MCF-7 cells to the highly aggressive MDA-MB-231 cells <sup>37</sup>. In accordance with these observations, we demonstrated that LD accumulation in breast cancer cells is essential for the Elovl5 silencing-mediated promotion of EMT and invasion, since repression of LD formation by

blockade of DGAT activity reversed the induction of EMT and cell invasion. Interfering with exogenous lipid uptake or lipogenesis also results in a decrease of LD abundance and affects the migratory and invasive properties of cancer cells<sup>38-40</sup>. LD are able to manage cell lipotoxicity and are a reservoir of FA for acetyl-CoA production during fatty acid oxidation<sup>41</sup>. Thus, we showed that Elov15-depleted breast cancer cells contained more acetyl-CoA, and as expected, abrogation of LD formation by DGAT inhibitors deprived cancer cells of acetyl-CoA. Availability in acetyl-CoA contributes to TGF- $\beta$  signaling through smad2 activation by acetylation, impacting EMT and metastasis<sup>17,40</sup> and this is in line with our data indicating that LD induced by downregulation of Elov15 expression mediated upregulation of EMT and cell invasion.

The present work suggests that treatment with DGAT inhibitors could block LD formation and subsequent TGF- $\beta$  signaling through repression of TGF- $\beta$  receptor expression in Elov15-depleted breast cancer cells. As we discussed above, drugs targeting actors of TGF- $\beta$  signaling (i.e. TGF- $\beta$  isoforms or TGF- $\beta$  receptors) have an interesting potential against metastasis. However, the use of DGAT inhibitors might also improve the efficacy of anti-cancer drugs for treatment of patients with a risk of metastases associated to low levels of Elov15 by suppressing the buffering of these anti-cancer drugs by LD<sup>42,43</sup>.

In conclusion, we demonstrate the role of Elov15 in breast cancer cells through regulation of lipid droplet content and expression of TGF- $\beta$  receptors. Furthermore, we show that low Elov15 expression increases the risk of metastatic breast cancer. Therefore, Elov15 expression might help in orienting patients for additional therapies in order to prevent metastasis.

## **Materials and methods**

### **Cell culture**

Human adenocarcinoma MCF7 (HTB-22-ATCC), MDA-MB-231 (HTB-26-ATCC), mouse 4T1 mammary carcinoma (CRL-2539-ATCC) and mouse NMuMG mammary cystadenoma (CRL-1636 – ATCC) cell lines were used within the first 25 passages and tested for mycoplasma contamination (Lonza, Mycoalert Detection Kit and Mycoalert Assay Control set). MDA-MB-231 and 4T1 cell lines were maintained in RPMI supplemented with 2 mM L-glutamine, 10% heat-decomplemented FBS, and antibiotics (Penicillin, streptomycin, and amphotericin B). MCF7 and NMuMG cell lines were maintained in DMEM supplemented with 2 mM L-glutamine, 10% heat-decomplemented FBS, and antibiotics (Penicillin, streptomycin, and amphotericin B). Cells were grown in a humidified 5% CO<sub>2</sub> incubator at 37°.

### **Plasmids, lentivirus production and MCF-7 cell line transduction**

The pLKO.5-puro lentiviral vectors expressing a control shRNA (SHC202) or an Elov15targeting shRNA (TRCN0000317892) were purchased from Sigma-Aldrich. Lentivirus were produced in HEK293T cells by transfection using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) of VSV-G envelope and PAX2 packaging vectors (Addgene). Lentivirus were collected 48 hours after transfection for transduction of MCF-7 cells with 8 µg/mL of polybrene (Sigma-Aldrich). After 2 days, transduced MCF-7 cells were selected with 2 µg/ml puromycin (Fisher scientific) to establish control MCF-7 cell line or cell line with a stable Elov15 knockdown.

### **Plasmids and Elov15-overexpressing 4T1 cell line.**

Linearized empty pcDNA3.1 or pcDNA3.1 encoding the ORF of the murine Elov15 gene sequence (Genscript) were transfected into 4T1 cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. 2 days post-transfection, non-clonal 4T1 cell lines that have stably integrated the plasmids were selected using 600 µg/ml G418 sulfate (Euromedex).

**Transient RNA interference and treatments**

Cells were reverse-transfected using Lipofectamine™ RNAiMAX transfection reagent (Thermo Fisher Scientific) with non-targeting siRNA (#4390846 ; Dharmacon) or siRNA targeting human ELOVL5 (#s34075 ; Dharmacon), or mouse ELOVL5 (#s87101 ; Dharmacon) according to the manufacturer's instructions. Treatments with 10 μM A922500 (SigmaAldrich), 10 μM PF-06424439 (Sigma-Aldrich), 1 μM LY2157299 (Selleck) and 1 μM LY2109761 (Sigma-Aldrich) were performed in complete culture medium. Breast cancer cells were incubated with 5 ng/ml recombinant human TGF-β1 (MiltenyiBiotec) in complete cell culture medium for the indicated times.

**Total fatty acid analysis by gas chromatography-mass spectrometry (GC-MS)**

Cell lines and tissue samples were homogenized and lipids were extracted in 1 ml of ethanol/butylated hydroxytoluene (50 mg/ml); 60 μl of 10 M potassium hydroxide and 25 μl of SI-internal standard (#SI S20-040; CDN Isotopes-Cayman) were added to the prepared suspensions followed by saponification at 56 °C for 45 minutes. The aqueous phase was collected after addition of 1 ml of 1.2M hydrochloric acid and 3ml of hexane. This was evaporated in vacuum dry centrifuge at 100°C, 2200g. The dry residues were resuspended in 10% v/v N, N-diisopropylethylamine, 10% v/v pentafluorobenzyl bromide diluted in acetonitrile and incubated at 37°C for 30 minutes to obtain pentafluorobenzyl esters. 1ml water and 2ml hexane were used to support the esters extraction. The hexane phase was collected and evaporated with liquid nitrogen. The fatty acid ester pellets were solubilized in hexane and 1 μl was injected into a GC-MS system (#7890A Gas Chromatograph, #7683 injector, #5975C Mass Selective Detector; Agilent Technologies) in pulsed split mode by using fused silica capillary column (#HP-5MS; Agilent Technologies). GC-MS running-mode contained helium gas with a flowrate at 1.8 ml/min, injector temperature at 250 °C, pulsed split 10, the oven temperature was set at 140 °C for 1 minute and then increased at a rate of 5 °C/min, then left at 300 °C for 7 minutes. The machine was set in negative chemical ionization mode, in the presence of methane as reactant gas,

with the temperature of ion source and quadrupole set at 150 °C. All the experimental steps were performed in glass-based containers. The FAs were first normalized using the closest internal standard by relative ratios using MassHunter and Qualitative software (Agilent Technologies). These FAs were then normalized to the total cell counts and the protein concentration from the initial sample.

### **Triacylglycerol (TAG) quantification**

Cells and tissue samples were homogenized in one volume of methanol. 4 volumes of dimethoxymethane were added and the samples were continuously vortexed for 1 hour. The organic solvents were evaporated under N<sub>2</sub> flow and TAG were resuspended in CHCl<sub>3</sub>-2% v/v Triton X-100. After CHCl<sub>3</sub> evaporation, deionized water was added to the Triton X-100 and TAG solution before TAG quantification using a Triglycerides FS enzymatic assay kit (Diagnosis systems) according to manufacturer's instruction. TAG content was normalized to the protein content of the initial sample, obtained by BCA assay quantification.

### **Nile red staining and quantification**

For fluorescence microscopy analysis, cells on coverslips were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT) and incubated with 1 μM Nile Red solution (Sigma-Aldrich) for 30 minutes. After washing with PBS, coverslips were mounted with Prolong™ diamond antifade mountant (Molecular Probes) containing 20 μM Hoechst (Thermo Fisher Scientific) and observed with Axio Imager 2 (Carl Zeiss Microscopy GmbH, Jena, Germany) connected to an Apotome 2 module (Carl Zeiss GmbH). Photos were taken with AxioCam MRm monochrome CCD camera (Carl Zeiss GmbH). The quantification of lipid droplets was based on the average of fluorescence intensity per cell in the recorded fields using imageJ software.

For flow cytometry analysis, cells were incubated for 15 minutes with 1 μM Nile Red solution (Sigma-Aldrich) at room temperature. Cells were washed and collected in PBS for analysis using an LSR

Fortessa cytometer and the BD FACSDiva software (BD Biosciences). Gating strategies were designed to exclude negative staining, cellular debris and duplexes. Data were analyzed using the FlowJo software (Tree Star, USA) and presented in mean of fluorescence intensity.

### **Phalloidin staining**

Cells on coverslips were washed with cold PBS and fixed with 4% PFA for 10 minutes at RT. After cell permeabilization with 0.1% v/v Triton X-100 (Euromedex) in PBS, cells were incubated with FITC-Phalloidin (50 µg/ml) for 40 minutes. After washing with PBS, cells were mounted with Prolong<sup>TM</sup> diamond antifade mountant (Molecular Probes) containing 20 µM Hoechst (Thermo Fisher Scientific) and observed with Axio Imager 2 (Carl Zeiss Microscopy GmbH, Jena, Germany) connected to an Apotome 2 module (Carl Zeiss GmbH). Photos were taken with an AxioCam MRm monochrome CCD camera (Carl Zeiss GmbH). The quantification of cellular morphology modification was based on the ratio between the number of cells with an epithelial-like morphology and the number of cells with a mesenchymal-like morphology in the recorded fields.

### **Immunofluorescence staining of TGF-β receptors**

For fluorescence microscopy analysis, cells were grown and treated on coverslips, washed with PBS and fixed with 4% paraformaldehyde (PFA) for 10 minutes. Cells were permeabilized with 0.2% saponin (Sigma-Aldrich) in PBS and saturated with 3% BSA in PBS for 20 minutes at RT. Cells were incubated with a primary antibody against TGF-β R1 (PA5-38718 ; Invitrogen) overnight at 4°C in 3% BSA in PBS. Samples were incubated with an Alexa568-conjugated anti-rabbit antibody (Invitrogen) for 30 minutes at RT in 3% BSA in PBS. Coverslips were mounted with Prolong<sup>TM</sup> diamond antifade mountant (Molecular Probes) containing 20 µM Hoechst (Thermo Fisher Scientific) and observed with Axio Imager 2 (Carl Zeiss Microscopy GmbH, Jena, Germany) connected to an Apotome 2 module (Carl Zeiss GmbH). Photos were taken with AxioCam MRm monochrome CCD camera (Carl Zeiss GmbH).

For Flow Cytometry analysis, cells were washed in cold-PBS and collected by scraping then saturated with 10% FBS in PBS for 10 minutes. After a washing step, cells were stained for 40 minutes on ice with an isotype control IgG-PE (Biolegend), or an anti-mouse TGF- $\beta$  R2-PE (R&D Systems) or an anti-human TGF- $\beta$  R2 PE (Biolegend). Flow cytometry analysis was performed with a BD LSR II flow cytometer (BD Biosciences). For TGF- $\beta$  R1 staining, human and mouse cells were incubated with anti-TGF- $\beta$  R1 (Merck Millipore) for 40 minutes on ice and after a washing step, cells were incubated with an isotype control Alexa568-conjugated IgG or a secondary Alexa568-conjugated anti-rabbit antibody (Invitrogen) for 20 minutes at RT. Cells were washed and collected in PBS for analysis on an LSR Fortessa cytometer and the BD FACSDiva software (BD Biosciences). Data were analyzed using the FlowJo software (Tree Star, USA) and presented in mean of fluorescence intensity.

### **RNA extraction and RT-qPCR**

Total RNA was extracted using TRIzol (Invitrogen) according to manufacturer's instructions. Reverse transcription was performed with an iScript cDNA Synthesis Kit (Bio-Rad). PCR was performed using Powerup SYBR Green master mix (Applied Biosystems, Life Technologies) on StepOnePlus™ Real-Time PCR System (Applied Biosystems). Relative gene expression was calculated using  $\Delta\Delta C_t$  values. Target mRNA levels were normalized to  $\beta$ -actin mRNA and 18S RNA. Primers (Supplementary Table 4) were purchased from Life technologies.

### **Cell proliferation assay**

Cells were washed with PBS and fixed with 100% cold-ethanol. Coloration with 4% crystal violet in 10% methanol was carried out to visualize adherent cells. After washing with water, crystal violet was dissolved in 33% acetic acid for OD quantification on a TECAN spectrophotometer at 540 nm.

**Western blot analysis**

Cells were lysed in RIPA supplemented with protease (#P8340; Sigma-Aldrich) and phosphatase (#P5726; Sigma-Aldrich) inhibitors. Protein lysates in Laemmli loading buffer were boiled for 5 minutes at 95°C and separated by SDS-PAGE electrophoresis. Proteins were wet-transferred onto nitrocellulose membranes (Amersham™ Protran™ 0,45 µm NC; Amersham). Membranes were saturated with 5% w/v non-fat milk or 5% BSA in TBST (Trisbuffered saline plus 0.1% Tween 20) for 1 hour at RT. Primary antibodies (listed in Supplemental Methods) were diluted in the appropriated saturation solutions for overnight incubation at 4°C. After TBST washes, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution; Cell Signaling Technologies). Membranes were imaged using Enhanced Chemi Luminescence (Clarity™ Western ECL Substrate; Bio Rad) in a sChemiDoc™ XRS<sup>+</sup> imaging system (Biorad). Images were analyzed with Image Lab 5.1 software (Biorad, France).

**Invasion assay**

Cell invasion assay was carried out in Tranwell insert (Greiner Bio-One) with a Matrigel-coated 8 µm-pore membrane (#356237; Corning). Cells in culture medium with 0.1% FBS were placed on top of the Matrigel coated-inserts and complete culture medium (10% FBS) was added in the lower chamber. After incubation, cells were fixed with 4% PFA and stained with a crystal violet solution (Sigma Aldrich) for 30 minutes. The inserts were washed to remove noninvasive cells. Images of randomly selected fields were recorded by an AxioScope A1 microscope (ZEISS Microscopy) coupled camera (Jenoptik; GRYPHAX NAOS) using the GRYPHAX software. Cell counting was performed using ImageJ software.

**Immunohistochemistry analysis**

Paraffin-embedded tumor breast tissue samples from breast cancer patients with or without metastasis were deparaffinized and rehydrated. Tissue sections were saturated with peroxidase blocking buffer

and incubated with a primary antibody against ELOVL5 (#HPA047752; Sigma-Aldrich) for 1 hour. After washing, HRP polymer-conjugated secondary antibodies (Dako) were then incubated with the tissue sections for 20 minutes followed by a 5 minutes coloration with 1g/ml of DM827-3, 3'-diaminobenzidine (DAB). Hematoxylin and Eosin staining was used to visualize nuclei. Tissue sections were mounted with an organic mounting solution from Dako. Quantification of ELOVL5 staining was analyzed with QuPath 0.2.3<sup>44</sup> after slides were scanned by Nanozoomer controlled by NDP.view scan software (Hamamatsu). Data are presented as average H-score of ELOVL5 expression in each slide comparing tumor and adjacent non-tumoral tissues.

### **MMTV-PyMT and Elov15 Mice**

Mice were bred and maintained in the animal facility of the University of Burgundy according to the center instructions, all the experiments were carried out following the instructions of the Federation of European Animal Science Associations. Experimental designs involving animals were approved by the Ethics Committee of the University of Burgundy (projects #17461, #14557 and #22358). *Elov15*<sup>-/-</sup> C57BL/6N mice were obtained from the Mutant Mouse Regional Resource Center at UC Davis (Davis, CA) and were generated by inserting a genetrapp cassette in the exon 3 of the *Elov15* gene. B6.FVB-Tg(MMTV-PyVT)634Mul/LelJ (MMTV-PyMT) mice were obtained from the Jackson Laboratory. Male MMTV-PyMT mice were crossed with female *Elov15*<sup>-/-</sup> to obtain MMTV-PyMT;*Elov15*<sup>+/-</sup> mice. Then, experimental female mice were obtained by crossing male MMTV-PyMT;*Elov15*<sup>+/-</sup> mice with female *Elov15*<sup>+/-</sup> mice. Mouse genotyping was performed using extraction solution (E7526; Sigma-Aldrich) and neutralization solution B (N3910; Sigma-Aldrich) and with PCR primer sets (Supplementary Table 1) for the detection of MMTV-PyMT transgene and *Elov15* invalidation. Female mice were monitored by palpation and tumor growth measured every 2 days. Tumor surface (mm<sup>2</sup>) were calculated by multiplying the measured length and width of the tumor. Upon reaching 180 days of age, mice were sacrificed, and tissues (lung and tumors) were collected for analysis.

**Mammary gland grafting and tail vein injection**

4T1 cells ( $0.5 \times 10^6$  cells in 100 $\mu$ l PBS per mice) were injected into the inguinal mammary fat pad of 8-week-old mice (BALB/cByJ; Charles River) using a 26Gx7/8'' needle and 1ml syringe. Tumors were measured three times per week and tumor size (mm<sup>2</sup>) was calculated by multiplying the measured length and width of the tumor. On the 23<sup>rd</sup> day post-injection, mice were sacrificed and tumors and lungs were collected for downstream experiments.

For tail vein injection of MCF-7 cells, female 8-week-old nude mice (NMRI; Charles River) were pre-treated with  $\beta$ -estradiol (Sigma-Aldrich) at 4 $\mu$ g/100 $\mu$ l PBS/mice by intraperitoneal injection 24 and 48 hours before cell injection. Elov15 knockdown and control MCF-7 cells ( $5 \times 10^6$  cells in 100 $\mu$ l PBS per mice) were injected into the tail vein using insulin syringes (30G x 1/2''). Injection with  $\beta$ -estradiol were carried out three times/week. Mice were sacrificed 35 days after MCF-7 cell injection and lungs were collected for metastase quantification. For tail vein injection of 4T1 cells,  $1 \times 10^5$  4T1 cells in 50 $\mu$ l PBS per mice were injected into the tail vein of 8-week-old mice (BALB/cByJ; Charles River) by using insulin syringes (30G x 1/2''). The mice were sacrificed 11 days post-cell injection and lungs were collected for metastase analysis.

Lungs were fixed in formalin and paraffin-embedded. Tissues were dewaxed and rehydrated for Hematoxylin and Eosin staining. Tissue sections were then mounted with an organic mounting solution. Scoring the invasive tumoral area over the total lung area was performed using QuPath 0.2.3 software after scanning the slides with a Nanozoomer using the NDP.view scan software (Hamamatsu). Data are presented as the ratio of total tumor area over the total lung area.

**Patients and METABRIC datasets**

Research on patient samples was conducted in agreement with the Declaration of Helsinki and approved by the Ethics Committee of the Centre Georges-François Leclerc, University Hospital

François Mitterrand Dijon-Bourgogne (Dijon, France) and the Burgundy Advisory Committee for the Protection of People in Biomedical Research. Patient's informed consent was obtained before project enrollment. The clinical and pathological characteristics of patients are summarized in Supplementary Tables 1-3.

Discovery Metabric datasets, generated by the Molecular Taxonomy of Breast Cancer International Consortium, was used as public dataset. Normalized data, obtained with the Illumina HT 12 platform, were requested and downloaded from the European Genomephenome Archive (EGA) under the identifiers EGAD00010000210 and EGAD00010000211. Overall survival and molecular classification were available.

### **Statistical analysis**

Statistical analyses were performed with the GraphPad Prism 9.1 software. After verifying data for normal distribution and variance, we applied one of the following statistical tests: Mann-Whitney test, Student's t test or one-way ANOVA. p values were determined as statistically significant with \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ . Data are presented as mean  $\pm$  SD or SEM.

<b>Application</b>	<b>Targeted protein</b>	<b>Reference</b>	<b>Producer</b>	<b>Dilution</b>
<b>Western blot</b>	DGAT1	A6857	AB clonal	1/1000
	DGAT2	A13890	AB clonal	
	ELOVL5	HPA047752	Sigma-Aldrich	
	E-cadherin	24E10-3195S	Cell Signaling Technologies	
	Hsc70	sc-7298	Santa Cruz Biotechnology	
	N-cadherin	ab18203	Abcam	
	Occludin	GTX114949	Genetex	
	Phospho ACC	s79-3661P	Cell Signaling Technologies	
	Total ACC	C83B10-3676P	Cell Signaling Technologies	
	p-smad 2/3	D27F4-8828	Cell Signaling Technologies	
	Total smad 2/3	D7G7-8685	Cell Signaling Technologies	
	Vimentin	D21H3-5741	Cell Signaling Technologies	
	SCD1	CDE10-sc58420	Santa Cruz Biotechnology	1/400
	TGF $\beta$ R2	MAB532	R&D Systems	1/500
$\beta$ -actin	sc-47778	Santa Cruz Biotechnology	1/2500	
<b>Immunofluorescence</b>	TGF $\beta$ R1	PA5-38718	Invitrogen	1/250
<b>Flow cytometry</b>	human TGFBR2-PE	W170555	BioLegend	1/100
	IgG control-PE	RTK4530	BioLegend	
	mouse TGFBR2-PE	FAB532P	R&D Systems	
	TGF $\beta$ R1	ABF17-I	EMD Millipore	

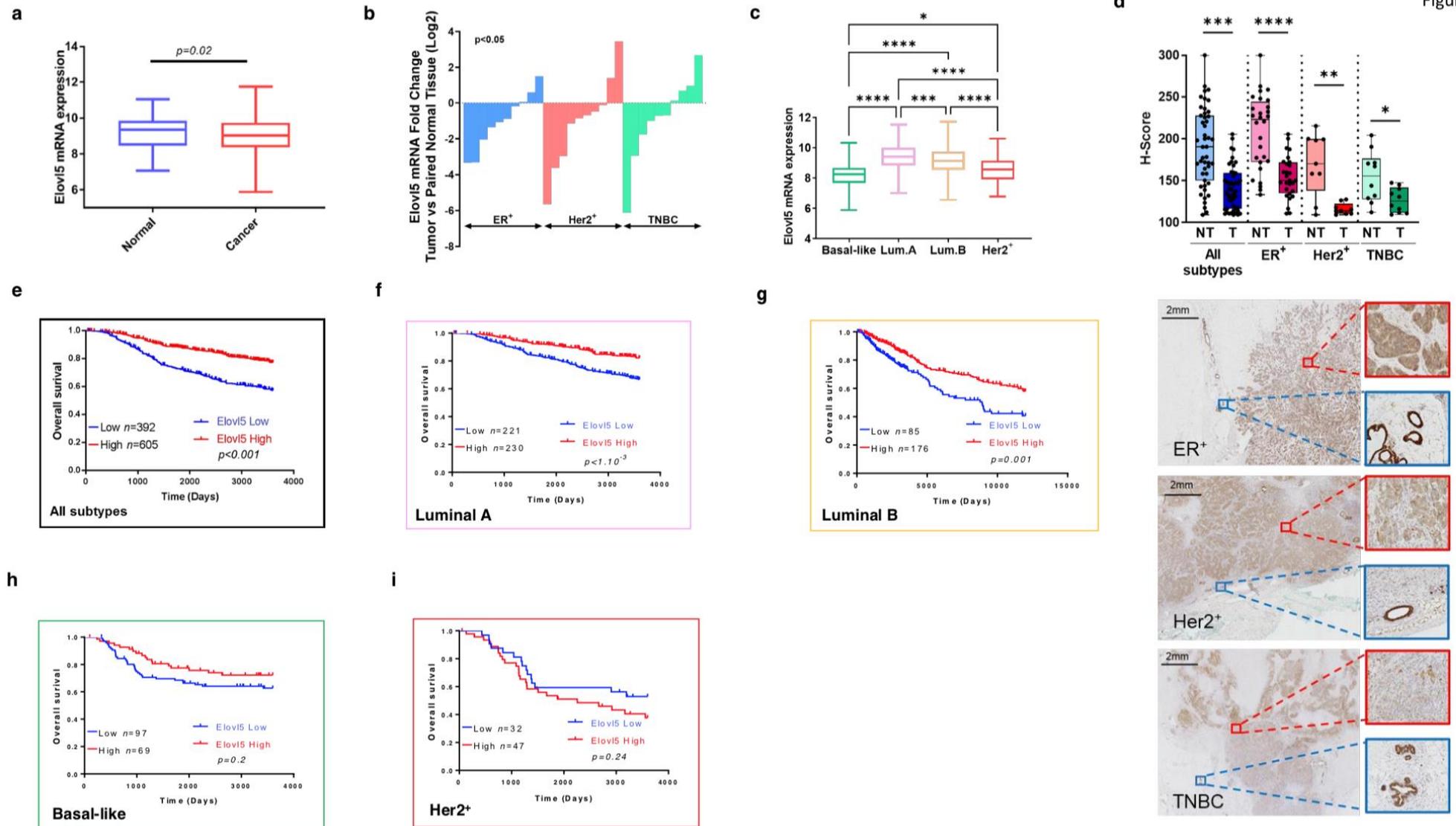
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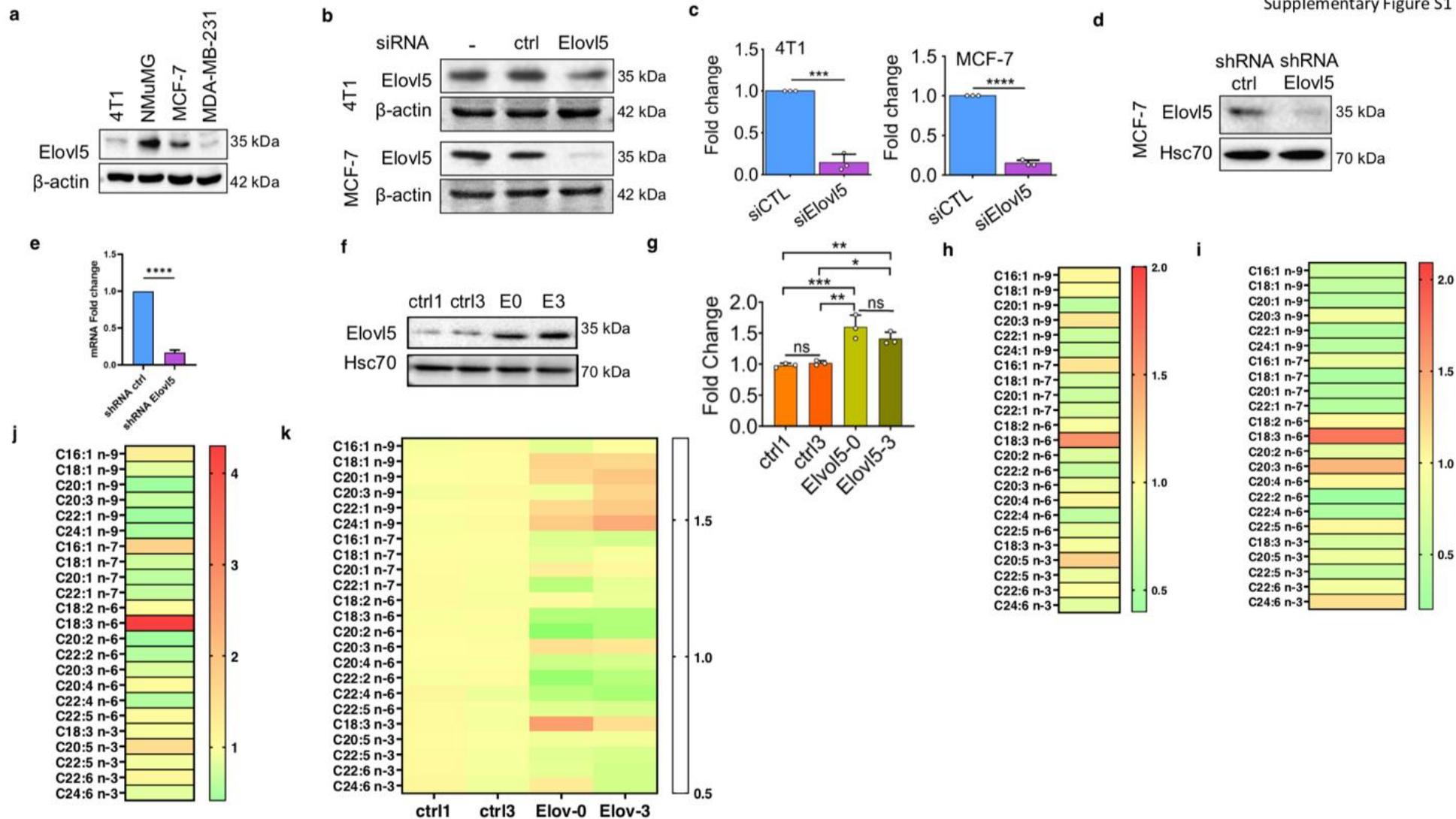
Figure 1



## Figure 1

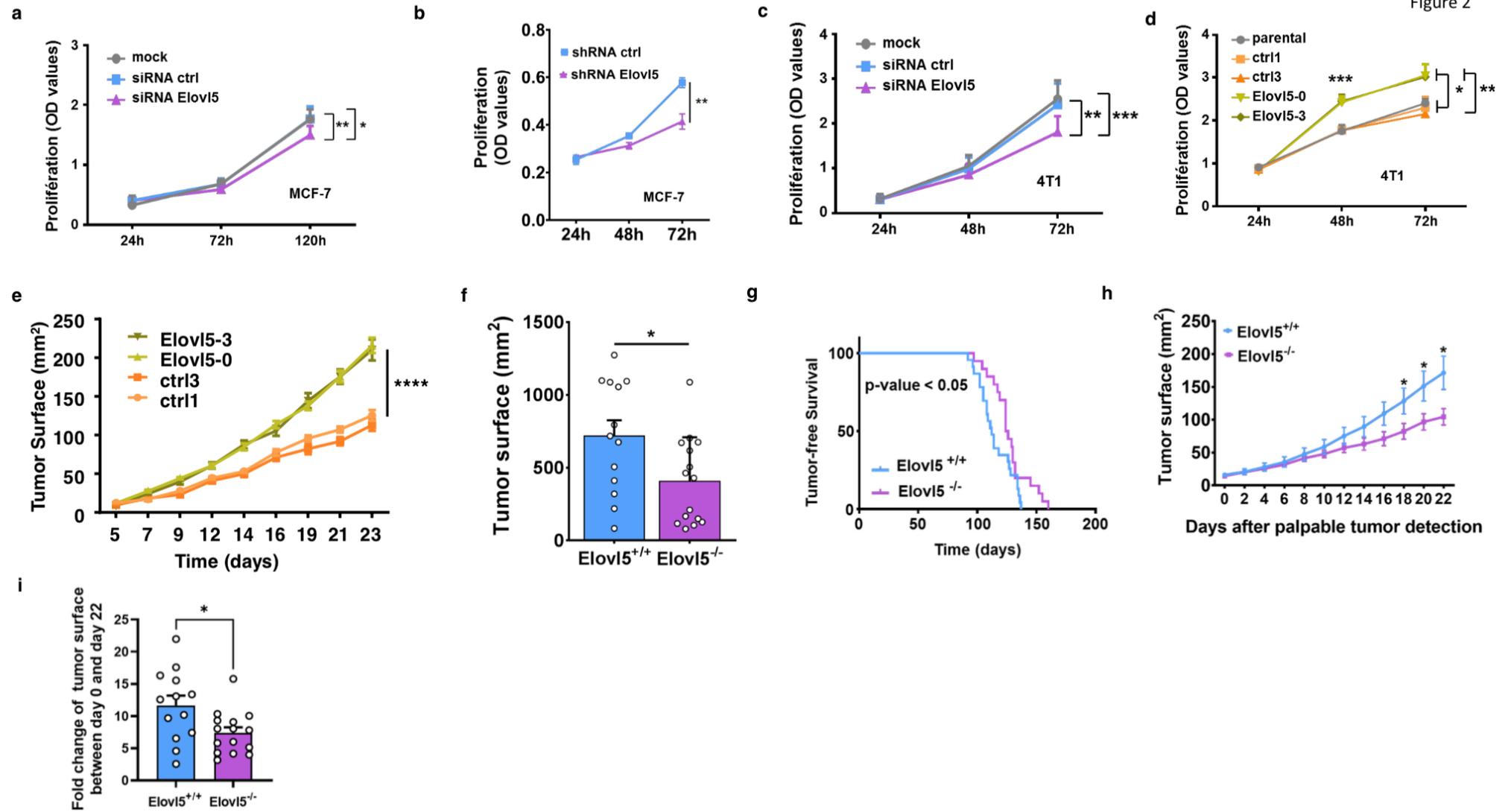
Expression of Elov15 in women breast cancers. a Comparison of Elov15 mRNA expression in normal (n=144) and breast cancer (n=957) tissues in the METABRIC cohort. The p-value obtained using a Wilcoxon rank test is indicated. b Elov15 mRNA expression in tumor sections relative to paired normal tissues are reported as Log2 of fold changes. The p-value was calculated using a Wilcoxon rank test. c Expression of Elov15 mRNA in breast cancer subtypes (Basal-like n=166, Her2<sup>+</sup> n=79, Luminal A n=451 and B n=261) according to the PAM50 classification in the METABRIC dataset. \* p<0.05, \*\*\* p<0.001 and \*\*\*\*p<0.0001 (one-way Anova with Tukey comparison test). d Box and whisker plots showing H-score of Elov15 IHC staining in non-tumoral adjacent (NT) and tumoral (T) breast tissues from patients with different breast cancer subtypes. Median values are indicated with the horizontal line. The whiskers show the lowest and highest values. \*\*p<0.01 and non-significant (ns) were obtained using a Wilcoxon rank test. Representative images are shown for each subtype with breast tumor tissues in red and normal adjacent tissues in blue. e-i Kaplan-Meyer curves for overall survival analysis in the METABRIC cohorts according to low (blue line) and high (red line) expression of Elov15 mRNA. p-value was calculated using the log-rank test.

Supplementary Figure S1



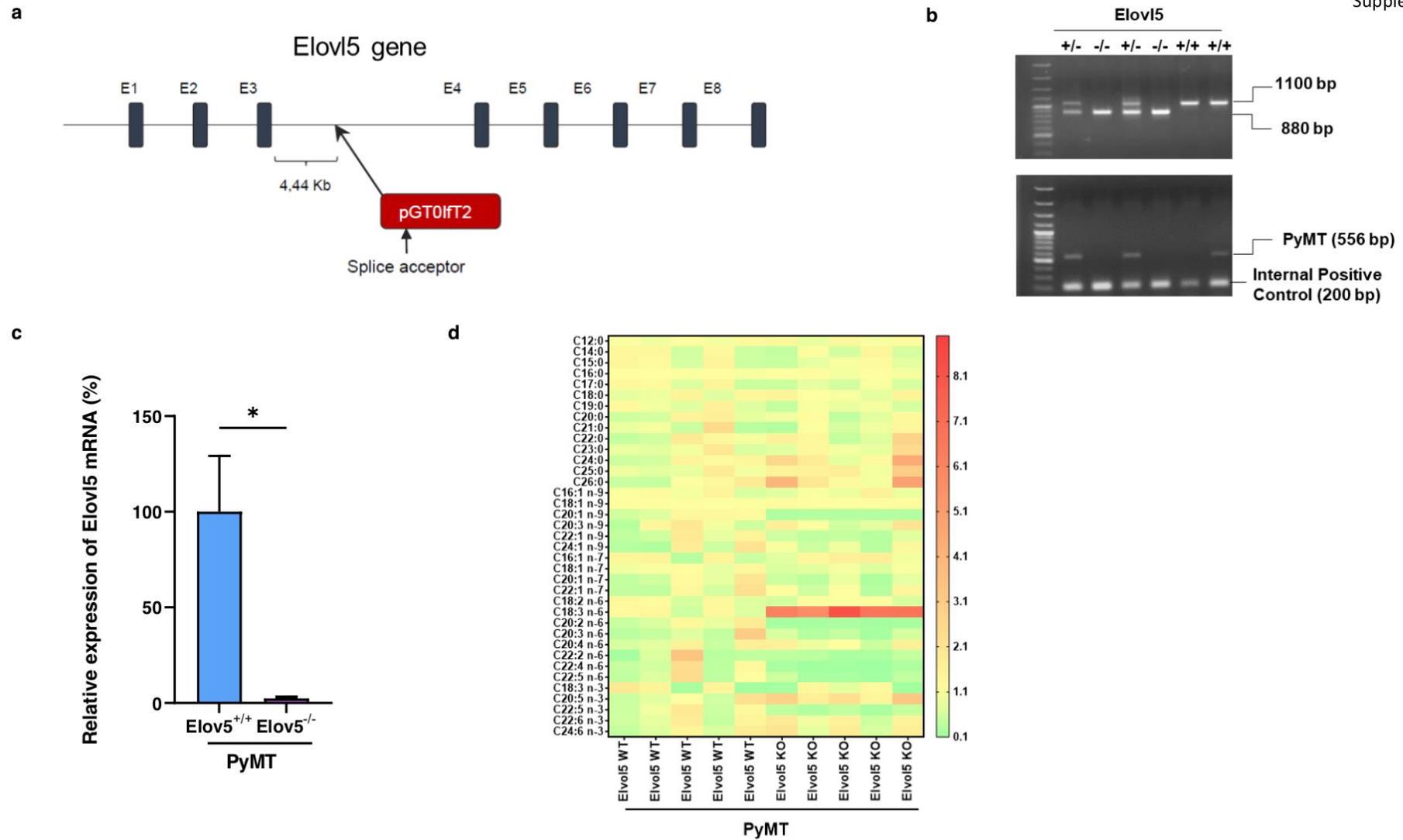
## Supplementary Figure S1.

Validation of breast cancer cell models with modulation of Elovl5 expression. a Elovl5 expression in murine 4T1 breast carcinoma cells, murine NMUMG normal mammary epithelial cells and human (MCF-7 and MDA-MB-231) breast cancer cell lines analyzed by western-blotting. b-c Elovl5 expression 48 hours after transfection with a control (ctrl) or Elovl5-targeting siRNA analyzed by western-blotting (b) and RT-qPCR (c). \*\*\* $p < 0,001$  and \*\*\*\* $p < 0,0001$  (Student's t test). d-e Validation of stable Elovl5 extinction using an Elovl5-targeting shRNA Elovl5 compared to a control shRNA (ctrl) in MCF-7 cells analyzed by western-blotting (d) and RT-qPCR (e). f Analysis by western-blotting of stable overexpression of Elovl5 in 4T1 cells (Elovl5-0 -E0- and Elovl5-3 -E3) compared to control (ctrl1 and ctrl3) 4T1 cells. g Expression of Elovl5 mRNA in Elovl5-overexpressing 4T1 cells (Elovl5-0 and Elovl5-3) and control (ctrl1 and ctrl3) 4T1 cells. \* $p < 0,05$ , \*\* $p < 0,01$ , \*\*\* $p < 0,001$  and non-significant (ns) were calculated using a one-way Anova analysis using Tukey's multiple comparison test. h-k Heatmaps showing the fold change of mono- and polyunsaturated fatty acid content in Elovl5 siRNA-treated MCF-7 cells relative to control siRNA-treated MCF7 cells (h), in Elovl5 shRNA MCF-7 cells relative to control shRNA MCF-7 cells (i), in Elovl5 siRNA-treated 4T1 cells relative to control siRNA-treated 4T1 cells and in Elovl5overexpressing 4T1 cells (Elovl-0 and Elovl-3) relative to the mean of control (ctrl1 and ctrl3) 4T1 cells.



## Figure 2

Cell proliferation and breast tumor growth under the control of Elov15 expression. a-d Proliferation analysis by crystal violet staining. Error bars represent the mean $\pm$ SD of independent experiments (n=5 (a), n=3 (b), n=4 (c) and n=3 (d)) with \* $p$ <0.05, \*\*  $p$ <0.01 and \*\*\* $p$ <0.001 obtained using a one-way Anova analysis with posthoc Tukey test for multiple comparison (a, c and d) and to a Student's t test at each time point for b. e Tumor growth of Elov15-overexpressing 4T1 (Elov15-0 and Elov15-3) and control 4T1 (ctrl1 and ctrl3) cells injected in the 4<sup>th</sup> mammary fat pad of female Balb-c mice (n=10 per group). Statistical significance \*\*\*\* $p$ <0.0001 was calculated for Elov15 and ctrl groups on day 23 using a one-way ANOVA with Tukey's multiple comparison test. f Aggregated tumor surface analysis in MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>) 6-month-old mice. Dots indicate the values of the cumulative surface of mammary tumor lesions for each individual mouse. Histograms and error bars indicate the mean $\pm$ SEM with \* $p$ <0.05 according to a Mann-Whitney test. g Kaplan-Meier curves of tumor-free survival percentage in PyMT-Elov15<sup>+/+</sup> (n=23) and PyMT-Elov15<sup>-/-</sup> (n=20) mice. A log rank (Mantel-Cox) statistical test was used. h Tumor growth analysis in MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>, n=13) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>, n=15) mice. Values used are the cumulative surface of tumors per mice. Curves and error bars represent the mean $\pm$ SEM with \* $p$ <0.05 according to Student's t tests calculated for each time point. i Fold change of aggregated tumor surface on day 22 compared to day 0 (day on which the tumor lesion was first palpable and measured) in MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>, n=23) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>, n=20) mice. Fold change for each individual mouse are indicated by dots, the histogram and error bars show the mean $\pm$ SEM with \* $p$ <0.05 according to Student's t test.



## Supplementary Figure S2.

Validation of the MMTV-PyMT;Elov15 murine model.

a *Elov15* gene targeting strategy. *Elov15*<sup>-/-</sup> mice were obtained from the Mutant Mouse Regional Resource Center at UC Davis (Davis, CA) and were generated by inserting a genetrapp cassette in the exon 3 of the *Elov15* gene. b Genotyping analysis of C57BL/6 mice by PCR. Elov15 primers amplified a 1100 bp DNA fragment for wild-type allele and a 880 bp DNA fragment for the knockout allele. PyMT primers amplified a 556 bp DN fragment in mice with the transgene. c Relative expression of Elov15 mRNA in mammary tumor of MMTV-PyMT;Elov15<sup>+/+</sup> (n=5) and MMTV-PyMT;Elov15<sup>-/-</sup> (n=5) mice. Shown are the mean±SEM of 5 mice per group. \*p<0.05 defined with Student's t test. d Heatmap showing the fold change of fatty acid content in MMTV-PyMT;Elov15 knockout (KO) mice (n=5) relative to MMTV-PyMT;Elov15 wild-type (WT) mice (n=5)

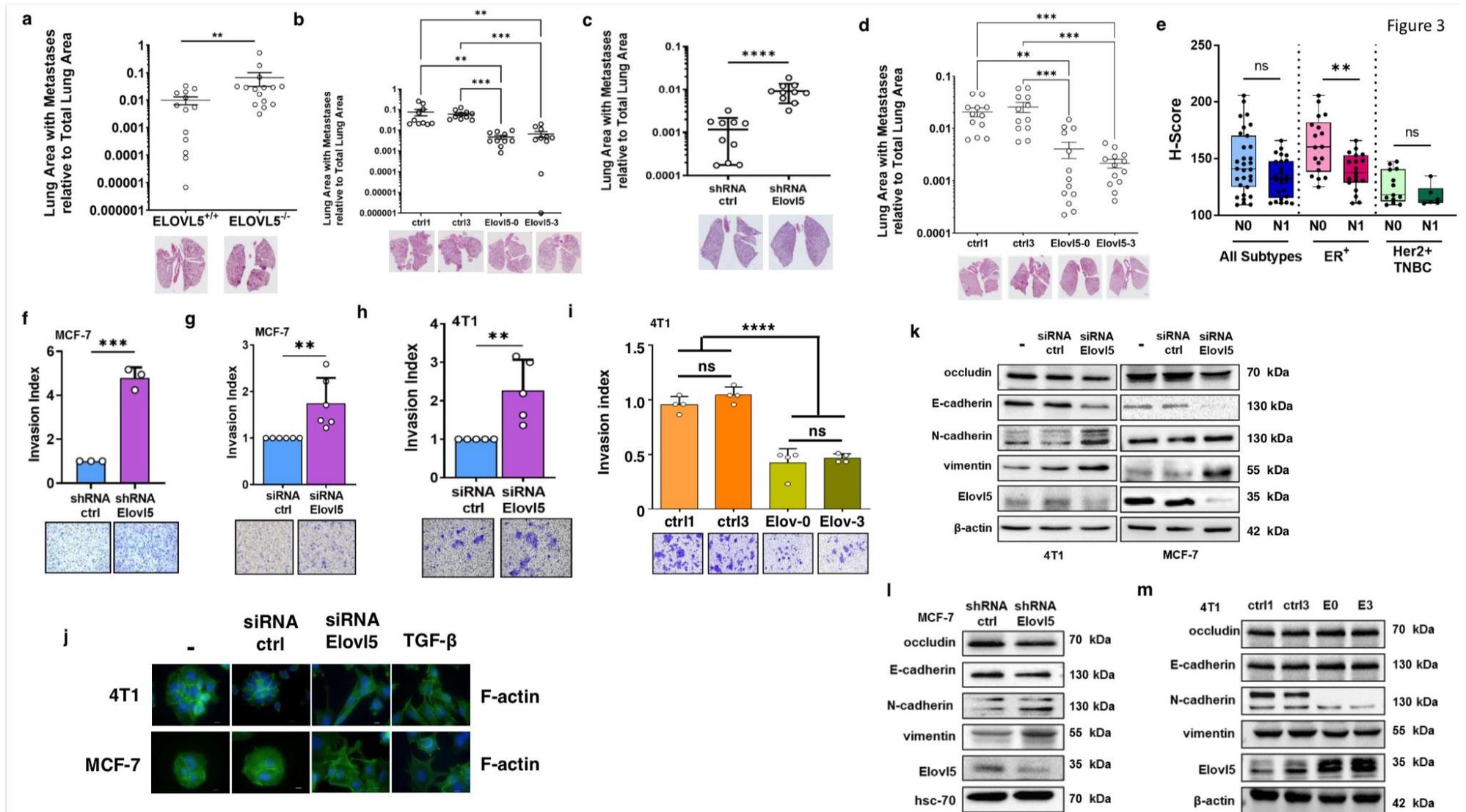
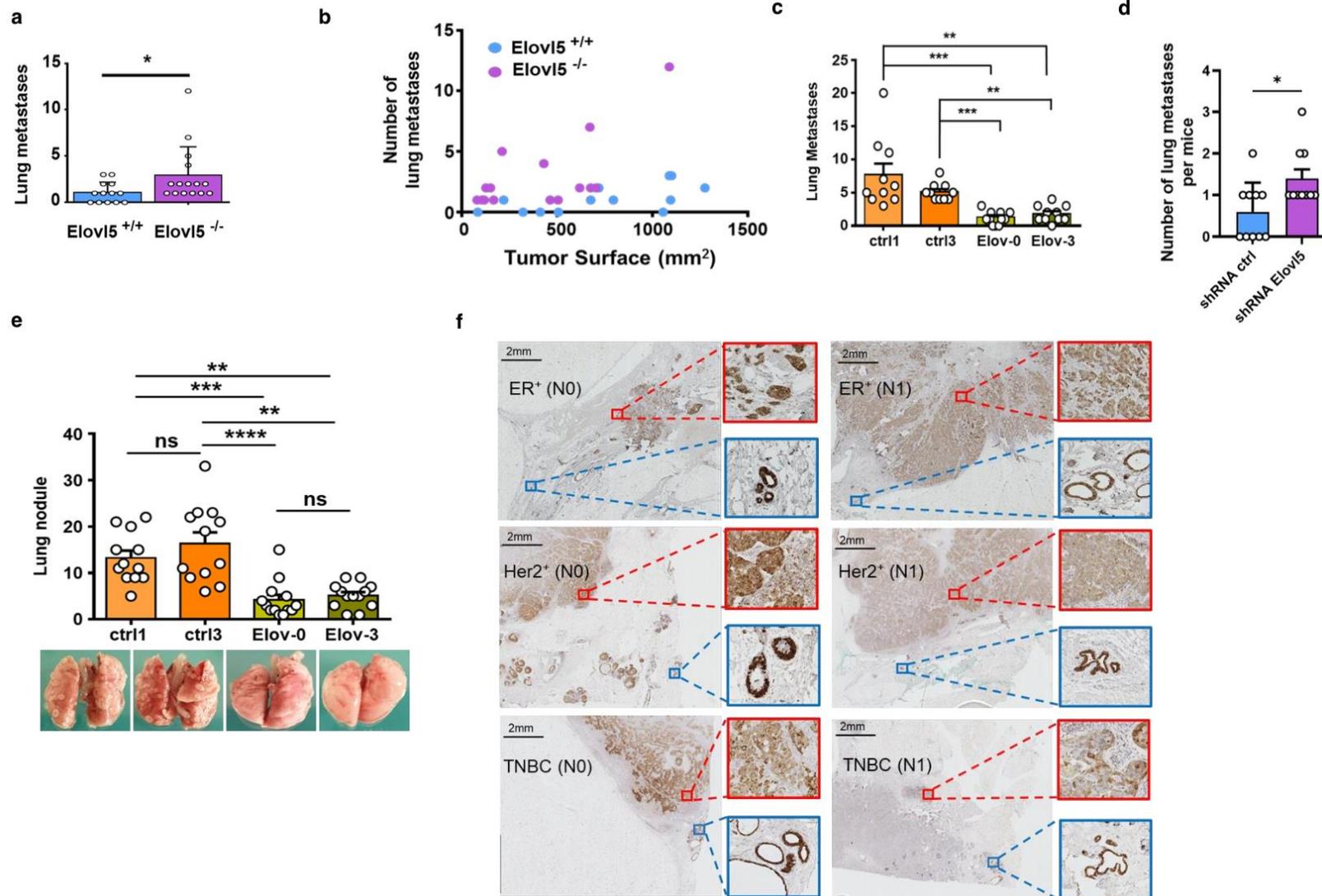


Figure 3

Elov15 regulates metastasis, cell invasion and expression of EMT markers. a-d QuPath analysis for quantification of lung surface with metastases in female MMTV-PyMT ; Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>) and MMTV-PyMT ;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>) 6-month-old mice (a), in lungs of female Ballb-c mice with fat pad injection of Elov15-overexpressing (Elov15-0 and Elov15-3) or control (ctrl1 and ctrl3) 4T1 cells (b), in lungs of female NMRI-nude mice with tail vein injection of shRNA Elov15 or shRNA control (ctrl) MCF-7 (c) and in lungs of female Ballb-c mice with tail vein injection of Elov15-overexpressing (Elov15-0 and Elov15-3) or control (ctrl1 and ctrl3) 4T1 cells (d). For a-d, dots show the values of ratio between lung metastase surface and total lung surface for each individual mouse. Representative images of H&E staining are shown, p-values were determined by Mann-Whitney test (a and c) and by Kuskall-Wallis analysis with Dunn's test (b and d). Data represent the mean±SEM with \*\**p*<0.01, \*\*\**p*<0.001 and \*\*\*\**p*<0.0001. e Box and whisker plots showing H-score for Elov15 IHC staining in breast cancer tissues of different subtypes (ER<sup>+</sup>, Her2<sup>+</sup> and TNBC) from women patients with lymph node invasion (N1) or without invasion (N0). Median values are indicated with the horizontal line. The whiskers show the lowest and highest values. \*\**p*<0.01 and non-significant (ns) were obtained using a Mann-Whitney test. f-g Fold change of cell invasion in Boyden chambers coated with Matrigel for MCF-7 cells with stable (shRNA Elov15) or transient (siRNA Elov15) silencing of Elov15 expression compared to respective control (shRNA ctrl or siRNA ctrl) MCF-7 cells. Invasive MCF-7 cells were stained with crystal violet for quantification. Statistical significance \*\**p*<0.01 and \*\*\**p*<0.001 were calculated using a Student's t test. Histograms and error bars represent the mean±SD. h Cell invasion analysis for Elov15-silenced 4T1 cells (siRNA Elov15) and non-targeting siRNA (siRNA ctrl). Data is represented as in f-g. i Cell invasion index for Elov15-overexpressing (Elov-0 and Elov-3) or control (ctrl1 and ctrl3) 4T1 cells. Data are represented as mean±SD with \*\*\*\**p*<0.0001 and non-significant (ns) according to one-way Anova analysis with Tukey's multiple comparison test. j Fluorescence staining of actin fibers with FITC-phalloidin in 4T1 and MCF-7 cells treated for 48 hours with Elov15

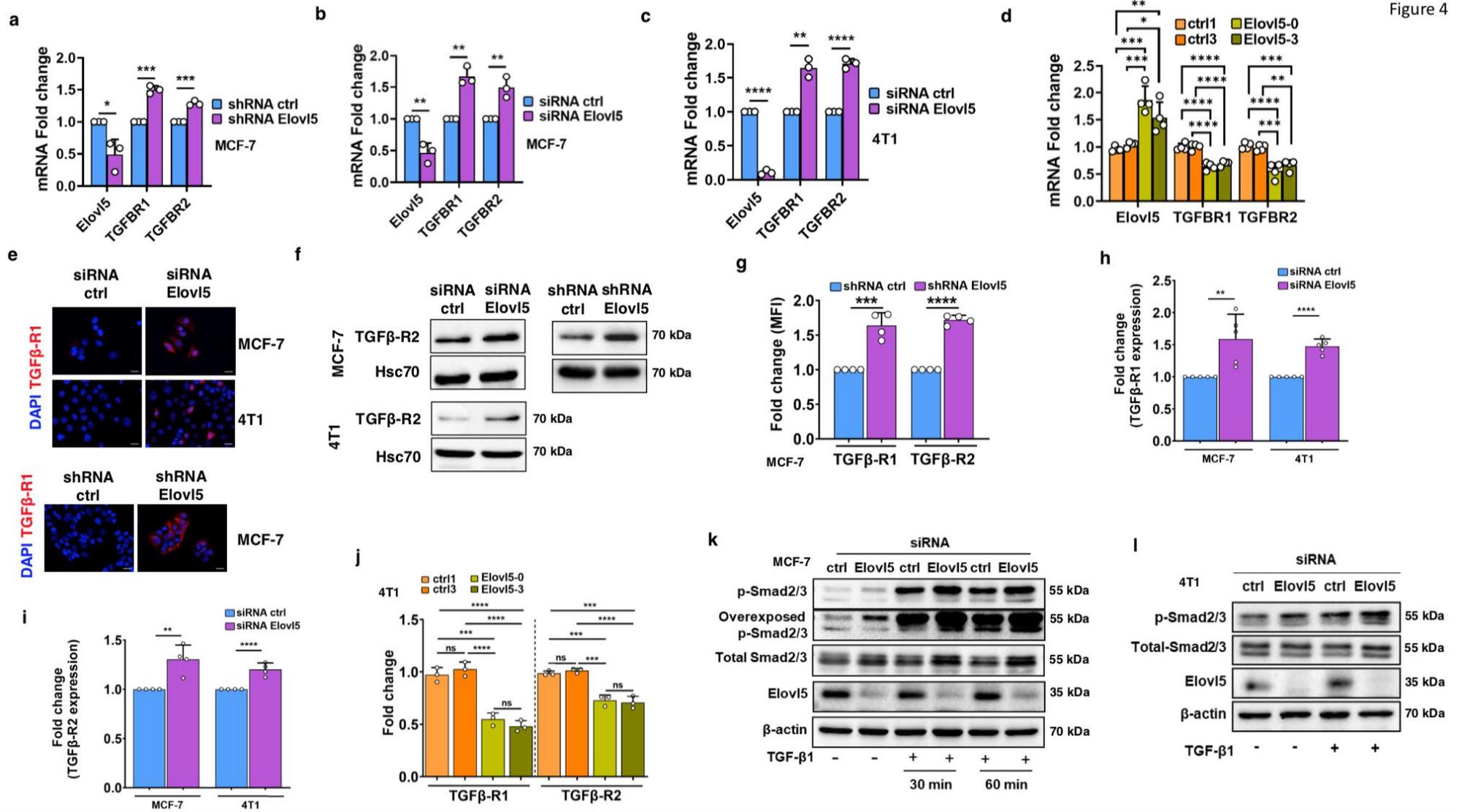
and non-targeting (ctrl) siRNA. Nuclei were counterstained with Hoechst. TGF- $\beta$  treatment is used as a positive control for induction of epithelial to mesenchymal transition. k-m Analysis of EMT markers by western-blotting in Elov15-silenced MCF-7 and Elov15-overexpressing 4T1 cells. Images are representative of at least two independent experiments.



## Supplementary Figure S3.

Development of lung metastases depends on Elov15 expression. a Number of metastases on the lung surface of 6-month-old MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>, n=13) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>, n=15) mice. Shown are the mean±SEM with \**p*<0.05 according to Student's t test. b Graph representing the number of lung metastases and the cumulative surface of the primary mammary tumor for each 6-month-old MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>, n=13) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>, n=15) mice. c Number of metastases on the lung surface of Elov15-overexpressing (Elov1-0 and Elov1-3) and control (ctrl1 and ctrl3) 4T1 cells transplanted in the fourth fat pad of female Balb-c mice. Shown are the mean±SEM with \*\**p*<0,01 and \*\*\**p*<0,001 values determined by a Kuskall-Wallis analysis with Dunn's test. d Number of metastases on the lung surface in female NMRI-nude mice with tail vein injection of Elov15 shRNA and control shRNA-expressing MCF-7 cells. Shown are the mean±SEM with \**p*<0.05 according to a Mann-Withney test. e Number of metastases on the lung surface of female Balb-c mice with tail vein injection of Elov15-overexpressing (Elov1-0 and Elov1-3) and control (ctrl1 and ctrl3) 4T1 cells. Shown are the mean±SEM with \*\**p*<0.01, \*\*\**p*<0,001, \*\*\*\**p*<0,0001 and non-significant (ns) according to a Kuskall-Wallis analysis with Dunn's test. f Representative images of Elov15 IHC staining for H-score analysis. The red insert shows breast tumor tissue and the blue insert shows normal adjacent tissue. Breast cancer subtypes are indicated.

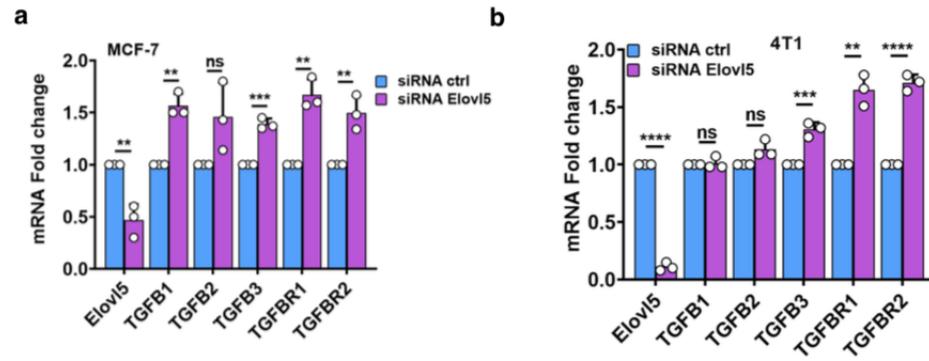
Figure 4



## Figure 4

Elov15 modulates expression of TGF- $\beta$  receptors. a-b Analysis of TGFBR1 and TGFBR2 mRNA expression at 24 hours by RT-qPCR in Elov15-silenced MCF-7 cells by shRNA (a) and siRNA (b) relative to control shRNA or siRNA-treated MCF-7 cells. Histograms and error bars represent the mean $\pm$ SD of three independent experiments with \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 according to Student's t test. c Analysis of TGFBR1 and TGFBR2 mRNA expression at 24 hours by RT-qPCR in Elov15-silenced 4T1 cells using an siRNA (c) relative to non-targeting siRNA-treated 4T1 cells. Histograms and error bars represent the mean $\pm$ SD of three independent experiments with \*\* $p$ <0.01 and \*\*\*\* $p$ <0.0001 according to Student's t test. d Analysis of TGFBR1 and TGFBR2 mRNA expression at 24 hours by RT-qPCR in Elov15-overexpressing 4T1 cells (Elov15-0 and Elov15-3) relative to the mean of control 4T1 cells (ctrl1 and ctrl3). Histograms and error bars represent the mean $\pm$ SD of five independent experiments with \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 according to one-way Anova analysis with Tukey's multiple comparison test. e Immunofluorescence staining of TGF- $\beta$  receptor 1 (TGF $\beta$ -R1) in Elov15-silenced MCF-7 and 4T1 cells compared to a control siRNA or shRNA at 48 hours. Nuclei are staining with Hoechst. Images are representative of three independent experiments. f Analysis of TGF- $\beta$  receptor 2 (TGF $\beta$ -R2) in Elov15-silenced MCF-7 and 4T1 cells (siRNA Elov15) compared to control siRNA or shRNA (ctrl) at 48 hours by western-blotting. Images are representative of three independent experiments. g Analysis of TGF- $\beta$  receptor 1 and 2 expression at 48 hours by flow cytometry in Elov15-targeting shRNA and control shRNA-treated MCF-7 cells. Values are the fold change in mean of fluorescence intensity (MFI) of shRNA Elov15 cells relative to shRNA control cells. Histograms and error bars represent the mean $\pm$ SD of four independent experiments with \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 according to Student's t test. h-i Analysis of TGF- $\beta$  receptor 1 and 2 expression at 48 hours by flow cytometry as in (g) in Elov15-depleted MCF-7 and 4T1 cells treated using an siRNA. Histograms and error bars represent the mean $\pm$ SD of at least four independent experiments with \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 according to

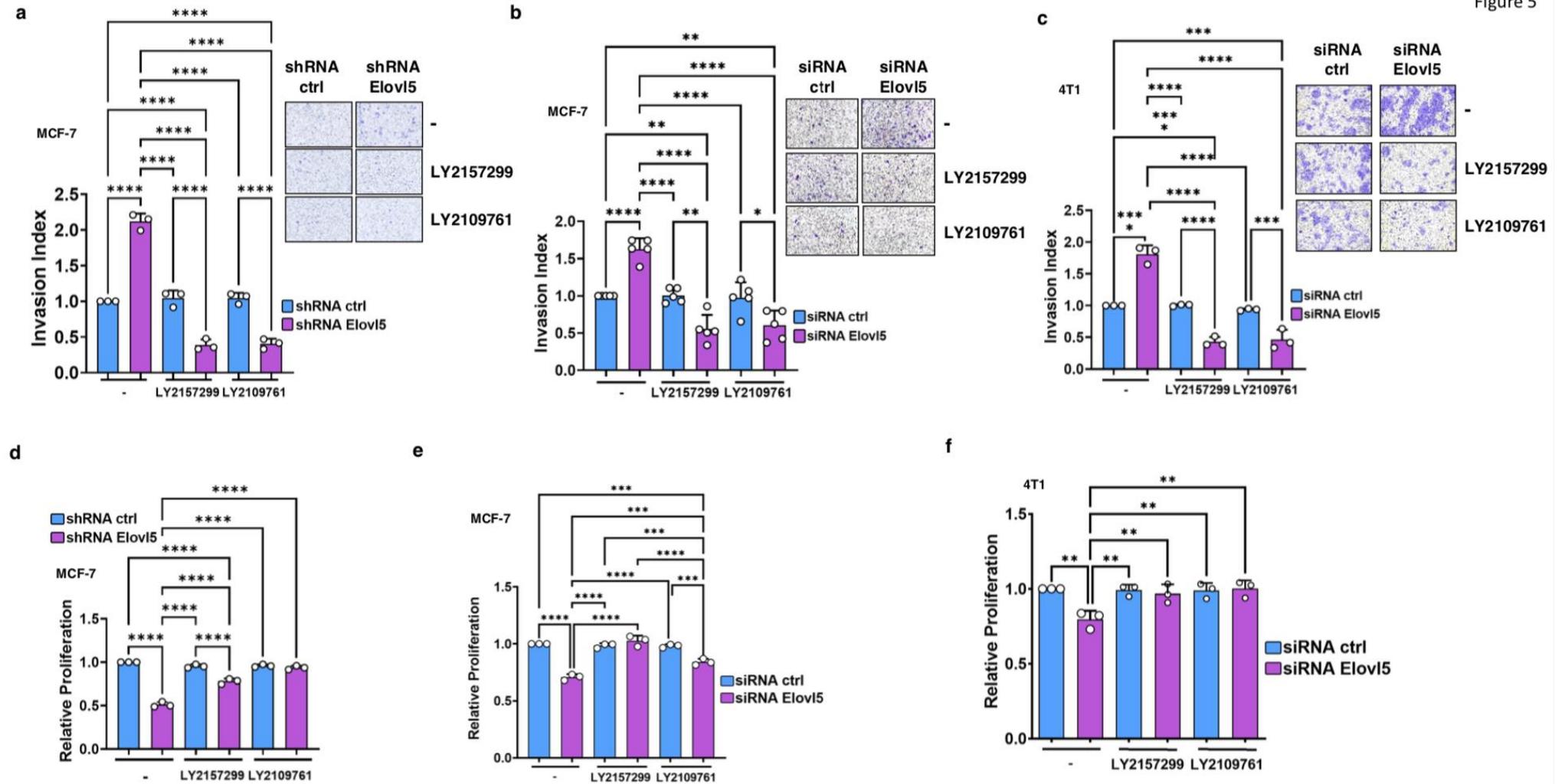
Student's t test. j Analysis of TGF- $\beta$  receptor 1 and 2 expression at 48 hours by flow cytometry as (g) in Elov15-overexpressing 4T1 cells (Elov15-0 and Elov15-3) relative to the mean of control 4T1 cells (ctrl1 and ctrl3). Histograms and error bars represent the mean $\pm$ SD of three independent experiments with \*\*\*\*p<0.001, \*\*\*\*p<0.0001 and non-significant (ns) according to one-way Anova analysis with Tukey's multiple comparison test. k Analysis of Smad2/3 phosphorylation (p-Smad2/3) and total Smad2/3 expression in Elov15-silenced MCF-7 cells treated with TGF- $\beta$ 1 (5 ng/ml) for the indicated times by western-blotting. l Analysis of Smad2/3 phosphorylation (pSmad2/3) and total Smad2/3 expression in Elov15-silenced 4T1 cells treated with TGF- $\beta$ 1 (5 ng/ml) for 30 minutes by western-blotting.



## Supplementary Figure S4

Regulation of the TGF- $\beta$  pathway by Elov15. a-b Analysis of TGFB1, TGFB2, TGFB3, TGFBR1 and TGFBR2 mRNA expression at 24 hours by RT-qPCR in Elov15-silenced MCF-7 and 4T1 cells using an Elov15 siRNA relative to a control siRNA. Shown are the mean $\pm$ SD of three independent experiments with non-significant (ns), \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 according to Student's t test.

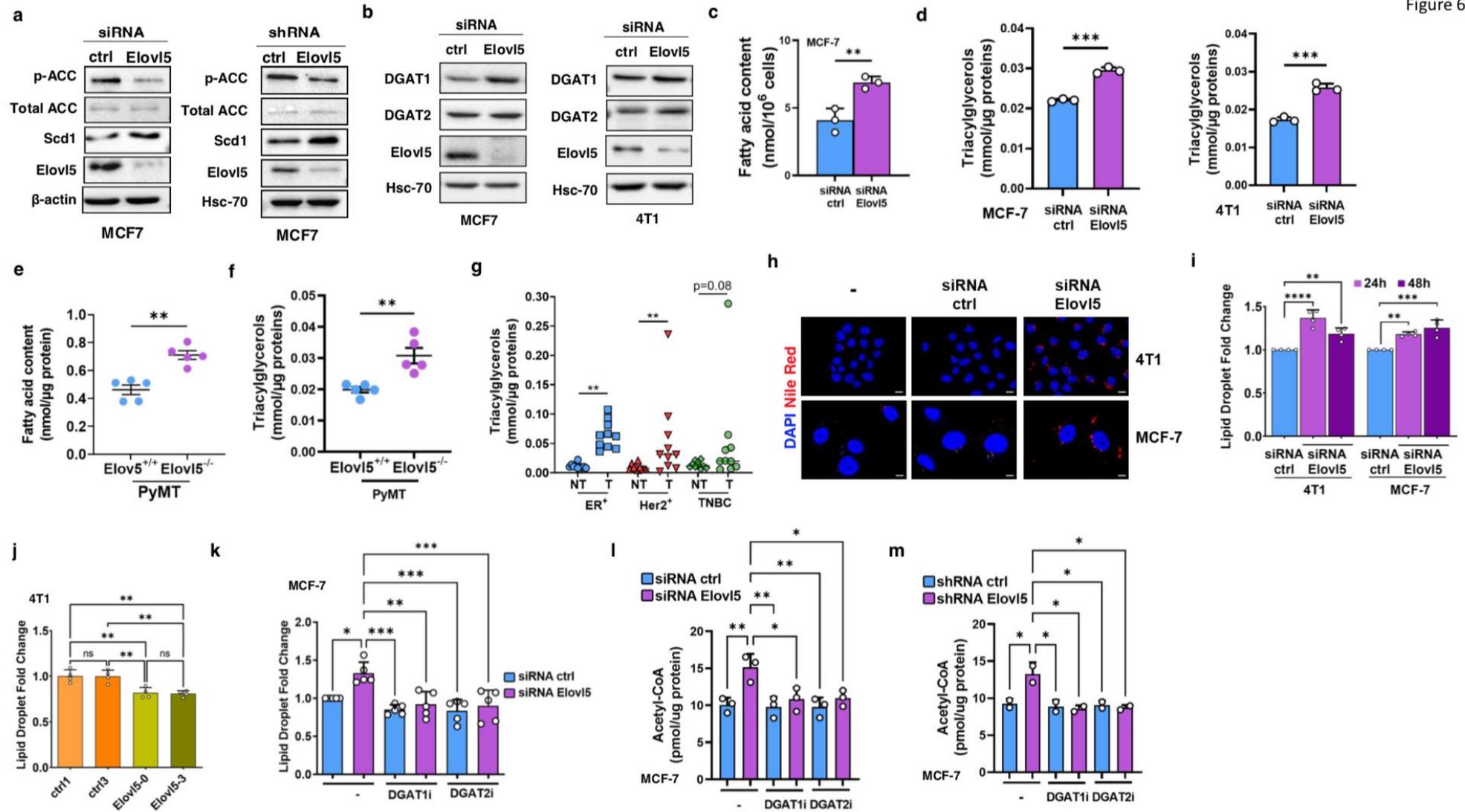
Figure 5



## Figure 5

TGF- $\beta$  receptors drive Elov15-dependent cell invasion and proliferation. a-b Analysis of cell invasion through a Matrigel-coated membrane for Elov15-depleted MCF-7 cells using shRNA (a) or transient siRNA (b) against Elov15 relative to MCF-7 cells treated with a control shRNA or siRNA (ctrl). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  were determined by One-way Anova analysis with Tukey's test. Histograms and error bars represent the mean $\pm$ SD of three independent experiments. Representative images are shown. c Analysis of cell invasion through a Matrigel-coated membrane for Elov15-depleted 4T1 cells with transient siRNA against Elov15 relative to 4T1 treated with a control siRNA (ctrl). \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$  were determined by one-way Anova analysis with Tukey's test. Histograms and error bars represent the mean $\pm$ SD of three independent experiments. Representative images are shown. d-e Analysis of proliferation by crystal violet staining in Elov15-depleted MCF-7 cells using an shRNA (a) or siRNA (b) against Elov15 relative to MCF-7 cells treated with a control shRNA or siRNA (ctrl). \*\*\* $p < 0.01$  and \*\*\*\* $p < 0.0001$  were determined by one-way Anova analysis with Tukey's test. Histograms and error bars are expressed as the mean $\pm$ SD of three independent experiments. f Analysis of proliferation by crystal violet staining in Elov15-depleted 4T1 cells using an siRNA against Elov15 relative to 4T1 cells treated with a control siRNA (ctrl). \*\* $p < 0.01$  was determined by One-way Anova analysis with Tukey's test. Histograms and error bars represent the mean $\pm$ SD of three independent experiments.

Figure 6

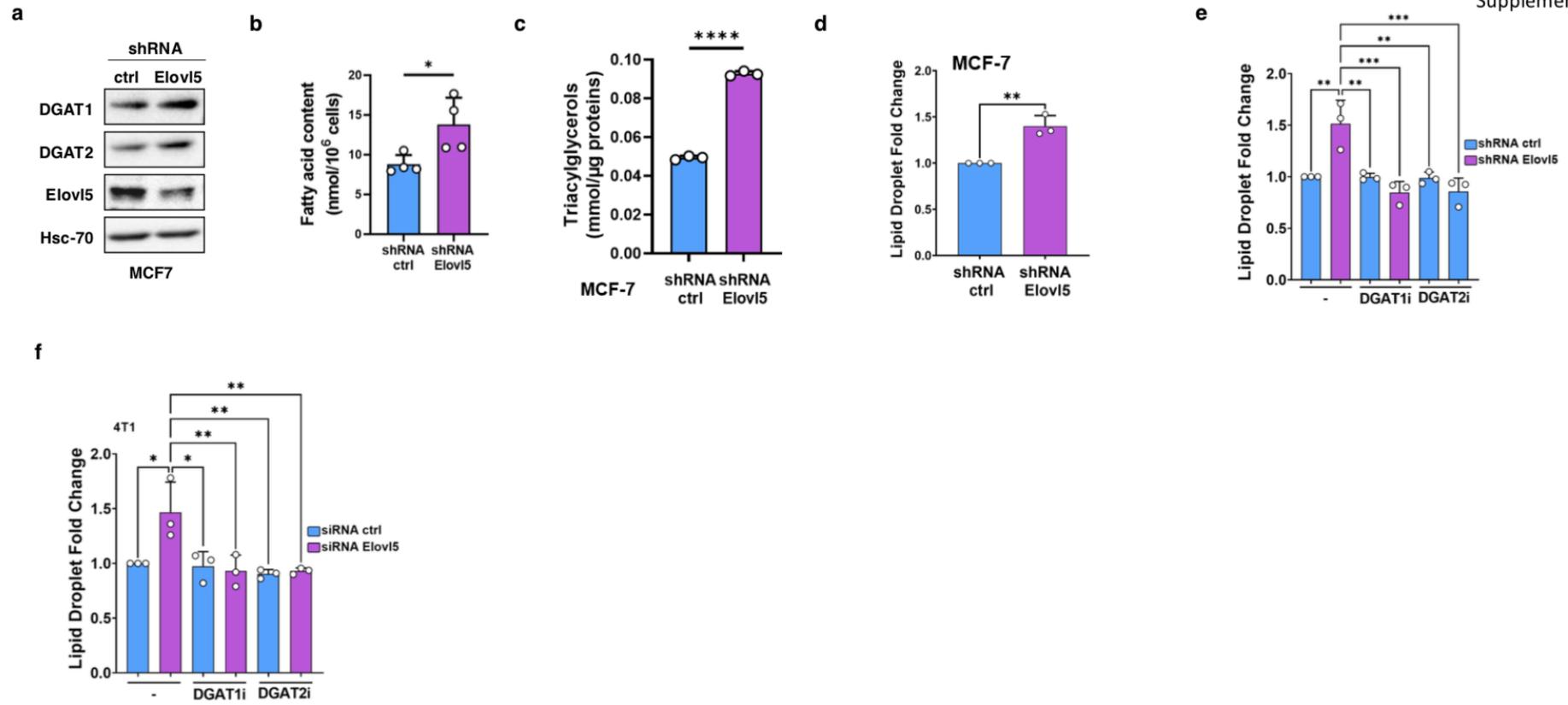


## Figure 6

Elov15 downregulation increases lipid droplet abundance. a Expression of lipogenic enzymes analyzed by western-blotting in Elov15-silenced MCF-7 cells. b Expression of DGAT1 and DGAT2 enzymes analyzed by western-blotting in MCF-7 and 4T1 cells transfected with a control or Elov15-targeting siRNA. c Determination of total fatty acid content by GC-MS in MCF-7 cells treated with a control or Elov15-targeting siRNA. Data are expressed as the mean $\pm$ SD of three independent experiments. \*\* $p$ <0.01 was determined by a Student's t test. d Analysis of triacylglycerol content by enzymatic assay in MCF-7 cells treated with siRNA targeting Elov15 or a control siRNA. Data are expressed as the mean $\pm$ SD of three independent experiments. \*\*\* $p$ <0.001 was determined by a Student's t test. e-f Determination of total fatty acid content by GC-MS (e) and triacylglycerol content by enzymatic assay (f) in tumors from MMTV-PyMT;Elov15<sup>+/+</sup> (Elov15<sup>+/+</sup>; n=5) and MMTV-PyMT;Elov15<sup>-/-</sup> (Elov15<sup>-/-</sup>; n=5). Data are expressed as the mean $\pm$ SEM. \*\* $p$ <0.01 was determined by Student's t test. g Analysis of triacylglycerol content in breast cancer subtypes (T) and paired non-tumoral breast (NT) tissues. \*\* $p$ <0.01 was determined according to Wilcoxon test. h Lipid droplet staining with Nile red in 4T1 and MCF-7 cells transfected with siRNA targeting Elov15 or a control siRNA (ctrl). Scale bar (10  $\mu$ m). i Lipid droplet quantification by flow cytometry after Nile red staining in Elov15-depleted MCF-7 and 4T1 cells using a siRNA. Histograms and error bars represent the mean $\pm$ SD of four independent experiments with \*\* $p$ <0.01, \*\*\* $p$ <0.001 and \*\*\*\* $p$ <0.0001 according to Student's t test. j Lipid droplet quantification by flow cytometry after Nile red staining in Elov15-overexpressing 4T1 cells (Elov15-0 and Elov15-3) relative to the mean of non-targeting control 4T1 cells (ctrl1 and ctrl3). Histograms and error bars represent the mean $\pm$ SD of three independent experiments with \*\* $p$ <0.01 and non-significant (ns) according to One-way Anova analysis with Tukey's multiple comparison test. k Fold change of lipid droplet content in Nile red stained-MCF-7 cells transfected with an siRNA targeting Elov15 or a control siRNA and treated with DGAT1 and DGAT2 inhibitors. Histograms and error bars represent the mean $\pm$ SD of five independent experiments with \* $p$ <0.05, \*\* $p$ <0.01 and

\*\*\* $p < 0.001$  according to one-way Anova analysis with Tukey's multiple comparison test. l-m Quantification of acetyl-CoA in Elov15-silenced MCF-7 cells using an siRNA (l) or shRNA (m) and treated with DGAT1 and DGAT2 inhibitors. Histograms and error bars represent the mean $\pm$ SD of five independent experiments with \* $p < 0.05$  and \*\* $p < 0.01$  according to one-way Anova analysis with Tukey's multiple comparison test.

Supplementary Figure S5



## Supplementary Figure S5

Elovl5 downregulation increases lipid droplet abundance. a Expression of DGAT1 and DGAT2 enzymes analyzed by western-blotting in MCF-7 cells with stable Elovl5 downregulation. b-c Determination of total fatty acid content by GC-MS and triacylglycerol concentration in MCF-7 cells with stable Elovl5 depletion. Data are expressed as the mean $\pm$ SD of three independent experiments. \* $p$ <0.05 and \*\*\*\* $p$ <0.0001 were determined by Student's t test. d Lipid droplet quantification by flow cytometry after Nile red staining in MCF-7 cells with stable Elovl5 depletion. Data represent the mean $\pm$ SD of four independent experiments with \*\* $p$ <0.01 according to Student's t test. e-f Lipid droplet quantification by flow cytometry after Nile red staining in Elovl5-silenced MCF-7 and 4T1 cells treated with DGAT1 and DGAT2 inhibitors. Shown are the mean $\pm$ SD of three independent experiments with \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001 according to One-way Anova analysis with Tukey's multiple comparison test.

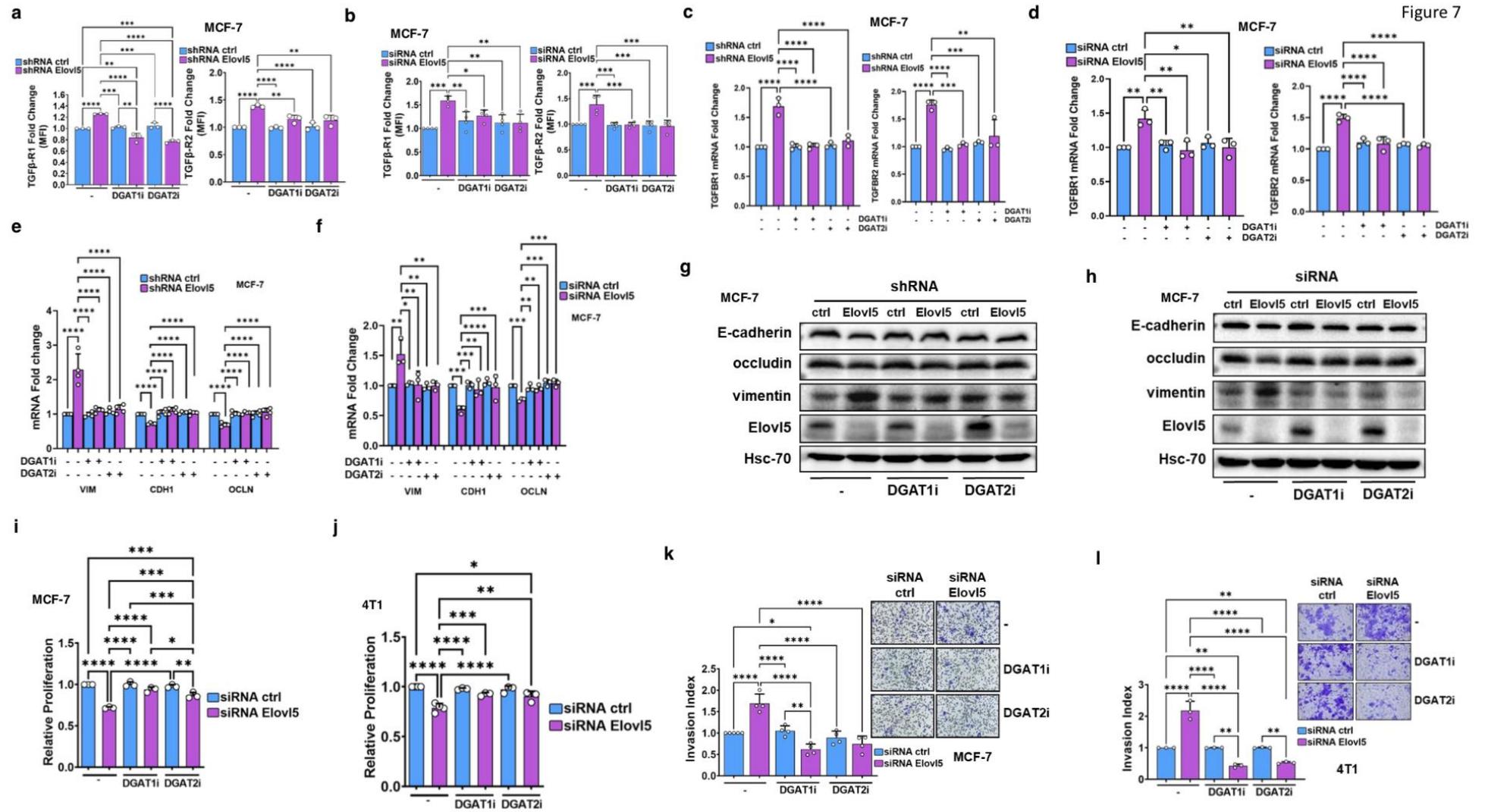
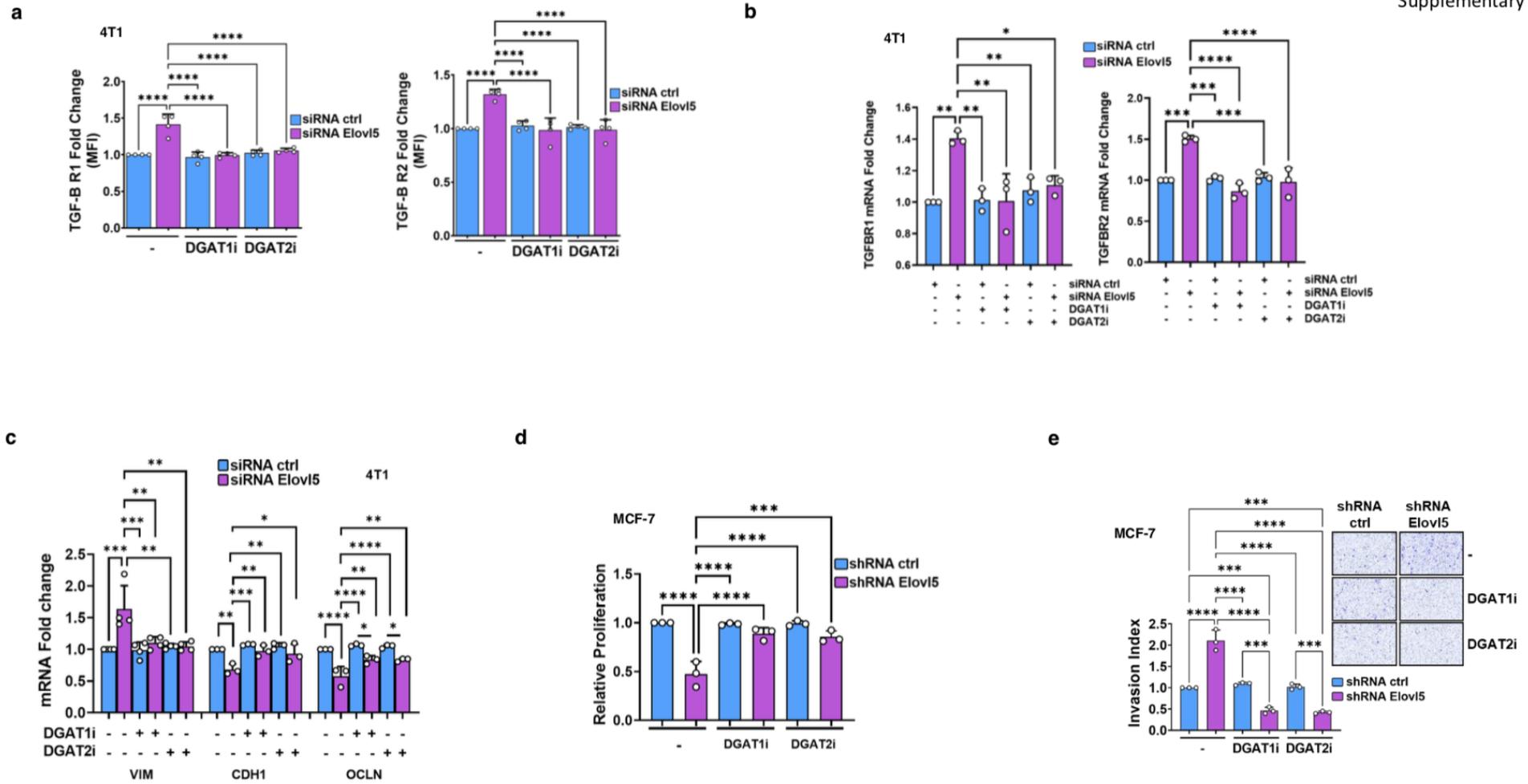


Figure 7

## Figure 7

Lipid droplets mediate the dependent expression of TGF- $\beta$  receptors and metastatic processes. a-b Analysis of TGF $\beta$ -1 and 2 receptor expression in MCF-7 cells by flow cytometry. MCF-7 cells with stable shRNA or siRNA silencing of Elov15 expression were treated for 48 hours with DGAT1 and 2 inhibitors and collected for anti-TGF $\beta$ -R1 and TGF $\beta$ -R2 staining. Values are fold change of mean fluorescence intensities (MFI) relative to MCF-7 cells treated with vehicle (DMSO) and control shRNA or siRNA (ctrl). Histograms and error bars are expressed as the mean $\pm$ SD of at least three independent experiments. \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. c-d Analysis of TGF $\beta$ -1 and 2 receptor mRNA expression in MCF-7 cells by RT-qPCR. MCF-7 cells with stable shRNA or transient siRNA silencing of Elov15 expression were treated for 48 hours with DGAT1 and 2 inhibitors and collected for RNA purification. Values used are fold changes of Elov15 mRNA expression relative to MCF-7 cells treated with vehicle (DMSO) and control shRNA or siRNA (ctrl). Histograms and error bars are expressed as the mean $\pm$ SD of three independent experiments. \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. e-f Expression of EMT marker mRNA (VIM, vimentin; CDH1, cadherin-1 and OCLN, occludin) analyzed by RT-qPCR in Elov15-silenced MCF-7 cells treated with vehicle (DMSO) or DGAT inhibitors (DGAT1i and DGAT2i) for 48 hours. Data are expressed as the mean $\pm$ SD of three independent experiments. \*p<0,05, \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. g-h Expression of EMT markers analyzed by western-blotting in Elov15-depleted MCF-7 cells using an shRNA (g) or siRNA (h) treated with vehicle (DMSO) or DGAT inhibitors (DGAT1i and DGAT2i) for 48 hours. Images are representative of two independent experiments. i-j Analysis of relative cell proliferation by crystal violet in MCF-7 (i) and 4T1 (j) cells treated with an siRNA and DGAT1 and 2 inhibitors. \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. Represented are the mean $\pm$ SD of three independent experiments. l Analysis of cell invasion through a Matrigel-coated

membrane for Elov15-depleted MCF-7 (k) and 4T1 (l) breast cancer cells silenced with an siRNA and treated with DGAT inhibitors (DGAT1i and DGAT2i) relative to the respective breast cancer cells treated with a control siRNA (ctrl) and vehicle (DMSO). \* $p < 0,5$ , \*\* $p < 0,01$ , \*\*\* $p < 0,001$  and \*\*\*\* $p < 0,0001$  were determined by one-way Anova analysis with Tukey's test. Represented the mean $\pm$ SD of three independent experiments. Representative images are shown.



## Supplementary Figure S6

Elovl5 dependent-LD accumulation controlled proliferation and invasion through expression of TGF- $\beta$  receptors. a Analysis of TGF $\beta$ -1 and 2 receptor expression in 4T1 cells by flow cytometry 4T1 cells with transient siRNA silencing of Elovl5 expression were treated for 48 hours with DGAT1 and 2 inhibitors and collected for anti-TGF $\beta$ -R1 and TGF $\beta$ -R2 staining. Values used are the fold change of mean of fluorescence intensity (MFI) relative to 4T1 cells treated with vehicle (DMSO) and a control shRNA or siRNA (ctrl). Data are expressed as the mean $\pm$ SD of three independent experiments. \*\*\*\*p<0,0001 was determined by One-way Anova analysis with Tukey's test. b Analysis of TGF $\beta$ -1 and 2 receptor mRNA expression in 4T1 cells by RT-qPCR. 4T1 cells transfected with siRNA were treated for 48 hours with DGAT1 and 2 inhibitors. Values are the fold change of Elovl5 mRNA expression relative to 4T1 cells treated with vehicle (DMSO) and a control siRNA (ctrl). Data are expressed as the mean $\pm$ SD of three independent experiments. \*p<0,05, \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. c Expression of EMT marker mRNA (VIM, vimentin; CDH1, cadherin-1 and OCLN, occludin) analyzed by RT-qPCR in Elovl5-silenced 4T1 cells treated with vehicle (DMSO) or DGAT inhibitors (DGAT1i and DGAT2i) for 48 hours. Data are expressed as the mean $\pm$ SD of at least three independent experiments. \*p<0,05, \*\*p<0,01, \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. d Analysis of relative cell proliferation by crystal violet staining in MCF-7 cells with stable Elovl5 downregulation treated for 72 hours with DGAT1 and 2 inhibitors. \*\*\*p<0,001 and \*\*\*\*p<0,0001 were determined by one-way Anova analysis with Tukey's test. Data are shown as the mean $\pm$ SD of three independent experiments. e Analysis of cell invasion through a Matrigel-coated membrane of Elovl5-depleted breast cancer cells silenced using an shRNA in MCF-7 cells and treated with DGAT inhibitors (DGAT1i and DGAT2i) relative to the same breast cancer cells treated with a control shRNA

(ctrl) and vehicle (DMSO). \*\*\* $p < 0,001$  and \*\*\*\* $p < 0,0001$  were determined by One-way Anova analysis with Tukey's test. Histograms and error bars are the mean $\pm$ SD of three independent experiments. Representative images are shown.

## Supplementary information

Supplementary Table 1

<b>Categories</b>	<b>Classification</b>	<b>Number of patients</b>
<b>Gender</b>	Male	0
	Female	30
<b>Age</b>	30-50	1
	51-70	15
	> 71	14
<b>Subtypes</b>	TNBC	10
	ER+/ HER2-	10
	ER-/HER2+	10
<b>Treatments</b>	Conservative surgery	30
	Radiotherapy	23
	Chemotherapy	23
	Immonotherapy	10
	Hormonotherapy	8
<b>TNM stage</b>	I	7
	II	10
	III	13
	IV	0
<b>Lymph node invasion</b>	0 (Negative)	16
	1 (Positive)	14
<b>Survival</b>	Alive	29
	Death	1

Supplementary table 2

<b>Categories</b>	<b>Classification</b>	<b>Number of patients</b>
<b>Gender</b>	Male	0
	Female	47
<b>Age</b>	30-50	8
	51-70	21
	> 71	18
<b>Subtypes</b>	TNBC	10
	ER+/ HER2-	28
	ER-/HER2+	9
<b>Treatments</b>	Conservative surgery	47
	Radiotherapy	41
	Chemotherapy	26
	Immunotherapy	2
	Hormonotherapy	31
<b>TNM stage</b>	I	20
	II	19
	III	8
	IV	0
<b>Lymph node invasion</b>	0 (Negative)	24
	1 (Positive)	23
<b>Survival</b>	Alive	46
	Death	1

Supplementary Table 3

<b>Categories</b>	<b>Classification</b>	<b>Number of patients</b>
<b>Gender</b>	Male	0
	Female	39
<b>Age</b>	30-50	7
	51-70	17
	> 71	15
<b>Subtypes</b>	TNBC	2
	ER+/ HER2-	28
	ER-/HER2+	9
<b>Treatments</b>	Conservative surgery	39
	Radiotherapy	35
	Chemotherapy	21
	Immunotherapy	1
	Hormonotherapy	31
<b>TNM stage</b>	I	17
	II	19
	III	3
	IV	0
<b>Lymph node invasion</b>	0 (Negative)	19
	1 (Positive)	20
<b>Survival</b>	Alive	38
	Death	1

Supplementary Table 4

Species	Gene name	Forward (5'-3')	Reverse (5'-3')
<b>human</b>	Actine	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
	ELOVL5	TGAGGCAGTGGTCAAACAGGT	AGATATGTCATGAGTGGTTCCAAGA
	Vimentin	GCAAAGATTCCACTTTGCGT	GAAATTGCAGGAGGAGATGC
	CDH2	TGTTTGA CTATGAAGGCAGTGG	TCAGTCATCACCTCCACCAT
	CDH1	GAAAGCGGCTGATACTGACC	CGTACATGTCAGCCGCTTC
	18s	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
	Occludin	TTTGTGGGACAAGGAACACA	TCATTCACTTTGCCATTGGA
	TGFβ1	GAGCCTGAGGCCGACTACTA	TCGGAGCTCTGATGTGTTGA
	TGFβ2	AAGAAGCGTGCTTTGGATGCGG	ATGCTCCAGCACAGAAGTTGGC
	TGFβ3	ACTTGCACCACCTTGGACTTC	GGTCATCACCGTTGGCTCA
	TGFβ1R	GCAGAGCTGTGAAGCCTTGAGA	TGCCTTCCTGTTGACTGAGTT
	TGFβ2R	ATGACATCTCGCTGTAATGC	GGATGCCCTGGTGGTTGA
<b>mouse</b>	Actine	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT
	Ncadherin	ATGTGCCGGATAGCGGGAGC	TACACCGTGCCGTCCTCGTC
	Vimentin	CTTGAACGGAAAGTGGAATCCT	GTCAGGCTTGAAACGTCC
	Ecadherin	AACCCAAGCACGTATCAGGG	GAGTGTTGGGGGCATCATCA
	ELOVL5	TTCGATGCGTCACTCAGTACC	TGTCCAGGAGGAACCATCCTT
	18s	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
	Occludin	CCACCCCATCTGACTATGC	TCGCTTGCCATTCACTTTGC
	TGFβ1	CCCAGTCTCCATACATTAACCC	CACCATCACCTTGA ACTCTGAC
	TGFβ2	TTGTTGCCCTCCTACAGACTGG	GTAAAGAGGGCGAAGGCAGCAA
	TGFβ3	CCTGGCCCTGCTGAACTTG	TTGATGTGGCCGAAGTCCAAC
	TGFβ1R	TCTGCATTGCACTTATGCTGA	AAAGGGCGATCTAGTGATGGA
	TGFβ2R	TTGGATTGCCAGTGCTAACCC	AACAAGCCACAGTAACATGACA
<b>Mouse Genotyping</b>	internal control	CAAATGTTGCTTGTCTGGTG	GTCAGTCGAGTGCACAGTTT
	O Reverse		GGCTAGACACACCTAGCAGAGC
	K Reverse		TGCGGTACCAGACTCTCCCAT
	C' Forward	AGAAGACAGACCTGGTTTGGCATA	

## **PART 4 Discussions**

## **1 Downregulation of Elov15 in breast cancer correlates with poor clinical outcome**

Our work demonstrates the association between Elov15 expression and the breast cancer aggressiveness in patients. The study was based on the METABRIC data set and breast cancer patient samples collected in GF Leclerc and CHU Dijon center.

In terms of general analysis between tumor and non-tumoral breast tissues, the mRNA and protein expression of Elov15 in primary breast tumor was significantly lower than in normal breast tissue in all subtypes including luminal (ER+), HER2 positive and triple negative breast cancer (TNBC-basal-like). In addition, the expression of Elov15 at both mRNA and protein levels were different between each molecular subtype, in which, the highest expression is detected in luminal, followed by HER2+ breast cancer and finally TNBC. Our results are not consistent with a recent finding, which demonstrates a higher expression of Elov15 mRNA in HER2+ and TNBC subtypes compared to normal breast tissue (Y. Yamashita et al. 2017). However, no concordance is detected between Elov15 mRNA and protein expression in this study. The expression of Elov15 is also modified in other types of cancer such as colorectal cancer (CRC) and prostate cancer. In CRC tumors, the DNA methylation of a specific subset of CpG islands contributes to the downregulation of Elov15 at both mRNA and protein levels (Boot et al. 2017). In addition, Elov12 expression is also down-regulated in high-aggressive basal-like subtype (TNBC) and contributed to metastasis (Y. P. Kang et al. 2019). Taken together, these finding indicate the link between Elov1 family proteins, especially Elov15 and breast cancer progression.

Interestingly, we also found that the low-mRNA expression of Elov15 is correlated to a shortER-overall survival time in breast cancer patients with a luminal and basal-like subtype but not in HER-2<sup>+</sup> subtype. In breast cancer, more than 75% of death are due to metastases (Dillekås, Rogers, and Straume 2019). Our breast cancer patient's data validated the relationship between Elov15 expression and metastasis. The expression of Elov15 is high in low-metastatic subtype (ER+) and subsequently reduced in high-metastatic subtypes (HER2+, TNBC), which suggests a correlation between Elov15 expression and the aggressiveness of breast cancer. Consistently, the decreases of overall survival in breast cancer patients are associated with Elov15 low-expression, which can be explained by the metastatic process induced by Elov15-down regulation. In CRC, high methylation of Elov15 correlates to low expression of Elov15 and reduce overall survival of CRC patients (Boot et al. 2017). In prostate cancer, high-Elov15 expression is associated with poor clinical outcome (Centenera et al. 2021). Recent research on Elov12

also comforts our data in terms of Elov1 family proteins and breast cancer patient survival. Indeed, low-expression of Elov12 correlated to a spheroid-induced metastatic phenotype in breast cancer cells, suggesting a poor prognosis in breast cancer patient (Y. P. Kang et al. 2019). Furthermore, the resistance against chemotherapy (tamoxifen) in Elov12-low expressing breast cancer patients indicates a poor clinical outcome (Jeong et al. 2021).

## **2 Low expression of Elov15 negatively regulates breast cancer tumor growth and positively induces lung metastasis**

### **2.1 Effect of Elov15 on proliferation and tumor growth**

We have shown the anti-proliferative effect of low Elov15 expression on different breast cancer cell lines (4T1 and MCF7 cells). Consequently, the invalidation of Elov15 delayed tumor appearance in MMTV-PyMT model and reduced tumor growth. In contrast, the overexpression of Elov15 in 4T1 cells enhances proliferation and 4T1-tumor growth in Balb/c mice. Altogether, these data show that Elov15 controlled proliferation and tumor growth in breast cancer. Our data also confirmed the data from a recent publication on Elov15 in prostate cancer context, the reduction of Elov15 expression is associated with the inhibition of cell proliferation and tumor growth (Centenera et al. 2021). In other Elov1 family protein, similar findings on cancer cell growth were reported. For example, Elov17 expression significantly decrease prostate cancer cells proliferation (Tamura et al. 2009). The glioblastoma cells with low expression of Elov12 reduce both cell proliferation and tumor growth (Saurty-Seerunghen et al. 2019). Knockdown Elov16 in liver cancer cells also leads to the inhibition of cellular proliferation and tumor growth (Su et al. 2018). However, the effect of Elov1 proteins in cancer context is not only tumor growth inducer but also suppressor, for instance, low expression of Elov12 in high MYCN-neuroblastoma cells increases the proliferation rate *in vitro* and tumor growth *in vivo* (Ding et al. 2019). The interesting common point of these findings is down-regulation of Elov1 protein family is often correlates with the modification of cancer development.

### **2.2 Effect of Elov15 on lung metastasis**

Based on the analysis of metastases in lungs of Elov15<sup>-/-</sup> and Elov15<sup>+/+</sup> MMTV-PyMT mice, and Elov15-silenced MCF7 cells injected in tail vein of nude mice, we observed that low-Elov15 expressing cells induced more metastases in lungs mice. Interestingly, the mammary fat pad transplantation of 4T1 overexpressing Elov15 in Balb/c mice also showed a lower lung-metastatic index (number of metastatic counts on total lung surface) despite the greater size of primary tumor compared to the

control group. In consistent with these findings, the tail vein injection of 4T1 cells stably overexpressing Elov15 in Balb/c mice resulted in a decrease of metastasis in lungs in comparison to control mice. The direct delivery of cancer cells into blood streams should be considered as an organ colonization assay, not a natural metastatic process due to the missing dissemination steps of cancer cells from primary tumor. Therefore, this is a limitation of our studies compared to literature. Different arguments highlight the lack of relationship between primary tumor size and metastasis, for example, the breast cancer cells inducing lung-metastasis is depending on gene-expression signature in primary breast tumors, not its size (Aceto et al. 2014a). No significant correlations are indicated between breast tumor size and metastatic rates, including risk of developing metastasis and the metastatic effectiveness (Sopik and Narod 2018). However, the intravenous injections were still our optimal option during the experiment to avoid any possible bias due the difference in primary tumor growth. This data was comforted by the consistent results of mammary fat pad injection models.

To evaluate a clinical relevance, we then analyzed the Elov15 expression in breast cancer patients with (N1) or without (N0) lymph node metastases. A significant decrease of Elov15 expression was found in ER+ breast cancer patients with metastasis (N1) compared to non-metastasis (N0) profile. However, no differences in Elov15 expression were observed between HER2+/TNBC breast cancers patients with metastasis (N1) compared to HER2+/TNBC breast cancers patients without metastasis (N0). Interestingly, the expression of Elov15 in N1-ER+ patients is similar to N0-HER2+ and N0-TNBC patients, which suggests the association between low expression of Elov15 and high degree of metastasis. Therefore, Elov15 low expression is highly correlated to breast cancer aggressiveness, in which, the downregulation of Elov15 expression associates with lymph node invasion in ER+ breast cancers and induces lung metastases formation in breast cancer mouse models.

In the *in vitro* study, we also showed that temporary or permanent downregulation of Elov15 significantly increased the invasive abilities of breast cancer cells through extracellular matrix (ECM) allowing initiation of metastasis. This data confirms our observations about lung metastasis in the *in vivo* model. The initiation of metastases requires the activation of cancer cells for migration and invasion to detach from the primary tumor (Riggio, Varley, and Welm 2021).

### **3 Low expression of Elov15 induces epithelial to mesenchymal transition of breast cancer cells**

We then investigated the correlation between Elov15 and EMT markers expressions. In temporary Elov15 down-regulated breast cancer cells (4T1, MCF7) and stable Elov15 silencing MCF7 cancer cells, there were a decrease of epithelial markers (E-cadherin and Occludin) and increase of

mesenchymal markers (Vimentin and N-cadherin) expression in both RNA and protein levels. In consistent with these findings, the 4T1 stably overexpressed-Elovl5 have high-expression of epithelial markers (E-cadherin and Occludin) and low-expression of mesenchymal markers (Vimentin and N-cadherin) also in RNA and protein levels. Among the molecular signaling induced-metastasis, EMT is one of the critical mechanisms. After EMT induction, cancer cells acquire mesenchymal properties to improve migrative and invasive properties, leads to the metastatic progression (Chaffer et al. 2016). Decrease of epithelial markers and increases of mesenchymal markers are widely observed in EMT as discussed in the above part (Dongre and Weinberg 2019; J. H. Yang et al. 2020). In breast cancer, it is frequently indicated the low-expression of epithelial protein and high-expression of mesenchymal proteins in low-metastatic subtype (ER+) and high-metastatic subtype (HER2+ and TNBC). For example, low-expression of E-cadherin is correlated to high-aggressiveness of breast cancer. E-cadherin expression is continuously decreased in ER+, then HER2+ and finally TNBC (J.-B. Liu et al. 2017; Horne et al. 2018). High expression of Occludin is found in ER+ while its low expression is observed in TNBC (T. A. Martin, Mansel, and Jiang 2010; T. A. Martin et al. 2016). In contrast, the mesenchymal marker Vimentin is significantly express in TNBC subtype (N. Yamashita et al. 2011). Therefore, these EMT markers are often used for breast cancer prognosis. Together with our finding on Elovl5-regulating these proteins, it is interesting to also consider Elovl-5 as a prognosis marker for breast cancer treatment.

In addition, we observed the modification of F-actin distribution in low Elovl5 expressing cells inducing a cytoskeleton structure change. These cells harbored a mesenchymal-like morphology, which comforts a relationship between Elovl5 expression and EMT in breast cancer cells. In fact, EMT is promoted by the disappearance of epithelial apical-basal polarity, reduction of cell-cell connections and cell-ECM interactions (J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020). In order to induce these effects, cytoskeleton rearrangement is necessary, which mainly control the expression of Vimentin and repression of E-cadherin and Occludin. Normally, cellular junctions' protein (E-cadherin, Occludin) are connect to the cytoskeleton, together with cortical Fibrous-actin (F-actin) and keratins to stabilize the structure (J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020; Leggett et al. 2021). The cytoskeletal organization is also fixed by cell-ECM adhesions. In the epithelial phenotype, cortical F-actin located at the periphery membrane and keratins form the connecting network from the nucleus to the cell membrane, which maintain the cells at “round intact shape”. In the mesenchymal phenotype, actin stress fibers are replaced cortical actin and keratin, together with loss of junctional

protein, which lead to the detachment of these cells from basement membrane and obtain the “spindle elongate shape” (Leggett et al. 2021; Svitkina 2018). The morphology modifications are visualized by F-actin staining in our experiment

#### **4 Elov15-controlled expression of TGF $\beta$ receptors regulates breast cancer cells proliferation and invasion**

The influences of TGF $\beta$  signaling on breast cancer cells are similar to our observations in low-expression Elov15 breast cancer cells models. Therefore, we decided to investigate the expression of TGF $\beta$  isoform and its receptors, especially TGF $\beta$ RI and TGF $\beta$ RII in breast cancer in low expression of Elov15 condition. From our data, TGF $\beta$ RI and TGF $\beta$ RII were highly expressed in Elov15-low expression breast cancer cells and we evidenced the activation of downstream pathway

Indeed, we analyzed the phosphorylated status of Smad2/3 (p-Smad2/3) and we observed in the presence (induction) or absence (basal) of exogenous TGF $\beta$ 1 that silencing of Elov15 in both 4T1 and MCF7 cell lines increase p-Smad2/3 expression. In fact, the activation of TGF $\beta$  signaling pathway leads to phosphorylation of Smad2/3 complex (Ikushima and Miyazono 2010; 2010). Smad proteins plays important function in cellular metastasis. For example, Smads associating Ras and p53 to form the Smad/mutant-p53/p63 protein complex, which inhibit the tumor suppressive protein p63, leading to the induction of breast cancer progression and metastasis (Adorno et al. 2009). TGF $\beta$  regulating Smads can promote EMT through EMT transcriptional factors (*i.e.* Snail1, Zeb1/2, Twist,  $\beta$ -catenin, Lef/TCF, Foxc2, and AP-1) (Fuxe, Vincent, and Garcia de Herreros 2010; Heldin and Moustakas 2012). Therefore, this data highlighted the activation of TGF $\beta$  signaling (via canonical pathway) and suggest the presence of TGF $\beta$  cytokine in low-expression Elov15 breast cancer cells; probably TGF $\beta$ 3 which is induced by silencing of Elov15 expression.

The blocking of TGF $\beta$  signaling with inhibitor of TGF $\beta$  receptors reduced low-Elov15 expression-induced cell invasion and partially rescued cell proliferation. This data is consistent to the effects of these inhibitors in clinical trials, which indicate the decrease of metastasis and result in promising outcome on metastatic cancer patients (*i.e.* breast cancer, pancreatic cancer) (Herbertz et al. 2015; Melisi et al. 2008). Our finding supported the evidence about high expression of TGF $\beta$  pathway components in breast cancers with poor outcome (de Kruijf et al. 2013; Joan Seoane and Gomis 2017b; Padua et al. 2008). Blocking TGF $\beta$  signaling by receptors inhibitor or cytokine neutralized antibodies in breast cancer xenograft mice model reduces the cellular metastasis (S. Liu, Ren, and ten Dijke 2021). Therefore, chemotherapies against TGF $\beta$  signaling are considered as the therapeutic response to cancer

cells dissemination; hence, several anti-pharmaceutical molecules inhibiting-TGF- $\beta$  pathway are developed for clinical studies (Peiris et al. 2015; S. Liu, Ren, and ten Dijke 2021).

## **5 Suppression of Elovl5 expression modulates fatty acid composition and drives accumulation of lipid droplets in a DGAT1/2-dependent manner**

There are seven very long-chain fatty acid elongases (Elovl1-7) in the elongase family, which identify the overall rate of fatty acid elongation and have substrate selectivity characteristics. Elovl5 enzymes catalyze long and very long chain-PUFA, long and very long chain-SFA with 18 and 22 carbon chain, especially focus on PUFA acylCoA with 18 and 20 carbon length (Leonard et al. 2000; Ohno et al. 2010). Our finding is consistent with several studies on Elovl5 expression and fatty composition regulation. For example, in liver tissue of Elovl5 knockout mice, there are the accumulation of C18 substrates and the reduction of C20 products (i.e arachidonic acid C20:4, n-6 and docosahexaenoic acid DHA, C22:6, n-3) (Moon, Hammer, and Horton 2009). In prostate cancer, down-regulation of Elovl5 leads to the decrease of C18 product (cis-vaccenic acid C18:1 n-7) and supplement with this FAs leads to the aggressive phenotype of prostate cancer cells (Centenera et al. 2021). Concerning other Elovl family members, the dysfunction of Elovl family protein also affects its corresponding products and substrates, results in the regulation of cancer development. For instance, Elovl7 is preferred to elongate SVLFA, C20:0, silencing Elovl7 in prostate cancer cells affected SVLFAs content in phospholipids and the neutral lipids, leads to the reduction of prostate cancer cells proliferation and tumor growth (Tamura et al. 2009). Elovl2 is responsible for the elongation of PUFA with 22 carbon length, low expression of Elovl2 reduces the ratio of C22:6n3 (docosahexaenoic acid, DHA) to C22:5n3 in spheroid breast cancer cells and promote their malignant phenotypes (Y. P. Kang et al. 2019).

Low-expression of Elovl5 in breast cancer cells increased C18:3 n-6 content (gamma-linolenic acid-GLA) while C18:3 n-6 content was consequently reduced in stable Elovl5 overexpressing 4T1 cells. In contrast, the C20:3 n-6 is decreased in Elovl5-silencing cells whereas this FAs is increased in Elovl5-overexpressing cells. This data confirmed the downregulation of Elovl5 is not only based on the protein expression but also the protein function.

GLA plays important role in cancer progression, the function of GLA as anti-proliferative factor is described. For example, as an anti-proliferative role, GLA reduces breast cancer cell proliferation *in vitro* and tumor growth *in vivo* (Rose, Connolly, and Liu 1995). Co-treatment of GLA and tamoxifen are a promising strategy endocrine-sensitive breast cancer in a clinical trial (Kenny et al. 2000). These findings suggest the suppressive role of GLA in cancer cell proliferation and breast cancer treatment.

However, it should be considered the argument that GLA supplementation can have metastasis effects, especially in breast cancer because GLA is metabolized to arachidonate-derived eicosanoids in the *in vivo* models, and these eicosanoids are widely involved in metastasis (Deng et al. 2021).

To determine the modification of lipid metabolism due to Elov15 dysregulation, the expression of lipogenic program enzyme (*i.e.* Acetyl-CoA carboxylase-ACC, Stearoyl-CoA desaturase-SCD1) and triacylglycerols (TAG) synthesis enzymes (*i.e.* Diacylglycerol Acyltransferases-DGAT 1 and 2) were studied. The increase of lipogenic program were indicated by the decreases of inhibitory p-ACC and increased of Scd1 expression in Elov15-silencing MCF7 cells. Metabolic alteration is considered as a hallmark of cancers, reprogramming of fatty acid (FA) metabolism is widely associated with the development of several cancer cells. In addition, breast cancer is correlated to quantitative and qualitative remodeling of FA composition (Hilvo et al. 2011). The modifications of FA contents in breast cancer are related to the high rate of de novo fatty acid synthesis; therefore, the elimination of lipogenesis reduces cancer cell proliferation and metastatic properties (Hilvo et al. 2011; Stoiber et al. 2018; Rios Garcia et al. 2017; 2017; J. Li et al. 2014). Exogenous fatty acids also involve in the proliferation of breast cancer cells and progression of metastases (J. Zhao et al. 2017; Pascual et al. 2017).

After that, we also observed the increase of intracellular total FA and high amount of TAG in low-Elov15 expression cells. Similar data was confirmed on patient samples. In fact, there were a significant increase of TAG content in low-Elov15 cancer tissues in ER+ and HER2+ subtypes, a non-significant induction was observed in TNBC subtype, the average TAG content is also highest in ER+ subtype. In fact, TAG is the main composition of lipid droplets (LD), which store fat and support resistance against lipotoxicity (Olzmann and Carvalho 2019). TAG synthesis is mainly occurred in endoplasmic reticulum (ER) under the catalytic activities of acyl-CoA:diacylglycerol acyltransferase (DGAT) (C.-L. E. Yen et al. 2008b). Metastatic cancer cells have high TAG content in the form of LD organelle accumulation (Cruz et al. 2019). High TAG content also correlates to aggressiveness of breast cancer. For example, the TAG content in breast cancer patient's plasma is significantly higher than healthy women (Potischman et al. 1991). In metastatic TNBC, higher content of TAG lead to greater overall mortality rate compared to TNBC with low TAG content. However, no significant difference in the correlation between TAG content and overall survival in luminal A and luminal B subtypes while high TAG amount in HER2+ can related to better survival rate (Lofterød et al. 2018).

Moreover, we observed the intracellular accumulation of lipid droplet (LD) in low-elov15 expressing breast cancer cells. The LD membrane contains several associated proteins such as DGAT (Dhiman et al. 2020). Therefore, treatment with DGAT inhibitors is used to study the involvement of DGAT

family proteins in Elov15-induced LD formation. As expected, there were a decrease the accumulation of LDs and prevention of intracellular Acetyl-CoA over-synthesis in low-Elov15 cancer cells. In general, several studies indicate the alteration of lipid metabolism supporting cancer development and target lipid metabolism for cancer treatments. Breast cancers cancer cells have high LD content, which may be due to the presence of estrogen/progesterone receptors or presence of adipocytes in microenvironment (Koundouros and Pouligiannis 2020; Y. Y. Wang et al. 2017). Interestingly, higher LD accumulation is a special characteristics of mesenchymal breast cancer cells compared epithelial-like and mesenchymal-like cells. In order to prepare for pre-metastatic process of breast cancer *in vitro*, the primary tumors up-regulated LDs (Giudetti et al. 2019). In addition, different breast cancer cell lines indicate the induction of LDs content, which correlates to metastatic stage, in which, the LDs accumulation is continuously increase from low-malignant cells (MCF10A), to the medium-malignant cells (MCF-7) and to the high-malignant cells (MDA-MB-231) (Abramczyk et al. 2015).

LDs have significant roles in risk and metastatic development in different types of cancer. Indeed, the LD accumulation is correlated to the aggressiveness of cancer and resistance against therapies. For example, cholesterol esterification and LD formation in glioblastoma (GBM) induces the GBM progression, leads to poor survival outcome of patient (Geng et al. 2016) (Geng F, 2016). In colorectal cancer (CRC), lysophosphatidylcholine acyltransferase 2 (LPCAT2) induced LDs accumulation, which promote the chemoresistance, results in a poor clinical outcome of patients (Cotte et al. 2018). In consistent with the previous studies, our study indicated the importance of LD accumulation for low-expression mediated promotion of EMT and invasion Elov15 in breast cancer.

DGAT inhibitors have potential for targeting low Elov15-inducing LD. Blocking Elov15-induced LD accumulations by DGAT inhibitors maintained epithelial phenotypes of breast cancer and reduced cellular invasion. In prostate cancer, inhibitor of DGAT1 regulates intracellular lipid modulation and microtubule-organizing center protein (MTOC-GM130), which is thereby used to reduce cell proliferation *in vitro* and decrease the tumor growth *in vivo* (Nardi et al. 2019). In colorectal cancer (CLC), treatment with DGAT1 inhibitor reduce the TAG synthesis (Dow et al. 2011), we know that carboxylesterase 1 (CES1) induces TAG catabolism, leads to the progression of colorectal carcinoma (Capece et al. 2021), therefore, using DGAT1 inhibitor can be a promising treatment method. Up to date, there are not many studies focusing on cancer treatment using DGAT2 inhibitor. In breast cancer, co-treatment of DGAT2 inhibitor with cisplatin or doxorubicin on different cancer cell lines induce cell death (Hernández-Corbacho and Obeid 2019). However, further studies must conduct to understand the molecular mechanism of DGAT2 increasing cell death, its side effects and effects on cancer cell metastasis.

In addition, the relationship between lipogenesis and LDs formation is interesting to study. In fact, product of lipogenesis is fatty acid CoA (FA-CoA), which is also the substrate for the final step of TAG production under the catalytic of DGAT1/2 enzyme (Schweizer and Hofmann 2004; C.-L. E. Yen et al. 2008a). In addition, LDs mainly contain TAG in neutral lipid core (Tauchi-Sato et al. 2002). These findings support the tight relationship between lipogenesis and LD accumulation. In cancer context, the induction of tumor lipolysis led to the accumulation of LDs in pre-malignant stage (M. Huang et al. 2012; Accioly et al. 2008; Hanahan and Weinberg 2011). LD accumulation and high expression of lipogenesis related-enzyme promote tumor growth in different cancers via SREBP1, mTORC1 and mTORC2 dependent pathways (Cruz et al. 2020). Interestingly, highest TAG content was detected in ER+ (luminal) subtypes among other subtypes in N0 clinical patient in our study, together with the evidence that luminal subtypes promote lipogenesis in pre-metastatic stage to prepare for cellular invasion and migration (Simeone et al. 2021), which suggest the promising linkage between lipogenesis and LD accumulation in clinical investigation at the early stage of cancer metastasis. This argument is enforced by our in vitro on high LD-content in MCF7 low-Elovl5 models (ER+ phenotype breast cancer cells). However, the precise studies on ACC and SCD1 in context of metastatic cancer-regulated LDs in lipogenesis dependent manner should be focused. In conclusion, lipogenesis is important during breast cancer progression and the roles of its enzymes in breast cancer have been widely discussed in the fourth chapter of the thesis. Therefore, targeting lipogenesis, especially its enzyme including SCD1, ACC, FAS are good candidates for anti-tumor and anti-metastatic drugs.

## **6 The accumulation of Elovl5-regulated lipid droplets regulates expression of TGF $\beta$ receptors, EMT markers and metastasis**

To understand the relationship between LD and TGF $\beta$ , we blocked the LD accumulation with DGAT inhibitors in low Elovl5 expression breast cancer cells. The treatment with DGAT inhibitors counteracted the induction of TGF $\beta$  signaling on these cells due to the loss of TGF $\beta$  receptor up-regulation. After that, we studied the effects of LD reduction on EMT markers in Elovl5 down-regulated breast cancer cells. Interestingly, DGAT inhibitors lead to the reduction of LD, results in the maintaining of cellular epithelial phenotype, cell proliferation restoration and cell invasion reduction. Taken together, the data indicate that LD accumulation promoted by Elovl5 downregulation regulate cellular proliferation, invasion and EMT through the increase of TGF $\beta$  receptors. TAG is the main neutral lipid of lipid droplets (LD), which are the FA storage organelles in the cells. As a reservoir of substrates for FA oxidation, LDs contribute to Acetyl-CoA production, and the formation of LDs is

depending on DGAT1-2 catalytic activities (Cruz et al. 2020). Indeed, the link between LDs and high expression of TGF $\beta$ RI (not TGF $\beta$ RII) are indicated in different cell types such as cancer cells and dendritic cells (Corbet et al. 2020; Trempolec et al. 2020). In fact, the low pH condition induces TGF $\beta$ 2 in an autocrine manner, which leads to the accumulation of LDs to escape anoikis. The induction of TGF $\beta$ 2 activates TGF $\beta$  signaling pathway through TGF $\beta$ RI and Smad2/3 regulation (phosphorylation and acetylation), which activate EMT responsive genes, results in the induction of cancer cell invasion and distant metastasis (Corbet et al. 2020). In breast cancer, ACC1 is phosphorylated due to the activation of TGF $\beta$ -activated kinase (TAK) 1, phosphorylation ACC1 corresponds to the inhibition of its activities, results in down-regulation of lipogenesis and accumulation of Acetyl-CoA. These effects activate Smad2 acetylation, which increase EMT in *in vitro* model, metastasis *in vivo* model and poor prognosis in breast cancer patients (Rios Garcia et al. 2017).

For the first time, our results indicate possible treatment using DGAT inhibitors to prevent the initiation of metastasis aggressiveness breast cancer cells (with EMT phenotype), which contain the low expression of Elov15. DGAT inhibitors reduce LD accumulation and consequently TGF $\beta$  signaling through the reduction of TGF $\beta$  receptors expression in Elov15-silencing cancer cells. In addition, pharmacological molecules to target TGF $\beta$  signaling including its cytokines and receptors are widely studied in clinic, which exhibit promising effects against breast cancer metastasis (Table + figure chapter 3). However, in breast cancer patients with high risk of metastasis, and poor prognosis correlated to low levels of Elov15 and high levels of TGF $\beta$  receptors, the applications of DGAT inhibitors can improve the efficacy of anti-cancer chemotherapies or post-operative surgery, and radiotherapies due to reducing high levels of LDs (Schlaepfer et al. 2012; Englinger et al. 2020).

# **PART 5    General conclusions and perspectives**

## 1 Conclusion

For the first time, we demonstrate the role of Elov15 in breast cancer development through lipid droplet regulation and TGF $\beta$  receptors (TGF $\beta$ RI and TGF $\beta$ RII) expression. In addition, we also indicate that low expression of Elov15 increases the aggressiveness of metastatic breast cancer in all the subtypes (Figure 40). Therefore, evaluating Elov15 expression is a promising method to orient breast cancer patients' treatments in order to prevent metastasis.

## 2 Perspectives

Due to the diverse range of molecular pathways that can associate to this study on Elov15 on breast cancer progression, there are variety of overall perspectives that is both describing in the discussion below and in Figure 40.

### 2.1 Fatty acid $\beta$ -oxidation in low-expression Elov15 model

Our studies demonstrated the accumulation of lipid droplets (LDs) and high Acetyl-CoA content which could be a product from fatty acid  $\beta$ -oxidation (FAO). In fact, FAO is the process that converts FAs into Acetyl-CoA and releases ATP molecules (Qu et al. 2016). Different studies indicate up regulation of FAO induces breast cancer metastasis and tumor progression (Camarda et al. 2016; C.-K. Lee et al. 2019; T. Wang et al. 2018). In addition, TGF $\beta$  signaling in mesenchymal-like breast cancer cells upregulates FAO and mitochondrial oxidative phosphorylation (OXPHOS) in p-AMPK dependent manner (J. Yang, Antin, Berx, Blanpain, Brabletz, Bronner, Campbell, Cano, Casanova, Christofori, Dedhar, Derynck, Ford, Fuxe, García de Herreros, et al. 2020). High FAO rate also activates MAPK pathway, which increases EMT of tumor cells, lead to metastasis (C. Wang et al. 2019). Therefore, it is interesting to determine the FAO rate in stable and transient Elov15-silencing cell lines (MCF7 and 4T1) by Seahorse experiment to understand the relationship between Elov15 expression and the rate of fatty acid breakdown to Acetyl-CoA. The results are expected to answer the question how metastatic cancer cells with low-Elov15 expression use the FAs storage in LDs to generate energy (ATP), initiate the metastasis process and support cellular survival in nutrient deficient conditions during circulation.

### 2.2 Acetylation of Smad2 in lipid droplets-regulate TGF $\beta$ receptors

Besides that, Elov15 silencing leads to the dysregulation of lipid metabolism in breast cancer cells including LDs accumulations. Therefore, it is possible that the conversion of TAG (in LDs) to Acetyl-

CoA via FAO activation can support the Elov15-low breast cancer cell metastasis. In fact, acetylation of Smad2 is the transfer process of acetyl groups from Acetyl-CoA to lysine residues on Smad2 protein by co-activator p300 modulates activin and TGF $\beta$  signaling, this transformation leads to the neutralization of the protein, which is also considered as Smad2 activation (Tu and Luo 2007; Corbet et al. 2020). As previously discussed, many studies demonstrate the involvement of Acetyl-CoA to activate TGF $\beta$  signaling *via* acetylation of Smad2, result in EMT and metastatic induction (Rios Garcia et al. 2017; Corbet et al. 2020). Therefore, to understand the direct linkage of Elov15-induced LDs activate TGF $\beta$  signaling via the induction of TGF $\beta$  receptors, the evaluation of acetylation status of Smad2 together with its effects on both active TGF $\beta$  receptor complexes and TGF $\beta$  receptor mRNA are necessary to perform.

### **2.3 Endoplasmic reticulum stress and lipid droplet formation in low-expression Elov15 model**

Elongase (Elovl) protein family is located on endoplasmic reticulum (ER), however, up to date, there is still no clue about the role of Elovl protein in maintaining ER homeostasis. Interestingly, LDs are emerged from endoplasmic reticulum (ER), therefore, the lipid monolayer of LDs also contain ER-related proteins on the membrane such as Diacylglycerol acyltransferases (DGAT1 and 2) (Dhiman et al. 2020). The accumulation of LDs in Elov15 low-expression breast cancer cells from our data suggest the abnormal function of ER during LDs synthesis and generation, for example, ER stress. In fact, ER stress appears when there is the saturation in the ability of the ER to fold proteins. The relationship between ROS production and ER stress is indicated in several diseases including breast cancer (Zeeshan et al. 2016; Perillo et al. 2020), as an ER-located protein, Elov15 and its regulation can be one of the origins for this relationship. Interestingly, LDs are emerged from endoplasmic reticulum (ER), therefore, the lipid monolayer of LDs also contain ER-related proteins on the membrane such as Diacylglycerol acyltransferases (DGAT1 and DGAT2) (Dhiman et al. 2020). ER stress induces the formation of LD formation, which isolate misfolded proteins and excess lipids from ER to reduce ER stress (Migdal and Serres 2011). LDs promote colorectal cancer cells and glioblastoma to metastasize and non-respond to therapies (Geng et al. 2016; Cotte et al. 2018), LDs also upregulate in breast cancer metastasis (Koundouros and Poulogiannis 2020), as an enzyme that is responsible for lipid elongation, role of Elov15 in metastatic inducing-lipid alteration and cellular resistance in breast cancer should be an interesting aspect. The accumulation of LDs in Elov15 low-expression breast cancer cells from our data suggest the abnormal function of ER during LDs synthesis and generation, for example, ER stress. In fact, ER stress appears when there is the saturation in the ability of the ER to fold proteins. The induction of ER stress involves the activation of different transcriptional factor such as activating transcription factor 4 (ATF4) (Lin, Walter, and Yen 2008). Therefore, experiments on verification of

ER stress status is necessary, especially the phosphorylation of ATF4 and its relationship with LD accumulations in Elov15 silencing breast cancer models.

#### **2.4 Elov15-silencing breast cancer models and ferroptosis**

Furthermore, dysregulation of lipid metabolism is a hallmark of cancer, various studies found that the survival of many aggressive cancer cells in fatty alteration condition by remodeling of lipid metabolism can affect their sensitivities against ferroptosis (Jiang et al. 2015; Viswanathan et al. 2017; Miess et al. 2018; Tsoi et al. 2018). Ferroptosis is a cell death program (necrosis), which depend on lipid-peroxidation mediating iron regulation (Dixon et al. 2012). We know that down regulation of E-cadherin improves cellular response to RSL3 (Erastin), which is a ferroptosis activator through the inhibitor of glutathione peroxidase 4 (GPX4), leads to the loss of cellular resistant against plasma membrane peroxidation. The study provides the insight about the high sensitivities of EMT cancer cells to ferroptosis (J. Wu et al. 2019). Interestingly, the high expression of Elov15 and fatty acid desaturase 1 (FADS1) is observed in mesenchymal-like gastric cancer cells (GCs), results in cellular ferroptosis sensitization. Low expression of Elov5 and FADS1 in Hs746T, SNU-484, and YCC-16 cells resists against cell death induced by RSL3 (J.-Y. Lee et al. 2020). Moreover, a recent study on hepatocytes demonstrates the degradation of intracellular LD can increase RSL3-promote cell ferroptosis. High LDs accumulation is observed at early ferroptosis while low LDs accumulation is detected at late ferroptosis in hepatocytes (Bai et al. 2019). LDs have anti-ferroptotic effects *via* nuclear factor erythroid 2-like 2 (NFE2L2) signaling regulation (D. Tang et al. 2021).

In this project, our data demonstrated the accumulation of LDs and activation of TGF $\beta$  signaling pathway, results in EMT including downregulation of E-cadherin in Elov15-silencing breast cancer cells. These data suggest the possible “ferroptotic-escape” phenotype of metastatic breast cancer cells, in which, the decrease of E-cadherin in EMT at the early stage of metastasis can activate ferroptosis signaling via TGF $\beta$  signaling. In fact, TGF $\beta$ 1-activated Smad3 leads to the decrease of system xc<sup>-</sup> activity (a cystine/glutamate exchange transporter) and increases lipid peroxidation driving a higher response of hepatocellular carcinoma cells to GPX4 inhibition and an improvement of ferroptosis (D. H. Kim et al. 2020). However, the presence of high LDs content in these mesenchymal breast cancer cells supports the resistant against ferroptosis. In this case, low-expressing Elov15 aggressive breast cancer cells can increase the chance to survive and enter to circulation for secondary tumor establishment. Therefore, it is interesting to evaluate the role between high LDs accumulation, FA elongation, loss of E-cadherin and activation of TGF $\beta$  signaling in Elov15-silencing models in response to ferroptosis signals. The understanding on new “cell death program” will provide an insight to know

how metastatic cancer cells can adapt to lipid alteration tumor microenvironment to and escape different programmed cell death. These findings can support propose possible treatment of aggressiveness breast cancer with low expression of Elov15, in which, the treatments will be targeted not only LD accumulations (*i.e.* DGAT inhibitors) but also ferroptosis in order to have a better outcome effect.

## **2.5 Supplement of fatty acid in cancer models with Elov15 regulated- lipid metabolism**

Moreover, it should be considered the nutritional supplementation of patient diet in order to prevent cancers progression or support cancer treatment. In fact, high expression of Elov15 in prostate cancer promote the aggressiveness of the cancer, leads to poor clinical outcome; hence, downregulation of Elov15 in this cancer is correlated to good prognosis. The addition of Elov15 catalytic product (monounsaturated fatty acid *cis*-vaccenic acid) in Elov15-low prostate cancer cells induce the metastatic phenotype, which associates with cell proliferation and migration (Centenera et al. 2021). This study highlights the important of FA supplement in Elov15-regulated cancer cell models and could be an additional treatment in breast cancer. In fact, we observed that low-expression Elov15 breast cancer cells (4T1 and MCF7) accumulated C18:3 n-6 FA and decreased C20:3 n-6 FA, which are corresponding to substrate and product of Elov15 enzymatic activities respectively. Therefore, it is interesting to first, supply of fatty acid (C20:3 n-6 FA) in Elov15-silencing cancer cells to understand whether we can counteract the effects of low-Elov15 expression on metastatic cancer cells characteristics (LD accumulations, TGF $\beta$  signaling activations and EMT) and their functions (invasive, proliferation). Secondly, the diet program with high C20:3 n-6 content can apply on either transgenic mice (MMTV-PYMT) or xenograft mice with Elov15 silencing cancer cells to either delay the initiation of metastasis or support the effects of DGAT inhibitors treatments.

## **2.6 Biomarker of breast cancer in clinical prognosis**

Finally, the clinical study on patients should be addressed in a larger cohort with high number of patients in each subtype, the correlation ratio between Elov15 and TGF $\beta$  receptors protein expression on primary breast tissues should be established to identify whether this ratio can be a precise prognosis. In addition, the association of Elov15 expression and Ki-67 is important to determine in order to confirm the effect of Elov15 on cancer cell proliferation and breast tumor growth. Based on these findings, a new standard to classify breast cancer aggressive phenotypes for each subtype can be established as following N0 (non-metastasis): Elov15+/TGF $\beta$ R-/ Ki-67 high; N1 (with metastasis) Elov15-/TGF $\beta$ R+/Ki-67 low.

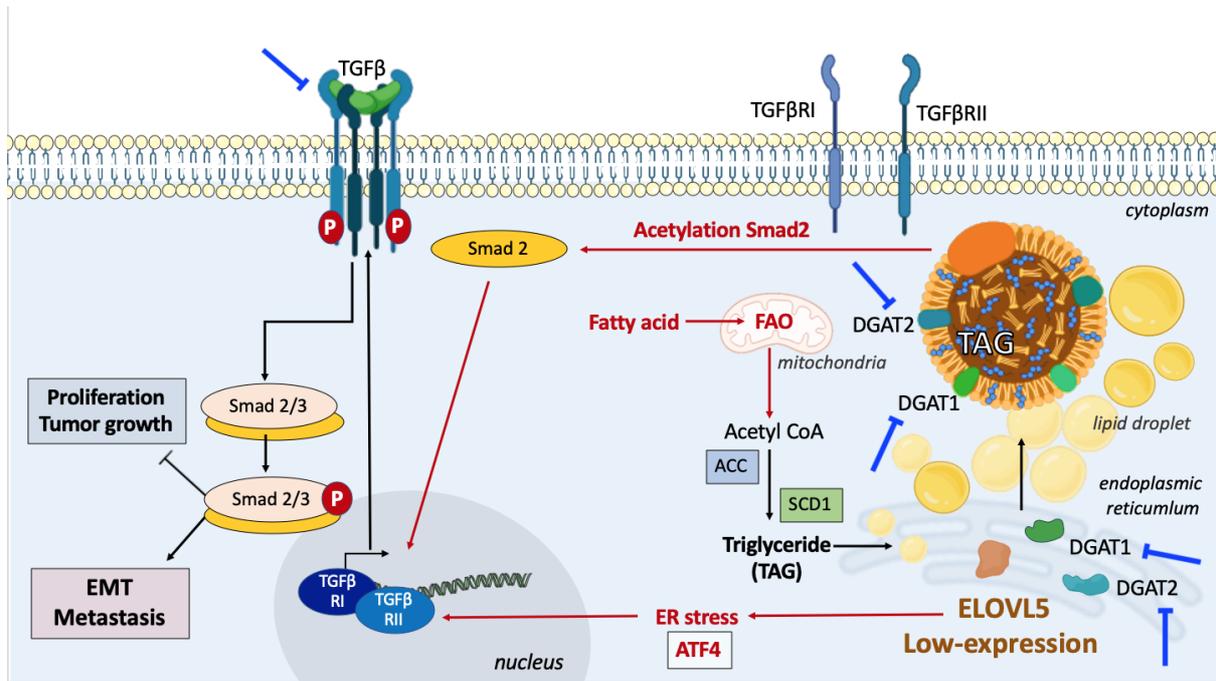


Figure 40: Summary scheme of the PhD project

The brown color (text) indicates origin of the regulation (down-regulation of Elov15). The black color (text and arrows) indicates molecular signaling and protein function that have been described and confirmed during the thesis. The red color (text and arrows) indicated the future perspectives that have been discussed above. The light-blue blocking-symbols illustrate the possible inhibitor targets for breast cancer treatment.

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# Annexes

## List of publications and presentations

### **Publications:**

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## Enzyme Elov15 dans le cancer du sein : régulation de la prolifération des cellules cancéreuses et du processus métastatique

**Mots clés :** cancer du sein, élongation des acides gras, métastases, gouttelettes lipidiques, TGF- $\beta$ , Elov15

**Résumé :** Le cancer du sein est le cancer le plus diagnostiqué et la principale cause de décès par cancer chez les femmes. Le taux de survie à 5 ans est inférieur à 30 % pour le cancer du sein métastatique et les métastases représentent plus de 75 % des décès par cancer du sein. Le métabolisme des acides gras est altéré dans les cancers et contribue à la progression tumorale. Nous nous sommes intéressés au rôle de l'enzyme Elov15 qui catalyse l'élongation des acides gras à longue chaîne par l'ajout de deux carbones. Nous avons observé que les tissus tumoraux de patientes atteintes d'un cancer du sein avaient une expression d'Elov15 plus faible que le tissu du sein normal apparié. De plus, une faible expression d'Elov15 est associée à un mauvais pronostic chez les patientes atteintes d'un cancer du sein de sous-type luminal (ER+) ou basal. Conformément à cette observation, nous avons montré que la diminution de l'expression d'Elov15 est plus prononcée dans les tumeurs mammaires ER+ de patientes présentant des métastases dans les ganglions lymphatiques. Bien que la régulation négative d'Elov15 limite la

progression tumorale, nous avons démontré que la répression de l'expression d'Elov15 favorisait la transition épithélio-mésenchymateuse et le développement de métastases pulmonaires dans des modèles murins de cancer du sein. Une répression de l'expression d'Elov15 dans les cellules cancéreuses du sein conduit à une augmentation de l'expression des récepteurs au TGF- $\beta$  induite par une accumulation de gouttelettes lipidiques. L'apparition de ces gouttelettes lipidiques est supprimée par un blocage de la synthèse des triglycérides avec des inhibiteurs pharmacologiques des enzymes DGAT1/2 et cette suppression a levé l'induction de l'expression des récepteurs au TGF- $\beta$ , de l'EMT et de l'invasion cellulaire activée par la répression de l'expression Elov15. Au total, nous avons montré que l'expression de l'enzyme Elov15 est un marqueur prédictif du risque de développement de métastases dans le cancer du sein et que cette enzyme régule le processus métastatique en modulant l'expression des récepteurs au TGF- $\beta$  induite par les gouttelettes lipidiques.

## Elov15 enzyme in breast cancer: regulation of cancer cell proliferation and metastasis

**Keywords:** breast cancer, fatty acid elongation, metastasis, lipid droplets, TGF- $\beta$  Elov15

**Abstract:** Breast cancer is the most diagnosed cancer and the leading cause of cancer death in women. The 5-year relative survival rate is less than 30% for metastatic breast cancer and metastases account for more than 75% of breast cancer deaths. Fatty acid metabolism is altered in cancer and contributes to tumor progression and metastasis. Here, we were interested in Elongation of very long chain fatty acids protein 5 (Elov15) which catalyses the elongation of long-chain fatty acids. We observed that the tumors from patients with a breast cancer had a lower expression of Elov15 than paired normal breast tissue. However, low expression of Elov15 is associated with a worse prognosis in breast cancer patients with a luminal (ER+) or basal-like subtype. In accordance with this finding, we showed that the decrease of Elov15 expression is more pronounced in ER+ breast tumors from patients with metastases in lymph nodes.

Although Elov15 downregulation limits breast cancer cell proliferation and cancer progression, we demonstrated that repression of Elov15 expression promoted EMT and lung metastases in murine breast cancer models. A repression of Elov15 expression in breast cancer cells led to an upregulation of TGF- $\beta$  receptors mediated by an accumulation of lipid droplets which is suppressed by a blockade of triacylglycerol synthesis with pharmacological drugs inhibiting DGAT1/2. Interestingly, the abolition of Elov15-regulated lipid droplet formation reversed the induction of TGF- $\beta$  receptors, EMT and cell invasion. Altogether, we showed that Elov15 expression is a predictive marker for the risk of metastases development in breast cancer and that Elov15 regulated the metastatic process through modulation of the expression of TGF- $\beta$  receptors mediated by lipid droplets.