UNIVERSITÉ DU QUÉBEC À MONTRÉAL

# CHARACTERIZATION OF THE MOLECULAR MECHANISMS UNDERLYING THE IMPACT OF OLEATE ON CELL MIGRATION

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# CARACTÉRISATION DES MÉCANISMES MOLÉCULAIRES IMPLIQUÉS DANS L'EFFET DE L'OLÉATE SUR LA MIGRATION CELLULAIRE DE CELLULES

THÈSE
PRÉSENTÉ
COMME EXIGENCE PARTIELLE
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PAR
ZHIQIANG GUO

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# REMERCIEMENTS

This journey—my journey—began with a single CV handed to Dr. Juneau in Shanghai more than five years ago, in 2019. It was my first time in that bustling city, my first step into the unknown. Little did I know then how profoundly that moment would reshape my life. Dr. Juneau introduced me to Catherine and Karl, two people who would later become my guiding stars. But between that meeting and the start of my Ph.D., there is an agonizing year of waiting—stranded in China as the world shut its doors under the shadow of COVID. Finally, in 2021, through the relentless snow and the raging pandemic, I arrived in Montreal, a lone traveler stepping into a new world. It was the first time I had ever left my home country.

The years that followed were a battlefield—not just of academia, but of the soul. I struggled against loneliness, against culture shock, against language barriers, against racism, and against bullying. There were nights when the weight of it all threatened to crush me, when I was certain I would not make it. But here I stand. I arrived at the end of this journey, not unscathed, but unbroken.

The first person I must thank is myself. The shy, introverted, timid boy who once doubted his every step has become a warrior. The battles I fought within my mind were a thousand times heavier than any challenge in my studies. Yet I endured. I survived the depths of depression, and in doing so, I ignited the hope of a new life.

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And to the countless others who lifted me up, in ways big and small—this triumph is yours as much as it is mine.

I came here as a boy. I leave as a fighter. And for that, I am proud.

Zhiqiang Guo

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# DÉDICACE

There is no prize to perfection, only an end to pursuit.

-Viktor

Sometimes taking a leap forward means leaving a few things behind.

-Ekko

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| | |
|---|---|
| AA | arachidonic acid |
| ABHD17 | α/β hydrolase domain-containing 17 proteins |
| ACC | acetyl-CoA carboxylases |
| ACLY | ATP citrate lyase |
| AJCC | American Joint Committee on Cancer |
| ANGPTL4 | angiopoietin-like 4 |
| AOM | azoxymethane |
| APT | acyl protein thioesterase |
| Arf | ADP-ribosylation factor |
| Arp | actin-related protein |
| BAX | Bcl-2-associated X protein |
| CAF | cancer-associated fibroblast |
| Cdc42 | Cell division control protein 42 homolog |
| CR | conserved regions |
| CRC | colorectal cancer |
| CTC | circulating tumor cells |
| ctDNA | circulating tumor DNA |
| DAG | diacylglycerol |
| DMH | dimethylhydrazine |
| DSS | dextran sodium sulfate |
| ECM | extracellular matrix |
| ECMELOVL | extracellular matrix fatty acid elongases |
| EGFR | epidermal growth factor receptor |

| | |
|---|---|
| EMT | epithelial to mesenchymal transition |
| ER | estrogen receptor |
| ERM | ezrin/radixin/moesin |
| EVOO | extra-virgin olive oil |
| FA | fatty acid |
| FABP | fatty acid-binding protein |
| FADS | fatty acid desaturase |
| FAK | focal adhesion kinase |
| FASN | fatty acid synthase |
| FTase | farnesyltransferase |
| GAP | GTPase-activating protein |
| GDP | guanosine diphosphate |
| GEF | guanine nucleotide exchange factors |
| GGTase | geranylgeranyltransferase |
| GPI | glycosylphosphatidylinositol |
| GPR/GPCR | G protein-coupled receptors |
| GTP | guanosine triphosphate |
| HAMLET | human alpha-lactalbumin made lethal to tumor cell |
| HER2/neu | human epidermal growth factor receptor 2 |
| HIF-1α | hypoxia-inducible factor-1 alpha |
| HNSCC | head and neck squamous cell carcinoma |
| ILDL | intermediate-density lipoprotein |
| ILK | integrin-linked kinase |
| $IP_3$ | inositol trisphosphate |
| LA | linoleic acid |
| LDE | Lipid-Dependent Enzymatic |

| | |
|---|---|
| LDL | low-density lipoprotein |
| LPL | lipoprotein lipase |
| MMP | matrix metalloproteinase |
| mTOR | mammalian target of rapamycin |
| MUFA | monounsaturated fatty acid |
| MβCD | methyl-β-cyclodextrin |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NNK | N-nitrosamine: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| NOX4 | NADPH oxidase 4 |
| NPF | family nucleation-promoting factor |
| OA | oleic acid / oleate |
| PA | phosphatidic acid |
| PC | phosphatidylcholine |
| PD-1 | programmed death-ligand 1 |
| PH | pleckstrin homology |
| PI | phosphoinositide |
| PI3K | phosphatidylinositol-3 kinase |
| PIP2 | Phosphatidylinositol 4,5-bisphosphate |
| PLC | phospholipase C |
| PLD | Phospholipase D |
| PM | plasma membrane |
| PPAR | peroxisome proliferator-activated receptor |
| PPT | palmitoyl protein thioesterase |
| PR | progesterone receptor |
| PTM | post-translational modifications |
| PX | phox homology |

| | |
|---|---|
| ROS | reactive oxygen species |
| RTK | receptor tyrosine kinases |
| SCD | stearoyl-CoA desaturases |
| SFA | saturated fatty acid |
| SFO | sunflower oil |
| SOCE | store-operated $Ca^{2+}$ entry |
| SPARC | secreted protein and rich in cysteine |
| Stat5 | signal transducers and activators of transcription 5 |
| TG | triacylglycerol |
| TNBC | triple-negative breast cancer |
| TSCC | tongue squamous cell carcinoma |
| VEGF | vascular endothelial growth factor |
| VLDL | very-low-density lipoprotein |
| WASP | Wiskott-Aldrich syndrome protein |
| WAVE | WASP family Verprolin-homologous protein |
| WHO | World Health Organization |
| zDHHC-PAT | zinc finger DHHC domain-containing protein acyl transferase |

# RÉSUMÉ

Le cancer du sein représente la forme de cancer la plus répandue et la deuxième cause de mortalité liée au cancer chez les femmes dans le monde. Le cancer du sein triple-négatif (TNBC), sous-type particulièrement agressif, se caractérise par des options thérapeutiques ciblées limitées et une forte propension aux métastases. Des données émergentes associent la stéaroyl-CoA désaturase 1 (SCD1) et son produit métabolique, l'oléate (OA), à la progression tumorale. Toutefois, les mécanismes moléculaires par lesquels l'OA influence la migration des cellules de TNBC demeurent incomplètement élucidés. Une récente étude de notre laboratoire a démontré que l'inhibition de l'activité de la SCD1, ainsi qu'un traitement par l'OA—mimant une suractivité de la SCD1—altèrent les propriétés migratoires des cellules MDA-MB-231 dérivées de TNBC, modulant leur vitesse, leur directionnalité et leur morphologie. Ces modifications sont médiées par une voie de signalisation intracellulaire spécifique impliquant la phospholipase D (PLD) et la voie mTOR, soulignant le potentiel thérapeutique de cibler le métabolisme SCD1-OA dans le TNBC.

Afin d'élucider les mécanismes moléculaires sous-tendant la migration et l'invasion induites par l'OA, nous avons examiné l'impact de l'OA sur la migration des cellules de TNBC, en nous focalisant sur le remodelage membranaire. En utilisant des lignées cellulaires de TNBC et des outils bioinformatiques, nous avons observé que la stimulation par l'OA induit rapidement la formation de plissements membranaires (*ruffles*) et accroît l'émergence de filopodes. Le traitement à l'OA favorise la translocation subcellulaire de Cdc42 et du complexe Arp2/3, avec un rôle central de Cdc42 dans la formation des filopodes et la migration cellulaire. L'inhibition pharmacologique de Cdc42—mais non du complexe Arp2/3—abolit la formation de filopodes et la migration induites par l'OA. Par ailleurs, nos résultats impliquent PLD2 dans la formation des filopodes dépendante de Cdc42, soulignant son rôle dans la motilité cellulaire induite par l'OA. Une surexpression de Cdc42 dans les tissus et lignées cellulaires de TNBC est corrélée à un faible taux de survie des patientes, confirmant sa pertinence dans la progression tumorale.

Sur le plan moléculaire, nous avons identifié un nouveau mécanisme par lequel l'OA active PLD2 via une S-acylation. Par microscopie confocale, isolement membranaire et tests d'acylation, nous démontrons que l'OA favorise la S-acylation de PLD2 aux résidus Cys223 et Cys224, entraînant sa dissociation des radeaux lipidiques et sa translocation vers des microdomaines

enrichis en phosphatidylinositol 4,5-bisphosphate ($PIP_2$). Cette relocalisation accroît l'activité GEF (*guanine nucleotide exchange factor*) de PLD2 envers Cdc42, facilitant le remodelage du cytosquelette d'actine et la formation de protrusions de type filopodes. La mutation des sites d'acylation de PLD2 ou la perturbation des radeaux lipidiques abolissent sa capacité à activer Cdc42 et à promouvoir les protrusions cellulaires.

Cette étude apporte un éclairage novateur sur la manière dont les modifications lipidiques induites par l'OA influencent la signalisation membranaire et la dynamique du cytosquelette, favorisant *in fine* la migration et la dissémination métastatique. En caractérisant les mécanismes moléculaires régissant l'activation de PLD2 par l'OA, nous contribuons à une meilleure compréhension des voies oncogéniques pilotées par les lipides. Par ailleurs, en évaluant le potentiel thérapeutique du ciblage conjoint de SCD1 et PLD2, nos travaux ouvrent la voie à de nouvelles stratégies thérapeutiques basées sur le métabolisme lipidique dans le TNBC. Compte tenu du pronostic défavorable associé au TNBC et des options thérapeutiques limitées, l'identification de vulnérabilités métaboliques des cellules cancéreuses offre une approche prometteuse pour le développement de thérapies ciblées.

**Mots clés :** Acide oléique ; cancer du sein triple négatif ; migration cellulaire ; filopodes ; Cdc42 ; phospholipase D2 ; S-acylation ; radeaux lipidiques ; facteur d'échange de nucléotides guanyliques

# ABSTRACT

Breast cancer is the most prevalent cancer and the second leading cause of cancer-related deaths among women worldwide. Triple-negative breast cancer (TNBC) is an aggressive subtype characterized by limited targeted therapeutic options and a high propensity for metastasis. Emerging evidence links stearoyl-CoA desaturase 1 (SCD1) and its product, oleate (OA), to cancer progression. However, the molecular mechanisms by which OA influences TNBC cell migration remain incompletely understood. A recent study from our lab demonstrated that both inhibition of SCD1 activity and OA treatment (mimicking SCD1 overactivity) altered migration properties of TNBC-derived MDA-MB-231 cells, affecting their speed, directionality, and morphology. These changes were mediated by a specific intracellular signaling axis involving phospholipase D (PLD) and mammalian target of rapamycin (mTOR) signaling, highlighting the potential of targeting MUFA metabolism in TNBC therapy.

To elucidate the molecular mechanisms underlying OA-induced migration and invasion, we investigated the impact of OA on TNBC cell migration with a particular focus on cell membrane remodeling. Using TNBC cell lines and bioinformatics tools, we found that OA stimulation rapidly induces membrane ruffling and enhances filopodia formation. OA treatment promotes the subcellular translocation of Cdc42 and the Arp2/3 complex, with Cdc42 playing a crucial role in filopodia formation and cell migration. Pharmacological inhibition of Cdc42—but not the Arp2/3 complex—abolishes OA-induced filopodia formation and migration. Furthermore, our findings implicate PLD in Cdc42-dependent filopodia formation, emphasizing its role in OA-driven cell motility. Elevated Cdc42 expression has been observed in breast tumor tissues and cell lines, and is correlated with poor survival in TNBC patients, supporting its relevance in cancer progression.

At the molecular level, we identified a novel mechanism by which OA enhances PLD2 activation via S-acylation. Using confocal microscopy, membrane isolation, and acylation assays, we demonstrate that OA promotes PLD2 S-acylation at Cys223 and Cys224, leading to its dissociation from lipid rafts and translocation to phosphatidylinositol 4,5-bisphosphate ($PIP_2$)-clusters. This relocalization enhances PLD2's guanine nucleotide exchange factor (GEF) activity toward Cdc42, facilitating actin cytoskeletal remodeling and filopodia-like protrusion formation.

Mutation of PLD2 acylation sites or disruption of lipid rafts abolishes its ability to activate Cdc42 and promote cell protrusion formation.

This study provides novel insights into how OA-driven lipid modifications influence membrane signaling and cytoskeletal dynamics, ultimately promoting cancer cell migration and metastasis. By delineating the molecular mechanisms underlying OA-induced PLD2 activation, we contribute to a broader understanding of lipid-mediated oncogenic signaling. Notably, our findings suggest that S-acylation plays a critical role in regulating PLD2 activity, pointing to lipidation as a potential therapeutic vulnerability. Targeting the S-acylation of PLD2 may therefore represent a novel strategy for interfering with its oncogenic function in TNBC. Given the poor prognosis and limited treatment options associated with TNBC, uncovering such metabolic vulnerabilities offers a promising avenue for the development of targeted therapies.

**Keywords:** Oleic acid; triple negative breast cancer; cell migration; filopodia; Cdc42; phospholipase D2; S-acylation; lipid raft, guanine nucleotide exchange factor

# CHAPITRE 1
# INTRODUCTION

## 1.1 Background

Breast cancer is the most frequently diagnosed cancer in women and remains the second leading cause of cancer-related deaths worldwide (Giaquinto *et al.*, 2022). Despite advancements in treatment, triple-negative breast cancer (TNBC), a highly aggressive subtype, continues to pose significant challenges due to its lack of hormone receptors and targeted therapeutic options (Landry *et al.*, 2022). Understanding the molecular mechanisms that drive TNBC progression is essential for identifying novel therapeutic targets.

Emerging evidence has revealed that alterations in lipid metabolism are strongly linked to cancer development and metastasis. Specifically, a metabolic shift toward increased monounsaturated fatty acid (MUFA) levels, mainly oleate (OA), is frequently observed in breast cancer cells (Guo, Z. *et al.*, 2023). This shift is mediated by stearoyl-coenzyme A desaturase-1 (SCD1), the enzyme responsible for converting saturated fatty acids (SFA) into MUFAs. Elevated SCD1 expression is a metabolic hallmark of several cancers, where it supports tumor cell proliferation, survival, and metastasis (Guo, Z. *et al.*, 2023). Importantly, elevated levels of MUFA contribute to plasma membrane remodeling by incorporating into membrane phospholipids and disrupting the structure of lipid rafts—specialized microdomains involved in organizing signal transduction (Lorent *et al.*, 2020). Such lipid-driven changes facilitate oncogenic signaling, influencing processes like cancer cell migration and invasion.

The previous study from our lab have demonstrated that inhibiting SCD1 or treating cells with OA (mimicking SCD1 overactivity) significantly altered the migration patterns of TNBC-derived MDA-MB-231 cells, including changes in migration speed, directionality, and cell morphology (Lingrand *et al.*, 2020a). Further investigation identified a specific intracellular signaling pathway involving phospholipase D (PLD) and the mammalian target of rapamycin (mTOR) (Lingrand *et al.*, 2020a). However, the precise molecular mechanism remains poorly understood.

## 1.2 Research Problem

Lipid metabolism reprogramming is a recognized hallmark of cancer (Yu, X. *et al.*, 2021), yet the molecular mechanisms linking altered lipid composition to cancer cell migration and metastasis remain insufficiently understood. Among these metabolic alterations, increased *de novo* lipogenesis, particularly the upregulation of SCD1 and its MUFA product, OA, has been strongly associated with breast cancer progression (Guo, Z. *et al.*, 2023). However, the precise mechanisms by which OA influences plasma membrane signaling to drive cancer cell migration remain largely unexplored.

PLD2 is a key regulator of cell membrane dynamics and is known to promote cancer cell migration by generating the lipid second messenger phosphatidic acid (PA) (Gomez-Cambronero, 2014). PA plays a central role in cytoskeletal remodeling, facilitating the formation of membrane ruffles and cellular protrusions, which are critical for cell motility (Bruntz *et al.*, 2014). While previous studies suggest that OA specifically activates PLD2 (Gibbs et Meier, 2000; Kasai *et al.*, 1998; Kim, J. H. *et al.*, 1999; Sarri *et al.*, 2003), the underlying molecular mechanism of this selective activation remains unclear.

OA has been shown to influence membrane lipid organization and microdomain dynamics, which are essential for the spatial regulation of signaling proteins (Lorent *et al.*, 2020). PLD2 activation involves membrane microdomain translocation (Petersen *et al.*, 2016). And S-acylation, specifically oleoylation, can regulate protein activity by translocation away from lipid raft (Nuskova *et al.*, 2021). Therefore, it is plausible that OA induces regulates PLD2 through S-acylation and its redistribution across membrane microdomains. Elucidating this mechanism could offer critical insights into how OA contributes to cancer cell migration and metastasis, potentially revealing novel therapeutic targets for breast cancer treatment.

## 1.3 Objectives

We aim to study the role of OA in breast cancer cell migration and the underlying molecular mechanisms, with particular attention to the signaling events associated with cell morphology changes. We also aim to study the underlying molecular mechanisms of PLD2 activation by OA with special attention towards protein S-acylation.

## 1.4 Working Hypothesis

### 1.4.1 Hypothesis 1: OA induces breast cancer cell morphological change to promote cell motility.

We hypothesize that in aggressive TNBC cells, OA induces cell membrane ruffling and the formation of cell protrusions, showing an attenuation of lamellipodia and increase of filopodia. The transition of lamellipodia-based migration to filopodia-based migration subsequently leads to a faster migration speed and migration directionality persistence, contributing to cell migration during tumor metastasis. As Cdc42 and Arp2/3 complex are the representative key regulators in filopodia and lamellipodia signaling pathways respectively, we hypothesize that OA could affect their activities.

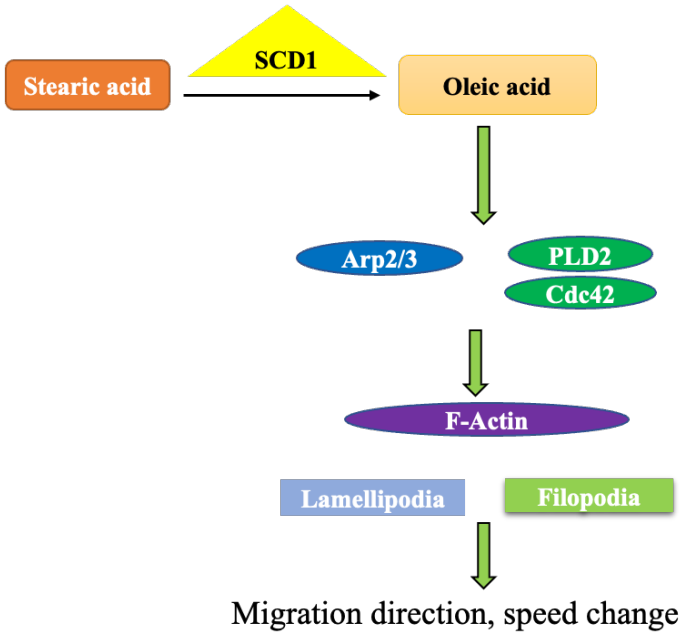


**Figure 1.1 Proposed model of hypothesis 1.**

### 1.4.2 Hypothesis 2: OA activates PLD2 via S-Oleoylation.

We hypothesize that OA activates PLD2 by inducing oleoylation at its S-acylation sites (Cys223 and Cys224), thereby regulating its subcellular localization and enzymatic activity. Specifically, we propose that OA-driven oleoylation displaces palmitoylation at these sites, causing PLD2 to dissociate from cholesterol-rich lipid rafts and translocate to PIP2 clusters to enhances PC

hydrolysis and promotes the generation of PA. The activation of PLD2 contributes to downstream signaling events that promote cell migration.

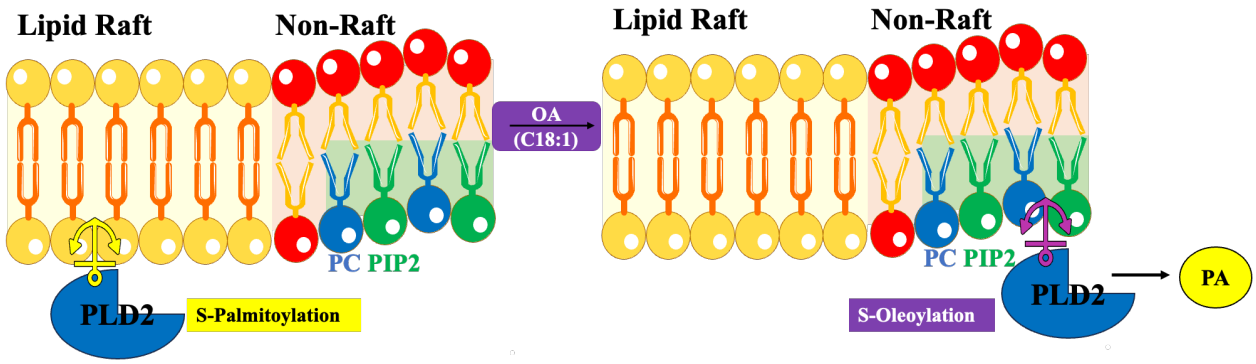


**Figure 1.2 Proposed model of hypothesis 2.**

## 1.5 Research Significance

Understanding the role of lipid metabolism in cancer progression is crucial for identifying new therapeutic targets, especially for aggressive and treatment-resistant cancers like TNBC. This study could provide novel insights into how OA-driven lipid modifications influence membrane signaling and cytoskeletal dynamics, subsequently promoting cancer cell migration in tumor metastasis.

By elucidating the molecular mechanisms governing OA-induced PLD2 activation, this research enhances our understanding of lipid-driven oncogenic signaling. Furthermore, by assessing the therapeutic potential of targeting MUFA metabolism and PLD2 signaling, this study could provide a foundation for novel lipid-based therapeutic interventions in TNBC. Given the poor prognosis associated with TNBC and its limited treatment options, identifying metabolic vulnerabilities within cancer cells offers a promising avenue for the development of targeted therapies.

## 1.6 Thesis Structure

This thesis is structured as follows:

- **Chapter 1: Introduction** – Provides the background, research problem, objectives working hypothesis, research significance, and thesis structure.
- **Chapter 2: Literature Review** – Covers relevant background studies on MUFA-cancer metabolism (based on our published **Review Article**), overview of breast cancer, tumor metastasis and cell migration, lipidation, cell membrane dynamics, and PLD.
- **Chapter 3: Research Article 1** – *Oleate Promotes Triple Negative Breast Cancer Cell Migration by Enhancing Filopodia Formation through a PLD/Cdc42-Dependent Pathway.*
- **Chapter 4: Research Article 2** – *Oleate Activates PLD2 Lipase and GEF Activity by Modulating Membrane Microdomain Dynamics via S-acylation.*
- **Chapter 5: General Discussion**– Integrates findings from the individual research aims and discusses their broader implications for cancer biology.
- **Chapter 6**: **Conclusion and Future Perspectives –** Final conclusion and perspectives on future research.

# CHAPITRE 2
# LITERATURE REVIEW

## 2.1 MUFA and Cancer[1]

The rise in overweight and obesity over the last decades has become a public health concern worldwide (Karpinska-Mirecka *et al.*, 2021). There is consistent evidence that a higher amount of body fat is associated with increased risk for several cancer types, including stomach, pancreatic, liver, colorectal, and breast cancers (Lauby-Secretan *et al.*, 2016; Stephenson et Rose, 2003). Obesity and cancer are linked in a complex and multi-factorial manner. Obesity is associated with several metabolic and hormonal alterations that increase the risk of cancer in patients. One main driver for obesity is believed to be an overall rise in caloric intake, through increased consumption of carbohydrates and fat (Stone *et al.*, 2018). Furthermore, it is becoming increasingly evident that the nutritional state of a patient plays a role in their response to cancer therapy (Kanarek *et al.*, 2020). In obesity, excess body fat leads to alterations in lipid metabolism, including increased levels of circulating lipids, such as cholesterol and triglycerides. These lipid metabolism states can contribute to the development of cancer by promoting inflammation, oxidative stress, insulin resistance, hormone imbalance as well as chronically activating growth factor signaling, which can all increase the risk of cancer (Dibaba *et al.*, 2019; Guevara-Aguirre et Rosenbloom, 2015; Lashinger *et al.*, 2014; Lauby-Secretan *et al.*, 2016; Stone *et al.*, 2018). Cancer cells derive most of their energy from the breakdown of lipids originating from *de novo* lipogenesis or the diet (Wang, W. *et al.*, 2020). There are changes in lipid metabolism that can support the growth and progression of cancer cells. For example, cancer cells have increased levels of some lipids, such as cholesterol, phospholipids, and fatty acids (FAs), which can contribute to rapid cancer cell growth and tumor formation (Fu, Y. *et al.*, 2021; Snaebjornsson *et al.*, 2020). Additionally, cancer cells display

[1] This section is based on our published review article (including all the figures): *Unveiling the MUFA-Cancer Connection: Insights from Endogenous and Exogenous Perspectives*. Manuscript published in *International of Molecular Sciences* 2023, 2023 Jun 8;24(12):9921. DOI: 10.3390/ijms24129921. All co-authors have given their consent for this article to be used in this thesis.

altered levels of FA metabolism, which can support the development of resistance to chemotherapy and radiation therapy. As such, modulation of lipid uptake and metabolism are gaining much interest in the field, and new cancer treatment strategies are expected to emerge from these studies (Kanarek *et al.*, 2020; Prendeville et Lynch, 2022; Snaebjornsson *et al.*, 2020; Sotgia *et al.*, 2013; Vander Heiden *et al.*, 2009).

### 2.1.1 **Lipid uptake and metabolism in cancer**

Lipids encompass a heterogeneous group of biomolecules that serve multiple essential functions in biological systems, including constituting the structural basis of biological membranes, serving as signaling molecules and energy sources (Fu, Y. *et al.*, 2021; Karagiota *et al.*, 2022; Snaebjornsson *et al.*, 2020). In mammals, the main lipid class of molecules comprises FAs, acylglycerols, phospholipids, sterols, and sphingolipids (Karagiota *et al.*, 2022; Pope *et al.*, 2019; Snaebjornsson *et al.*, 2020). Endogenous lipogenesis and exogenous (dietary) uptake are the main lipid supply sources for either normal or cancer cells (see Figure 2.1). Firstly, except for liver and adipose tissue, most tissues possess little capacity for *de novo* FA synthesis and depend on FA uptake for their needs (Su et Abumrad, 2009). Circulating lipids provided by the liver or adipose tissues can be taken in through the receptor-mediated endocytosis of low- or very-low-density lipoproteins (LDL/VLDL). Lipids are also imported via specific transmembrane transporters, such as the fatty acid-binding proteins (FABPs) and CD36 FA translocase, as well as members of the FA transport proteins (FATP1-6) and solute carrier family 27 family (SLC27A1-6) (Anderson et Stahl, 2013; Su et Abumrad, 2009). In addition, during *de novo* lipogenesis, FAs are synthesized from cytoplasmic acetyl-CoA. Citrate, produced in the mitochondrial tricarboxylic acid cycle, is the main source of acetyl groups for FA biosynthesis. Acetyl-CoA is activated by acetyl-CoA carboxylases (ACC1/2) to form malonyl-CoA, which can be subsequently condensed via several steps catalyzed by the fatty acid synthase (FASN) to form the 16-carbon saturated FA palmitic acid. Palmitic acid can then be elongated by FA elongases (ELOVLs) and/or desaturated by SCDs or fatty acid desaturases (FADSs) to form unsaturated FAs, such as the 16- and 18-carbon MUFAs palmitoleic acid and OA (Currie *et al.*, 2013; Hilvo *et al.*, 2011; Karagiota *et al.*, 2022; Pope *et al.*, 2019; Prendeville et Lynch, 2022; Snaebjornsson *et al.*, 2020).

Altered metabolism is one of the most prominent hallmarks of cancer. The most understood metabolic change in cancer cells is the Warburg effect, which is the use of fermentation, even in

the presence of oxygen, to generate ATP. It is characterized by an increase in glucose uptake and consumption, a decrease in oxidative phosphorylation, and the production of lactate. As a corollary to this metabolic modification, cancer cells use carbon from glucose to build other biomolecules instead of completely oxidizing it to carbon dioxide (Currie *et al.*, 2013; Warburg, 1956). In rapidly proliferating cancer cells, among other metabolic fuels, fatty acids are also an important source of energy. Rapid cancer cell growth and tumor formation demand increased lipid metabolism to meet their energy needs (Fu, Y. *et al.*, 2021; Yu, X. *et al.*, 2021). In non-cancer cells, a balance is maintained between lipogenesis and lipid degradation. Whereas in cancer cells, lipids derived from *de novo* lipid synthesis are an important source of energy, therefore expression and activity of enzymes involved in lipid synthesis and transformation are increased, making them more independent from externally provided lipids (Medes *et al.*, 1953; Rohrig et Schulze, 2016; Swinnen *et al.*, 2006). Moreover, rapid cancer cell proliferation and tumor formation also demand increased lipid metabolism to meet cell membrane synthesis needs (Fu, Y. *et al.*, 2021; Yu, X. *et al.*, 2021). Several studies have demonstrated that an increase in ATP citrate lyase activity (catalyzing formation of cytosolic acetyl-CoA from mitochondria-derived citrate) and ACC1/2 activity are found in many cancers, such as breast, liver, ovarian and colorectal cancer (Brown, J. M. et Rudel, 2010; Chajes *et al.*, 1999; Flowers et Ntambi, 2009; Long *et al.*, 2016; Ntambi *et al.*, 2004; Popeijus *et al.*, 2008; Scaglia et Igal, 2008). Similarly, FASN also shows increased expression in cancers such as breast and prostate cancer, and correlates with poor disease prognosis (Brown, J. M. et Rudel, 2010; Chajes *et al.*, 1999; Flowers et Ntambi, 2009; Ntambi *et al.*, 2004; Popeijus *et al.*, 2008; Scaglia et Igal, 2008). The increase in *de novo* FA synthesis in cancer cells alters cellular lipid composition and can be used for diagnostics (Pala *et al.*, 2001). The limiting step in the synthesis of *de novo* MUFAs, SCD activity, has also been found to be elevated in cancer cells (Guo, S. *et al.*, 2014; Popeijus *et al.*, 2008). Thus, the proportion of MUFAs could also be used as an important biomarker in cancer screening (Chavarro *et al.*, 2013; Ntambi *et al.*, 2004; Zureik *et al.*, 1995). In parallel to the role of lipogenesis, cellular FA uptake was also implicated in the progression of some carcinomas (Flowers et Ntambi, 2009; Popeijus *et al.*, 2008). The relative contribution of *de novo* synthesis and uptake depends on the availability of different lipid species within the extracellular milieu. While this can be influenced by the lipid composition of the diet, heterogeneity in the tumor microenvironment, due to ongoing vascularization, also has a major effect on local lipid availability (Fu, Y. *et al.*, 2021; Snaebjornsson *et al.*, 2020). In addition to FA

synthesis and uptake, altered lipid metabolism in cancer cells also impacts energy production. For instance, overexpression of fatty acid oxidation (FAO) enzymes has been observed in various cancer types (Ma *et al.*, 2018). Inhibition of FAO has been shown to reduce tumor growth in multiple experimental tumor models (Fu, Y. *et al.*, 2021; Snaebjornsson *et al.*, 2020). Certain enzymes involved in β-oxidation, such as α-methylacyl-CoA racemase (AMACR) and carnitine palmitoyl transferase 1B (CPT1B), are specifically upregulated in colorectal, hepatic, and prostate cancers, whereas CPT1A is elevated in breast cancer (Koundouros et Poulogiannis, 2020; Ma *et al.*, 2018; Monaco, 2017; Wu, X. *et al.*, 2014). Furthermore, altered FA metabolism is also involved in oncogenic signaling, cancer epigenetic alterations, supporting tumorigenesis and cancer progression, and driving cancer stem-like cell phenotypes (see review (Koundouros et Poulogiannis, 2020) and (Snaebjornsson *et al.*, 2020)). Considering the extensive roles of FAs in cancer pathogenesis via the interplay between oncogenic signaling and lipid metabolism, regulating processes involved in cancer cell growth, survival, dissemination and metastases formation, there is potential for treatment strategies that leverage the selective metabolic vulnerabilities caused by these changes (Fu, Y. *et al.*, 2021; Koundouros et Poulogiannis, 2020; Snaebjornsson *et al.*, 2020; Wang, W. *et al.*, 2020).

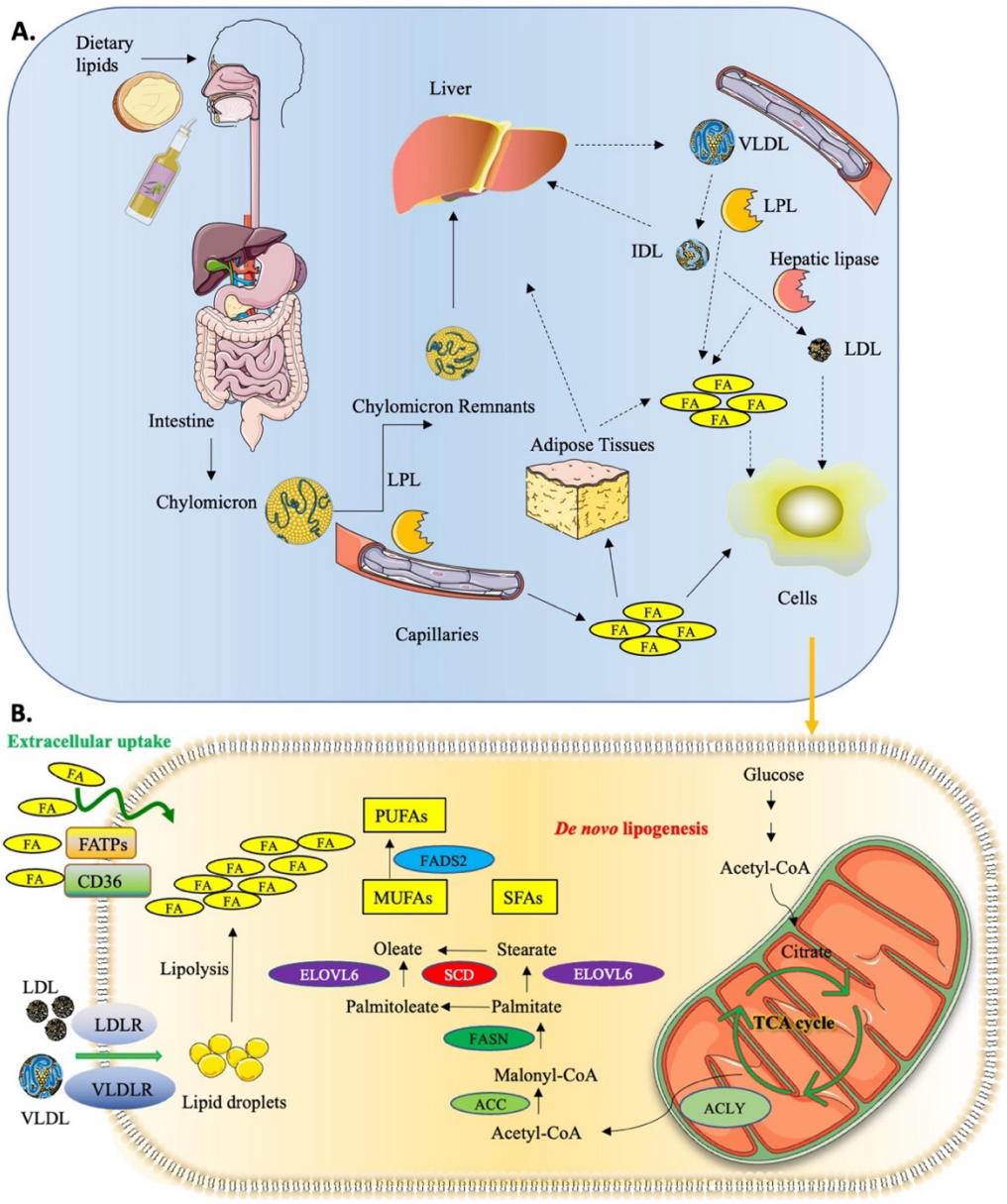


**Figure 2.1 Dietary lipid uptake and lipid metabolism.**

**(A)** Dietary lipids are digested by the digestive system, absorbed in the intestine, and converted to triglycerides and cholesterol, which are then incorporated into chylomicrons. Lipoprotein lipase (LPL) hydrolyzes the triglycerides in chylomicrons to fatty acids (FA), allowing them to enter the lymphatic and circulatory system. The released FAs can be stored in adipose tissues or directly taken up by cells. The chylomicron remnants are cleared from circulation by the liver, which in turn releases very-low-density lipoproteins (VLDL) into the circulation where they are hydrolyzed into intermediate-density lipoproteins (IDL) by LPL. IDL is then converted to low-density lipoprotein (LDL) by hepatic lipase or taken up by liver via the LDL receptor. Circulating lipoproteins and released FAs can be taken up by cells. Dotted lines indicate that endogenous lipid sources are involved. **(B)** At the cellular level, circulating FAs might enter the cell through simple diffusion and/or via some membrane transporters, such as fatty acid transport proteins (FATPs) and FA binding protein CD36. Cells can internalize circulating lipoproteins via their cognate receptors (LDLR/VLDLR) and release FAs from them by intracellular lipolysis. FAs can also be

biosynthesized intracellularly by *de novo* lipogenesis from acetyl-CoA produced by the mitochondrial tricarboxylic acid (TCA) cycle. ATP citrate lyase (ACLY) provides acetyl groups to the acetyl-CoA carboxylase (ACC) and the fatty acid synthase (FASN), allowing the synthesis of saturated fatty acid (SFA) palmitate. Palmitate can then be elongated by the fatty acid elongases (ELOVL) and/or desaturated by stearoyl-CoA desaturases (SCDs). Monounsaturated fatty acids (MUFAs) produced by SCD can be further desaturated by fatty acid desaturases (FADS) to yield non-essential polyunsaturated fatty acids (PUFAs). The figure was created using Servier Medical Art image templates under a Creative Commons Attribution 3.0 Unported License. This figure is from (Guo, Z. *et al.*, 2023).

### 2.1.2 **MUFA biosynthesis**

The primary desaturases that involved in *de novo* synthesis of MUFAs in human are SCDs, which are a family of enzymes localized in the membrane of the smooth endoplasmic reticulum (ER) (Castro *et al.*, 2011; Ntambi et Miyazaki, 2004). They are essential enzymes for the survival of organisms, from bacteria to mammals (Alloatti *et al.*, 2011; Horikawa *et al.*, 2008; Igal, 2011; Ntambi, 1999). There are five SCD isoforms (SCD1-5) in vertebrates (Castro *et al.*, 2011). In humans, only two variants exist: SCD1 and SCD5 (Ntambi et Miyazaki, 2004). The main isoform, SCD1, is expressed in most tissues (Castro *et al.*, 2011) while SCD5 is mainly expressed in embryonic tissues, but also in the brain and pancreas of adults (Wang, J. *et al.*, 2005). The SCDs catalyze the formation of a double bond in the delta-9 position of saturated fatty acids (SFAs), creating a single unsaturation. The main products of SCD1, OA and palmitoleic acid, are formed from stearic acid and palmitic acid respectively (Wang, J. *et al.*, 2005). The regulation of human SCD expression and functional activity has been discussed in many comprehensive reviews (see (Igal, 2016; Igal et Sinner, 2021)). Briefly, SCD1 expression is modulated by a variety of lipogenic transcriptional factors that bind to the gene promoters. Sterol regulatory element binding protein 1 (SREBP1) and carbohydrate response element binding protein (CREBP) act synergistically in the induction of *SCD1* expression (and of other lipogenic genes) in response to insulin and glucose, respectively (Dentin *et al.*, 2004; Tracz-Gaszewska et Dobrzyn, 2019). Regulation of expression is complexified by the binding of other transcription factors, such as PPARα, liver X receptor, CCAAT/enhancer binding protein α, nuclear transcription factor Y, neurofibromin 1 and specificity protein 1, all of which are activated by various growth factors, cytokines, hormones, as well as nutritional status (Igal, 2016; Tracz-Gaszewska et Dobrzyn, 2019). Of note, phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling was found to enhance SCD1 expression as part of the mechanism of lipogenesis activation in cancer cells (Igal, 2016; Igal et Sinner, 2021; Qiang *et al.*, 2015; Ricoult *et al.*, 2016). Interestingly, *SCD1*

is directly targeted by the tumor suppressor p53, suggesting that an increase in SCD1 expression and activity can be a key event in the development of cancer (Igal, 2016; Igal et Sinner, 2021; Kirschner *et al.*, 2015).

Despite the central role of canonical SCDs and their products OA and palmitoleate in MUFA studies, recent research has found other relatively rare MUFA isomers in certain tissues and cancer cells. These unusual MUFA are catalyzed by other FADS (Cheng *et al.*, 2023; Vriens *et al.*, 2019; Young *et al.*, 2021). For example, a recent study identified elevated sapienate, desaturated from palmitate by FADS2, in some cancer lines (Vriens *et al.*, 2019). Sapienate supported cancer cell membrane biosynthesis and proliferation in a SCD-independent way, which increased cancer plasticity (Vriens *et al.*, 2019). An in-depth lipidomic study of prostate cancer cells revealed a diversity of unusual MUFAs, such as n-5, n-13, n-8, n-10, and n-12 FAs, which are related to FADS1/2 activity (Young *et al.*, 2021). In addition, a lipidomic study in breast cancer cells reported that the inhibition of SCD1 led to the increase of n-10 MUFA isomers that depended on FADS2. Interestingly, high FAO activities were found in some specific subtypes of human breast cancer cell lines, which are correlated with cancer metastasis and invasiveness (Cheng *et al.*, 2023). These results indicate that a diversity of alternative MUFA metabolic pathways is involved in lipid metabolism to promote cancer plasticity.

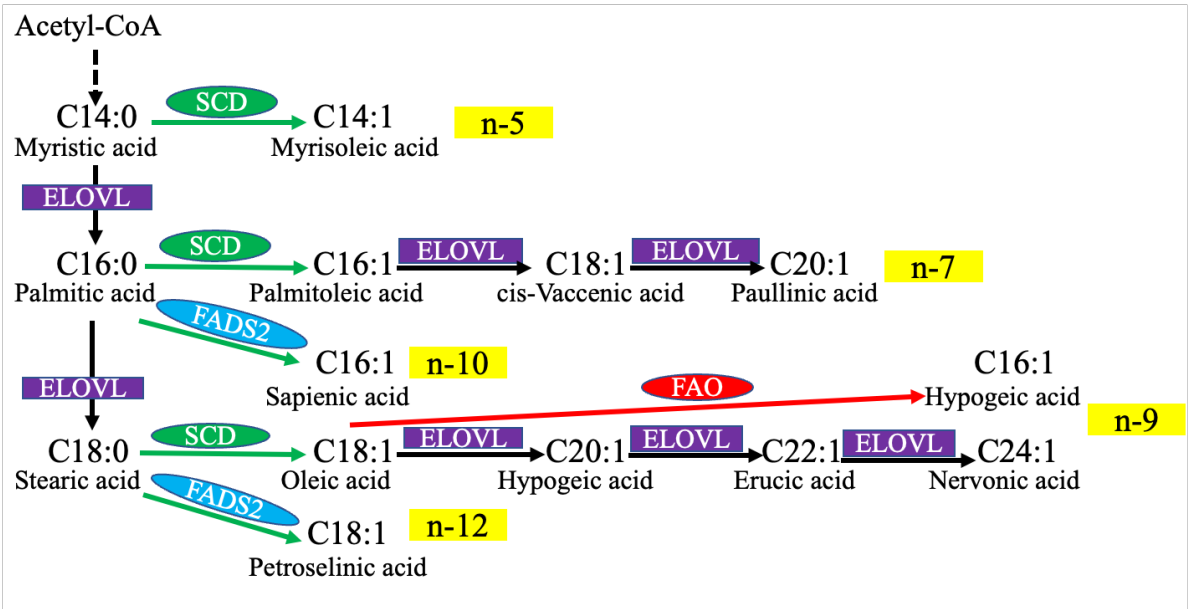

**Figure 2.2 Biosynthetic pathways of main cis-monounsaturated fatty acids.**

SCD, stearoyl-CoA desaturase (1 and 5); FADS2, fatty acid desaturase 2; FAO, fatty acid oxidation; ELOVL, fatty acid elongase**.** Black arrows indicate fatty acid elongation. Green arrows indicate fatty acid desaturation. The red arrow indicates fatty acid oxidation. The figure is modified from (Young *et al.*, 2021) and (Cheng *et al.*, 2023) , and published in (Guo, Z. *et al.*, 2023).

### 2.1.3 **Exogenous MUFA and cancer**

An understanding of the impact of MUFA on cancer progression must consider the role of circulating MUFA, most prominently derived from the diet. They are commonly found in foods such as olive oil, avocados, nuts, and seeds, and have been the subject of extensive research due to their potential health benefits (Snaebjornsson *et al.*, 2020). The common MUFAs and their outcomes on cancer have been summarized in Table 2.1.

**Table 2.1 Summary of common MUFAs and effects on cancer.**

| MUFA | | Source | Main outcome | Reference |
|---|---|---|---|---|
| Myristoleic acid | 14:1 (n-5), cis | Small amounts in nutmeg and nutmeg butter | Anti-cancer effects in prostate cancer cells | (Iguchi *et al.*, 2001) |
| Palmitoleic acid | 16:1 (n-7), cis | Nuts, meats, animal fats | Related to cancer death and rescued SCD1 blockade anti-cancer effects | (Bermudez *et al.*, 2022; Byberg *et al.*, 2014; Hess *et al.*, 2010; Scaglia et Igal, 2008) |
| Hypogeic acid | 16:1 (n-9), cis | Human milk | Limited studies | (Astudillo *et al.*, 2020; Bermudez *et al.*, 2022) |
| Sapienic acid | 16:1 (n-10), cis | Human sebum | Increased in lung and liver carcinomas and contributed to SCD inhibition resistance | (Bermudez *et al.*, 2022; Kucuksayan *et al.*, 2022; Vriens *et al.*, 2019) |
| cis-Vaccenic acid | 18:1 (n-7), cis | Sea buckthorn oil | Inhibited colon cancer cell growth | (Awad, A. B. *et al.*, 1995) |
| Vaccenic acid | 18:1 (n-7), trans | Human milk, dairy products | Inhibited cancer cell growth, proliferation, and induced apoptosis | (Lim *et al.*, 2014; Song, J. *et al.*, 2019) |

| | | | | |
|---|---|---|---|---|
| Paullinic acid | 20:1 (n-7), cis | The seed oil of the plant *Pangium edule* | Limited studies | (Avato *et al.*, 2003) |
| Oleic acid | 18:1 (n-9), cis | Vegetable oils, such as olive oil, rapeseed oil and sesame oil | Both cancer-promoting and anti-cancer effect effects | See Section 2.1.3 |
| Elaidic acid | 18:1 (n-9), trans | Small amounts in caprine, bovine milk and some meats | Promoted survival, growth, and invasion of the colorectal cancer cell lines | (Lim *et al.*, 2014; Ohmori *et al.*, 2017; Song, J. *et al.*, 2019) |
| Petroselinic acid | 18:1 (n-12), cis | Several animal and vegetable fats and oils | Limited studies | (Delbeke *et al.*, 2016) |
| Gondoic acid | 20:1 (n-9), cis | Plant oils and nuts, such as jojoba oil. | Limited studies | (Awad, N. A. *et al.*, 2022; Farag et Gad, 2022) |
| Gadoleic acid | 20:1 (n-11), cis | Some fish oils, such as cod liver oil | Limited studies | (Christiansen *et al.*, 1977; Farag et Gad, 2022) |
| Erucic acid | 22:1 (n-9), cis | Brassica seeds, Indian mustard, rapeseed | Anti-cancer activity in brain cancer and glioblastoma | (Altinoz *et al.*, 2018; Altinoz *et al.*, 2021; Peng, Y. *et al.*, 2009) |
| Brassidic acid | 22:1 (n-9), trans | Seeds of certain brassica crops, such as mustard, rapeseed, and kale. | Limited studies | (Christiansen *et al.*, 1977) |
| Nervonic acid | 24:1 (n-9), cis | Animal brain, plant seed oil | Limited studies | (Farag et Gad, 2022; Liu, F. *et al.*, 2021; Umemoto *et al.*, 2021) |

This table is from (Guo, Z. *et al.*, 2023).

#### 2.1.3.1 Dietary MUFA and cancer risk – evidence from human studies

OA is the most abundant MUFA in the human diet, accounting for around 20% of FAs in most fat sources. It is highly enriched in olive oil, where it reaches almost 80% of FAs (Gunstone, 1996; Prendeville et Lynch, 2022). High consumption of olive oil is a main feature of the Mediterranean diet, which is renowned for its health benefits and protective effect on cardiovascular diseases, diabetes, obesity, as well as cancer (Assy *et al.*, 2009; Davis *et al.*, 2015; Gunstone, 1996; Morze *et al.*, 2021; Psaltopoulou *et al.*, 2011; Schwingshackl *et al.*, 2017). A recent comprehensive meta-analysis of 117 studies including 3,202,496 participants evaluated the association between the Mediterranean diet and cancer risk (Morze *et al.*, 2021). The highest adherence score to a Mediterranean diet was inversely associated with mortality in cases of breast, colorectal, head and neck, respiratory, gastric, bladder, and liver cancers. However, cancers risk of blood, esophageal, pancreatic, and prostate was not modified (Morze *et al.*, 2021; Schwingshackl *et al.*, 2017). Furthermore, individual component analyses showed that the protective effects towards cancer risk were mostly attributed to lower alcohol consumption, whole grain intake, as well as fruit and vegetable intake. No clear association was identified for olive oil through this approach (Morze *et al.*, 2021; Schwingshackl *et al.*, 2017). These results hint to a complex, multi-target impact of the Mediterranean diet, of which olive oil is only one component. To better understand the role of dietary MUFA in cancer risk, we reviewed the recent epidemiological and clinical studies focusing on MUFA-enriched diets, mostly based on olive oil.

Several studies have shown that MUFA intake was associated with a decreased risk of cancer. A meta-analysis of 38 studies found that olive oil consumption was positively associated with lower odds of developing breast cancer and digestive cancers (colorectal, oral cavity, pharynx, oesophagus, and pancreatic cancers) (Psaltopoulou *et al.*, 2011). A randomized clinical trial performed in Spain found a significant inverse association between consumption of a Mediterranean diet supplemented with extra-virgin olive oil and breast cancer incidence. A high consumption of extra-virgin olive oil (≥15% of total energy intake) is instrumental for obtaining this significant protection (Toledo *et al.*, 2015). Similarly, another research study also reported an inverse association between OA intake and breast cancer (Binukumar et Mathew, 2005). In the *European Prospective Investigation into Cancer and Nutrition* cohort, dietary total MUFA was inversely associated with colon cancer, but not rectal cancer (Aglago *et al.*, 2021). A cohort study

using 7-day food diaries in England assessed dietary OA in the etiology of pancreatic cancer. They found a large, dose-dependent, inverse associations between OA intake and pancreatic cancer risk (Banim *et al.*, 2018). Another case-control study of 462 cases of pancreatic cancer and 4721 controls from 8 Canadian provinces, reported that dietary MUFAs were associated with 28% reduced risk of pancreatic cancer (Nkondjock *et al.*, 2005). Furthermore, the results from the French prospective cohort *NutriNet-Santé* showed that MUFA intake was associated with a decreased risk of digestive cancers (oesophagus, liver, stomach, pancreas and colorectal cancers) (Sellem *et al.*, 2019). A New Zealand study showed that increasing intake of MUFA-enriched vegetable oil was associated with a progressive reduction in prostate cancer risk (Norrish *et al.*, 2000). In addition to olive oil, another study found that avocado intake, as a source of dietary MUFA, was associated with reduced risk of prostate cancer (Jackson *et al.*, 2012). Erucic acid (C22:1) is rich in the Chinese diet. This might contribute to the lower incidence of brain cancer in Chinese children as high levels of erucic acid have been found in the breast milk of Chinese women (Peng, Y. *et al.*, 2009).

On the other hand, some studies have demonstrated either no correlation or an elevated risk of cancer development with MUFA consumption. A meta-analysis of 10 studies, including 8 case-control studies and 2 prospective studies, examined the association between olive oil intake and breast cancer risk. Although it suggested a potential inverse association between MUFA consumption and breast cancer, it was not statistically significant (Sealy *et al.*, 2021). Similarly, other meta-analysis studies also reported that there was no significant positive or negative trend on breast cancer risk with dietary MUFA intake (Binukumar et Mathew, 2005; Cao *et al.*, 2016; Lodi *et al.*, 2022; Xin *et al.*, 2015). A Korean colorectal adenoma study found that there was no significant association with MUFA intake in adults (Kim, J. *et al.*, 2017). Observational studies including 13 case-control studies and 7 prospective studies showed no significant difference between high *versus* low MUFA intake and pancreatic cancer risk (Yao et Tian, 2015). No correlation between the intakes of MUFA and pancreatic cancer was observed in a large cohort of US women during the subsequent 18 years of follow-up in the *Nurses' Health Study* (Michaud *et al.*, 2003). A case-control study from southeast China based on questionnaire also failed to show a statistically significant association between MUFA intake (including C14:1, C18:1, C20:1 and C22:1) and incidence of oral cancer (Fan *et al.*, 2022). Interestingly, in a hospital-based large-scale case–control study, replacement of MUFAs with carbohydrates, SFAs and PUFAs for every 50

kcal of energy associated with an increased odds of breast cancer (Sasanfar *et al.*, 2022). Dietary MUFA was also reported to be associated with an increased risk of pancreatic cancer in a case-control study from the San Francisco Bay and a large prospective cohort from NIH-AARP Diet and Health Study (Gong *et al.*, 2010; Thiebaut *et al.*, 2009). And a population-based cohort study performed on Chinese men showed that dietary intake of MUFA was associated with an increased risk for liver cancer (Ji *et al.*, 2021). Lastly, a recent follow-up study from the Nurses' Health Study and the Health Professionals showed a positive association between MUFA intake and colorectal cancer risk (Wan *et al.*, 2022).

Overall, the evidence from human studies regarding the association between dietary MUFA and cancer risk is ambiguous, particularly in pancreatic and colorectal cancers where reported results are contradictory. One possible reason is that dietary MUFAs come from different sources, such as plant and animal sources, with the presence of additional others dietary components that might potentially obscure the associations between MUFAs and health outcomes (Bojkova *et al.*, 2020). In two large prospective cohorts of U.S. men and women, total MUFAs and MUFA intake from plants were inversely associated with total mortality after adjusting for potential confounders, whereas MUFA intake from animal sources were associated with higher mortality (Guasch-Ferre *et al.*, 2019). A recent study also showed that intake of MUFA tended to be positively associated with the risk of colorectal cancer while this positive association was mainly driven by dietary MUFAs coming from animal sources (Wan *et al.*, 2022). The specific FA composition may also influence the effects. For example, an increased risk of breast cancer was associated with increasing levels of the trans-MUFAs palmitoleic acid and elaidic acid while cis-MUFAs were unrelated to breast cancer risk (Chajes *et al.*, 2008). A diversity of minor compounds is contained in dietary sources of MUFA. In olive oil, although OA is the primary component, there are other FAs and many minor compounds in the unsaponifiable fraction. Of note, some of them have been defined as “bioactive compounds” and have been shown to exert chemopreventative effects on cancer, such as hydroxytyrosol (Cruz-Lozano *et al.*, 2019; Lu, H. Y. *et al.*, 2019), oleuropein (Lu, H. Y. *et al.*, 2021; Lu, H. Y. *et al.*, 2019), oleanolic acid (Chakravarti *et al.*, 2012), oleocanthal (Elnagar *et al.*, 2011), and pinoresinol (Lopez-Biedma *et al.*, 2016). Another study showed that OA and the representative minor components of olive oil have opposite effects. Treatment of colorectal cancer cell Caco-2 with OA (1-100 μM) induced DNA synthesis and cell growth, while minor compounds (hydroxytyrosol, oleuropein, pinoresinol, squalene, and maslinic acid; 0.1-10 μM) reverted these

effects. These results suggest that different sources of dietary MUFA, containing various minor compounds, can have different effects on cancer (Storniolo *et al.*, 2019). Lastly, the dosage of MUFA might also play an important role. For example, low OA concentrations increased $Ca^{2+}$ entry (related to cell proliferation) while higher OA concentrations inhibited it in HT29 human colorectal adenocarcinoma cells (Carrillo *et al.*, 2012). In a commentary paper, the authors expressed their concerns that high concentration and long-time treatment of OA could lead to apoptosis (Lin, 2021). The inconclusive results of the human studies described above highlight the need for more research in this area, using rigorous study design and methodology, to fully understand the potential relationship between dietary MUFA intake and cancer risk.

#### 2.1.3.2 Exogenous MUFA and cancer risk – evidence from animal models

As the evidence from human diet studies is inconclusive, animal studies could offer a more controlled environment in which to investigate the potential impact of MUFA consumption on cancer development. Access to animal tissues also allows for the investigation of the biological mechanisms involved. As such, experimental studies assessing the effects of dietary MUFA on cancer progression have been conducted in several animal models (see Table 2.2). These studies typically rely on feeding tumor-bearing animals a diet enriched with MUFA from sources such as olive oil. Occasionally, MUFA is directly injected into the animal, bypassing the digestive system.

MUFA-enriched diets have shown anti-cancer effects in animal models of colorectal and lung cancers. In a dextran sodium sulphate (DSS)-induced colon cancer mouse model, mice were put on 10 % sunflower oil (SFO) or 10% extra virgin olive oil (EVOO) diets. EVOO-fed mice showed less incidence and multiplicity of tumors than SFO-fed mice. β-catenin immunoreactivity, proinflammatory cytokine production (TNF alpha, IL-6, INF gamma), cyclooxygenase-2 (COX-2) and inducible nitric oxidase synthase expression were significantly lower in the colon tissue of animal group fed with EVOO than in the SFO group, which is indicative of lowered inflammation and colorectal carcinogenesis progression (Sanchez-Fidalgo *et al.*, 2010). In a dimethylhydrazine (DMH)-induced rat model of colon cancer, olive oil treatment lowered tumor incidence, multiplicity, and size, compared with treatment with DMH alone. Olive oil also reduced the expression of inflammatory and angiogenic markers (nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB), vascular endothelial growth factor (VEGF), and matrix metalloproteinase-9 (MMP-9)) and elevated the expression of pro-apoptotic markers (caspase-3

and 9) in DMH-treated rats (Nanda *et al.*, 2019). In an azoxymethane/DSS-induced model of intestinal cancer on mice where the *Scd1* gene is specifically knocked out of intestinal tissue, an OA-rich diet reduced intestinal inflammation and significantly decreased the number and size of tumors (Ducheix *et al.*, 2018). In a murine lung adenocarcinoma LAC-1 transplantation mouse model, a diet enriched with olein (a palm oil fraction rich in OA) significantly delayed adenocarcinoma progression, increasing tumor latency and mice survival (Piegari *et al.*, 2017).The effect of high OA (C18:1) peanut oil and high linoleic acid (LA; C18:2) SFO was investigated in a mouse model of lung tumorigenesis (induced by a nicotine-derived NNK (N-nitrosamine: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone). After 20 weeks of feeding, all LA-enriched diet fed mice showed lung tumors (6.3 tumors in average per mouse). In comparison, the OA-enriched diet fed mice presented a 25% lower incidence of lung tumors and a 31% reduction in the number of tumors per mouse, suggesting that OA specifically suppresses lung tumorigenesis in this model (Yamaki *et al.*, 2002).

However, other studies have suggested that a high MUFA diet may increase the risk of certain types of cancer. For instance, in a mouse model of pancreatic cancer, mice xenografted with HPAF cells were fed for 14 weeks with four different high-fat diets (15% fat, 4 kcal/g): SFA, MUFA, n-3 PUFA and n-6 PUFA. Except for the n-3 PUFA diet that decreased tumor viability, mice fed with the other diets, including the MUFA diet (15% olive oil), showed an increase in tumor weight compared with an isocaloric control diet (5% fat, 4 kcal/g) (Yu, M. *et al.*, 2015). In another study, nude mice implanted with cervical cancer HeLa cells were fed with a high olive oil diet (45% kcal fat). Compared to the control diet (10% kcal fat), the olive oil group showed a significantly increase in tumor weight, by more than 6-fold. Xenograft tumor tissues from the olive oil group exhibited poor differentiation and higher heterogeneity. Immunohistochemistry analyses of these sections further uncovered a significant increase in cell proliferation (PCNA-positive cells) following olive oil treatment (Zhang, X. *et al.*, 2019). Another study from this group also showed that the high olive oil diet aggravated cervical cancer metastasis. They injected HeLa cells into the tail vein to cause metastasis in the liver. Mice in the olive oil group displayed a higher metastasis incidence and a significant increase in the size of the metastatic nodules, suggesting an association between dietary OA and cancer progression (Yang, P. *et al.*, 2018). Lastly, a breast cancer study, using a female MMTV-neu(ndl)-YD5 transgenic mouse model (overexpression of *Erbb2*/*Neu*/*Her2*), compared the tumors effects of different dietary FA-enriched diets: 10%

safflower oil (n-6 PUFA), 3% menhaden oil + 7% safflower oil (marine-derived n-3 PUFA), 3% flaxseed + 7% safflower oil (plant-based n-3 PUFA), 10% olive oil (MUFA), or 10% lard (SFA). Marine n-3 PUFA best mitigated tumor outcomes, and MUFA, SFA, and plant n-3 PUFA showed performed similar intermediary outcomes, while n-6 PUFA-fed mice had the poorest outcomes. Examination of tumor tissue revealed that the phospholipid fractions (phosphatidylcholine and phosphatidylethanolamine composition) were enriched in the FA families included in each experimental diet, suggesting that dietary FAs may exert their biological effects through cell membrane-mediated mechanisms (Hillyer *et al.*, 2020).

Intriguingly, there are a few studies testing administration of refined OA to mouse models of cancer. In a tongue squamous cell carcinoma (TSCC) xenograft mouse model (CAL27 cells), intraperitoneal injection of OA had a marked inhibitory action on tumor growth. Immunohistochemical analyses of xenograft tumors showed that OA strongly inhibited p-Akt, p-mTOR and p-S6K expression, and induced caspase-3 cleavage, indicating that OA could have valuable anticancer effects on TSCC via autophagy and apoptosis (Jiang, L. *et al.*, 2017). In a recent study, OA was incorporated into nanoparticles and either intravenously injected or administered by gavage to a breast cancer xenograft mouse model (4T1 cells). OA nanoparticles accumulated in tumors and triggered significant inhibition of tumor growth (Fu, J. *et al.*, 2022). In a colorectal cancer xenograft mouse model (HCT116 cells), intragastric OA injection had no effect on tumor volume. However, tumor size was increased upon insulin injection and this effect was potentiated by OA (Zhang, Y. *et al.*, 2021). In another colorectal cancer xenograft mouse model (HC29 cells), nude mice were treated with elaidic acid and OA by gavage. The elaidic acid-treated group showed both increased subcutaneous tumor growth and metastases while the OA-treated group only showed increased peritoneal metastasis (Kishi *et al.*, 2018). And the enhanced metastasis results induced by elaidic acid could be attributed to the increased HT29 growth and stemness through the activation of EGFR in lipid rafts (Kishi *et al.*, 2018). Furthermore, in a lung colonization model where head and neck squamous carcinoma TU183 cells were injected into the tail vein of mice, preliminary injection of OA (mimicking high circulating free FAs) significantly increased metastatic seeding of the lungs (Shen, C. J. *et al.*, 2017).

The results from animal studies presented here suggest a complex relationship between MUFA intake and cancer risk, that might depend on a range of factors such as the type of tumor,

the specific type and dose of MUFA consumed, as well as other aspects of the diet. The mixed results from the few studies using purified OA have so far failed to clarify this complexity.

**Table 2.2 MUFA and mouse models of cancer.**

| Cancer type | MUFA source | Mouse model | Main outcome | Reference |
|---|---|---|---|---|
| Breast cancer | 10% olive oil diet | MMTV-neu(ndl)-YD5 transgenic mouse model | Mitigated tumor outcome (though not as efficiently as a 3% menhaden oil + 7% safflower oil mix) | (Hillyer *et al.*, 2020) |
| Breast cancer | OA nanoparticles, intravenous injection, and gavage | Xenograft of 4T1 cells | Inhibited tumor growth | (Fu, J. *et al.*, 2022) |
| Cervical cancer | high olive oil diet (45 kcal % fat) | Xenograft of HeLa cells | Increased tumor growth | (Zhang, X. *et al.*, 2019) |
| (Liver metastasis) | | (Subcutaneous and tail vein injection) | Increased tumor metastasis | (Yang, P. *et al.*, 2018) |
| CRC | OA, injected intragastrically at a dose of 2.0 g/kg/day | Xenograft of HCT116 cells | No difference compared with controls | (Zhang, Y. *et al.*, 2021) |
| CRC | 10% extra virgin olive oil diet | Chemically induced (DSS) | Reduced incidence and multiplicity of tumors | (Sanchez-Fidalgo *et al.*, 2010) |
| CRC | Olive oil 1g/kg through oral gavage | Chemically induced (DMH) | Inhibited tumor growth | (Nanda *et al.*, 2019) |
| CRC | Fatty acid-rich diet, 75% OA | Chemically induced (AOM/DSS) | Reduced body weight loss, number, and size of tumors | (Ducheix *et al.*, 2018) |
| CRC | Oral intake of OA and elaidic acid | Xenograft of HT29 cells (subcutaneous, spleen, tail vein, and peritoneum) | Increased tumor growth and metastasis | (Kishi *et al.*, 2018) |
| HNSCC/ Lung metastasis | OA, tail vein injection | Xenograft of TU183 cells (tail vein injection) | Induced metastasis | (Shen, C. J. *et al.*, 2017) |
| Lung cancer | 6% OA-enriched diet | LAC1 tumor transplantation | Inhibited tumor growth but no impact on metastasis | (Piegari *et al.*, 2017) |

| | | | | |
|---|---|---|---|---|
| Lung cancer | AIN-76A diet containing 10% OA | Chemically induced (NNK) | Reduced incidence and level of tumors | (Yamaki *et al.*, 2002) |
| Pancreatic cancer | 15% olive oil diet | Xenograft of HPAF cells | Increased tumor weight | (Yu, M. *et al.*, 2015) |
| TSCC | OA, injected intraperitoneally at 2/4 mg/kg | Xenograft of CAL27 cells | Reduced tumor volume and weight | (Jiang, L. *et al.*, 2017) |

CRC, colorectal cancer; HNSCC, head and neck squamous cell carcinoma; TSCC, tongue squamous cell carcinoma; DSS, dextran sulfate sodium; AOM, azoxymethane; LAC-1, lung adenocarcinoma 1; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. This table is from (Guo, Z. *et al.*, 2023).

#### 2.1.3.3 Exogenous MUFA and cancer cell behavior – evidence from cellular models

*In vitro* cell culture models afford the possibility of direct exposure to known concentrations of specific MUFA. As such, this approach has yielded strong data characterizing the influence of these specific FA on cancer cell behavior. Furthermore, various signaling pathways have been identified that underlie the mechanisms of these effects. The well delineated pathways triggered by OA are summarized in Figure 2.3.

##### 2.1.3.3.1 Effect on cell proliferation

Several studies have shown that OA significantly promotes the proliferation of breast cancer cells via signaling pathways dependent on the activation of G protein-coupled receptors (GPR) 40 and 120 (Hardy *et al.*, 2005; Marcial-Medina *et al.*, 2019; Yonezawa *et al.*, 2004). In the triple-negative breast cancer cell line MDA-MD-231, OA binds to GPR40, which is coupled to $G_i/G_o$ and $G_q$, results in the activation of PLC/PKC/$Ca^{2+}$, PI3K/Akt, and MEK1/2/Src pathways, promoting cell growth and proliferation (Hardy *et al.*, 2005). These pathways also are also implicated in promoting proliferation in prostate cancer cell lines PC3 and DU-145 (Liotti *et al.*, 2018). GPR40 and GPR120 are expressed in poorly invasive MCF-7 breast cancer cells. Cell proliferation of these cells was also induced following stimulation with OA. This effect is dependent on Src kinase activation and EGFR transactivation, ERK1/2 phosphorylation on Thr-202 and Tyr-204, and DNA binding of AP-1 (Soto-Guzman *et al.*, 2008). In renal cell carcinoma 786-O cells, OA activates integrin-linked kinase (ILK) via GPR40, resulting in the activation of Akt and COX-2, and subsequently promoting cell proliferation (Liu, Z., Xiao*, et al.*, 2013).

Moreover, OA treatment stimulated cell proliferation in a dose and time-dependent manner in the cervical cancer HeLa cell line. OA treatment increased the percentage of cells in S phase and decreases cells in G2 phase as well as higher number of colonies in colony formation assay. This proliferation effect is associated with CD36 up-regulation, the best characterized FA transporter. Inhibiting CD36 prevented the effect of OA on cell proliferation while overexpressing it mimicked the effects of OA (Yang, P. *et al.*, 2018). Furthermore, OA activates Src kinase and the downstream ERK1/2-dependent signaling pathway in a CD36-dependent manner (Yang, P. *et al.*, 2018). These results suggest that OA can promote cancer cell growth by inducing the expression of CD36, resulting in the activation of the Src/ERK signaling pathway (Yang, P. *et al.*, 2018).

2.1.3.3.2 Effect on cell survival

In HepG2 hepatocellular carcinoma cells, OA was found to facilitate survival through FABP5 – hypoxia-inducible factor-1 alpha (HIF-1α) axis, which plays a pivotal role in response to hypoxic stress. Under hypoxic conditions and following OA exposure, HIF-1α was activated and FABP5 was upregulated. OA treatment improved cell survival according to a colony-formation assay with increased colony number and size. This phenomenon was suppressed when FABP5 or HIF-1α were silenced, indicating that the FABP5/HIF-1α axis is involved in OA-driven hepatocellular carcinoma cell growth (Seo *et al.*, 2020). In addition, a role for OA in prolonging breast cancer cell survival has also been described. OA can protect human breast cancer cells (MDA-MB-231 and MCF10A) against palmitate-induced apoptosis in part by increasing the esterification of this free FA (FFA) into triacylglycerol (TG). And OA can protect cells against apoptosis induced by serum withdrawal by the upregulation of the TG–FFA cycle (Przybytkowski *et al.*, 2007). Another study showed that OA treatment following serum deprivation specifically promotes cancer cell survival, growth, and migration in highly aggressive carcinoma cell lines, including gastric carcinoma HGC-27 and breast carcinoma MDA-MB-231 cells, via AMPK activation (Li, S. *et al.*, 2014). In addition, in a co-culture system of adipocyte-breast cancer cells, OA secreted from adipocytes inhibited lipid peroxidation and ferroptosis of triple-negative breast cancer cells (Xie, Y. *et al.*, 2022). In a recent study, OA treatment promoted H460 lung cancer cell survival under glucose-deficient conditions by activating lipid metabolism and inhibit autophagy (Hwang *et al.*, 2022). In esophageal squamous carcinoma cells, high expression of the transcription factor BACH1 induced ferroptosis by inhibiting MUFA synthesis. OA significantly attenuated the

ferroptosis phenotypes and reversed the cell death of BACH1-overexpressing cells. OA was found to be incorporated in the cell membrane and to protect the tumor cell from ferroptosis (Xie, X. *et al.*, 2023). OA also plays a role in chemoresistance. OA treatment of PC3 and DU-145 prostate cancer cells had interfered with the decline of cell viability induced by docetaxel, the first-line chemotherapeutic agent for the treatment of androgen-independent prostate cancer. This effect was mediated by the GPR40 receptor, suggesting that OA and GPR40 might represent a new prognostic factor and a molecular target for the treatment of advanced prostate cancer (Liotti *et al.*, 2018). Another study found that elaidic acid, a *trans* form of OA, significantly enhanced survival of CT26 and HT29 colorectal cancer cell lines. Elaidic acid enhanced cell proliferation and bestowed drug resistance to 5-fluorouracil, demonstrating tumorigenic potential (Ohmori *et al.*, 2017). Nervonic acid (C24:1), a long-chain MUFA produced by OA elongation, was also reported to protect PC-12 pheochromocytoma cells from oxidative stress (Umemoto *et al.*, 2021).

2.1.3.3.3 Effect on cell migration and invasion

GPR40/120, EGFR and Akt-dependent pathways have been heavily involved in OA-induced migration in many cancer cell lines. In PC3 and DU-145 prostate cancer cells, OA was found to increase cell proliferation and migration via GPR40 and PI3K/Akt signaling (Liotti *et al.*, 2018). In MCF-7 and MDA-MB-231 breast cancer cells, OA induced cell migration and invasion. Cell migration was dependent on GPR40/120, EGFR, PI3K and Akt activity, whereas invasion was mediated though PI3K and Akt. Furthermore, OA promoted relocalization of paxillin to focal contacts in a PI3K and EGFR-dependent manner (Marcial-Medina *et al.*, 2019). Another study performed in MDA-MB-231 breast cancer cells found that OA induces MMP-9 secretion through a PKC, Src and EGFR dependent pathway, whereas it induced invasion via an EGFR, $G_i/G_o$ proteins, MMPs, PKC and Src. In contrast, OA did not induce an increase of MMP-9 secretion in MCF10A and MCF12A mammary non-tumorigenic epithelial cells. This suggests that OA has an important role in the invasion process and metastasis in breast cancer (Soto-Guzman *et al.*, 2010). In addition, an arachidonic acid (AA) dependent pathway was implicated in OA-triggered breast cancer cell migration (Navarro-Tito *et al.*, 2010; Soto-Guzman *et al.*, 2013). In MDA-MB-231 breast cancer cells, OA mediates the production of AA from membrane phospholipids through the activation of GPR40/120. AA metabolites then mediate focal adhesion kinase (FAK) phosphorylation and cell migration (Navarro-Tito *et al.*, 2010). Free AA is metabolized by COX-

2 and LOXs to produce eicosanoids. Eicosanoids bind and activate GPRs, which mediate EGFR transactivation, activation of MMPs and Src. OA can then promote cell migration through the signal transducers and activators of transcription 5 (Stat5), of which the activation requires Src, MMPs, COX-2 and LOXs (Soto-Guzman *et al.*, 2013). Furthermore, a recent study in our lab highlighted a phospholipase D2 (PLD2)/mTOR-dependent signaling pathway in OA-induced breast cancer cell migration. In wound healing assays, OA treatment increased wound recovery of MDA-MB-231, T47D and MCF-7 breast cancer lines. Analysis of migratory dynamics revealed OA increased the speed and the directionality of the migration of MDA-MB-231 cells. Further Transwell migration and invasion analysis showed that these changes were associated with the activation of PLD2 and mTOR (Lingrand *et al.*, 2020a).

In both colorectal cancer and head and neck squamous cell carcinomas (HNSCCs), OA was found to enhance cancer metastasis via angiopoietin like 4 (ANGPTL4) pathways (Shen, C. J. *et al.*, 2017; Shen, C. J. *et al.*, 2020). In HNSCC cell lines TU183 and HMEC-1, OA induced ANGPTL4 protein expression and secretion in PPAR dependent manner. The expression of ANGPTL4 induced epithelial-mesenchymal transition (EMT) markers vimentin, MMP-9, as well as fibronectin and its downstream effectors Rac1/Cdc42, which significantly promoted cell migration and invasion (Shen, C. J. *et al.*, 2017). In SW480 colorectal cancer cells, OA promoted cell migration and invasion by the induction of NADPH oxidase 4 (NOX4), accompanied with increased levels of ROS and MMP-1/9. NOX4 induction and activation were ANGPLT4 dependent (Shen, C. J. *et al.*, 2020). In addition, in 786-O renal carcinoma cells, OA was demonstrated to increase cell invasion in a dose-dependent manner, which was dependent on the ILK/COX-2/MMP-9 pathway (Liu, Z., Xiao*, et al.*, 2013). A study using a two-dimensional co-culture system to simulate the crosstalk between adipocytes and gastric cancer cells has shown that after co-culture with isolated omental adipocytes, gastric cancer cells exhibited significantly enhanced invasiveness. A lipidomic analysis showed that gastric cancer cells accumulated higher levels of OA during the co-culture. Further analysis in chick chorioallantoic membrane assays showed that OA treatment significantly promoted the invasiveness of gastric cancer cells and induced the expression of MMP-2 in gastric cancer cells by activating the PI3K/Akt signaling pathway in a PTEN-independent manner (Xiang *et al.*, 2017).

2.1.3.3.4 Effect on cancer suppression

Though the studies presented above support the view that OA promotes cancer progression, several studies demonstrated a more complicated association between OA and cancer. In low metastatic carcinoma cells, such as SGC7901 gastric carcinoma and MCF-7 breast carcinoma cell lines, OA inhibited cancer cell growth and survival (Li, S. *et al.*, 2014). Moreover, it was reported that relatively high concentration (1 mM) of OA could inhibit DNA and protein synthesis in Lewis lung carcinoma cells, while slightly increasing their adherence to human microvascular endothelial cell (Kimura, 2002). In human colorectal adenocarcinoma HT29 cells, OA both enhanced and inhibited store-operated $Ca^{2+}$ entry (SOCE), which is associated with proliferation. At low concentrations (1 and 10 uM), OA increased SOCE, but at higher concentrations OA potently inhibited it, suggesting that different concentrations of OA might trigger different mechanisms (Carrillo *et al.*, 2012). In TSCC, OA effectively inhibited cell proliferation in a dose- and time-dependent manner, which was associated with lower activation of specific downstream signaling pathways as indicated by the phosphorylation level of key proteins (p-Akt, p-mTOR, p-S6K, p-4E-BP1 and p-ERK1/2). In OE19 and OE33 human esophageal cancer cells, OA downregulated cell proliferation, adhesion, and migration via activation of tumor suppressor genes p27, p21 and p53. OA also increased AMPK phosphorylation but decreased p70S6K activation (Moon *et al.*, 2014). In TSCC cells CAL27 and UM1, OA treatment significantly induced cell cycle G0/G1 arrest and increased the proportion of apoptotic cells as shown by decreased CyclinD1 and Bcl-2 expression and increased p53 and cleaved caspase-3 expression. OA also induced the formation of autolysosomes and decreased the expression of p62 as well as the LC3 I/LC3 II ratio (Jiang, L. *et al.*, 2017). A recent study investigated the effects of OA treatment in two hepatocellular carcinoma cell lines (Hep3B and Huh7.5) and in a healthy liver-derived human cell line (THLE-2). OA treatment reduced cell migration and invasion. It also increased cell death by apoptosis and necrosis, while it had no effects on healthy cells (Giulitti *et al.*, 2021). However, the high concentration used (300 μM) and long exposure (48 h) raised concerns that the inhibition of cell migration and invasion could be due to OA-induced apoptosis (Lin, 2021). In addition, OA potently inhibited telomerase activity, which plays an important part in the cellular immortalization of cancers (Mizushina *et al.*, 2012). Furthermore, OA and its metabolite, oleoylethanolamide, inhibited programmed death-ligand 1 (PD-1) expression, and induced apoptosis via STAT phosphorylation in several cancer cell lines, namely A549, HuH-7, MCF-7, DLD-1, and LoVo cells (Yamagata *et al.*, 2021). In

addition to OA, myristoleic acid (C14:1) extracted from *Serenoa repens,* induced LNCaP prostate cancer cell death by apoptosis and necrosis (Iguchi *et al.*, 2001). It is also reported that both cis- and trans-vaccenic acid inhibited cancer cell growth (Awad, A. B. *et al.*, 1995; Lim *et al.*, 2014; Song, J. *et al.*, 2019). And erucic acid (C22:1) inhibited glioblastoma cell C6 proliferation, inhibiting DNA synthesis via PPAR activation (Altinoz *et al.*, 2018; Altinoz *et al.*, 2021).

Lastly, OA was found to potentially interact with cancer therapy agents. In BT-474 and SKBr-3 breast cancer cells, OA downregulated the expression of the Her-2/neu (erbB-2) oncogene and concurrent exposure to OA and trastuzumab synergistically enhanced the growth inhibition effects of this chemotherapy drug (Menendez *et al.*, 2005; Nelson, 2005). Because of the pH responsiveness, newly developed OA-based nanostructures have the potential to efficiently target tumors, combining drug delivery with the therapeutic potential of OA. This could become a powerful strategy for targeted treatment of metastatic melanoma (Rinaldi *et al.*, 2022). A recent study found that MUFA radiosensitized cervical cancer cells through a novel p53-dependent mechanism. MUFAs activated $PPAR_{\gamma}$ and p53 to promote lipid uptake, storage, and metabolism after radiotherapy (Muhammad *et al.*, 2022). Furthermore, OA interacts with some anti-cancer proteins such as α-lactalbumin and lactoferrins. For example, HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cell), a molecular complex of human α-lactalbumin and OA, is known to have selective cytotoxic activity against certain types of tumors and OA might play a key role in HAMLET-induced tumoricidal action (Brinkmann *et al.*, 2011; Chaudhuri *et al.*, 2016; Jung *et al.*, 2016). In patients with advanced cancer, a combination of OA and Gc protein-derived macrophage activating factor was shown to have significant influence on immune system stimulation and reduction of tumor mass while avoiding harmful side-effects (Ruggiero *et al.*, 2014). In addition, OA was found to increase absorption of drugs by decreasing breast cancer resistance protein or P-glycoprotein mediated efflux (Aspenstrom-Fagerlund *et al.*, 2012; Houshaymi *et al.*, 2019).

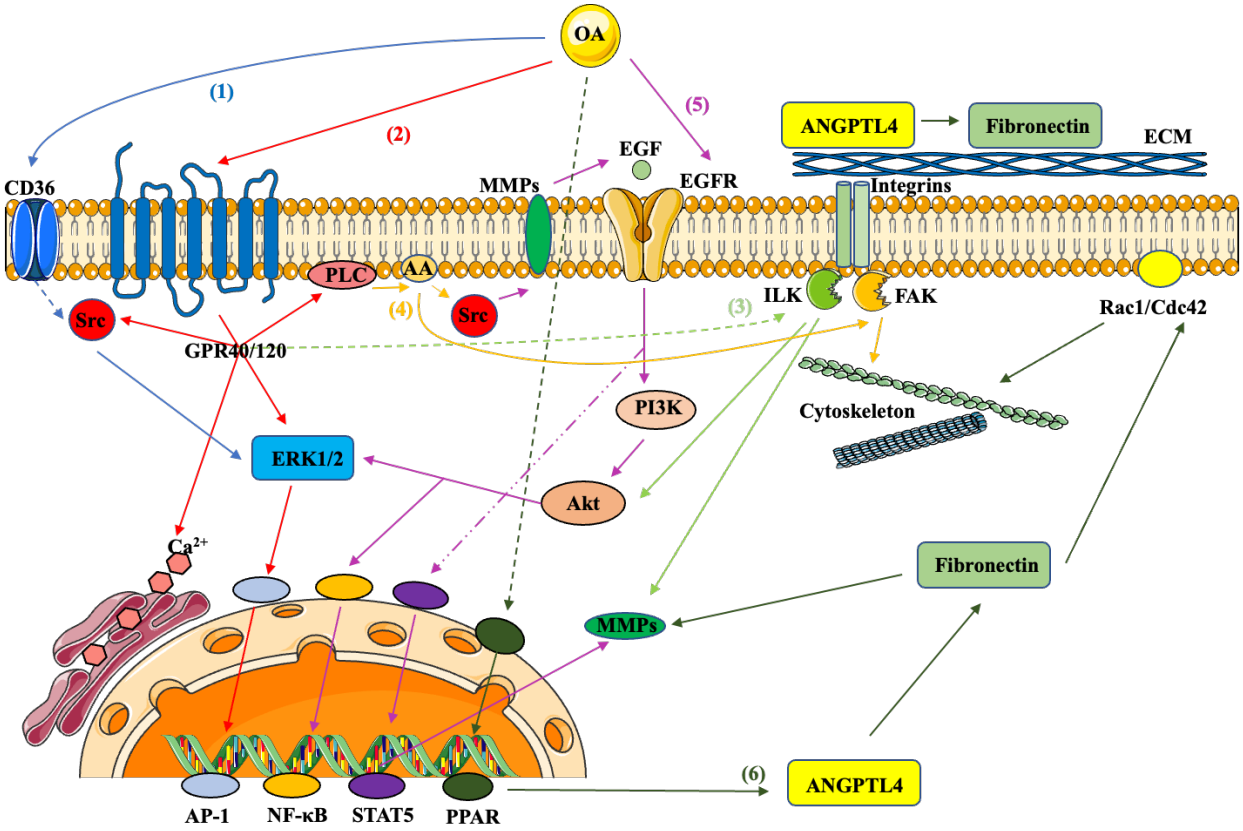


**Figure 2.3 Overview of tumor-promoting signaling triggered by OA.**

(1) OA increases the expression of CD36, promoting cell growth and invasion via Src and ERK1/2. (2) OA promotes cell proliferation and migration via GPR40/120 activity, Ca2+ release from the ER and downstream activation of the ERK1/2/AP-1 signaling pathway. (3) OA promotes cell proliferation through a GPR40/ILK/Akt pathway and enhances cell invasion via ILK/MMP-9. (4) OA promotes cell migration through an arachidonic acid (AA)-dependent pathway associated with Src and FAK activation, while GPR and PLC are necessary for AA accumulation. (5) OA mediates EGFR transactivation (or Src/MMPs/EGF signaling), activating ERK1/2/NF-κB via the PI3K/Akt pathway. In addition, OA induces cell migration through a Stat5-dependent pathway with EGFR and MMPs involved. (6) OA promotes cell migration and invasion via a ANGPTL4/Fibronectin pathway, activating MMPs, Rac1 and Cdc42. PPAR is involved in the activation of ANGPTL4 by OA. Dotted lines indicate indirect activation by mechanisms that remain to be clarified. The figure was created using Servier Medical Art image templates under a Creative Commons Attribution 3.0 Unported License. This figure is from (Guo, Z. *et al.*, 2023).

### 2.1.4 Role of endogenously synthesized MUFA in cancer

As the limiting step in MUFA synthesis, SCD activity (and its modulation using pharmacological inhibitors or by over/under-expression) provides insights into the impact of endogenously synthesized MUFA on cancer.

#### 2.1.4.1 SCD activity and cancer – evidence from human studies

Extensive clinical and epidemiological research were performed to study the role of SCD in cancer as well as its association with cancer progression and death rates. A recent study evaluated the expression of *SCD1* in different cancer types utilizing The Cancer Genome Atlas database. Compared with normal tissues, *SCD1* expression was upregulated in most types of cancer including bladder urothelial carcinoma, cervical squamous cell carcinoma, colon adenocarcinoma, esophageal carcinoma, head and neck squamous cell carcinoma, kidney chromophobe, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, and stomach adenocarcinoma. On the contrary, in thymoma, pheochromocytoma and paraganglioma, lung adenocarcinoma, glioblastoma multiforme and breast invasive carcinoma, SCD1 was downregulated. Gastric cancer patients with higher SCD1 expression have relatively less-optimistic prognostic outcome and relatively shorter overall survival (Wang, C. *et al.*, 2020). A study performed SCD1 immunohistochemistry on 11 different tumors: breast, colon, lymphoid, prostate, gastric, ovary, brain, kidney, liver, skin, and lung. SCD1 expression was detectable in >75% of tumors tested, and more than 50% of tumors showed strong staining while corresponding normal tissues showed relatively low SCD1 expression (Roongta *et al.*, 2011). Our laboratory also investigated the impact of SCD1 expression in metastatic breast cancers by generating Kaplan–Meier plots over a period extending up to 180 months by using available gene expression dataset records. These analyses reveal that high *SCD1* expression in the primary tumor is significantly associated with an increased proportion of metastasis-related deaths in patients suffering from breast cancers. Of note, this association appears even stronger in triple negative cancer, as reflected by the elevated hazard ratio associated with this cancer subtype (Lingrand *et al.*, 2020a). An exploratory study measuring *SCD1* expression levels in primary tumors also found a higher expression level in HER2+ and HR+ breast cancers. In this study, *SCD1* expression was associated with shorter relapse-free survival and shorter overall survival by multivariable analysis (Holder *et al.*, 2013). Another group performed immunohistochemical staining of a tissue microarray containing a total of 192 cores from different breast cancer subtypes. SCD1 expression was higher in cancer tissues compared with normal adjacent breast tissue. The expression of SCD1 was also found to be correlated with tumor grade and was associated with low overall survival in patients (Peck *et al.*, 2016). The association between high SCD1 expression in cancer tissue samples and poor clinical prognosis was also observed in bladder cancer (Presler *et al.*, 2018), prostate cancer (Fritz, V. *et al.*, 2010), pancreatic

cancer (Gao *et al.*, 2021), ovarian cancer (Tesfay *et al.*, 2019), lung cancer (Huang, J. *et al.*, 2016), colorectal cancer (Ran *et al.*, 2018), clear cell renal cell carcinoma (von Roemeling *et al.*, 2013; Wang, J. *et al.*, 2016), and cervical cancer (Wang, L. *et al.*, 2022). A Swedish men study also showed the association between single nucleotide polymorphisms in the SCD-1 gene and cancer death (Byberg *et al.*, 2014). However, studies on SCD5 expression in cancer drew different conclusions. Analysis of samples from public databases showed that SCD5 expression in different cancers could be either upregulated or downregulated. It was downregulated in breast cancer, and low expression of SCD5 was associated with more aggressive breast cancer phenotypes, such as high histological grade, late stage and HER2 overexpression. Survival analysis revealed that there was no correlation between SCD5 expression and overall survival, while upregulated SCD5 expression was related to longer breast cancer-free survival (Zhao, W. *et al.*, 2021). In addition, SCD5 was reported to be significantly higher in primary and low-invasive melanoma than in metastatic cell lines or in five independent cultures of normal melanocytes, at both the mRNA and protein levels (Bellenghi *et al.*, 2015). However, compared with the widely studied SCD1, SCD5 has limited expression and is poorly characterized. More research is needed to fully understand its role in cancer.

In accordance with the abnormal expression of SCD, unbalanced amount of SFA and MUFA has been observed in blood and tissue samples from cancer patients (Bougnoux *et al.*, 1992; Ruggieri *et al.*, 1979; Williams, C. M. et Maunder, 1993). A study analyzed the FA composition of phospholipids in membranes of red blood cells from cancer patients and found that these phospholipids have a lower percentage of SFA and a higher percentage MUFA compared to controls (Amezaga *et al.*, 2018). Lipid imaging profiling of 6 different cancer types (breast, lung, colorectal, esophageal, gastric, and thyroid cancer) showed a significant increase in MUFA and monounsaturated phosphatidylcholines levels in the cancer microenvironment compared with the adjacent normal tissue (Guo, S. *et al.*, 2014). A study on Swedish men showed an association between increased circulating palmitoleic acid in serum lipids and future cancer death (Byberg *et al.*, 2014). Breast cancer studies reported higher levels of MUFA in blood/plasma lipids (Preethika *et al.*, 2020) and breast adipose tissues (Conceicao *et al.*, 2016). A prospective study also found that blood levels of MUFA were related to prostate cancer incidence and this association was even stronger for high grade (Gleason ≥7) tumors (Chavarro *et al.*, 2013). Compared to normal hepatocytes, SCD expression levels and concentration of its MUFA products were increased in

aggressive hepatocellular carcinoma and were associated with poor survival times and tumor recurrence (Budhu *et al.*, 2013). Another study showed that the quantity of MUFAs in cholesterol esters was positively correlated with a higher patient death rate (Zureik *et al.*, 1995).

The studies above discussed presented elevated MUFA levels as detrimental to cancer patients. However, a few additional studies showed contradictory results. A nested case-control study examined the FA composition of erythrocytes membranes from prostate cancer patients and found no significant association between MUFA and cancer risk (Park, S. Y. *et al.*, 2009). In addition, a higher MUFA:SFA ratio was positively associated with decreased colon cancer risk in the Singapore Chinese Health Study (Butler *et al.*, 2017). Also, genotype data from 9254 colorectal cancer cases and 18,386 controls of European ancestry allowed one research group to correlate predicted plasma MUFA levels and reduction in the risk of colorectal cancer (May-Wilson *et al.*, 2017). Statistically significant inverse associations were found between high plasma levels of MUFAs and the risk of pancreatic cancers from a nested case–control study in Iran (Ghamarzad Shishavan *et al.*, 2021). A prospective analysis showed an inverse association for MUFA levels, especially OA, with T-cell non-Hodgkin lymphoma risk (Chiu *et al.*, 2018). Furthermore, some breast cancer studies revealed an inverse association with cancer aggressiveness. Lipid composition quantification analysis from the tumors freshly excised from breast cancer patients found that MUFAs concentrations, in lymphovascular invasion positive (LVI) breast carcinoma, were significantly lower than that in LVI negative tumors (Cheung, S. M. *et al.*, 2021). Fatty acid analysis from the mammary adipose tissue of postmenopausal women showed patients with malignant lesions had significantly lower MUFA compared to those with benign disease and history of breast cancer (Chajes *et al.*, 2011).

#### 2.1.4.2 SCD activity and cancer – evidence from animal studies

There are several potentially redundant SCD isoforms in mice (SCD1-4) (Man *et al.*, 2006), making it challenging to study SCD activity in these animal models. Furthermore, SCD depletion (expression and activity) causes phenotypes including dry skin, alopecia, and sebocyte hypoplasia (Ntambi *et al.*, 2002; Sampath *et al.*, 2009; Scaglia et Igal, 2008). These phenotypes, though seemingly innocuous at first, become more severe with age and can lead to blindness. Consequently, genetically modified xenograft models (*SCD* gene knockdown or overexpression) as well

pharmaceutical inhibition of SCD activity are commonly used to study the role of this enzyme family in tumor development and progression (see Table 2.3).

SCD1 plays a key role in mouse tumor growth. One study determined the role of SCD1 in lung tumor growth by subcutaneous injection of SCD1-deficient and control A549 lung cancer cells into athymic nude mice. Compared with control, SCD1-deficient mice showed increased tumor latency and reduced tumor growth rate, with about 40% in contrast to 100% tumor formation in the control group (Scaglia et Igal, 2008). Another study observed lighter and smaller tumors compared with controls through implantation of SCD1-knockdown H1650 lung cancer cells in mice (Huang, J. *et al.*, 2016). In addition, treatment with the SCD1 inhibitor A939572 in a A549 lung cancer xenograft mouse model attenuated tumor growth and showed enhanced anti-tumor activity in combination of amodiaquine (an anti-malarial drug) (Hu, X. *et al.*, 2022). The tumor inhibition effects of A939572 were also documented in xenograft tumors of LOVO colorectal cancer cells (Chen, L. *et al.*, 2016), GA16 and (SCD1-overexpressing) MKN45 gastric cancer cells (Roongta *et al.*, 2011; Wang, C. *et al.*, 2020), Panc02 pancreatic cancer cells (Hackney *et al.*, 2021), and FT-t ovarian cancer cells (Tesfay *et al.*, 2019). In a C4-2 prostate cancer xenograft mouse model, treatment with BZ36, a specific pharmaceutical SCD1 inhibitor, also significantly reduced tumor volume and tumor growth rate. Interestingly, BZ36 treatment induced tumor regression and resulted in a significant and dose-dependent increase in life span in comparison with control mice (Fritz, V. *et al.*, 2010). Another group implanted DU145 prostate cancer cells expressing doxycycline-inducible shRNAs into the prostates of immunocompromised mice and found that SCD1 ablation resulted in a significant increase in life span and a substantial attenuation of tumor growth in the early doxycycline treatment regimen (Peck *et al.*, 2016). In line with this, ectopic expression of SCD1 facilitated tumor formation and growth in a SCD1 overexpressing LNCaP prostate cancer cell model (Kim, S. J. *et al.*, 2011) and MKN45 gastric cancer cell model (Wang, C. *et al.*, 2020). In a bladder cancer study, tumor growth was suppressed by treatment with SCD1 inhibitor A37062 in UMUC-14 xenografts models and by doxycycline-inducible knockdown of SCD1 in a SW780 tumor model. These results were associated with a decrease in the ratio of MUFAs to saturated FAs in the tumors and liver tissues, as well as an induction of apoptosis as determined by caspase-3 cleavage (Du, X. *et al.*, 2012). Of note, the novel piperidine derivative SCD1 inhibitor T-3764518 showed dose-dependent growth inhibition of xenograft mouse models of HCT116 colorectal carcinoma, MSTO-211H lung mesothelioma carcinoma as well as 786-O

renal cell adenocarcinoma (Imamura *et al.*, 2017; Nishizawa *et al.*, 2017). This inhibitor showed great pharmacokinetic properties in mice according to oral absorption and tumor distribution. Lipidomic profile analysis revealed a lower desaturation index in T-3764518-treated mouse tumor tissues, suggesting efficient *in vivo* inhibition of SCD1 activity (Nishizawa *et al.*, 2017). Interestingly, in a liver tumor xenograft mouse model, conditional knockdown of SCD2 in primary hepatic stellate cells (the major isoenzyme in these cells) significantly slowed tumor formation and development (Lai *et al.*, 2017).

Furthermore, animal studies have shown that SCDs are involved in cancer metastasis. The incidence of lung metastasis decreased in mice having undergone tail vein injection of colorectal cancer HCT116 cells where *SCD1* was silenced as compared to controls. Histological analysis showed decreased size and number of lung metastatic tumors following *SCD1* suppression (Ran *et al.*, 2018). Similar metastasis inhibition results were also reported in a SCD1-knockdown hepatocellular carcinoma model (Liu, H. H. *et al.*, 2022). In a gastric cancer xenograft model, Twist1 (a key transcription factor driving metastasis) positive cells were found to overexpress *SCD1*, implicating it in the metastasis process (Wang, C. *et al.*, 2020). Interestingly, in a SW1 melanoma mouse model, treatment with the SCD inhibitor A939572 inhibited tumor growth but promoted a substantial increase in lung metastases (Vivas-Garcia *et al.*, 2020). Another study on nude mice injected with SCD5-overexpressing A375M melanoma cells and 4T1 mammary carcinoma cells showed significantly fewer metastasis formation in the lung compared to the mice injected with control cells. Primary tumors derived from SCD5-overexpressing cells showed diminished fibrotic morphology and fewer extracellular bundles, suggesting reduced extracellular matrix (ECM) deposition, which is associated with fewer metastases (Bellenghi *et al.*, 2015). In a 4T1 triple negative breast cancer mouse model, SCD5 overexpression hampered metastatic spreading via blocking SPARC (Secreted Protein and Rich in Cysteine) secretion, which plays a role in decreasing ECM deposition and reverting the EMT (Bellenghi *et al.*, 2022).

**Table 2.3 SCD activity in mouse cancer models.**

| Cancer type | SCD model | Mouse model | Main outcome | Reference |
|---|---|---|---|---|
| Bladder cancer | SCD1 knockdown, SCD1 inhibitor A37062 | Xenograft of SW780, UMUC-14 cells | Inhibited tumor growth and progression | (Du, X. *et al.*, 2012) |

| | | | | |
|---|---|---|---|---|
| Breast cancer | SCD5 overexpression | Xenograft of 4T1 cells | Reduced tumor aggressiveness. | (Bellenghi *et al.*, 2022) |
| Colorectal cancer | SCD1 inhibitor A939572 | Xenograft of LOVO cells | Reduced tumor volume and tumor weight | (Chen, L. *et al.*, 2016) |
| Colorectal cancer | SCD1 knockdown | Tail vein injection xenograft of HCT116 cells | Decreased the size and number of lung metastatic tumors | (Ran *et al.*, 2018) |
| Colorectal/Lung/Renal cancer | SCD1 inhibitor T-3764518 | Xenograft of HCT116/MSTO-211H/786-O cells | Inhibited tumor growth. | (Imamura *et al.*, 2017; Nishizawa *et al.*, 2017) |
| Gastric cancer | SCD1 overexpression, SCD1 inhibitor A939572 | Xenograft of MKN45 cells | Overexpression of SCD1 enhanced proliferation and metastasis while inhibition reduced both tumor volume and tumor weight | (Wang, C. *et al.*, 2020) |
| Gastric cancer | SCD1 inhibitor A939572 | Xenograft of GA16 cells | Inhibited tumor growth | (Roongta *et al.*, 2011) |
| Liver cancer | SCD1 knockdown | Xenograft of HepG2 cells | Inhibited tumor size and metastasis. | (Liu, H. H. *et al.*, 2022) |
| Liver fibrosis | SCD2 conditional knockout, SCD inhibitor A939572 | SCD2 conditional knockout | Reduced liver fibrosis, tumor formations, tumor size and tumor multiplicity | (Lai *et al.*, 2017) |
| Lung cancer | SCD1 knockdown, SCD1 inhibitor A939572 | Xenograft of A549 cells | Reduced tumor growth, less tumor formations and increase in tumor latency | (Scaglia et Igal, 2008) |
| Lung cancer | SCD1 knockdown | Xenograft of H1650 cells | Reduced tumor weight and volume | (Huang, J. *et al.*, 2016) |
| Lung cancer | SCD1 inhibitor A939572 | Xenograft of H460 cells | Inhibited tumor growth | (Hu, X. *et al.*, 2022) |
| Melanoma | SCD1 inhibitor A939572 | Xenograft of B16F1, SW1 cells | Inhibited primary tumors growth but increased lung metastases | (Vivas-Garcia *et al.*, 2020) |

| | | | | |
|---|---|---|---|---|
| Melanoma | SCD5 overexpression | Xenograft of A375M, 4T1 cells | Reduced metastases | (Bellenghi *et al.*, 2015) |
| Ovarian cancer | SCD1 inhibitor A939572 | Xenograft of FT-t cells | Reduced tumor number and mass | (Tesfay *et al.*, 2019) |
| Pancreatic cancer | SCD1 inhibitor A939572 | Xenograft of Panc02 cells | Reduced tumor size | (Hackney *et al.*, 2021) |
| Prostate cancer | SCD1 inhibitor BZ36 | Xenograft of LNCaP, C4-2 cells | Inhibited tumor volume and tumor growth rate | (Fritz, V. *et al.*, 2010) |
| Prostate cancer | SCD1 knockdown | Xenograft of DU145 cells | Inhibited tumor growth | (Peck *et al.*, 2016) |
| Prostate cancer | SCD1 overexpression | Xenograft of LN cells | Increased tumor formation and growth | (Kim, S. J. *et al.*, 2011) |

This table is from (Guo, Z. *et al.*, 2023).

#### 2.1.4.3 SCD activity and cancer – evidence from cellular models

Generally, high MUFA concentrations are a result of increased SCD activity. As such, elevated MUFA levels in cell membranes and corresponding extracellular vesicles of PC3 human prostatic adenocarcinoma cells, as compared to the less aggressive LNCaP cells (Ferreri *et al.*, 2020), implies enhanced SCD activity in the most aggressive cell line. However, SCD activity is not limited in its impact to cancer cell biology. For example, cancer stem cells contain a distinctive lipid profile, with higher free MUFA and lower free SFA levels than bulk cancer cells, which suggests that increased lipid desaturation is essential to stem-like characteristics in cancer cells (Choi, S. *et al.*, 2019; Mukherjee *et al.*, 2017).

##### 2.1.4.3.1 Role of SCD1 in cell proliferation and cell cycle

In H460 lung cancer cells, use of the SCD1 inhibitor CVT-11127 significantly decreased cell proliferation. An effect that could be reversed by addition of exogenous MUFAs (OA, palmitoleic or cis-vaccenic acid) (Hess *et al.*, 2010; Scaglia *et al.*, 2009). It was also reported that the population of H460 cells in S-phase was decreased by almost 75% with a concomitant increase

in $G_1$ phase following treatment with CVT-11127, with no changes in G2/M phase. However, a 50% decrease in G2/M-phase was observed in cells exposed to the SCD inhibitor in serum-deficient media. This indicated that MUFA-containing lipids in serum possibly sustained the passage of SCD1-deficient cells through mitosis. Exogenous OA reversed the cell cycle changes induced by SCD1 inhibition, confirming that SCD1 impacts cell cycle progression through its MUFA product (Hess *et al.*, 2010). In A549 lung cancer cells where *SCD1* was suppressed, the MUFA/SFA ratio in total lipids lowered and cell proliferation and growth were considerably decreased (Scaglia et Igal, 2008). Interestingly, two different SCD1 inhibitors were found to suppress cell growth in A549 lung cancer cells but only following EGFR activation. Further analysis found that SCD1 phosphorylation on Y55 by EGFR kinase activity was critical for it to enhance lung cancer growth (Zhang, J. *et al.*, 2017). In HeLa cervical cancer cells, *SCD1* knockdown was found to decrease cell proliferation and reduce colony formation ability (Wang, L. *et al.*, 2022). In line with this, *SCD1* overexpression in HEK293 cells led to significant promotion of colony formation while cell growth and colony formation were inhibited in H1650 cells where *SCD1* was suppressed (Huang, J. *et al.*, 2016). In SW780 and UMUC-14 bladder cancer cells, *SCD1* knockdown by siRNA inhibited cell proliferation in a FA desaturation-dependent manner while this effect was reversed by exogenous addition of OA (Du, X. *et al.*, 2012). In three different bladder cancer cell lines (UMUC-14, TCC-97-7, and SW780), DNA synthesis suppression was observed following *SCD1* knockdown. Further analyses in SW780 cells revealed a reduced percentage of cells in the $G_2$ and S-phases and an increased percentage in $G_1$ 48 hours after *SCD1* knockdown (Du, X. *et al.*, 2012). In LNCaP and C4-2 prostate cancer cells, inhibition of SCD1 activity by BZ36 induced a dose dependent decrease in cell proliferation, reaching 100% inhibition at the maximal dose used. Flow cytometry analysis showed accumulation of LNCaP cells in the $G_0/G_1$ phase of the cell cycle and a decrease in the S phase upon BZ36 treatment. However, no effect on proliferation was observed in the non-cancerous prostate cell line PNT2, even at a maximal dose. Similarly, *SCD1* knockdown resulted in a decrease in the proliferation while SCD1 overexpression increased cell proliferation in both LNCaP, and C4-2 cells (Fritz, V. *et al.*, 2010).

#### 2.1.4.3.2 Role of SCD1 in cell migration and invasion

It was reported that *SCD1* expression increased cell membrane fluidity as well as fibroblast-induced EMT and migration in poorly (MCF-7) and highly (MDA-MB-231) invasive breast cancer

cells. Inhibition of SCD1 by siRNA and inhibitor A939572 both resulted in a significant inhibition of MCF-7 and MDA-MB-231 cell migration promoted by fibroblast-released soluble factors (Angelucci *et al.*, 2015). Further study showed that the effects of SCD1 inhibition could be rescued by addition of OA (Angelucci *et al.*, 2018). Our laboratory also reported that SCD1 activity was implicated in the transformation of MDA-MB-231 cells from an epithelial to a mesenchymal phenotype. Silencing *SCD1*was associated with increased GSK3 activity, reduction of β-catenin nuclear localization and transactivation activity. It also modified cell shape and their invasive potential by the reduction of cell spreading and cell–cell junctions (Mauvoisin *et al.*, 2013). In MDA-MB-231 cells, inhibition of SCD1 induced a rounded, less elongated phenotype, while OA treatment resulted in elongated, spindle-shaped cells, which was associated with increased speed and diminished directional changes during migration (Lingrand *et al.*, 2020b). In HeLa cells, *SCD1* knockdown significantly inhibited cell migration and invasion abilities in wound healing and Transwell assays and the expression level of EMT-related proteins were decreased (Wang, L. *et al.*, 2022). In the 786-0 clear cell renal cell carcinoma cells, depletion of *SCD1* diminished cell migration and colony formation ability (Zhang, Y. *et al.*, 2013). In HCT116 and SW116 colorectal cancer cells, stable *SCD1* knockdown impaired migration and invasion ability while ectopically expressed SCD1 in Caco2 cells significantly increased migration and invasion rates. These effects were associated with increased MUFA levels and suppression of PTEN expression and Akt activity (Ran *et al.*, 2018). Similarly, overexpression of SCD1 in HEK293 promoted cell invasion and migration while knockdown of *SCD1* in lung cancer H1650 cells had opposite effects (Huang, J. *et al.*, 2016). However, the expression of SCD5 was lower in more aggressive metastatic breast cancer and melanoma cells than primary breast cells and low-invasive melanoma (Puglisi *et al.*, 2018; Zhao, W. *et al.*, 2021). And supplementation with OA reduced A375M melanoma cell malignancy by reducing the dissemination capability, impairing tumor spread (Bellenghi *et al.*, 2015). In MDA-MB-231 cells, cell migration was not significantly affected by siRNA-mediated SCD5 depletion (Angelucci *et al.*, 2018).

2.1.4.3.3 Role of SCD1 in cell death

Studies have reported that the inhibition of SCD1 induced ER stress and cell death through several mechanisms, including apoptosis and ferroptosis. In SW780 and UMUC-14 bladder cancer cells, *SCD1* knockdown significantly increased levels of the apoptotic cell-surface marker

Annexin-V. It was also associated with cleavage of caspases-3 and 7, and activation of the caspase-3 substrate PARP suggesting a stimulation of apoptosis (Du, X. *et al.*, 2012). In U2OS and SW480 colorectal cancer cells, *SCD1* depletion induced high-level induction of caspase-3 activity and PARP cleavage as well as unfolded protein response hallmarks such as Xbp1 mRNA splicing, phosphorylation of eIF2α and increased expression of the apoptosis-related protein C/EBP homologous protein (Minville-Walz *et al.*, 2010). In Caki1 and A498 clear cell renal cell carcinoma cells, both genetic knockdown and pharmacologic inhibition of SCD1 decreased tumor cell proliferation and induced apoptosis. Induction of ER stress response signaling was also observed upon inhibition of SCD1 activity (A939572) while OA treatment reversed these effects (von Roemeling *et al.*, 2013). A recent study performed in ovarian cancer cells showed that SCD1 depletion or inhibition lowered MUFA levels and triggered the ER stress response with the activation of IRE1α/XBP1 and PERK/eIF2α/ATF4 pathways. The induction of long-term mild ER stress or short-time severe ER stress led to cell death by apoptosis and supplementation with OA rescued these effects (Zhao, G. *et al.*, 2022). Furthermore, in an ovarian cancer study, inhibition of SCD1 by inhibitors MF-438 or CAY10566, and *SCD1* knockdown reduced cell viability and increased cell death. This was restored by providing cells with either SCD1's product OA or Fer-1, an inhibitor of ferroptosis. In addition, cell death triggered by the ferroptosis inducer RSL-3 could be rescued by MUFAs (palmitoleic acid or OA) but not by SFAs (palmitic or stearic acid) (Tesfay *et al.*, 2019). A recent study found the transcription factor BTB and CNC homology 1 (BACH1) induced ferroptosis in esophageal squamous cell carcinoma cells by negatively regulating *SCD1* through binding to its intron 2 region. *SCD1* knockdown significantly increased lipid ROS accumulation in KYSE150 and KYSE170 cells, while OA addition significantly attenuated oxidative stress and ferroptosis (Xie, X. *et al.*, 2023). In MKN45 and HGC27 gastric cancer cells, SCD1 overexpression enhanced anti-ferroptosis markers SLC7A11 and GPX4. SCD1 overexpression also prevented Erastin-induced ferroptotic cell death and characteristic lipid oxidation (Wang, C. *et al.*, 2020). Interestingly, a study showed that depletion of SCD5 in MCF-7 breast cancer cells induced necrosis. The double *SCD1* and *SCD5* knockdown did not worsen cell viability compared to single *SCD5* silencing. And this necrotic effect was rescued by a 48-h treatment of cells with OA, suggesting SCD5 maintains cell survival via the production of OA (Angelucci *et al.*, 2018).

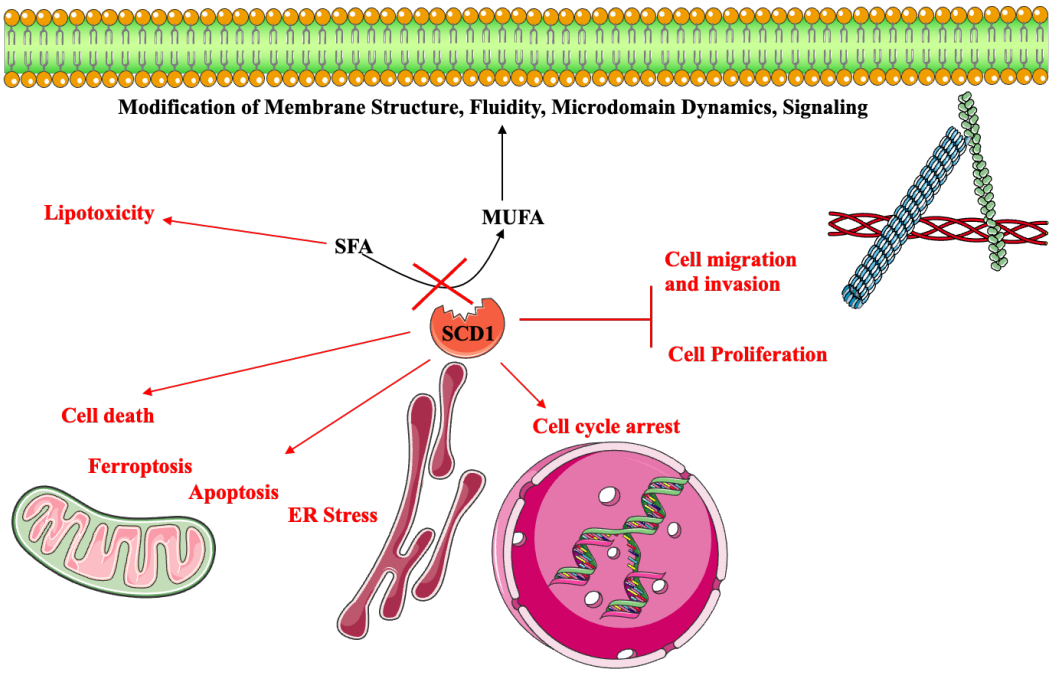


**Figure 2.4 Impact of Stearoyl-CoA desaturase 1 activity in cancer cells.**

Impact of Stearoyl-CoA desaturase 1 activity in cancer cells. Stearoyl-CoA desaturase 1 (SCD1) modifies cell membrane structure, membrane fluidity, microdomain dynamics and signaling through synthesis of monounsaturated fatty acids (MUFA). Inhibition of SCD1 increases the SFA/MUFA ratio, causing lipotoxicity, ER stress and cell death. SCD1 inhibition diminishes tumor cell proliferation, migration, and invasion. Black lines indicate the consequences of SCD1 activity in cancer cells while red lines indicate the anticancer effect of SCD1 inhibition. This figure is from (Guo, Z. *et al.*, 2023).

## 2.2 Breast Cancer

### 2.2.1 Breast cancer epidemiology: a global challenge

Breast cancer persists as a leading oncologic challenge, with 2.3 million new cases and 685,000 deaths annually (from Global Cancer Observatory – World Health Organization (WHO) *https://gco.iarc.fr/en*), reflecting its status as the most prevalent malignancy worldwide. Disparities in incidence, mortality, and survival rates highlight the interplay of healthcare infrastructure, screening accessibility, and sociodemographic factors. In Canada, an estimated 28,000 new cases are diagnosed yearly, with a 5-year survival rate of 88%, attributable to nationwide mammography programs and advanced therapeutic protocols (from Canadian Cancer Society, 2024 *https://cancer.ca/en/cancer-information/cancer-types/breast/statistics*). Conversely, China reports ~420,000 annual cases, with a younger median age at diagnosis (45–55 years) and a lower survival rate (83%), driven by late-stage detection and regional inequities in medical resources (National Cancer Center of China, *http://www.ncc.org.cn/en/*). This divergence underscores the critical role of early detection in high-income nations, whereas low- and middle-income regions face systemic barriers, including limited screening infrastructure and public awareness (Giaquinto *et al.*, 2022). Furthermore, lifestyle transitions—such as urbanization, delayed childbirth, and Westernized diets—contribute to rising incidence in developing economies (Giaquinto *et al.*, 2022). Addressing these disparities necessitates evidence-based, region-specific interventions, emphasizing preventive strategies, equitable healthcare access, and genomic research to mitigate the growing global burden of breast cancer.

### 2.2.2 Morphological characteristics and development

Breast cancer typically originates from the epithelial lining of terminal duct lobular units, giving rise to two principal histological patterns: ductal and lobular carcinomas (Tan *et al.*, 2020). Ductal carcinomas, representing 70-80% of cases, demonstrate cohesive tumor cells forming glandular structures, while lobular carcinomas (10-15%) exhibit discohesive growth patterns with characteristic loss of E-cadherin expression (Badowska-Kozakiewicz *et al.*, 2017; Luveta *et al.*, 2020). The transition from *in situ* to invasive carcinoma involves progressive genetic alterations, including activation of oncogenes (e.g., HER2/neu), inactivation of tumor suppressors (e.g., TP53), and expression of EMT markers (Risom *et al.*, 2022).

The current WHO classification recognizes over 20 distinct histological subtypes, with invasive ductal carcinoma being most prevalent. By immunohistochemistry for the presence of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu), breast cancers are commonly classified into different molecular subtypes (Eliyatkin *et al.*, 2015). Luminal-like breast cancer is defined as tumors with detectable ER, PR, or both, with or without HER2 amplification (Harbeck et Gnant, 2017). HER2+ breast cancer is defined as tumors with HER2 overexpression, but not ER or PR (Loibl et Gianni, 2017). TNBC is defined by a lack of expression of all three receptors (Marra *et al.*, 2020). Luminal-like breast cancers are the most common molecular subtype of breast cancers and are generally responsive to endocrine-based therapies, making effective treatment options more widely available (Yuksel *et al.*, 2022). About 20% of breast cancers are HER2+ breast cancer, which is often a more aggressive tumor subtype but generally responsive to HER2-targeted therapies (Kyriazoglou *et al.*, 2022). Although TNBC only accounts for 15–20% of breast cancers, this subtype is highly aggressive and prone to metastasize. It has the worst clinical outcomes, with greater recurrence and lower overall survival rate. And there are no targeted therapies yet (Devericks *et al.*, 2022; Landry *et al.*, 2022). According to the American Cancer Society, in America, the overall 5-year relative survival rate for TNBC is 77% compared 90% with non-TNBC breast cancer (*https://www.cancer.org* ).

### 2.2.3 **Staging, risk factors, and treatment of breast cancer**

Breast cancer staging is standardized through the American Joint Committee on Cancer (AJCC) 8th edition TNM system, which provides a framework for classifying disease progression based on tumor characteristics (Giuliano *et al.*, 2018). The T category describes the primary tumor size and local invasion, where T1 tumors measure ≤2 cm and show excellent prognosis, while T4 tumors involve chest wall or skin invasion and indicate advanced disease (Amin *et al.*, 2017). Lymph node involvement (N category) significantly impacts outcomes, with N0 indicating no nodal spread and N3 representing metastasis to supraclavicular nodes (Jang *et al.*, 2019). Distant metastasis (M1) most commonly occurs in bone, liver, and lungs, substantially reducing survival rates (Bidard *et al.*, 2014).

The multifactorial etiology of breast cancer involves complex interactions between genetic predisposition and environmental exposures. Germline mutations in BRCA1 and BRCA2 genes account for 5-10% of cases and confer a 45-72% lifetime risk of developing breast cancer

(Kuchenbaecker *et al.*, 2017). Other significant genetic factors include PALB2 mutations (33% lifetime risk) and TP53 mutations (Li-Fraumeni syndrome) (Antoniou *et al.*, 2014; Masciari *et al.*, 2012). Hormonal influences play a crucial role, with early menarche (<12 years), late menopause (>55 years), and nulliparity each independently increasing risk by 15-20% (Mishra *et al.*, 2017; Pompei et Fernandes, 2020). Environmental factors such as therapeutic radiation exposure before age 30 elevates risk 3-7 fold, while lifestyle factors including obesity (postmenopausal women) and alcohol consumption (>1 drink/day) demonstrate dose-dependent risk increases (Singletary, 2003).

Current treatment paradigms are tailored to molecular subtypes and disease stage. For local therapy, breast-conserving surgery with adjuvant radiation achieves equivalent survival to mastectomy in early-stage disease while preserving breast tissue (Fisher *et al.*, 2002). Sentinel lymph node biopsy has become standard for axillary staging, reducing lymphedema complications by 70% compared to complete axillary dissection (Giuliano *et al.*, 2017). Systemic therapies have advanced significantly, with endocrine treatments like tamoxifen administration reducing recurrence by 50% in ER+ cancers (Early Breast Cancer Trialists' Collaborative, 2015, 2022). The development of CDK4/6 inhibitors such as palbociclib extended progression-free survival in metastatic HR+ disease to 24 months compared to 14 months with letrozole alone (Hortobagyi *et al.*, 2016; Jeong *et al.*, 2021). For HER2-positive tumors, dual blockade with trastuzumab and pertuzumab combined with docetaxel demonstrated a 16-month improvement in overall survival (Kawajiri *et al.*, 2015).

Emerging therapies are transforming treatment landscapes, particularly for aggressive subtypes. Antibody-drug conjugates like sacituzumab govitecan showed a 35% objective response rate in heavily pretreated TNBC (Bardia *et al.*, 2019; Seligson *et al.*, 2021). PARP inhibitors such as olaparib improved progression-free survival from 4.2 to 7.0 months in BRCA-mutated metastatic disease (Liu, H. *et al.*, 2025; Tutt *et al.*, 2021). Immunotherapy combinations with chemotherapy are now first-line treatments for PD-L1 positive triple-negative breast cancer, demonstrating a 13-month improvement in event-free survival (Neven *et al.*, 2025; Schmid *et al.*, 2024). Liquid biopsy techniques detecting circulating tumor DNA enable real-time monitoring of treatment response and minimal residual disease (Garcia-Murillas *et al.*, 2015), while artificial intelligence algorithms now achieve 97% accuracy in detecting lymph node metastases on pathology slides (Ehteshami Bejnordi *et al.*, 2017).

## 2.3 Breast Tumor Metastasis and Cancer Cell Migration

### 2.3.1 The metastatic cascade

Tumor metastasis is a highly organized, non-random biological process that remains the primary cause of mortality in breast cancer patients. This complex cascade begins with local invasion, where malignant cells breach the basement membrane through coordinated degradation of extracellular matrix (ECM) components. In breast cancer, this process is particularly facilitated by the upregulation of MMPs), with MMP-2 and MMP-9 showing elevated expression in aggressive TNBC (Kwon, M. J., 2022). The subsequent EMT involves dramatic phenotypic alterations, including downregulation of E-cadherin mediated by transcription factors such as Twist and Snail, which have been specifically implicated in ductal carcinoma progression (Wang, Y. et Zhou, 2011). Following stromal invasion, tumor cells intravasate into blood or lymphatic vessels, a process significantly enhanced by VEGF-mediated angiogenesis. Clinical studies have demonstrated that VEGF expression correlates with increased microvessel density in invasive ductal carcinomas (Madu *et al.*, 2020). During hematogenous dissemination, circulating tumor cells (CTCs) employ sophisticated survival strategies, including platelet aggregation and immune evasion through PD-L1 expression, with CTC enumeration now recognized as a prognostic marker in metastatic breast cancer (Strati *et al.*, 2023). The final metastatic colonization exhibits remarkable organotropism in breast cancer, with bone, lung, liver and brain being predominant sites. Bone metastasis, occurring in approximately 70% of advanced cases, involves a vicious cycle wherein tumor cells activate osteoclasts through RANKL signaling while osteoclast-derived growth factors further promote tumor growth (Wu, X. *et al.*, 2020). This reciprocal interaction has been particularly well-characterized in ER+ breast cancers, which demonstrate particular affinity for bone marrow niches (Chen, F. *et al.*, 2021; Pang *et al.*, 2022; Wu, X. *et al.*, 2020).

### 2.3.2 Molecular regulation of metastatic progression

The molecular orchestration of metastasis involves dynamic interplay between tumor-intrinsic alterations and microenvironmental cues. Genomically, HER2-amplified breast cancers frequently exhibit hyperactivation of the PI3K/AKT/mTOR pathway, which not only drives primary tumor growth but also enhances metastatic competence through increased cell survival during circulation (Murthy *et al.*, 2020). BRCA1/2-deficient tumors, while initially sensitive to

DNA-damaging agents, often develop compensatory mechanisms that facilitate metastatic spread, including upregulation of error-prone DNA repair pathways (Patel *et al.*, 2024). Epigenetic reprogramming plays an equally critical role, with DNA methylation silencing key metastasis suppressors such as CDH1 in invasive lobular carcinomas. Whole-genome bisulfite sequencing has revealed distinct methylation patterns between metastatic and primary tumors, suggesting epigenetic evolution during disease progression (Dopeso *et al.*, 2024). The tumor microenvironment further modulates metastatic behavior through paracrine signaling, where cancer-associated fibroblasts (CAFs) secrete TGF-β to induce EMT in ER+ tumors while simultaneously remodeling the ECM to create migration-permissive tracks (Karagiannis *et al.*, 2012).

### 2.3.3 **Mechanisms of cancer cell migration**

Breast cancer cells exhibit remarkable plasticity in their migratory strategies, adapting to varying physical constraints and microenvironmental conditions. Mesenchymal migration, characterized by elongated cellular morphology and integrin-mediated adhesion, predominates in collagen-rich stroma typical of mammary tissue. This process is orchestrated by Rho GTPases, with RhoA promoting actomyosin contractility while Rac1 regulates lamellipodial protrusion (Zegers et Friedl, 2014). In contrast, the amoeboid mode, observed in CTCs, involves Rho/ROCK-dependent membrane blebbing that facilitates navigation through narrow vascular spaces (Wu, J. S. *et al.*, 2021).

Collective migration represents another clinically relevant pattern, particularly in luminal breast cancers where tumor cells invade as cohesive clusters (Cheung, K. J. *et al.*, 2013). These multicellular units maintain E-cadherin junctions while collectively degrading ECM barriers, potentially explaining the frequent observation of tumor emboli in lymphovascular spaces (Yang, Y. *et al.*, 2019). Signaling pathways such as Wnt/β-catenin and Notch exhibit subtype-specific regulation of migration, with Wnt activation promoting single-cell dissemination in basal-like tumors while Notch mediates tumor-stromal interactions during bone colonization (Zhang, Y. *et al.*, 2025; Zhu *et al.*, 2022).

### 2.3.4 Therapeutic targeting of metastatic pathways

Current therapeutic strategies increasingly focus on intercepting metastatic progression at multiple levels. The RANKL inhibitor denosumab has demonstrated efficacy in preventing skeletal-related events in bone-metastatic breast cancer, highlighting the clinical relevance of microenvironmental targeting (Steger et Bartsch, 2011). Novel FAK inhibitors such as defactinib show promise in disrupting mesenchymal migration, particularly in TNBC where FAK overexpression correlates with poor prognosis (Wu, C. C. *et al.*, 2023). Emerging approaches include CTC-directed therapies and microenvironment-modifying agents that disrupt pre-metastatic niche formation. Liquid biopsy technologies now enable real-time monitoring of metastatic risk through detection of circulating tumor DNA (ctDNA), with specific methylation signatures showing predictive value for organ-specific recurrence (Mazzitelli *et al.*, 2023). These advances underscore the transition from empirical to mechanism-based strategies in managing metastatic breast cancer.

## 2.4 Cell Membrane Ruffling and Cell protrusions

In cancers, abnormal cell migration is an essential process and a classical feature in tumor cell metastasis (Lou *et al.*, 2021). During the metastasis cascade, cancer cells frequently exhibit collective migration patterns, maintaining cell-cell contacts while moving as cohesive groups, with distinct morphological features distinguishing leader from follower cells. Collective cell migration plays an essential role in this process, as evidenced by characteristic morphological changes in individual cells (Bischoff *et al.*, 2021). For individual cell migration to occur, cells must first acquire a characteristic polarized morphology in response to extracellular signals, which are known to elicit various intracellular responses in the organization of both actin and microtubule cytoskeletons. At the cell front, actin assembly drives the extension of flat membrane protrusions called lamellipodia and finger-like protrusions called filopodia. At the leading edge of the lamellipodium, cells form adhesions that connect the extracellular matrix to the actin cytoskeleton to anchor the protrusion and tract the cell body. Finally, to move forward, cells retract their trailing edge by combining actomyosin contractility and disassembly of adhesions at the rear (Le Clainche et Carlier, 2008; Mahankali, Peng, Cox*, et al.*, 2011). Although migration varies from one cell type to another, it is generally accepted that the role and the regulation of actin dynamics associated

with membrane protrusion and cell-matrix adhesion are the driving forces and the common features of cell migration. Therefore, the spatial regulation of the actin cytoskeleton is a critical component in the regulation of cell migration (Bonfim-Melo *et al.*, 2018; Caswell et Zech, 2018; Randzavola *et al.*, 2019).

Preceding the formation of these definitive protrusions, cancer cells often exhibit dynamic membrane ruffling – undulating waves of actin-rich membrane that sample the local environment. Ruffles are of similar morphology to lamellipodia, but non-adherent and often protruding dorsally (Hoon *et al.*, 2012). These ruffles represent exploratory structures that frequently evolve into more stable lamellipodial or filopodial extensions (Le Clainche et Carlier, 2008). In breast cancer cells, membrane ruffling is particularly prominent in response to growth factor stimulation, such as EGF in TNBC or heregulin in HER2+ cancers (Adam *et al.*, 1998; Boulay *et al.*, 2008). The formation of these actin-rich membrane structures is absolutely dependent on precise spatial and temporal regulation of the actin cytoskeleton, making this system a critical control point for metastatic dissemination (Alexandrova *et al.*, 2020).

Lamellipodia are the most iconic form of cell protrusion, which is the large fan-like structures at the leading edge. They adhere weakly to the substratum of motile cells (He et Ji, 2017). They are primarily formed by Arp2/3 complex-dependent actin filament, which are not only a hallmark of the leading edge but also the driving force in migrating single cells (Innocenti, 2018; Pal *et al.*, 2020; San Miguel-Ruiz et Letourneau, 2014; Shakir *et al.*, 2008; Wu, C. *et al.*, 2012). Arp2/3 complex is an actin filament nucleation and branching complex of seven proteins, including two actin-related proteins (Arp2 and Arp3), and five scaffolding subunits, (actin-related protein complex, ARPC1,2,3,4,5) (Fregoso *et al.*, 2022; San Miguel-Ruiz et Letourneau, 2014). The activation of the Arp2/3 complex increases its binding to the sides of actin filaments and induces the formation of an actin branch. This pushes against the plasma membrane causing lamellipodial protrusions, which are critical for directional cell migration and polarity (Liu, Z., Yang*, et al.*, 2013). Several proteins regulate these processes by activating or inhibiting Arp2/3 complex and by stabilizing or disassembling branched networks. Among these proteins, Wiskott-Aldrich syndrome protein (WASP)-family nucleation-promoting factors (NPFs) activate Arp2/3 complex by inducing a conformational change that prompts the binding of the complex to the side of a pre-existing (mother) filament and formation of a new (branch) filament that grows at a ~70° angle relative to the mother filament (Fregoso *et al.*, 2022; Le Clainche et Carlier, 2008; Liu, Z., Yang*, et al.*, 2013;

Randzavola *et al.*, 2019; San Miguel-Ruiz et Letourneau, 2014). A positive feedback loop regulated by the small GTPase Rac1 and involving Arp2/3 complex activation by the NPF, WASP family Verprolin-homologous protein (WAVE) controls the assembly of the branched networks at the cell cortex in order to drive cell motility (Carabeo *et al.*, 2007; Pal *et al.*, 2020; Shakir *et al.*, 2008; Tang *et al.*, 2016; Ten Klooster *et al.*, 2006). Arp2/3 complex is the central pillar of lamellipodia and might not be necessary for filopodia. Depletion of Arp2/3 complex in fibroblasts generates a stable line lacking lamellipodia with defective random cell motility relying only on a filopodia-based protrusion system (Wu, C. *et al.*, 2012).

Filopodia are finger-like actin-rich membrane protrusions that extend out from the cell edge, mediated by proteins such as formins and regulated by various small GTPases of the Rho family such as Cdc42. They are thought to be more explorative in sensing local environment, controlling directionality but also maintain persistence of migrating cells by promoting cell-matrix adhesiveness at the leading edge (Arjonen *et al.*, 2011; Bischoff et Bogdan, 2021; Bischoff *et al.*, 2021; Bray *et al.*, 2013; Gat *et al.*, 2020; Jacquemet *et al.*, 2015; Kishimoto *et al.*, 2020; Kiso *et al.*, 2018; Sakabe *et al.*, 2017). Cdc42 (Cell division control protein 42 homolog) is a member of the Ras homolog (Rho) family of the Ras superfamily. It functions as a binary molecular switch between guanosine triphosphate (GTP)- and guanosine diphosphate (GDP)-bound active states, triggering downstream signaling cascades (Ubukawa *et al.*, 2020). Overexpression of Cdc42 is observed in several cancers, where it is associated with poor prognosis (Bray *et al.*, 2013). Cdc42 regulates the formation of the actin cytoskeleton and microtubule assembly through several effector proteins playing a role in cell polarization, division, and movement (Geiger et Zheng, 2014; Ubukawa *et al.*, 2020). Therefore, deregulation of Cdc42 is also linked to tumor metastasis (Sakabe *et al.*, 2017; Yang, W. *et al.*, 2020). Several extracellular receptors (such as TRPV4, EGFR, RET and CD44) activate Cdc42 signaling. Theses receptors activate guanine nucleotide exchange factors (GEF) proteins, catalyzing the nucleotide exchange, resulting in the production of Cdc42-GTP. While GTPase-activating proteins (GAPs), which assist with hydrolysis of the GTP nucleotide, switch off the signal by promoting the formation of Cdc42-GDP (Murphy *et al.*, 2021). Membrane localization of Cdc42 is crucial for its downstream signaling. For example, Rho-GDI proteins can sequester Cdc42 in the cytoplasm, stabilizing the inactive GDP-bound form, shuttling Cdc42 between the plasma membrane and the Golgi apparatus. Of note, the C- terminal region of

Cdc42 encompasses a CAAX box which is a site for post-translational lipid modification, which regulate its membrane localization and activity (Nishimura et Linder, 2013).

Despite intense studies of how cells generate these cell protrusions, especially in cancer cells, the exact mechanisms by which ruffling is regulated are not fully understood (Mahankali, Peng, Cox*, et al.*, 2011). It is generally accepted that actin polymerization regulators Rac1 and Arp 2/3 are involved in the formation of lamellipodia and ruffles, and that Cdc42 is required in filopodia generation (Rolo *et al.*, 2016). Targeting cytoskeletal regulators such as Cdc42 and the Arp2/3 complex has emerged as a promising strategy for limiting tumor metastasis. Several small-molecule inhibitors have demonstrated efficacy in preclinical cancer models. The small-molecule inhibitor AZA197 selectively inhibits Cdc42 and suppresses colon cancer cell proliferation, migration, and invasion (Zins *et al.*, 2013). In xenograft mouse models, AZA197 significantly reduced tumor growth and prolonged survival by downregulating PAK1 and ERK signaling (Zins *et al.*, 2013). MBQ-167, a potent dual inhibitor of Cdc42 and Rac1, impairs migration, proliferation, and mammosphere formation in TNBC cells. In vivo, MBQ-167 significantly reduced tumor growth and metastasis in both immunocompromised and immunocompetent TNBC models (Cruz-Collazo *et al.*, 2021). Pimozide, an FDA-approved antipsychotic, has been identified as an Arp2/3 inhibitor through its interaction with ARPC2. In cancer models, it suppresses migration and invasion, and *in vivo* studies show its ability to reduce metastatic spread (Choi, J. *et al.*, 2019). Ongoing research continues to refine our understanding of how these cytoskeletal systems adapt during different stages of metastatic progression and in response to therapeutic pressures, offering new opportunities for intervention in this lethal aspect of cancer biology.

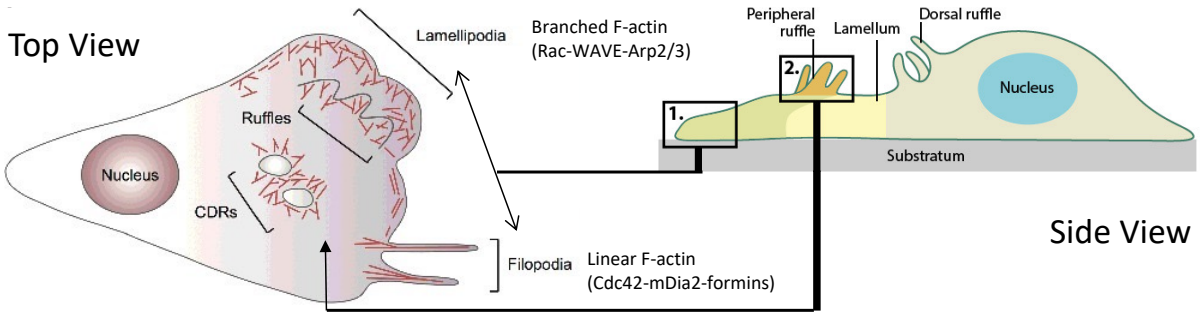

**Figure 2.5 Actin-based cell protrusions in cells.**

Top view and side view schematic of the actin-based protrusions in a cell showing in red filamentous F-actin. Lamellipodia, filopodia, (peripheral and dorsal) ruffles and circular dorsal ruffles (CDRs) are depicted. Different pathways control actin nucleation and organization dictating the formation of different cell protrusions. Image is adapted from MBINFO and (Bischoff *et al.*, 2021).

## 2.5 Lipidation

Post-translational modifications (PTM) can regulate diverse activities of many proteins, which occurs at all stages of human life, and abnormal PTM often leads to various disease (Hou *et al.*, 2022; Wang, R. et Chen, 2022). Well-studied PTMs include protein glycosylation, methylation, hydroxylation, amidation, phosphorylation, acetylation, and ubiquitination (Wang, R. et Chen, 2022). These various modifying groups can affect charge, hydrophobicity, and other aspects of protein chemistry, modifying protein activity and function and thereby linking cell metabolism to cell signaling (Counihan *et al.*, 2022; Wang, R. et Chen, 2022). Protein lipidation is a significant PTM that usually refers to various lipids or lipid-like group covalently attached to proteins, although it has not well been studied as other modifications such as phosphorylation or acetylation. To date, studies have shown that many types of protein lipidation exist, including fatty acylation, N-lipoylation, S-prenylation, C-terminal phosphatidylethanolaminylation, C-terminal cholesterolyation, C-terminal GPI Anchoring, and LDE acylation (Lipid-Dependent Enzymatic acylation, which is dependent on specific membrane lipid environments such as $PIP_2$ and cholesterol)) (Chamberlain et Shipston, 2015; Nuskova *et al.*, 2021; Vrljic *et al.*, 2011; Wang, R. et Chen, 2022). Among various lipidation types, S-acylation and prenylation are particularly noteworthy, in the context of cysteine residue modification (Kouba et Demaurex, 2024).

### 2.5.1 **S-acylation**

S-acylation is the reversible attachment of FAs to cysteines via a thioester bond (Chamberlain et Shipston, 2015). Historically, this modification was frequently referred to as palmitoylation since palmitate (C16:0) is the most common FA attached to S-acylated proteins (Chamberlain et Shipston, 2015; Schulte-Zweckel *et al.*, 2019). This modification enhances the hydrophobicity of proteins, facilitating their association with cell membranes and influencing their stability and trafficking (Kouba et Demaurex, 2024). The dynamic nature of S-acylation allows for rapid regulation of protein function in response to cellular signals. In addition, recent studies have

identified a variety of other different FAs that can be attached to proteins via S-acylation, including C16:1 (Schulte-Zweckel *et al.*, 2019; Zheng, B. *et al.*, 2016), C18:0 (Liang *et al.*, 2002), C18:1 (Montigny *et al.*, 2014; Nuskova *et al.*, 2021), C20:4 (Liang *et al.*, 2001), and trans-2-hexadecenal (t-2-hex) (Cohen *et al.*, 2020). Furthermore, a recent proteomic analysis of human S-acylated proteins showed that most S-acylated proteins can be modified by different FAs on single cysteine residues, indicating that S-acylation is a universal modification and competitive S-acylation with different FAs is a general phenomenon (Nůsková *et al.*, 2022).

S-acylation is a reversible modification. Many S-acylated proteins undergo cycles of S-acylation and deacylation due to the labile nature of the thioester bonds in the intracellular environment. The creation of a thioester bond between a FA and a cysteine residue of a protein is catalyzed mainly by the family of the zinc finger DHHC domain-containing protein acyl transferases (zDHHC-PATs), with 23 members in humans (Rana *et al.*, 2019). All zDHHC family members characterized to date, are predicted to be polytopic membrane proteins with the catalytic DHHC domain facing the cytosol (Chamberlain et Shipston, 2015). The majority of zDHHC proteins are localized to ER and Golgi membranes, with a small number of them present on post-Golgi compartments (Malgapo et Linder, 2021). The reverse process, protein deacylation that leads to the removal of a FA, is catalyzed by thioesterases, which include palmitoyl protein thioesterases (PPTs), acyl protein thioesterases (APTs) and α/β hydrolase domain-containing 17 proteins (ABHD17s) (Li, J. *et al.*, 2022; Swarbrick *et al.*, 2020). Acylated proteins therefore undergo continuous cycles of acylation and deacylation due to the opposing activities of acyltransferases and thioesterases. Deacylation occurs everywhere in the cell whereas acylation occurs mainly at the Golgi (Li, J. *et al.*, 2022; Swarbrick *et al.*, 2020).

*S*-acylation has been implicated in the control of multiple stages of the life cycle of transmembrane and peripheral- membrane proteins, from protein assembly to trafficking and final degradation (Chamberlain et Shipston, 2015; Chen, J. J. *et al.*, 2021). Firstly, S-acylation is found to mediate stable membrane attachment. For example, mutation of the S-acylation site(s) in Ras proteins leads to a weak association with membranes, whereas mutation of the farnesylation signal leads to a loss of both S-acylation and membrane binding (Chen, J. J. *et al.*, 2021; Hancock *et al.*, 1989). Secondly, S-acylation is involved in protein targeting to membrane microdomains. The adhesion protein CD44, a key regulator of cell migration, is enriched in cholesterol-enriched membrane microdomains termed lipid rafts. When CD44 is associated with lipid rafts via

palmitoylation on its cysteine residues, it is sequestered from binding its cytoplasmic binding partners and thus migration is restrained. However, when CD44 translocates outside of lipid rafts in its de-palmitoylated state, its cytoplasmic tail is free to bind its cytoskeletal partners, subsequently facilitating cell migration (Babina *et al.*, 2014; Donatello *et al.*, 2012; Sun *et al.*, 2020; Wei *et al.*, 2014). In addition, a recent study showed that GNAI proteins can be differentially acylated by either SFAs-C16:0/C18:0 or unsaturated MUFAs-C16:1/C18:1 on one amino acid, Cys3 (Nuskova *et al.*, 2021). Acylation with SFAs shifts GNAI proteins into detergent-resistant fractions of the cell membrane (lipid raft) where they potentiate EGFR signaling, while acylation with MUFAs causes GNAI proteins to re-localize out of lipid raft. Acylation with either SFAs or MUFAs on Cys3 depends on the relative abundance of the FAs in the medium, thereby linking cellular lipid availability to a regulatory effect on GNAI function and EGFR signaling (Nuskova *et al.*, 2021). Lastly, a recent study reported S-acylation of Bcl-2-associated X protein (BAX) by t-2-Hex to enhances its pro-apoptotic function by modulating its conformation and membrane association (Cohen *et al.*, 2020). BAX is S-acylated at Cys126, which is crucial for its insertion into the mitochondrial membrane to facilitate its oligomerization into pore-forming structures. Of note, this S-acylation is crucial in inducing conformational change and activation of BAX. In its cytosolic state, BAX is in an auto-inhibited conformation where the α9 helix is tucked inside the protein core. Upon S-acylation, structural changes occur, exposing its BH3 domain and transmembrane α9 helix, leading to membrane insertion and oligomerization. This modification stabilizes the active conformation of BAX, which is necessary for its apoptotic function (Cohen *et al.*, 2020).

### 2.5.2 **Other types of cysteine lipidation: Prenylation**

Beyond S-acylation, cysteine residues can undergo prenylation, another critical lipid modification. This modification entails the attachment of isoprenoid groups, such as farnesyl (C15) or geranylgeranyl (C20) moieties, to cysteine residues near the C-terminus of proteins. Prenylation is critical for the membrane association and protein-protein interactions of various signaling proteins, including members of the Ras superfamily (Berndt *et al.*, 2011). The prenylation motif, known as the conserved CaaX (C is cysteine, a represents aliphatic amino acids, and X determines the type of prenylation) box. The specific prenylation type is dictated by the identity of the terminal X residue in this motif, with farnesyltransferase (FTase) catalyzing farnesylation when X is serine,

methionine, alanine, or glutamine, and geranylgeranyltransferase I (GGTase I) mediating geranylgeranylation when X is leucine or phenylalanine (Zhang, F. L. et Casey, 1996). Following prenylation, the protein undergoes additional processing where the -aaX residues are cleaved by the protease Rce1 and the newly exposed C-terminal cysteine is methylated by isoprenylcysteine carboxyl methyltransferase (ICMT), modifications that collectively enhance the protein's hydrophobicity and promote its membrane association (Winter-Vann et Casey, 2005). Prenylation plays an essential role in the membrane localization and function of numerous signaling proteins, particularly those in the Ras and Rho GTPase families. While H-Ras undergoes farnesylation, K-Ras (specifically the K-Ras4B isoform) and most Rho family GTPases including Cdc42, Rac, and RhoA are geranylgeranylated (Hancock *et al.*, 1990; Parker et Mattos, 2018). The longer 20-carbon geranylgeranyl chain confers stronger membrane affinity compared to the 15-carbon farnesyl group, explaining why geranylgeranylated K-Ras4B shows more stable plasma membrane localization and broader subcellular distribution than farnesylated H-Ras (Parker et Mattos, 2018; Silvius *et al.*, 2006). This biochemical difference has profound implications for cancer biology, as K-Ras mutations drive approximately 90% of pancreatic cancers, 50% of colorectal cancers, and 30% of lung adenocarcinomas, while H-Ras mutations are relatively rare in human malignancies (Huang, L. *et al.*, 2021; Molina-Arcas et Downward, 2024). The essential role of prenylation in Ras membrane localization and oncogenic signaling has made it an attractive therapeutic target, though clinical development of prenylation inhibitors has faced significant challenges. FTase inhibitors showed initial promise but ultimately failed in cancers with K-Ras mutations due to K-Ras's ability to undergo alternative geranylgeranylation by GGTase I when FTase is inhibited (Novotny *et al.*, 2017; Whyte *et al.*, 1997). GGTase inhibitors have demonstrated preclinical efficacy but suffer from toxicity issues related to their broad effects on multiple geranylgeranylated proteins (Cox *et al.*, 2015; Philips et Cox, 2007).

In addition to Ras proteins, the geranylgeranylated GTPase Cdc42 plays critical roles in cancer progression by regulating cytoskeletal dynamics and cell migration. Membrane-anchored Cdc42 controls the formation of actin-rich filopodia protrusions that enable cancer cells to sense and respond to environmental cues during invasion (Mattila et Lappalainen, 2008). Through its effectors including WASP, N-WASP, and IRSp53, Cdc42 coordinates the assembly of linear actin bundles that drive filopodia extension, while also establishing cell polarity by regulating the Par6/aPKC complex (Jacquemet *et al.*, 2015). In breast and pancreatic cancers, hyperactivation of

Cdc42 promotes EMT and enhances metastatic potential by increasing tumor cell motility and facilitating interactions with the tumor microenvironment (Razidlo *et al.*, 2018; Zhang, Y. *et al.*, 2019). The critical dependence of both Ras and Rho family GTPases on prenylation for their oncogenic functions continues to motivate the development of novel therapeutic strategies targeting this modification pathway, including combination approaches that may overcome the limitations of single-agent prenylation inhibitors (Berndt *et al.*, 2011).

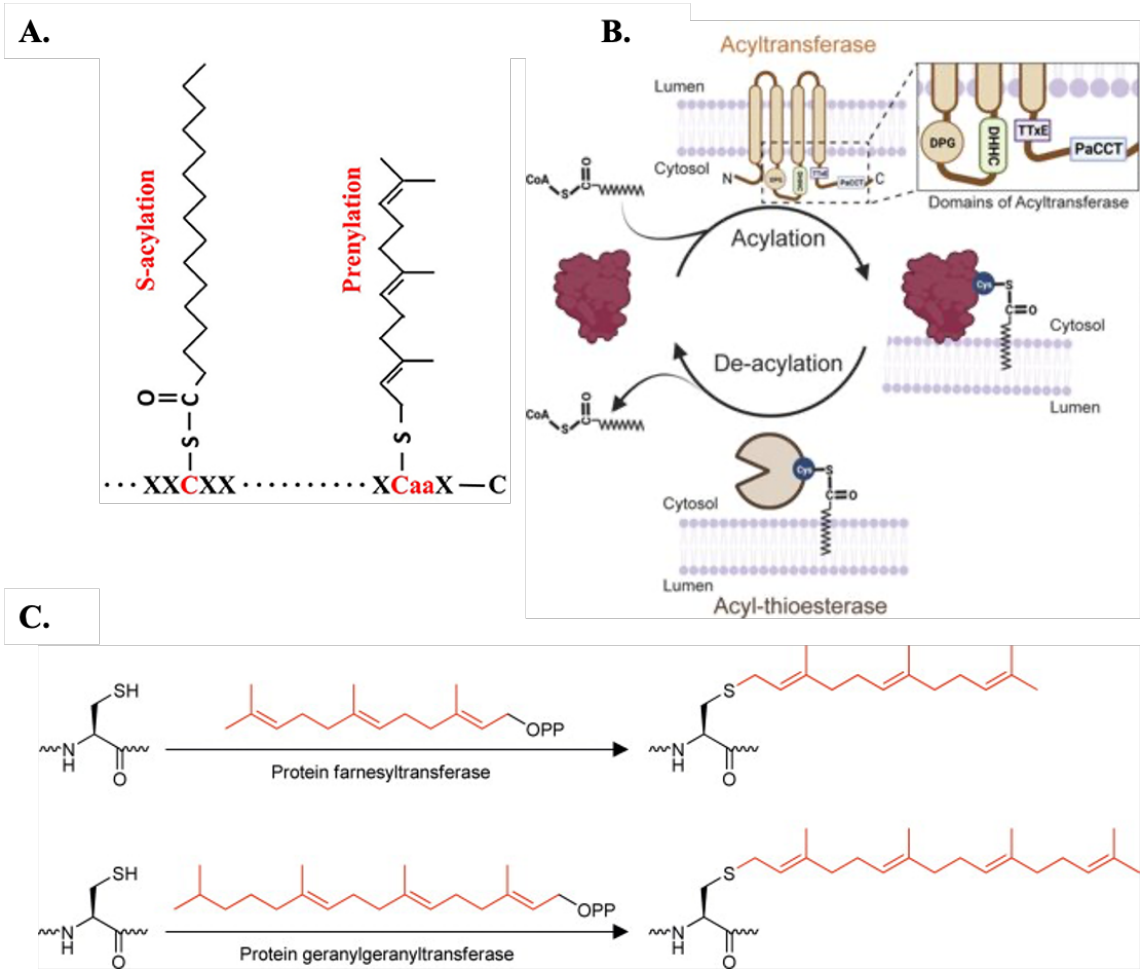


**Figure 2.6 Cysteine lipid modifications in membrane protein regulation.**

(A) Structural comparison of S-acylation and S-prenylation. S-acylation (left) forms a thioester bond between a cysteine thiol (–SH) and a medium/long-chain fatty acyl group (e.g., palmitate, C16:0). S-prenylation (right) attaches either a farnesyl (C15) or geranylgeranyl (C20) isoprenoid group via a thioether linkage. Image adapted from (Li, Y. et Qi, 2017). (B) Dynamic cycle of S-acylation. A cytosolic cysteine residue of the target protein is modified by a membrane-bound zDHHC acyltransferase, which transfers an acyl group from acyl-CoA to form a labile thioester bond. The reaction is reversed by acyl-protein thioesterases (APTs), which hydrolyze the bond. Inset: Domain architecture of a zDHHC acyltransferase (catalytic DHHC motif, transmembrane domains, and cytosolic ankyrin repeats). Image adapted from (Anwar et van der Goot, 2023). (C) Protein prenylation. Cysteine residues near the C-terminus are modified

by farnesyltransferase (FTase) or geranylgeranyltransferase (GGTase), attaching farnesyl (3 isoprene units) or geranylgeranyl (4 units) groups. This anchors proteins (e.g., small GTPases) to membranes. This figure is adapted from (Liu, H.-w. et Begley, 2020).

## 2.6 Cell Membrane Microdomains in Cancer Signaling

### 2.6.1 Lipid raft and its role in Cancer

The classic fluid mosaic model of the cell membrane by Singer–Nicolson describes the lipid bilayer as a neutral two-dimensional solvent in which proteins diffuse freely (Singer et Nicolson, 1972). This concept has since been modified by emerging evidence of membrane organization where membrane heterogeneity might play critical roles (Nicolson, 2013). The dynamic nature of cell membranes together with an uneven distribution of lipids leads to the formation of specialized membrane domains, where proteins are selectively included or excluded. Thus, cell membranes are structurally heterogeneous and contain compartmentalized microdomains, which are called rafts (Mollinedo et Gajate, 2020). Lipid rafts were firstly defined as dynamic membrane microdomains of sphingolipids and cholesterol. These microdomains are enriched in glycosphingolipids and cholesterol as well as in phospholipids acylated with saturated FAs. Lipid rafts float freely within the bilayer of cellular membranes and act as platforms for signal transduction (Simons et Ikonen, 1997). A consensus definition of a lipid raft emerged in 2006 as small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small lipid rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006). Lipid rafts can be classified into the flat type (also referred to as planar lipid rafts or non-caveolar) and caveolae type (Allen *et al.*, 2007). Caveolae lipid rafts have a concave configuration, and their key components are the caveolin proteins which has three isoforms. Caveolin-1 and caveolin-2 are widely expressed in epithelial cells, and caveolin-3 is highly expressed in striated and smooth muscle cells (Williams, T. M. et Lisanti, 2004). Flat lipid rafts are not invaginated and maintain a typical flat structure with flotillin proteins as an indispensable component. Flotillin-1 and -2 (also known as reggie-2 and reggie-1, respectively), are ubiquitously expressed (Kwiatkowska *et al.*, 2020).

Although lipid rafts have received much attention in the recent years, there is still some controversy around the techniques used to study them (Allen *et al.*, 2007; Lichtenberg *et al.*, 2005). There are two common techniques to isolate lipid rafts, the detergent-resistance-based method, and

the non-detergent-resistance-based method. Classically, lipid raft membranes are defined as being insoluble in cold non-ionic detergents such as Triton X-100, therefore they are often referred as detergent-resistant membranes. This method of isolation has received criticism because the use of detergent is thought to introduce undue artefacts (Abdelmaseeh *et al.*, 2021; Allen *et al.*, 2007). The non-detergent-based isolation of raft membranes is based on pH and carbonate resistance. Sodium carbonate, at high pH, separates proteins that are firmly attached to membranes from those that are more peripherally associated. As is the case with detergent-resistant preparations, the sonication for cell disruption involved in this method also thought to alter the relationship between membrane and proteins that normally exists in cells (Abdelmaseeh *et al.*, 2021; Allen *et al.*, 2007). Therefore, a better methodology is needed to isolate lipid rafts.

Regardless of this controversy, accumulated studies have suggested that lipid rafts serve as a spatial and a temporal platform for signalling molecules. They are involved in migration, invasion, and metastatic processes of cancer cells (Codini *et al.*, 2021; Greenlee *et al.*, 2021; Li, B. *et al.*, 2022; Mollinedo et Gajate, 2020; Vona *et al.*, 2021). Cancer cells have been reported to show elevated levels of membrane lipid rafts. Several human prostate and breast cancer cell lines (PC-3, LNCaP, MCF-7, and MDA-MB-231) show stronger cholesterol and GM1 (a raft component) staining compared with their non-tumorigenic cell line counterparts (PZ-HPV7 and MCF- 10A) (Li, Y. C. *et al.*, 2006). A higher cholesterol and lipid raft levels in tumorigenic versus non-tumorigenic melanoma cells have also been reported (Levin-Gromiko *et al.*, 2014). These results indicate the potential of lipid raft as a hallmark of cancer. In addition, invadopodia are localized where enrichment and trafficking of lipid rafts occur. And caveolin-1 is an essential regulator of MT1-MMP function in invadopodia-mediated breast cancer cell invasion (Yamaguchi *et al.*, 2009). Similarly, another study found that disruption of lipid rafts by depletion of membrane cholesterol using methyl-β-cyclodextrin (MβCD) suppressed either invadopodia formation or tumor invasion, decreased low shear stress induced activation of caveolin-1 as well as activation of PI3K/Akt/mTOR signaling cascades. Caveolin-1 knockdown significantly suppressed tumor colonization in the lungs and distant metastases in animal models (Yang, H. *et al.*, 2016). Besides, MβCD treatment reduced colocalization of the GPI-anchored membrane protein uPAR and MMP-9 in lipid rafts, inhibited phosphorylated forms of Src, FAK, Cav, Akt and ERK, leading to decreased migration of breast cancer cells (Raghu *et al.*, 2010). Finally, as described above in 2.6.1, many studies support the pro-migratory role of CD44 localized to lipid rafts. Enhanced

palmitoylation of CD44 drives its colocalization with rafts, limiting associations with its cytoskeletal linker binding partner ezrin to suppress migration in invasive breast cancer cells (Donatello *et al.*, 2012). Indeed, a further study showed that raft affinity of CD44 was also regulated via $PIP_2$ membrane concentration, which decreases raft affinity thereby accelerating formation of the CD44-adaptor complex (Sun *et al.*, 2020). Therefore, this relationship introduces a new idea for lipid rafts and lipidation acting as platforms for cell signaling. That is, while proteins may exist within rafts by palmitoylation in an "inactive" state, rapid depalmitoylation or other lipidation change may induce raft dissociation and protein translocation, subsequently activating downstream signaling. The role of lipid rafts in regulating signaling molecules involved in cancer progression is worthy of investigation.

### 2.6.2 **$PIP_2$ clusters and their role in cancer**

$PIP_2$ is a minor yet essential phospholipid component of the inner leaflet of the plasma membrane, accounting for approximately 1–2% of membrane phospholipids (Gambhir *et al.*, 2004; Ko *et al.*, 2024). It plays a pivotal role in various cellular functions, including signal transduction, cytoskeletal organization, and membrane trafficking (Mandal, 2020). $PIP_2$ can form clusters within the membrane through electrostatic interactions with polybasic proteins, influencing the localization and activity of numerous signaling molecules (Brown, D. A., 2015).

Super-resolution microscopy has revealed that $PIP_2$ forms nanoclusters ranging from approximately 50 to 200 nm that dynamically associate with receptors, such as the epidermal growth factor receptor (EGFR), and signaling effectors (Wang, J. et Richards, 2012). These clusters are stabilized by several mechanisms, including electrostatic shielding by $Ca^{2+}$ and polyamines, cytoskeletal anchoring via ERM (ezrin/radixin/moesin) proteins, and lipid phase separation in cholesterol-enriched domains (Levental *et al.*, 2009; Shabardina *et al.*, 2016; Wang, J. et Richards, 2012). The clustering of $PIP_2$ serves as a platform for the recruitment and regulation of proteins involved in actin cytoskeleton remodeling, vesicle trafficking, and other signaling pathways (Mandal, 2020).

In the context of cancer, dysregulation of $PIP_2$ metabolism and clustering has been linked to malignant transformation and tumor progression (Gozzelino *et al.*, 2020; Mandal, 2020). Alterations in $PIP_2$ levels can affect the activity of various signaling molecules, leading to enhanced

cell migration, invasion, and metastasis (Gozzelino *et al.*, 2020; Mandal, 2020). For example, changes in $PIP_2$ dynamics have been associated with the modulation of pathways that control cell proliferation and survival (Mejillano *et al.*, 2001; Singh *et al.*, 2024). $PIP_2$ interacts with proteins such as ezrin, which serves as a linker between the plasma membrane and the actin cytoskeleton (Shabardina *et al.*, 2016). Ezrin's activity is regulated by its binding to $PIP_2$ and subsequent phosphorylation, processes that are crucial for maintaining cell shape, adhesion, and motility (Shabardina *et al.*, 2016). In cancer cells, elevated $PIP_2$-dependent ezrin activation has been observed, enhancing cell motility and contributing to tumor progression via activating FAK/Src and Akt signaling (Shabardina *et al.*, 2016; Song, Y. *et al.*, 2020).

$PIP_2$ also plays a role in the regulation of PLD signaling (Oude Weernink *et al.*, 2007).The activity of PLD2 is regulated by its association with lipid rafts and $PIP_2$ clusters (Petersen *et al.*, 2016). Cholesterol-dependent interactions with lipid rafts sequester PLD2 away from its substrate, PC. Upon binding to $PIP_2$, PLD2 undergoes a conformational change that enhances its catalytic activity, leading to increased PA production (Petersen *et al.*, 2016). $PIP_2$ modulates small GTPase signaling pathways downstream of PLD2, including Rac2 and RhoA, which are essential for actin cytoskeletal reorganization and cell migration. (Gozzelino *et al.*, 2020; Oude Weernink *et al.*, 2007; Wertheimer *et al.*, 2012). In breast cancer cells, $PIP_2$ clustering enhances PLD2-mediated PI3K-AKT signaling, promoting cancer cell survival and proliferation (Chen, Y. *et al.*, 2005). Given its central role in cancer progression, targeting $PIP_2$ clusters and PLD2 signaling has emerged as a potential therapeutic strategy. Further research into the molecular interplay between $PIP_2$ and PLD2 may provide new insights into targeted therapies for metastatic cancers.

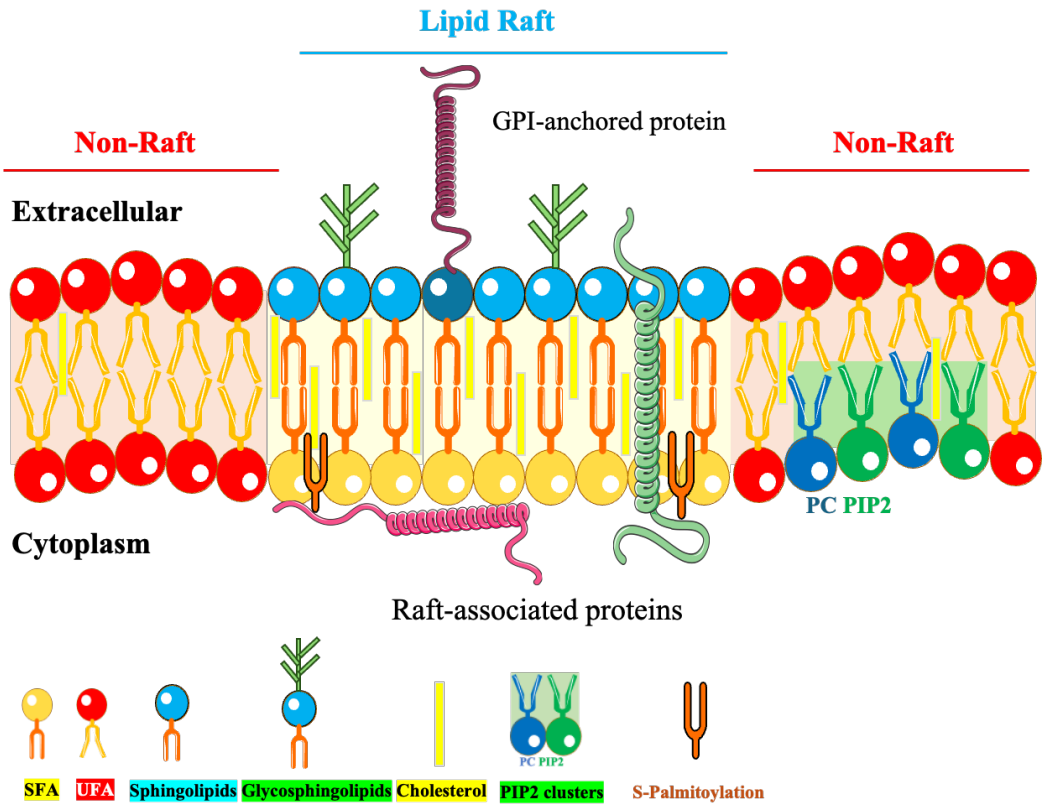


**Figure 2.7 Simplified model of membrane microdomain organization.**

Cholesterol-enriched lipid rafts are stabilized by saturated fatty acids (SFAs), which promote tight packing of phospholipids, while unsaturated fatty acids (UFAs) introduce kinks that disrupt raft formation. GPI-anchored proteins preferentially partition into rafts due to their saturated lipid tails, while S-palmitoylated proteinsare dynamically anchored to rafts via thioester-linked palmitate. In contrast, phosphatidylinositol 4,5-bisphosphate ($PIP_2$) clusters form distinct membrane microdomains enriched in UFAs, serving as platforms for signaling proteins such as PC. This image is adapted from (Chichili et Rodgers, 2009).

## 2.7 PLD and Cancer

### 2.7.1 PLD family

PLD hydrolyzes PC to yield PA and free choline (Brown, H. A. *et al.*, 2017; Bruntz *et al.*, 2014). They were first identified from carrots in 1947 (Hanahan et Chaikoff, 1947a, 1947b) and the existence of mammalian PLD was not discovered until 1973 (Gomez-Cambronero et Carman, 2014; Saito et Kanfer, 1973). In the 1990s, two human PLD enzymes, PLD1 and PLD2 were cloned and identified (Hammond *et al.*, 1995; Lopez *et al.*, 1998; Park, S. H., Chun, *et al.*, 1998; Park, S. H., Ryu, *et al.*, 1998). PLDs are known to be ubiquitously expressed, and their activities have been

described in almost all organisms from viruses and prokaryotes up to fungi, plants, and human (Gomez-Cambronero, 2014).

There are over 4000 sequences for PLD in the National Center for Biotechnology Information (NCBI) GenBank. The overall sequence homology between plant, yeast, and mammalian enzymes is quite low, with only four small regions of sequence similarity termed conserved regions (CR) CRI, CRII, CRIII, and CRIV. CRII and CRIV contain duplicate catalytic sequences, commonly termed the HKD domain (Morris *et al.*, 1996). HKD domains are characterized by the sequence $HxKx_4Dx_6G(G/S)$ (X represents any amino acid). The two best characterized mammalian isoforms are PLD1 and PLD2 with about 50% homology including two highly conserved phosphatidyltransferase HKD catalytic motifs that are required for catalytic activity. PLD1 and PLD2 also have phox homology (PX) and pleckstrin homology (PH) domains (Bowling *et al.*, 2021). There are other non-classical PLD isoforms. Most HKD-bearing PLD have 2 HKD motifs, whereas PLD6 has only one HKD motif. PLD6 is located in the mitochodrial membrane and uses cardiolipin as substrate to generate PA (Choi *et al.*, 2006). Some PLDs have no HKD motif, such as GPI-PLD, which uses glycosylphosphatidylinositol (GPI) as substrate instead of PC (Metz *et al.*, 1991). There are some PLD isoforms bearing HKD but without phospholipase activity, such as K4, Nuc, Bfil, PLD3, PLD4, mPLD5, and Zuc (Gomez-Cambronero, 2014). However, the role of these noncanonical PLDs are not well characterized. The PLD active site contains conserved histidine and lysine residues within the HKD domain, while aspartate residues are positioned externally. PLD catalyzes the hydrolysis of PC using a ping-pong mechanism (Stuckey et Dixon, 1999). One histidine acts as a nucleophile, attacking the phosphorus of PC, releasing choline, and forming a covalent intermediate with PA. The second histidine activates a water molecule, which hydrolyzes the phosphoryl-histidine bond, regenerating the enzyme and releasing PA. Lysine residues help in substrate binding by interacting with the phosphate group. In human PLD1 and PLD2, aspartic acid and glutamic acid assist in orienting histidines for catalysis (Bowling *et al.*, 2021; Bowling *et al.*, 2020).

Human PLD1 has four known splice variants termed PLD1a, PLD1b, PLD1c and PLD1d (McDermott *et al.*, 2020). PLD1b is 38 amino acids shorter than PLD1a and appears to have similar regulatory and catalytic properties (Hammond *et al.*, 1995). PLD1c contains an early truncation mutation and has been theorized to function as an inhibitor of endogenous PLD activity (Steed *et al.*, 1998). PLD1d has a 10 amino acids substitution in the C-terminal domain (962-1074). Human

PLD2 also has been reported to have 3 splice variants. PLD2a was the first identified variant. PLD2b possesses an 11aa C-terminal deletion (RFALSLRKHCFG → S), and PLD2c contains a 56 bp insertion resulting in a truncated protein (337-933 sequence missing compared with PLD2a) of unknown biological significance, since it lacks the catalytic HKD motifs, and is catalytically inactive (Di Fulvio et Gomez-Cambronero, 2005; Steed *et al.*, 1998). Although PLD1 and PLD2 share high structural similarity, their cellular localization and regulatory mechanisms differ significantly. PLD1 is highly enriched in the human heart, brain, pancreas, uterus, and intestine while PLD2 is highly enriched in the brain, placenta, lung, thymus, prostate, and uterine tissue (Lopez *et al.*, 1998). Under resting conditions, PLD1 resides on perinuclear, intracellular membranes of secretory vesicles, lysosomes, endosomes, Golgi, and ER (Freyberg *et al.*, 2001). Extracellular stimulation of cells results in PLD1 relocalization to the plasma membrane suggesting that PLD1 activation might require plasma membrane localization (Du, G. *et al.*, 2003; Kim, Y., Kim, J. E.*, et al.*, 1999). In contrast, PLD2 primarily localizes to the plasma membrane under basal conditions and is found to be enriched in lipid-raft fractions (Zheng, X. et Bollinger Bollag, 2003). Stimulation of fibroblasts with serum or mast cells with antigen, results in PLD2 relocalization to filopodia and membrane ruffles, respectively (Colley *et al.*, 1997; O'Luanaigh *et al.*, 2002). While cell type and expression levels can influence these localization patterns, this fundamental difference in membrane distribution plays a crucial role in their distinct cellular functions.

In terms of basal activity, PLD2 is constitutively active, whereas PLD1 requires protein activators to become fully functional (Bowling *et al.*, 2021; Jenkins et Frohman, 2005). Structural differences between the two isoforms may explain their distinct activation mechanisms. A key difference lies in a loop near the substrate tunnel entrance. In PLD1 (residues 827–836), this region remains flexible. In PLD2 (residues 687–696), the same region forms an alpha helix, positioned away from the active site entrance. This alpha helix formation is the most notable structural distinction between the isoforms. It results in a wider active site entrance in PLD2, which may underlie its constitutive activity. Since PLD1 lacks bound activators in its available structures, it is believed to be captured in an inactive state. Activation may involve a conformational change that reorganizes the loop into an alpha helix, similar to PLD2, leading to increased enzyme activity. Despite these structural differences, the loop's amino acid sequence is conserved in both isoforms. However, in PLD2, the alpha helix displaces an adjacent flexible loop that contains non-conserved

residues, which might contribute to the isoform-specific regulation of PLD1 and PLD2 (Bowling *et al.*, 2021).

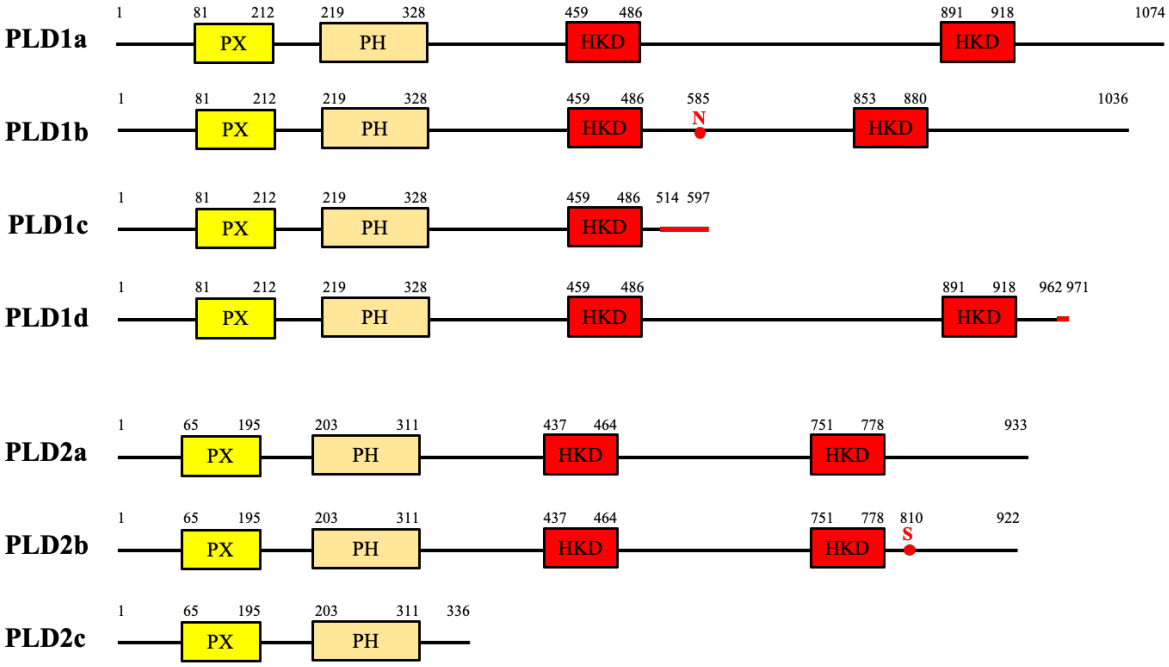


**Figure 2.8 Structure of main human PLD isoforms and splice variants.**

Human PLDs encode amino-terminal PX and PH domains followed by two catalytic HKD domains. PLD1a and PLD1b vary by 38 amino acids between 585-623. The PLD1c splice variant contains an early truncation mutation resulting in an inactive protein. PLD1d has a 10 amino acids substitution for C-terminal 962-1074. Human PLD2 also have been reported to have 3 splice variants. Compared with PLD2a, PLD2b possesses an 11aa C-terminal deletion (RFALSLRKHCFG→S), and PLD2c contains 337-933 sequence missing. Numbers indicate amino acid positions.

### 2.7.2 **Regulation of PLD2**

Studies have reported the regulation of PLD by various ways, including cell surface receptors, protein activators, phosphorylation, and lipids (Bowling *et al.*, 2021; Bruntz *et al.*, 2014; Kang *et al.*, 2014; McDermott *et al.*, 2020).

#### 2.7.2.1 Cell surface receptors

PLD activity is modulated by various cell surface receptors, including GPCRs and receptor tyrosine kinases (RTKs). Upon ligand binding, these receptors initiate signaling cascades that activate PLD. PLD activation by GPCRs has been widely observed across different cell types. Both

PLD1 and PLD2 respond to GPCR signaling, with activation reported for receptors including OA receptor GPCR40/120 (Brandenburg *et al.*, 2014; Du, G. *et al.*, 2000; Liotti *et al.*, 2018; Wang, L. *et al.*, 2003). GPCR-mediated PLD activation occurs through multiple mechanisms, including the PLC-PKC pathway, direct regulation by $G_{\beta\gamma}$, and small GTPase-mediated activation. GPCR activation leads to Gα-mediated activation of phospholipase $C_\beta$ ($PLC_\beta$). Firstly, hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI_{(4,5)}P_2$) by $PLC_\beta$ generates diacylglycerol (DAG) and inositol trisphosphate ($IP_3$). DAG and $IP_3$ synergistically activate PKCα, which subsequently stimulates PLD activity (Brandenburg *et al.*, 2014). The $G_{\beta\gamma}$ subunit can also activate $PLC_\beta$, further contributing to PLD activation via PKC-dependent signaling. While $G_{\beta\gamma}$ can indirectly enhance PLD activity via PLC-PKC signaling, it can also directly inhibit PLD by interacting with its catalytic domain, suppressing both basal and ADP-ribosylation factor (Arf)-stimulated activity (Brandenburg *et al.*, 2014; Preininger *et al.*, 2006). The $G_{\alpha12/13}$ subunits regulate PLD via small GTPases: $G_{\alpha12}$ activates RhoA through Pyk2, a focal adhesion kinase, leading to direct stimulation of PLD1. $G_{\alpha13}$ activates PI3Kγ, increasing PIP3 levels. PIP3 recruits ARNO and Rho GEF, which facilitate GTP exchange on Arf and RhoA, leading to PLD activation (Brandenburg *et al.*, 2014; Plonk *et al.*, 1998). RTKs regulate PLD activity through downstream signaling cascades, involving phosphoinositide metabolism, small GTPases, and protein kinases. Upon ligand binding, RTKs (such as EGF, PDGF, insulin) undergo autophosphorylation, triggering multiple pathways including PLC/DAG/PKC and GTPase Ras/Ral-dependent signaling cascade, ultimately modulating PLD function (Oude Weernink *et al.*, 2007; Voss *et al.*, 1999).

#### 2.7.2.2 Protein activators

Small GTPases from the Rho and ARF families are significant regulators of PLD activity. Members such as Arf1, RalA, RhoA directly bind to and allosterically activate PLD1, enhancing its enzymatic function (Bae *et al.*, 1998; Kim, J. H. *et al.*, 1998; Liotti *et al.*, 2018; Yamazaki *et al.*, 1999). Additionally, the translocation of cytosolic Arf to the plasma membrane is essential for PLD activation. Other small GTPases like Rac1, Cdc42 can also activate PLD1 (Bae *et al.*, 1998; Henage *et al.*, 2006).

PLD2 appears to be less directly regulated by GTPases (Gomez-Cambronero, 2014; Powner et Wakelam, 2002). In fact, it acts upstream and regulates the activity of some small GTPases (Chae *et al.*, 2010; Jeon *et al.*, 2011; Mahankali *et al.*, 2012; Mahankali, Peng, Henkels*, et al.*, 2011).

PLD2 functions as both a phospholipase and a GEF, regulating small GTPases such as Rac2 and RhoA (Jeon *et al.*, 2011; Mahankali, Peng, Henkels*, et al.*, 2011). PLD2's dual functionality is organized across its structure, with GEF activity residing in the N-terminal region and lipase activity in the C-terminal domain. Interestingly, these two functions are interconnected, with the products of one activity influencing the other. PA, the product of PLD2's lipase reaction, Rac2, the small GTPase, and $PIP_2$, the cofactor, all have a regulatory effect on its GEF and lipase function, suggesting a dynamic balance between the two activities over time (Mahankali *et al.*, 2013). The discovery of its GEF activity has added complexity to the understanding of its distinct roles, making it essential to differentiate between its lipase-dependent and GEF-dependent functions. Through mutational studies, key residues responsible for GEF activity—Phe-107, Phe-129, Leu-166, Arg-172, and Leu-173—were identified (Mahankali *et al.*, 2012). These findings allow for the use of lipase-inactive or GEF-inactive PLD2 mutants to distinguish between the enzyme's various cellular functions. Notably, PLD2's GEF function is linked to Ras activation in aggressive breast cancer cells, a crucial aspect of tumor progression, as Ras hyperactivation, along with mutations, drives oncogenesis (Henkels, Mahankali*, et al.*, 2013).

#### 2.7.2.3 Phosphorylation

Phosphorylation plays a significant role in regulating PLD activity, including serine, threonine, and tyrosine phosphorylation. The effects of phosphorylation vary depending on cell type, stimulus, and kinase involved (Bruntz *et al.*, 2014).

##### 2.7.2.3.1 Serine and Threonine Phosphorylation

PKC regulates PLD1 and PLD2, with phosphorylation primarily serving to modulate activity rather than being essential for catalytic function. For instance, PKCα-mediated phosphorylation of PLD1 (T147, S561) is associated with receptor activation and actin cytoskeleton interactions (Farquhar *et al.*, 2007; Kim, Y., Han, J. M.*, et al.*, 1999). Similarly, phosphorylation of PLD2 (S243, T252) in COS-7 cells does not impact its activity but affects its interactions (Chen, J. S. et Exton, 2005). PKC-mediated phosphorylation often leads to PLD activity downregulation over time, as seen with overexposure of PMA-induced activation (Chen, J. S. et Exton, 2004; Hu, T. et Exton, 2003). Other serine/threonine kinases, including p90 ribosomal S6 kinase (T147-PLD1), AMPK (S505-PLD1), and Cdk5 (S134-PLD2), regulate PLD

in response to different cellular stimuli, affecting processes like exocytosis and insulin secretion (Kim, J. H. *et al.*, 2010; Lee *et al.*, 2008; Liotti *et al.*, 2018; Zeniou-Meyer *et al.*, 2008).

#### 2.7.2.3.2 Tyrosine Phosphorylation

PLD phosphorylation is a key mechanism in cancer, influencing proliferation and signaling pathways such as Ras/MAPK (Bruntz *et al.*, 2014; Gomez-Cambronero, 2014). Elevated PLD activity, often linked to tyrosine kinase overexpression, contributes to cancer progression. PLD is regulated by tyrosine kinases such as EGFR, JAK3, and Src. EGF stimulation leads to phosphorylation of PLD2 at Y11 and Y296, which inhibits its activity (Henkels *et al.*, 2010; Slaaby *et al.*, 1998). JAK3 and Src phosphorylate PLD2 at Y415 and Y511, respectively, with opposing effects on its activity (Henkels *et al.*, 2010; Slaaby *et al.*, 1998). Additionally, PLD2 interacts with Grb2 at Y169, promoting Ras activation (Di Fulvio *et al.*, 2006). In cells overexpressing the nucleophosmin-anaplastic lymphoma kinase, PLD1/2 had increased phosphorylation at Y711 and Y573 (Wu, F. *et al.*, 2010).

### 2.7.2.4 Lipids

Phosphoinositides (PIs) regulate mammalian PLD by influencing their localization and catalysis activity via an interaction with $PI_{(4,5)}P_2$ in the N-terminal PH domain of PLD. This domain is required for proper cell localization but not for catalytic activity (Hodgkin *et al.*, 2000). When arginine 236 and tryptophan 237 of PLD2 were mutated, the resulting protein was catalytically inactive *in vivo* but displayed similar catalytic activity to wild-type protein when this mutant was immunoprecipitated and assayed *in vitro* (Sciorra *et al.*, 2002). PLD2 resides primarily in detergent-insoluble membrane fractions but mutation of R237 and W238 resulted in its relocalization to detergent-soluble membrane fractions. And this mutation also resulted in a relocalization from plasma membranes to intracellular localizations (Sciorra *et al.*, 2002). Mutation of the PH domain in PLD1 (K252/R253/W254) also reduced basal and stimulated activity and disrupted its normal localisation in COS-1 cells (Bowling *et al.*, 2021; Hodgkin *et al.*, 2000). In addition, $PIP_2$ is a critical activator of both PLD1 and PLD2 as an essential cofactor. It binds a membrane facing polybasic pocket, stabilizing the enzyme and facilitating substrate access (Bowling *et al.*, 2021; Sciorra *et al.*, 1999).

Lipid rafts and MUFAs have also been shown to play key roles in regulating PLD2 activity by sequestering the enzyme from its substrate. Disruption of the lipid rafts activates PLD2 by mixing the enzyme with its activator $PIP_2$ and substrate to produce the signalling lipid PA (Pavel *et al.*, 2020; Petersen *et al.*, 2016). The role of $PIP_2$ *in vivo* might be to recruit PLD to specific membranes, enhancing its catalytic activity by promoting substrate binding to the active site. In addition, several lines of evidence suggest that PLD2 is the isoform stimulated by unsaturated FAs. PLD activity is highly stimulated by oleate in Jurkat T cells but not in HL-60 cells (Kasai *et al.*, 1998). mRNA analysis suggests that Jurkat T cells express only PLD2, whereas HL-60 cells express PLD1. OA stimulates PLD activity in RBL-2H3 mast cells when PLD2, but not PLD1, is overexpressed (Sarri *et al.*, 2003). The *in vivo* relevance of unsaturated FA stimulation of PLD2 is not fully understood. Furthermore, both PLD1 and PLD2 undergo lipid modification. Labeling cells with tritiated FAs and subsequent measurement of lipid incorporation onto PLD protein shows that PLD1 contains a covalent palmitoylation (Manifava *et al.*, 1999). Later studies concluded that cysteines 240 and 241 are the amino acids responsible for attachment (Sugars *et al.*, 1999). In COS-7 cells, PLD1 is normally localized to punctate intracellular membranes. However, when the palmitoylated cysteines were mutated to alanine, the levels of punctate intracellular PLD1 decreased with a concomitant increase in plasma membrane localized protein. The mutant protein was less active *in vivo* but showed no differences in activity compared with wild- type protein *in vitro*, suggesting that the palmitoylation promotes accessibility of substrate lipids to PLD in the cell (Sugars *et al.*, 1999). Similar to human PLD1, rat PLD2 is also palmitoylated on C223 and C224. Mutation of the cysteine residues decreases *in vivo* activity and also results in a smaller fraction of membrane-associated PLD2 (Xie, Z. *et al.*, 2002). According to SwissPalm database (Blanc *et al.*, 2019), human PLD2 is predicted to have 2 S-acylation sites (C223 and C224), but no experimental study has been performed on human PLD2 yet.

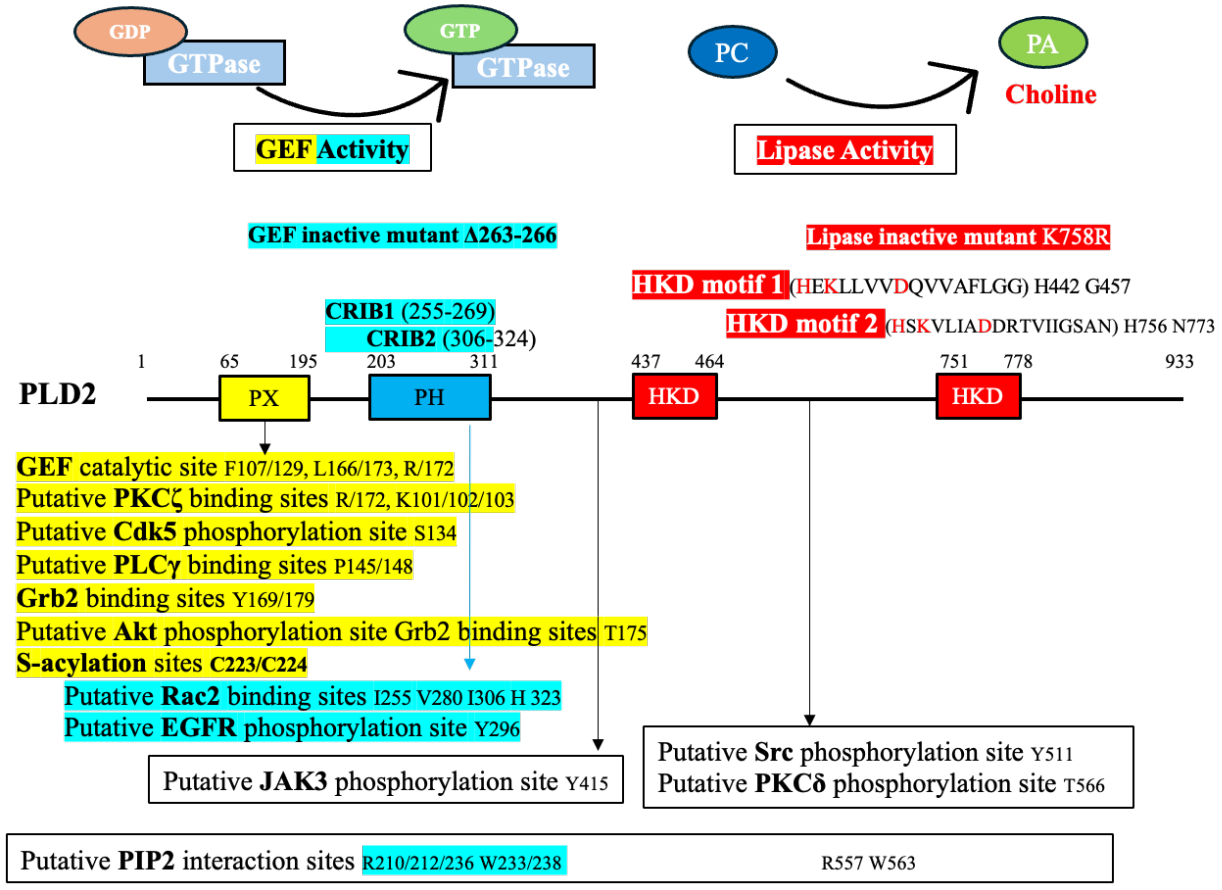


**Figure 2.9 Key regulatory sites of human PLD2.**

Schematic representation of the major structural domains and critical regulatory residues (e.g., phosphorylation, protein-binding, and catalytic sites) in human PLD2, based on data primarily from (Egea-Jimenez et Zimmermann, 2018; Mahankali *et al.*, 2015). Note: Amino acid positions may vary slightly depending on the prediction model used.

### 2.7.3 Role of PLD2 in cell migration and cancer metastasis

Elevated expression and activity of PLD, especially PLD2, have been detected in various human cancer tissues and cells, including breast cancer (Bowling *et al.*, 2021; Brown, H. A. *et al.*, 2017; Cho et Han, 2017; Gomez-Cambronero, 2014; Henkels, Boivin, *et al.*, 2013; Henkels *et al.*, 2016; Kang *et al.*, 2014; Wang, Z. *et al.*, 2017). Elevated expression of PLD2 in low invasive breast cancer cells has been shown to induce a highly aggressive phenotype, with primary tumors that formed following xenotransplantation being larger, growing faster, and developing lung metastases more readily (Henkels, Boivin, *et al.*, 2013; Henkels *et al.*, 2016). Xenotransplantation of overexpressed PLD2 in MCF-7 cells formed larger, faster-growing primary tumors and developed

lung metastases more readily, indicating a conversion to a more aggressive phenotype (Henkels, Boivin*, et al.*, 2013). Silencing of PLD2 in highly metastatic aggressive TNBC cells and osmotic pumps delivering PLD inhibitors (FIPI and NOPT) decreased tumor size and metastases formation *in vivo* (Henkels, Boivin*, et al.*, 2013; Henkels *et al.*, 2016). The recent study from our lab also reported that PLD2 expression was associated with an increased proportion of metastasis-related deaths among TNBC patients by Kaplan–Meier DMFS survival analyses and PLD activity was involved in OA induced MDA-MB-231 cell migration and invasion (Lingrand *et al.*, 2020a). Similarly, small-molecule PLD inhibitors and PLD siRNA decrease invasion of the MDA-MB-231 human breast cancer cell line and the mouse metastatic breast cancer line model 4T1 and PMT cells (Scott *et al.*, 2009). Of note, PLD enzymes, especially PLD2, are frequently localized to the leading edge of motile cells in membrane ruffles and lamellipodia. Elevated expression of PLD2 substantially increased the length of cell protrusions and a catalytically inactive PLD2 mutant abolished these cell protrusions (Colley *et al.*, 1997; O'Luanaigh *et al.*, 2002; Shen, Y. *et al.*, 2002). Furthermore, PLD2 plays an important role for cell migration via Rac-regulated cytoskeletal rearrangements. The PH domain of PLD2 encodes a Cdc42/Rac interactive binding domain that directly interacts with Rac proteins, located between amino acids 255 and 269. PLD2 potently stimulates GDP-GTP exchange on Rac2 and silencing PLD2 leads to decreased Rac2 activation and chemotaxis in neutrophils (Mahankali, Peng, Henkels*, et al.*, 2011). It has also been reported that PLD2 interaction with Grb2 via Y169 in the PX domain, and its further association to Sos, is implicated in the regulation of cell ruffling, chemotaxis, and phagocytosis of leukocytes (Gomez-Cambronero, 2011; Kantonen *et al.*, 2011). However, a more direct relationship between PLD2 and cancer cell migration remains to be established.

## 2.8 General Conclusion and Perspective

Understanding the complex interplay between lipid metabolism and cancer progression requires a comprehensive look at how MUFAs, membrane architecture, and post-translational lipid modifications coordinate cellular behavior in cancer biology. The sections above outlined the central roles of MUFAs—especially OA—and their metabolic regulators like SCD1, alongside emerging insights into the structural and functional reorganization of cell membranes in cancer. In particular, lipid-driven processes such as S-acylation, membrane microdomain remodeling, and lipidation-dependent protein trafficking converge on the regulation of signaling enzymes like

PLD2, which have been implicated in breast cancer progression and metastasis. Together, these themes provide the conceptual and mechanistic foundation of this study. Below, we summarize the broader cancer context of MUFA metabolism, and then narrow the focus to breast cancer, where our project explores how OA modifies PLD2 activity through S-acylation and membrane microdomain translocation to regulate cell membrane ruffling and cell migration.

### 2.8.1 **MUFA Metabolism and Cancer Perspective**

The exploration of MUFA and the key SCD enzymes in the context of cancer research is a relatively recent area and is still evolving. The numerous studies discussed above in *Section 2.1* highlight the intricate relationship between MUFA metabolism in the regulation of cancer development and progression. Regarding dietary MUFA, the results from epidemiologic and animal studies are inconclusive. Largely influenced by the well-known Mediterranean diet, some epidemiological studies have reported a protective role for dietary MUFA consumption in breast, colorectal, digestive, pancreatic, and prostate cancers. However, there are some contradictory findings suggesting no association in breast, colorectal, and pancreatic cancers, and even increased risks in colorectal, liver, oral, and pancreatic cancers. In a similar contradictory fashion, animal studies based on MUFA-enriched diets have shown an inhibitory effect on tumor growth in breast, colorectal, and lung cancers while tumor-promoting effects were observed in cervical and pancreatic cancers. Many factors, including the dietary source, MUFA type, and conformations (cis-/trans-FA), could contribute to the controversial relationship between MUFA intake and cancer risk. MUFAs derived from plant sources, such as extra-virgin olive oil, have been negatively correlated with cancer risk. While MUFAs from animal sources, such as meat, seem to have more adverse effects. Interestingly, MUFAs that are derived from trans-fatty acids such as elaidic acid, are also positively correlated with cancer risk. In addition, the substantial studies on SCD1, the most well-characterized enzyme implicated in MUFA synthesis, have shown a strong correlation between its expression and activity, and the development of a variety of cancer types, including breast, bladder, cervical, colorectal, esophageal, gastric, lung, ovarian, pancreatic, and prostate cancers. Deprivation of SCD1 has been shown to have antiproliferative and pro-apoptotic effects in both animal and cellular studies. In accordance with aberrant SCD1 activity, imbalanced MUFA level have been observed in various cancer patients. Of note, a series of key players in cell signaling,

including GPR, $Ca^{2+}$, PKC, ERK, EGFR, MMP, PI3K/Akt and PLD2/mTOR, have also been implicated in OA-induced cancer cell proliferation, migration, invasion, and survival.

As a result, SCD1 appears to be a promising potential target for cancer therapy. Several SCD1 inhibitors (including A939572, CAY10566, MF-438, CVT-11127 and T-3764518) have already been tested as anticancer agents in different cancer models, both *in vivo* and *in vitro*. These SCD1 inhibitors slow cancer development and progression by inducing cell death as well as inhibiting angiogenesis (Assy *et al.*, 2009; Binukumar et Mathew, 2005; Chajes *et al.*, 2008; Chakravarti *et al.*, 2012; Escrich *et al.*, 2007; Jackson *et al.*, 2012; Michaud *et al.*, 2003; Nkondjock *et al.*, 2005; Norrish *et al.*, 2000; Psaltopoulou *et al.*, 2011; Thiebaut *et al.*, 2009; Toledo *et al.*, 2015; Xin *et al.*, 2015). These inhibitors can also improve chemotherapy and radiation therapy responses by reducing inflammation, oxidative stress, and insulin resistance as well as enhancing the efficacy of other cancer therapeutic agents (Ji *et al.*, 2021; Schwingshackl *et al.*, 2017). Although the results of the preclinical SCD1 inhibitor studies are promising, inhibiting SCD1 could disrupt lipid metabolism, potentially affecting normal cellular functions and leading to side effects. In fact, severe adverse effects have been observed from animal studies, such as eye and skin dryness, hair loss, and cold-induced hypothermia (Cao *et al.*, 2016; Lodi *et al.*, 2022; Sealy *et al.*, 2021; Xin *et al.*, 2015), which is the primary challenge preventing these inhibitors from being applied to cancer therapy. Therefore, new strategies are needed before SCD1 inhibitors can be fully translated into clinical trials. From this perspective, an intriguing alternative would be to use dietary MUFAs to potentially overcome the side effects of SCD1 inhibition, as cancer cells are more dependent on SCD1 activity than normal cells. By incorporating dietary MUFAs, it might be possible to compensate for the reduced endogenous production of MUFAs to some extent, maintaining lipid homeostasis as well as supporting membrane stability and function. In addition, MUFAs, particularly those found in olive oil and avocados, have been associated with anti-inflammatory and antioxidants properties (Carvajal-Zarrabal *et al.*, 2014; de Oliveira Marques *et al.*, 2022), which potentially alleviate the inflammatory and oxidative damage caused by SCD1 inhibition. Interestingly, newly developed OA-based nanostructures showed the potential to efficiently target tumors (Ascenzi *et al.*, 2021; Chen, L. *et al.*, 2016; Gan *et al.*, 2022; Xie, X. *et al.*, 2023), and could be used to deliver SCD1 inhibitors. Thus, the combination of dietary modifications of MUFA intake and SCD1 inhibitors targeting MUFA synthesis, hold promise as powerful approaches to cancer therapy. Ongoing endeavors to identify and optimize these inhibitors are necessary to

determine the optimal dose and combination strategies to overcome side effects and improve efficiency. Conducting well-designed clinical trials across different cancer types can also provide valuable data on the potential benefits and limitations of targeting SCD and modulating MUFA metabolism in cancer patients.

Notably, there are still unresolved questions regarding the role of MUFA metabolism in cancer biology. Firstly, while the impact of dietary MUFAs on cancer *in vivo* is debated, exploring dietary interventions for cancer prevention and therapy is potentially valuable. However, controlling for confounding factors in epidemiological studies is challenging, making it difficult to isolate the specific effects of MUFA intake on cancer risk. Animal studies using purified MUFAs offer better control and focused investigations. Secondly, understanding the role of SCD and MUFAs in individual tumors and patient responses to treatment can pave the way for personalized therapeutic approaches. Identifying specific molecular biomarkers and genetic characteristics associated with SCD dysregulation or MUFA metabolism may also help identify patient subgroups that are more likely to respond to SCD inhibition or benefit from dietary modifications involving MUFAs. In addition, the existing understanding of the influence of MUFA on cancer primarily stems from studies focused on SCD1 and OA. However, recent studies have revealed alternative fatty acid desaturation pathways independent of SCD1 activity in cancer cells, involving unconventional MUFAs and desaturases (Ascenzi *et al.*, 2021; Oatman *et al.*, 2021; She *et al.*, 2019; Tracz-Gaszewska et Dobrzyn, 2019; Zhao, W. *et al.*, 2021). Recent investigations into SCD5 in metastatic melanoma cells have shown distinct expression patterns and roles in cancer progression, which is contrary to SCD1 (Ntambi *et al.*, 2002; Sampath *et al.*, 2009; Scaglia et Igal, 2008). These discoveries could explain the contradictory results of cancer cells reacting to SCD1 deprivation to some extent. Future studies on these less common desaturases, such as SCD5 and FADS2, as well as on other MUFAs, like palmitoleate and their isomers, could lead to new strategies targeting MUFA metabolism in cancer therapy. Lastly, how exogenously supplemented MUFA and endogenously synthesized MUFA from desaturases act differently on cancer development and progression remains to be clarified. Consequently, further studies are warranted to expand our knowledge in these areas and gain a more comprehensive understanding of the effects of different desaturases and MUFAs on cancer.

### 2.8.2 **Breast Cancer Perspective and Link to Present Study**

Breast cancer remains a biologically heterogeneous disease, with TNBC representing one of its most aggressive and treatment-resistant subtypes. A defining characteristic of invasive and metastatic breast cancer cells is their ability to undergo cytoskeletal remodeling and membrane reshaping, processes that are tightly regulated by signaling molecules and the physical properties of the plasma membrane (*Section 2.4*). In this context, lipid composition, membrane microdomains, and lipid-mediated signaling have emerged as crucial factors driving tumor cell plasticity, migration, and invasion.

Recent studies have emphasized the role of cell membrane microdomains, particularly lipid rafts and $PIP_2$-enriched domains, in spatially organizing signaling molecules that regulate cell motility, including PLD2 (*Section 2.6 and 2.7*). Lipid rafts, enriched in cholesterol and saturated lipids, serve as organizing centers for proteins involved in adhesion and growth factor signaling (Greenlee *et al.*, 2021; Murai, 2012; Vona *et al.*, 2021). However, MUFAs like OA could disrupt lipid raft integrity, leading to redistribution of key proteins into more dynamic and signal-responsive domains (Lindwasser et Resh, 2002; Shaikh *et al.*, 2009). This reorganization facilitates localized signaling events at the leading edge of migrating cells, including the assembly of filopodia and lamellipodia, which are hallmarks of migratory behavior in cancer cells (Bi *et al.*, 2018; Gomez-Mouton *et al.*, 2001; Vassilieva *et al.*, 2008; Wang, R. *et al.*, 2013).

One such protein whose activity is shaped by membrane microdomain localization is PLD2. PLD2 is increasingly recognized as a multifaceted player in cancer biology, functioning not only as a lipase that generates PA, but also as a non-canonical guanine GEF for small GTPases like Cdc42, which drive actin polymerization and filopodia formation (*Section 2.7*). Recent research, including our own (*Chapter 4*), has shown that PLD2 activity and localization are dynamically regulated by S-acylation, a reversible lipid modification that attaches fatty acids to cysteine residues via thioester bonds (*Section 2.5 and 2.7*). Notably, S-acylation at Cys223 and Cys224 near the PH domain of PLD2 determines its association with lipid rafts or $PIP_2$-rich regions.

Crucially, the type of FA used for S-acylation—traditionally assumed to be palmitate (C16:0)—can vary. OA (C18:1), a MUFA, has now been shown to serve as a functional S-acylation substrate (Montigny *et al.*, 2014; Nuskova *et al.*, 2021). Unlike palmitoylation, which retains proteins within lipid rafts, oleoylation tends to promote protein dissociation from lipid rafts and

favors their localization to $PIP_2$-enriched, cholesterol-independent domains, where signaling activity is often heightened (Nuskova *et al.*, 2021). Thus, OA-induced S-acylation of PLD2 could serve as a mechanism to relocate it from inactive zones to active signaling microdomains, enhancing both its lipase and GEF functions.

These processes could have direct relevance to cell migration and metastasis in breast cancer. Membrane ruffling, such as filopodia, is critical for directional cell movement, are regulated by Cdc42 activity and require finely tuned coordination of membrane dynamics and cytoskeletal remodeling (*Section 2.4*). Through its dual enzymatic roles, PLD2 integrates membrane lipid remodeling with signaling cascades that drive invasive behaviors. Our work focuses on how OA stimulates PLD2 through S-acylation, leading to its translocation from lipid rafts to $PIP_2$-rich microdomains, activation of Cdc42, and subsequent filopodia formation—a key step in cancer cell migration (*Chapter 3 and 4*).

In summary, this study builds on the growing understanding that membrane microdomains, lipid modifications like S-acylation, and dynamic protein localization are not merely structural phenomena but central regulators of oncogenic signaling. By elucidating how OA remodels PLD2 activity and localization, we offer a novel perspective on how metabolic inputs—particularly from the MUFA metabolism—can directly influence cancer cell behavior. These insights pave the way for targeting lipid-protein interactions and post-translational modifications in future therapeutic strategies for TNBC and potentially other cancers.

# CHAPITRE 3

# Article 1: Oleate Promotes Triple Negative Breast Cancer Cell Migration by Enhancing Filopodia Formation through a PLD/Cdc42-dependent Pathway

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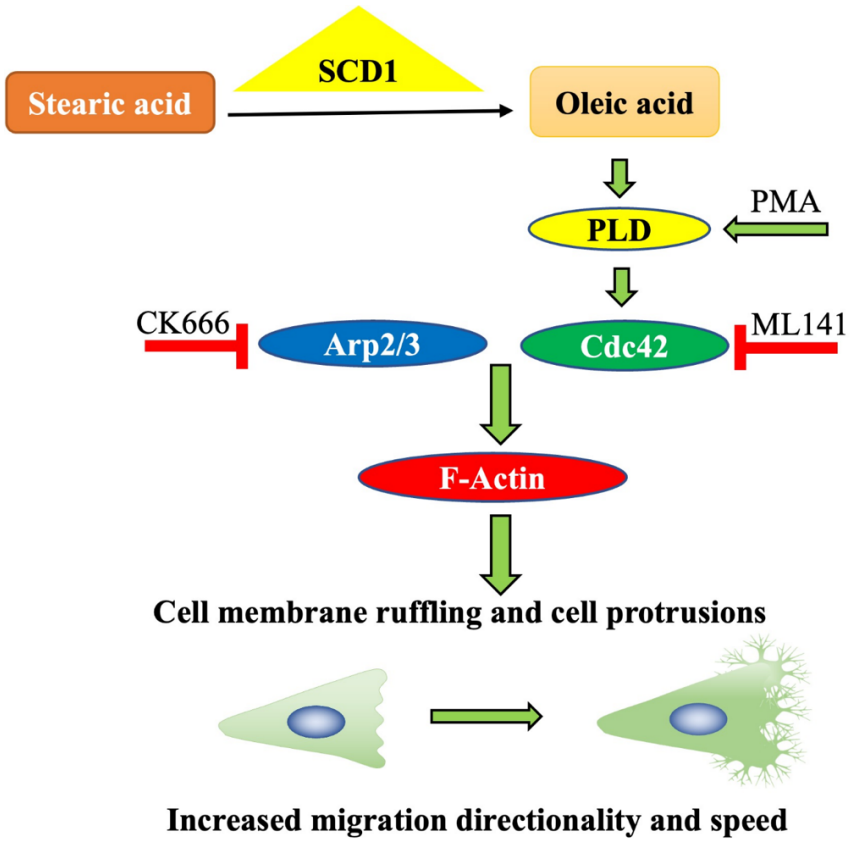
## 3.1 Abstract

Breast cancer, particularly triple-negative breast cancer (TNBC), poses a global health challenge. Emerging evidence has established a positive association between elevated levels of stearoyl-CoA desaturase 1 (SCD1) and its product oleate (OA) with cancer development and metastasis. SCD1/OA leads to alterations in migration speed, direction, and cell morphology in TNBC cells, yet the underlying molecular mechanisms remain elusive. To address this gap, we aim to investigate the impact of OA on remodeling the actin structure in TNBC cell lines, and the underlying signaling. Using TNBC cell lines and bioinformatics tools, we show that OA stimulation induces rapid cell membrane ruffling and enhances filopodia formation. OA treatment triggers subcellular translocation of Cdc42 and Arp2/3 complex. Inhibiting Cdc42, not Arp2/3 complex, effectively abolishes OA-induced filopodia formation and cell migration. Additionally, our findings suggest that phospholipase D is involved in Cdc42-dependent filopodia formation and cell migration. Lastly, the elevated expression of Cdc42 in TNBC tissues and cell lines is associated with a lower survival rate in patients. Our study outlines a new signaling pathway in OA-induced migration of TNBC cells, via promotion Cdc42-dependent filopodia formation, providing novel insight for therapeutic strategies in TNBC treatment.

**Keywords:** oleic acid; triple negative breast cancer; cell migration; filopodia; Cdc42; Arp2/3 complex; phospholipase D




**Graphic Abstract 3.1**

## 3.2 Introduction

Breast cancer is one of the most commonly diagnosed cancers in the world, presenting a significant global health challenge (Giaquinto *et al.*, 2022). By immunohistochemistry for the presence of the ER, PR, and HER2/neu, breast cancers are commonly classified into different molecular subtypes (Eliyatkin *et al.*, 2015). TNBC is defined by the lack of expression of all three receptors (Marra *et al.*, 2020). Although TNBC only accounts for 15–20% of breast cancers, this subtype is highly aggressive and prone to metastasis. It has the worst clinical outcomes with greater recurrence and lower overall survival rate. And there are no targeted therapies available yet (Devericks *et al.*, 2022; Landry *et al.*, 2022). According to the American Cancer Society, the overall 5-year relative survival rate for American patients with TNBC is 77%, compared with 90% for non-TNBC breast cancer.

OA, constitutes the most prevalent MUFA in the human diet, comprising approximately 20% of all dietary fat sources. It is also the principal component of olive oil, accounting for nearly

80% of total oil content (Gunstone, 1996; Prendeville et Lynch, 2022). The consumption of olive oil is a defining feature of the Mediterranean diet, renowned for its health-promoting attributes and traditionally associated with protective effects against cardiovascular diseases, diabetes, obesity, and cancer (Assy *et al.*, 2009; Davis *et al.*, 2015; Gunstone, 1996; Morze *et al.*, 2021; Psaltopoulou *et al.*, 2011; Schwingshackl *et al.*, 2017). MUFA, particularly OA, has been the subject of extensive research exploring its potential impact on cancer, including breast cancer (Guo, Z. *et al.*, 2023). However, the outcomes of these investigations remain inconclusive, yielding both pro-cancer and protective effects (Guo, Z. *et al.*, 2023).

In cancer progression, abnormal cell migration is a pivotal and classical aspect of tumor metastasis, encompassing a multifaceted sequence of events including tumor cell migration, invasion, intravasation, survival in the circulatory system, extravasation, and regrowth in a new environment (Bergers et Fendt, 2021; Greenlee *et al.*, 2021; Yang, H. *et al.*, 2016). Unlike normal cell migration, cancer cells can spread and move through various alternative mechanisms, such as amoeboid cell migration, mesenchymal cell migration, and collective cell migration (Wu, J. S. *et al.*, 2021). While migration patterns may differ among tumor microenvironment context, the consensus is that the regulation of actin dynamics associated with membrane protrusion serves as a fundamental and shared driver of cell migration (Wu, J. S. *et al.*, 2021). Consequently, the spatial control of the actin cytoskeleton stands as a critical factor in governing cell migration (Bonfim-Melo *et al.*, 2018; Caswell et Zech, 2018; Randzavola *et al.*, 2019). Cell membrane ruffling is the formation of actin rich membrane structures, such as lamellipodia, filopodia and membrane ruffles, and plays a key role in cell motility (Le Clainche et Carlier, 2008). Lamellipodia, large fan-like structures at the leading edge, are the most iconic form of cell protrusion. In motile cells, they adhere weakly to the substratum (He et Ji, 2017). They are formed by Arp2/3 complex-dependent actin filaments, which are not only a hallmark of the leading edge but also the driving force in single migrating cells (Innocenti, 2018; Pal *et al.*, 2020; San Miguel-Ruiz et Letourneau, 2014; Shakir *et al.*, 2008; Wu, C. *et al.*, 2012). The Arp2/3 complex, comprising seven subunits and including two actin-related proteins (Arp2 and Arp3), plays a key role in producing branched networks of actin filaments (Nolen *et al.*, 2004). Ruffles are of similar morphology to lamellipodia, but non-adherent and often protruding dorsally (Hoon *et al.*, 2012). Filopodia are finger-like actin-rich membrane protrusions that extend out from the cell edge, mediated by actin-bundling proteins such as formins, and regulated by various small GTPases of the Rho family such as Cdc42. They

are thought to be explorative, sensing the local environment, and controlling directionality but also maintaining persistence by promoting cell-matrix adhesiveness at the leading edge (Arjonen *et al.*, 2011; Bischoff et Bogdan, 2021; Bischoff *et al.*, 2021; Bray *et al.*, 2013; Gat *et al.*, 2020; Jacquemet *et al.*, 2015; Kishimoto *et al.*, 2020; Kiso *et al.*, 2018; Sakabe *et al.*, 2017). Our current understanding of the molecular mechanisms of cell migration has been largely influenced by studies in non-cancerous contexts, such as embryonic development, immune response, and tissue repair (Merino-Casallo *et al.*, 2022). However, cancer cells exhibit distinct metabolic reprogramming leading to changes in cell migration and invasion compared to normal cells (Han *et al.*, 2013).

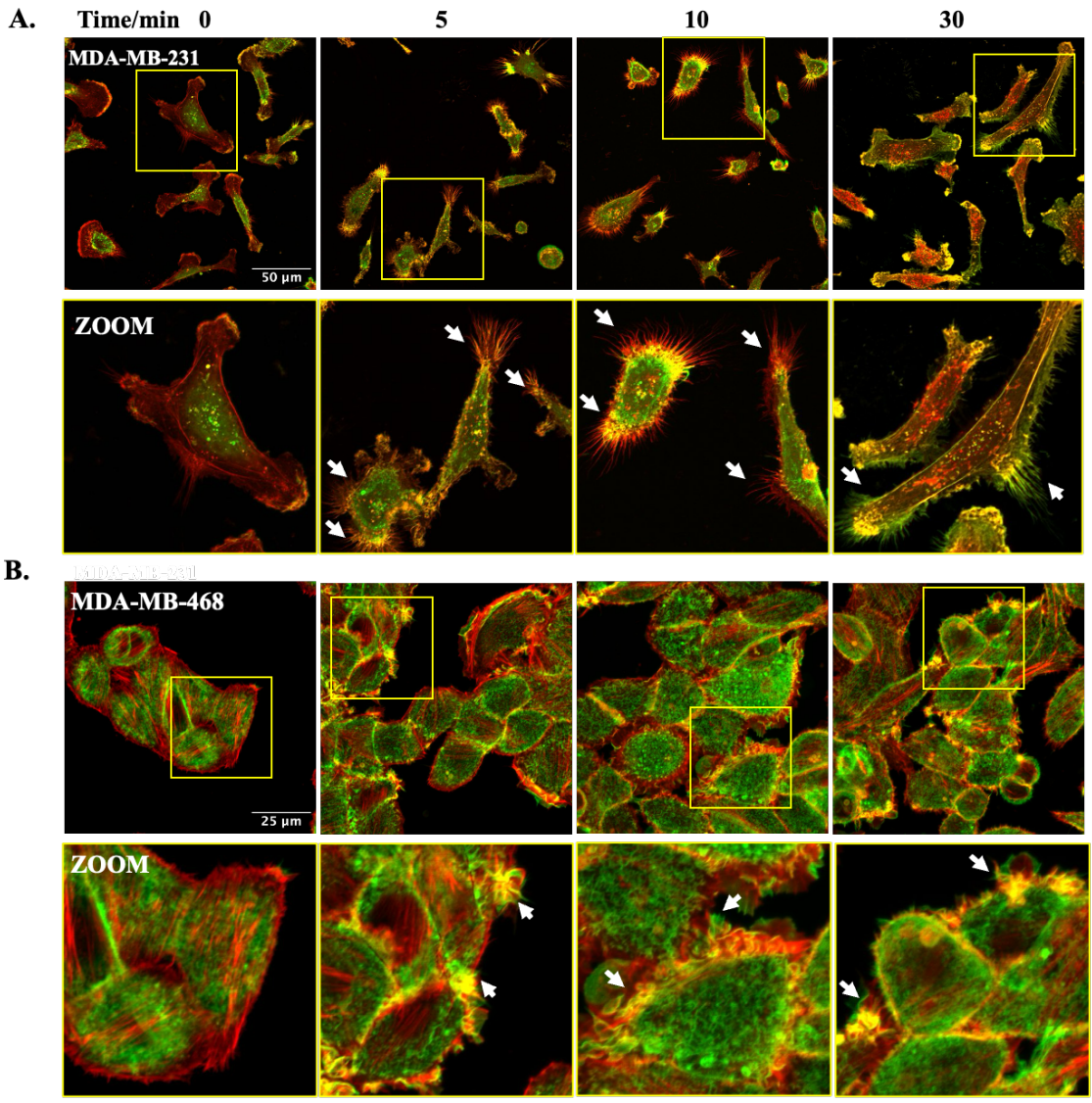
Several studies have demonstrated that OA promotes breast cancer cell migration and invasion via GPR40/120, EGFR, PI3K/Akt dependent pathways (Marcial-Medina *et al.*, 2019). OA also influences cell adhesion mechanisms, including integrin signaling and focal adhesion kinase activity (Liu, Z., Xiao*, et al.*, 2013; Liu, Z. H. *et al.*, 2020; Navarro-Tito *et al.*, 2010; Soto-Guzman *et al.*, 2010). In addition, it has been shown that OA is involved in extracellular matrix remodeling by regulating paxillin, MMPs), and fibronectin activity (Marcial-Medina *et al.*, 2019; Shen, C. J. *et al.*, 2017; Soto-Guzman *et al.*, 2010; Soto-Guzman *et al.*, 2013). The previous study from our lab revealed initial insights into the link between OA and TNBC cell migration through a PLD-mTOR pathway (Lingrand *et al.*, 2020a). However, the precise molecular mechanism involved remains elusive. This prompted us to investigate the impact of OA on remodeling the actin structure in TNBC cell lines, and the underlying signaling pathways. Our research highlighted the pivotal role of Cdc42-dependent filopodia formation in promoting TNBC cell migration, shedding light on a novel avenue for developing strategies in the treatment of TNBC.

## 3.3 Results

### 3.3.1 OA-induced cell membrane ruffling in TNBC cells

Firstly, we investigated the impact of OA treatment on TNBC cell morphology. OA treatment induced rapid cell membrane ruffling in both MDA-MB-231 and MDA-MB-468 cells (Fig. 3.1A and B). Ruffling area and intensity were both increased, with peak responses at 10 min following the onset of OA treatment (Fig. 3.1C). Moreover, OA treated TNBC cells displayed prominent filopodia or filopodia-like protrusions (Zoom panels in Fig. 3.1A, and Supplementary

Fig. S1). Quantitative assessment confirmed substantial formation of filopodia, with increased length and density, particularly at the 10 min time point (Fig. 3.1D). These findings underscore the dynamic and time-dependent alterations in cell morphology elicited by OA treatment in TNBC cells.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Time/min 0
5
10
30
MDA-MB-231
50 μm
ZOOM
B.
MDA-MB-468
25 μm
ZOOM

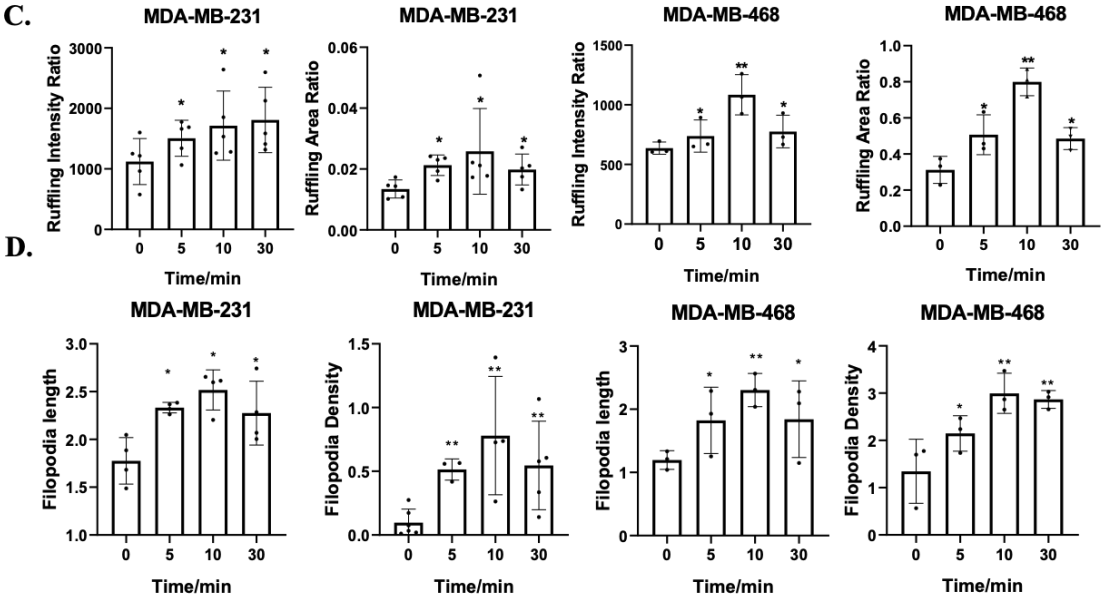
C.
MDA-MB-231
Ruffling Intensity Ratio
MDA-MB-231
Ruffling Area Ratio
MDA-MB-468
Ruffling Intensity Ratio
MDA-MB-468
Ruffling Area Ratio
Time/min
D.
MDA-MB-231
Filopodia length
MDA-MB-231
Filopodia Density
MDA-MB-468
Filopodia length
MDA-MB-468
Filopodia Density
Time/min

**Figure 3.1 OA-induced morphological changes in TNBC cell lines.**

**(A and B)** Representative fluorescence microscopy images of MDA-MB-231 and MDA-MB-468 cells. Cells were treated with 100 μM OA for the indicated times (5, 10, and 30 min), followed by Phalloidin-TRITC staining. Z-stack projection is pseudo colored: bottom cell F-actin in red, and ruffles (upper cell F-actin) in green. The scale bar depicted in the leftmost image applies uniformly to all images within the same set. The white arrows point to representative filopodia structures. **(C)** Quantitative assessment of the extent of dorsal ruffling induced by OA treatment. **(D)** Quantification of OA-induced filopodia formation from Fig. S1. Data was acquired from at least 60 cells in 3 independent experiments. Statistical significance was evaluated via the Student's t-test (*P < 0.05, **P < 0.01).

### 3.3.2 OA-induced translocation of Cdc42 and Arp2/3 complex in TNBC cells

Considering the actin-rich cell protrusion changes induced by OA, we delved deeper into the mechanisms underlying these alterations, particularly focusing on the roles of Cdc42 and the Arp2/3 complex. These two molecular players are pivotal in orchestrating cytoskeletal dynamics, including the formation of filopodia and lamellipodia respectively (Innocenti, 2018; Murphy *et al.*, 2021). In both MDA-MB-231 and MDA-MB-468 cells, the localization of Cdc42 was primarily observed in the cytoplasmic and nuclear regions in the absence of OA treatment. Following OA exposure, a distinctive perinuclear distribution pattern of Cdc42 was observed (Fig. 3.2A and B). Quantitative analysis unveiled an increased ratio of cytoplasmic to nuclear fluorescence intensity (Fig. 3.2C), suggesting a dynamic translocation of Cdc42 in response to OA treatment. To locate Arp2/3 complex, we labeled TNBC cell with an antibody against its Arp2 subunit. In MDA-MB-231 cells, Arp2 exhibited a nucleus-to-cytoplasm translocation pattern following OA treatment (Fig. 3.2D). However, this translocation was not as observed in MDA-MB-468 cells. Interestingly, MDA-MB-468 cells rather exhibited a reduction in Arp2 localization to the plasma membrane, with a greater proportion of Arp2 found within the cytoplasm (Fig. 3.2E). Quantification further revealed an increased ratio of cytoplasmic to nuclear fluorescence intensity in MDA-MB-231 cells but not in MDA-MB-468 cells (Fig. 3.2F). Our findings underscore the involvement of Cdc42 in TNBC cell response to OA treatment, while the involvement of the Arp2/3 complex appears to be contingent on the cell line.

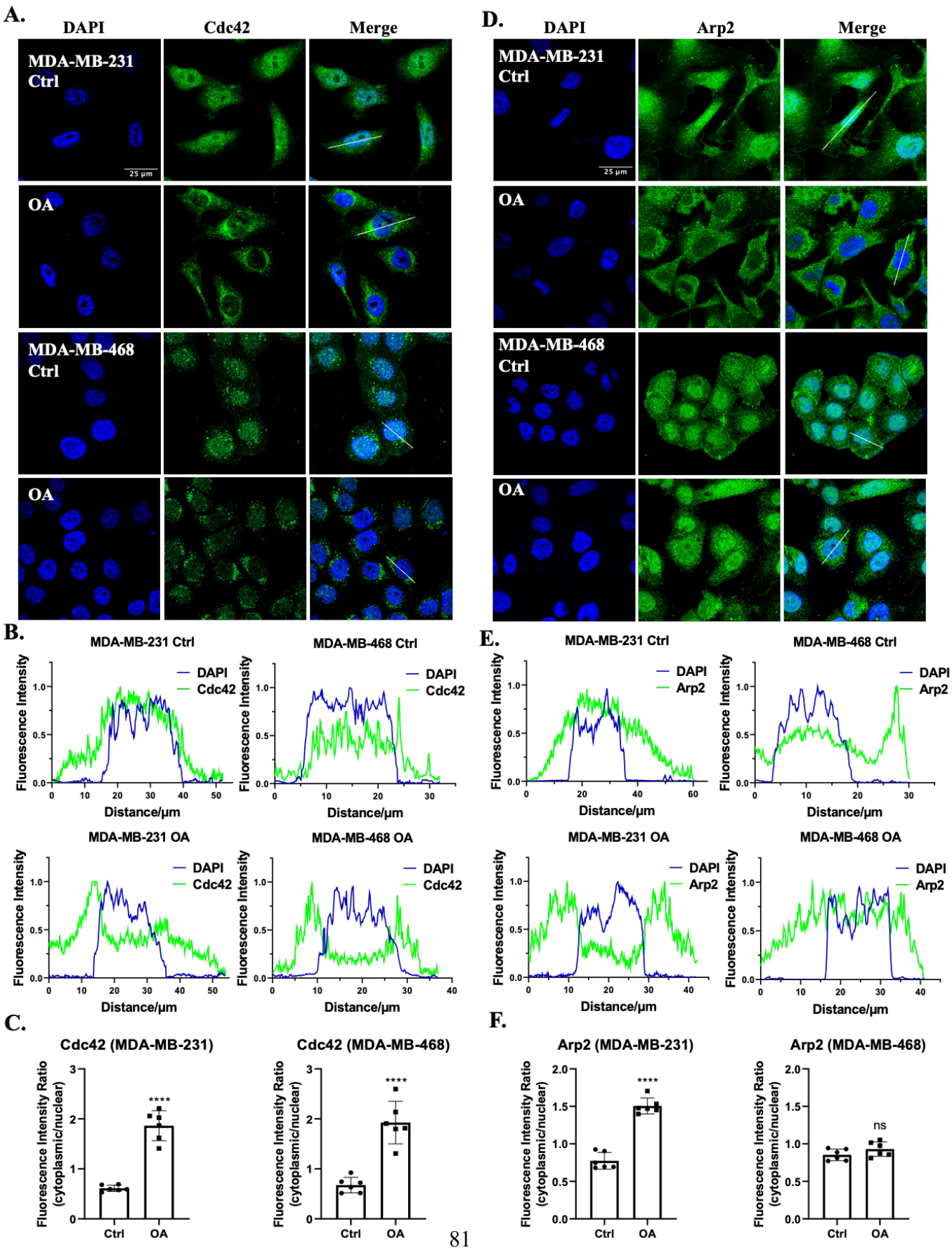
A.
DAPI
Cdc42
Merge
MDA-MB-231 Ctrl
OA
MDA-MB-468 Ctrl
OA
25 μm
D.
DAPI
Arp2
Merge
MDA-MB-231 Ctrl
OA
MDA-MB-468 Ctrl
OA
25 μm
B.
MDA-MB-231 Ctrl
MDA-MB-468 Ctrl
MDA-MB-231 OA
MDA-MB-468 OA
DAPI
Cdc42
Fluorescence Intensity
Distance/μm
E.
MDA-MB-231 Ctrl
MDA-MB-468 Ctrl
MDA-MB-231 OA
MDA-MB-468 OA
DAPI
Arp2
Fluorescence Intensity
Distance/μm
C.
Cdc42 (MDA-MB-231)
Cdc42 (MDA-MB-468)
Fluorescence Intensity Ratio (cytoplasmic/nuclear)
****
Ctrl
OA
F.
Arp2 (MDA-MB-231)
Arp2 (MDA-MB-468)
Fluorescence Intensity Ratio (cytoplasmic/nuclear)
****
ns
Ctrl
OA

**Figure 3.2 Subcellular localization of Cdc42 and Arp2/3 complex in response to OA treatment in TNBC cells.**

**(A and D)** Representative immunofluorescence confocal microscopy images of Cdc42 (A) and Arp2 (D) in MDA-MB-231 and MDA-MB-468 cells. Cells were treated with BSA (Ctrl) or 100 μM OA for 10 mins then stained with specific human Cdc42/Arp2 antibodies (green) and counterstained with DAPI (blue). The scale bar shown in the first image is applicable to all images within the same panel. **(B and E)** Fluorescence intensity histograms of DAPI (blue) and Cdc42 (B)/Arp2 (E) (green) from the lines shown in (A) and (D) (see Merge columns). **(C and F)** Quantitative assessment of Cdc42 and Arp2 fluorescence intensity within the nucleus relative to the cytoplasmic region. Statistical significance was determined using the Student's t-test (ns, not significant; ****$P < 0.0001$).

### 3.3.3 Cdc42 activity is required for OA-induced filopodia formation in TNBC cells

To further elucidate the involvement of Cdc42 and the Arp2/3 complex in OA-induced cell protrusion formation, we conducted colocalization analyses of Arp2/3 complex and Cdc42 with cell protrusions in TNBC cell lines. Our results showed that both Arp2 and Cdc42 exhibited notable colocalization with F-actin-rich cell protrusions (Fig. 3.3A). Particularly in MDA-MB-231 cells, Cdc42 exhibited a more pronounced association with filopodia, while Arp2 demonstrated a greater localization in lamellipodia, which is consistent with established literature (Jacquemet *et al.*, 2015; Wu, C. *et al.*, 2012). Subsequently, we evaluated the impact of disrupting the activity of Cdc42 and the Arp2/3 complex using specific pharmaceutical inhibitors: ML141 and CK666, respectively. MDA-MB-231 and MDA-MB-468 cells were pre-treated with either DMSO (control) or inhibitors for 1 h prior to OA exposure. MTT assays showed no significant cytotoxicity induced by the inhibitors under the conditions used in the experiments (Supplementary Fig. S2). As both Cdc42 and Arp2/3 complex are critical for the regulation of the cell cytoskeleton, both inhibitors led to a subtle disruption of F-actin structures in control cells (Fig. 3.3B). In OA treated cells, ML141 (but not CK666), resulted in a decreased percentage of cells with filopodia (Fig. 3.3B and C). These findings strongly suggest that Cdc42 activity, but not Arp2/3 complex activity, is a requisite factor for the induction of filopodia formation in response to OA treatment.

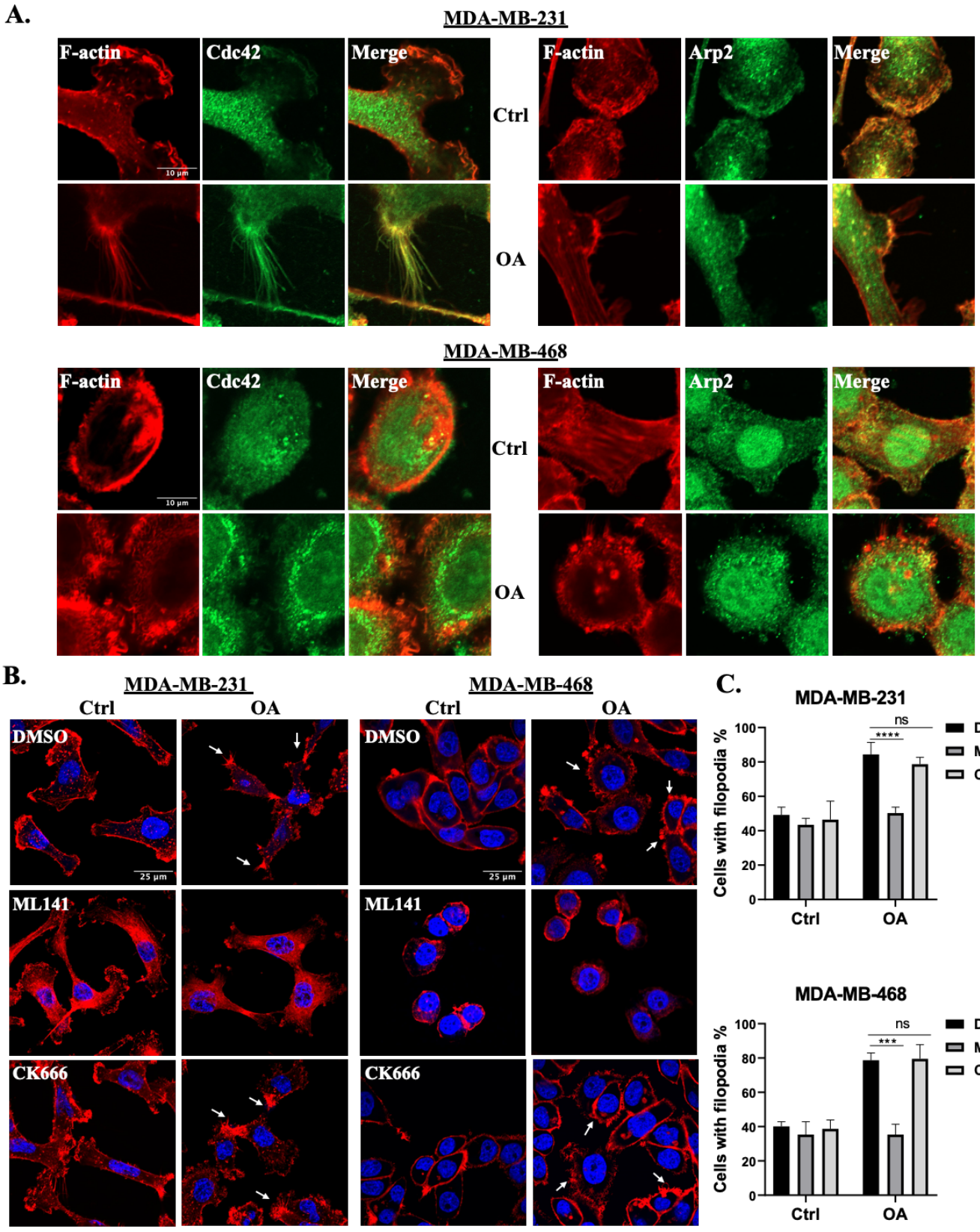
A.
MDA-MB-231
F-actin
Cdc42
Merge
F-actin
Arp2
Merge
Ctrl
OA
MDA-MB-468
F-actin
Cdc42
Merge
F-actin
Arp2
Merge
Ctrl
OA
B.
MDA-MB-231
MDA-MB-468
Ctrl
OA
Ctrl
OA
DMSO
ML141
CK666
C.
MDA-MB-231
Cells with filopodia %
ns
****
DMSO
ML141
CK666
Ctrl
OA
MDA-MB-468
Cells with filopodia %
ns
***
DMSO
ML141
CK666
Ctrl
OA

**Figure 3.3 OA promotes filopodia formation in TNBC cells via Cdc42 activation.**

**(A)** Representative immunofluorescence confocal microscopy images of the localization of Cdc42 and Arp2/3 complex in MDA-MB-231 and MDA-MB-468 cells. Cells were treated with BSA (Ctrl) or 100 μM OA for 10 min, and subsequently stained with specific Cdc42/Arp2 antibodies (green) and counterstained with Phalloidin-TRITC (red). **(B)** Effects of Cdc42 inhibition (ML141) and Arp2/3 complex inhibition (CK666) on OA-induced cell protrusions. MDA-MB-231 and MDA-MB-468 cells were pretreated with either DMSO (Ctrl), 20 μM ML141 or CK666 for 1 h, and subsequently exposed to BSA (Ctrl) or 100 μM OA for 10 min. The cells were then stained with DAPI (blue) and Phalloidin-TRITC (red). White arrows indicate representative filopodia structures. The scale bars featured in the first image apply uniformly to all images within the same set. **(C)** Images in (B) were quantified by counting the percentage of cells presenting filopodia. Data are aggregate of three experiments with at least 50 cells per experiment. Statistical significance was determined using the Student's t-test (ns, not significant; ***$P < 0.001$, ****$P < 0.0001$).

### 3.3.4 Cdc42 activity is required for OA-induced cell migration in TNBC cells

To gain a more comprehensive understanding of the interplay between OA and cell protrusions, and their combined impact on breast cancer cell migration, we conducted wound healing assays in the presence of the two distinct inhibitors for Cdc42 and Arp2/3 complex (ML141 and CK666). The MTT assay results confirmed the absence of significant cell toxicity induced by the inhibitors, as well as the absence of significant proliferation effects induced by OA under the experimental conditions in our wound healing assays (Fig. S2). In both MDA-MB-231 and MDA-MB-468 cells, OA treatment promoted wound closure, confirming the pro-migratory effects of OA. Intriguing differences emerged when Cdc42 and Arp2/3 complex inhibitors were introduced. Treatment with ML141, had a significant inhibitory effect on OA-induced wound closure in both TNBC cell lines (Fig. 3.4A and B). However, CK666, demonstrated a variable impact. It effectively inhibited OA-promoted wound closure in MDA-MB-231 cells but only at a higher concentration (10 μM). In contrast, in MDA-MB-468 cells, CK666 had no effect (Fig. 3.4C and D). Collectively, our results provide compelling evidence that Cdc42 activity is an essential requirement for OA-induced cell migration, while the contribution of Arp2/3 complex activity appears to be context-dependent, contingent on the specific characteristics of the cell line involved.

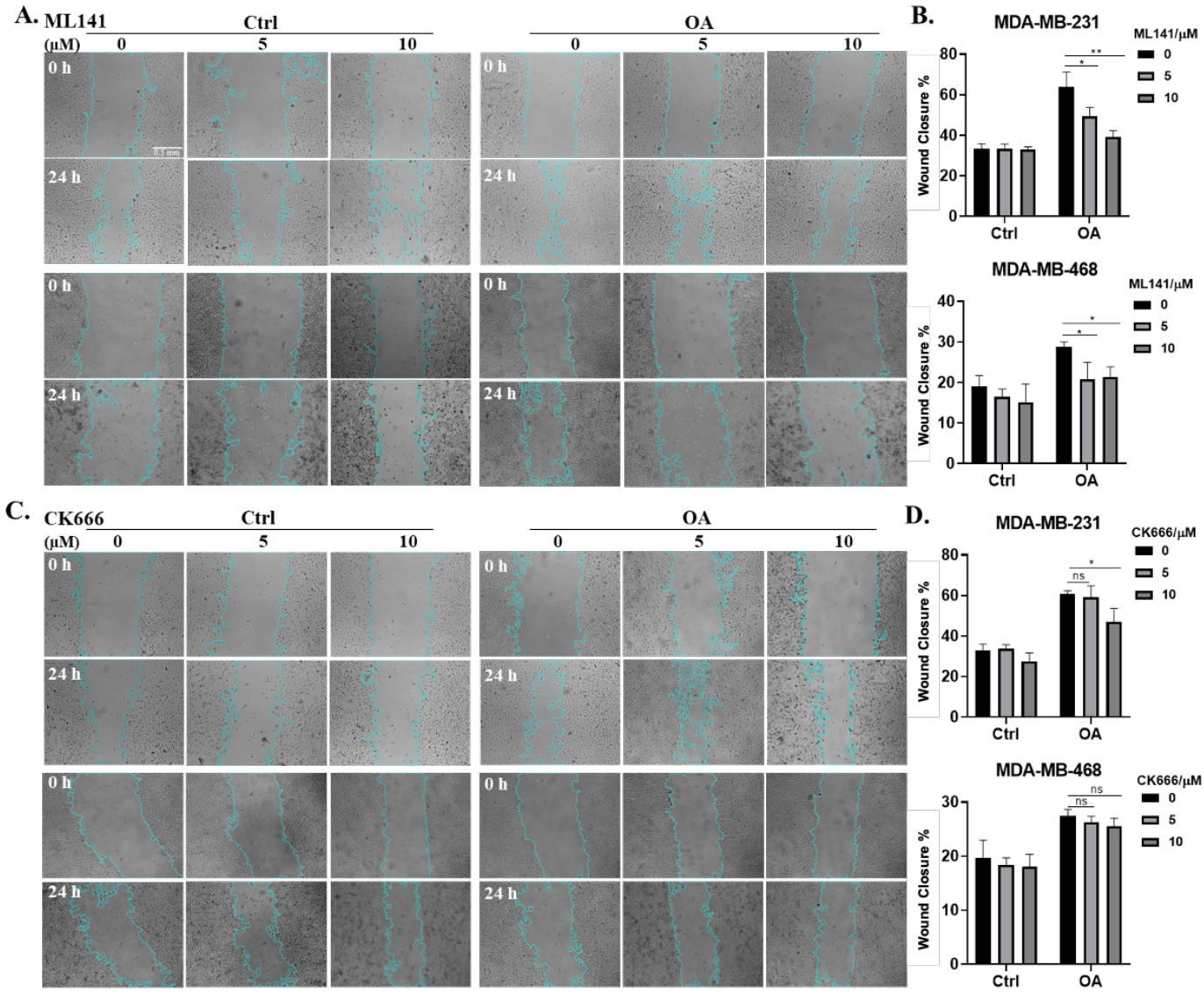


**Figure 3.4 Effect of Cdc42 and Arp2/3 complex inhibitions on OA-induced TNBC cell migration.**

**(A and C)** Representative light microscopy images of wound healing assays for MDA-MB-231 and MDA-MB-468 cells. Wound healing was evaluated over a 24-hour period following BSA (Ctrl) or 50 μM OA treatment in the presence of indicated concentrations of inhibitors (0 μM: DMSO; 5/10 μM: ML141 (A)/CK666 (C)). The scale bar featured in the first image applies uniformly to all images in the figure. **(B and D)** Quantification of wound closure in (A) and (C). The average (± standard deviation) percentage of wound closure was calculated from 3 independent experiments. Significance was determined by comparing the inhibitor subgroups (5/10 μM) with the control subgroups (0 μM) within the OA treatment groups using the Student's t-test (*P < 0.05, **P < 0.01).

### 3.3.5 **PLD is involved in OA-induced filopodia formation and cell migration**

The previous study from our lab reported that OA stimulated MDA-MB-231 cell migration in a PLD, most likely PLD2, dependent pathway (Lingrand *et al.*, 2020a). Therefore, we explored the potential involvement of PLD2 in OA-induced filopodia formation and cell migration signaling. Using a highly sensitive and specific phosphatidic acid (PA) sensor known as GFP-PASS, we confirmed that in both MDA-MB-231 and MDA-MB-468 cells, OA activated PLD activity as shown by the recruitment of GFP-PASS to the cell membrane (Fig. 3.5A and B) (Lu, M. *et al.*, 2016). We then examined the colocalization of Cdc42 and PLD2 in TNBC cells, revealing the colocalization on the cell membrane region of untreated cells. And OA treatment did not influence the expression of Cdc42 and PLD2 (Fig. S3), while it prompted the translocation of Cdc42 from the nucleus to the cytoplasm, consequently increasing the degree of colocalization of the two proteins (Fig. 3.5C and D). Much like the effect of OA, treatment with PMA (a strong PLD activator (Bruntz *et al.*, 2014; Vinggaard et Hansen, 1991)) also induced filopodia formation in both MDA-MB-231 and MDA-MB-468 cells (Fig. 3.5E). The previous research from our lab had already demonstrated that the inhibition of PLD activity effectively blocked the stimulatory effect of OA on cell migration (Lingrand *et al.*, 2020a). Building upon this, we confirmed that the activation of PLD activity by PMA led to a similar stimulating effect on wound healing recovery in TNBC cells (Fig. 3.5F and G). The wound closure stimulating effect induced by PMA was effectively abrogated by ML141, signifying the indispensable role of Cdc42 activity in PMA-induced cell migration. Taken together, our results suggest that PLD activity is required for OA-induced Cdc42-dependent filopodia formation and cell migration in TNBC cells.

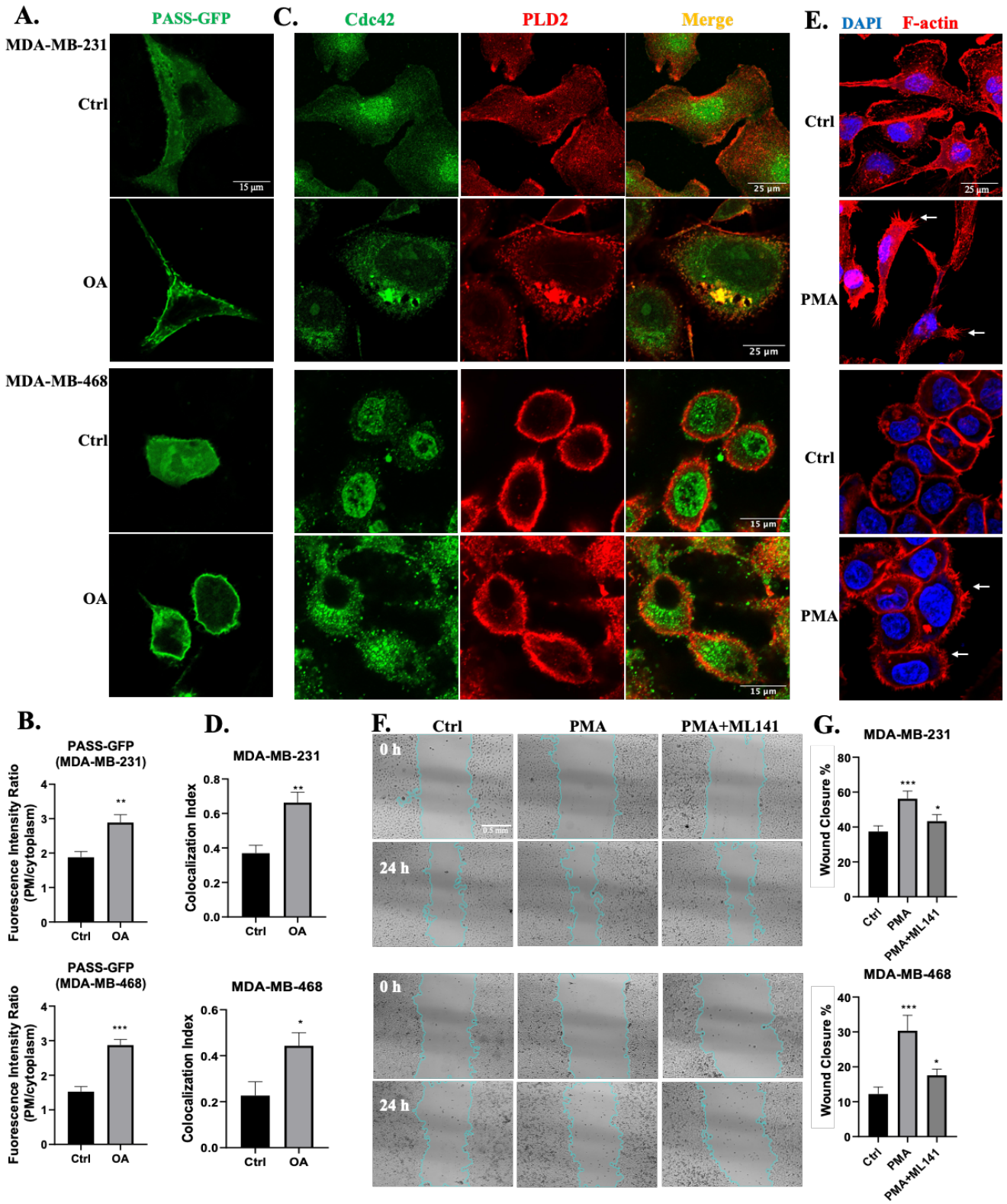
A.
PASS-GFP
MDA-MB-231
Ctrl
OA
MDA-MB-468
Ctrl
OA
15 μm
C.
Cdc42
PLD2
Merge
25 μm
15 μm
E.
DAPI
F-actin
Ctrl
PMA
Ctrl
PMA
25 μm
15 μm
B.
PASS-GFP (MDA-MB-231)
Fuorescence Intensity Ratio (PM/cytoplasm)
Ctrl
OA
PASS-GFP (MDA-MB-468)
Fuorescence Intensity Ratio (PM/cytoplasm)
Ctrl
OA
D.
MDA-MB-231
Colocalization Index
Ctrl
OA
MDA-MB-468
Colocalization Index
Ctrl
OA
F.
Ctrl
PMA
PMA+ML141
0 h
24 h
0 h
24 h
G.
MDA-MB-231
Wound Closure %
Ctrl
PMA
PMA+ML141
MDA-MB-468
Wound Closure %
Ctrl
PMA
PMA+ML141

**Figure 3.5 Involvement of PLD in OA-induced filopodia formation and TNBC cell migration.**

**(A)** Representative confocal microscopy images of PASS-GFP in response to BSA (Ctrl) or 100 μM OA treatment for 10 min in MDA-MB-231 and MDA-MB-468 cells. **(B)** Quantification of the fluorescence intensity of PASS-GFP on the plasma membrane (PM) relative to the cytoplasm. **(C)** Colocalization of Cdc42 and PLD2 in TNBC cell lines. Cells were treated with BSA (Ctrl) or 100 μM OA for 10 min, and subsequently stained with PLD2 antibody (red) and Cdc42 antibody (green). **(D)** Quantification of Cdc42 and PLD2 colocalization using Pearson's coefficient. **(E)** Induction of filopodia formation in TNBC cells by PMA. Cells were treated with BSA (Ctrl) or 10 ng/mL PMA for 10 min, and subsequently stained with DAPI (blue) and Phalloidin-TRITC (red). **(F)** Representative light microscopy images of wound healing assays for MDA-MB-231 and MDA-MB-468 cells. Wound healing was evaluated over a 24-hour period following treatment with BSA (Ctrl) or in the presence of PMA (10 ng/mL) and Cdc42 inhibitor ML141 (5 μM). **(G)** Quantification of wound closure in (F). The average (± standard deviation) percentage of wound closure was calculated from 3 independent experiments. Statistical significance was determined via the Student's t-test (*$P < 0.05$, ***$P < 0.001$). The scale bars featured apply uniformly to all images within their sets.

### 3.3.6 Elevated CDC42 expression and its association with survival in TNBC patients

To determine the expression profiles of Cdc42 (gene: CDC42) and Arp2 (gene: ACTR2) across various cancer types, we performed gene expression analysis utilizing the GEPIA (Gene Expression Profiling Interactive Analysis) platform based on tumor and normal samples from the TCGA and the GTEx databases (Li, C. *et al.*, 2021). Gene expression levels of both CDC42 and ACTR2 were notably elevated in breast cancer tissues compared to their expression in normal tissues, and were ranked among the highest in terms of expression across various cancer types (Fig. S4). Further exploration through individual cancer stages revealed that breast cancer patients exhibited heightened expression of both CDC42 and ACTR2 across all AJCC (American Joint Committee on Cancer) stages, distinguishing them from normal patients (Fig. S5). We also investigated the expression of these 2 genes in 62 breast cancer cell lines, leveraging data from the Human Protein Atlas database. Interestingly, our analysis revealed that both CDC42 and ACTR2 mRNA expression were markedly higher in TNBC cell lines in contrast to non-TNBC cell lines (Fig. 3.6A-C).

To assess the potential significance of ACTR2 and CDC42 expression in breast cancer, we generated Kaplan–Meier survival plots for Relapse Free Survival (RFS) and Distant Metastasis-Free Survival (DMFS) using available gene expression dataset records, spanning a period of up to 180 months. Intriguingly, our analysis unveiled a significant correlation between high CDC42 expression in tumor tissues and increased mortality rates in TNBC patients compared to all breast

cancer patients (Fig. S6 and Fig. 3.6D). In contrast, the association between high ACTR2 expression and mortality was notably weaker in TNBC patients compared to the broader cohort of breast cancer patients (Fig. S6 and Fig. 3.6D). The elevated mortality rate observed in the TNBC subset suggests that Cdc42 plays a more crucial role in the development and progression of TNBC compared to non-TNBC types, whereas ACTR2 exhibits the opposite trend. In sum, our bioinformatic analyses bolster our *in vitro* data supporting a role for Cdc42 in cell migration-related TNBC risk.

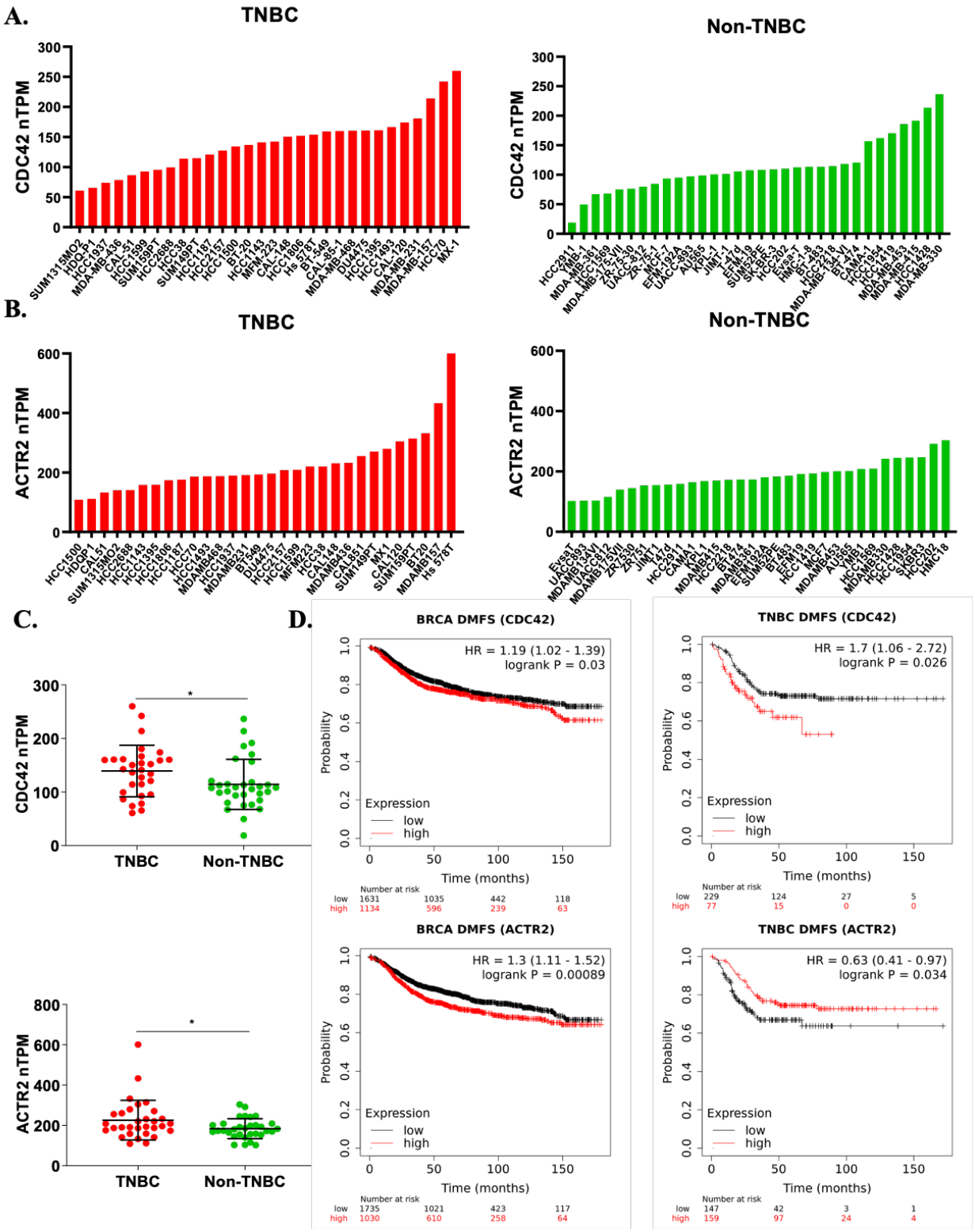
A.
TNBC
Non-TNBC
CDC42 nTPM
B.
ACTR2 nTPM
C.
D.
BRCA DMFS (CDC42)
HR = 1.19 (1.02 - 1.39)
logrank P = 0.03
TNBC DMFS (CDC42)
HR = 1.7 (1.06 - 2.72)
logrank P = 0.026
BRCA DMFS (ACTR2)
HR = 1.3 (1.11 - 1.52)
logrank P = 0.00089
TNBC DMFS (ACTR2)
HR = 0.63 (0.41 - 0.97)
logrank P = 0.034
Probability
Expression
low
high
Time (months)
Number at risk
TNBC
Non-TNBC

**Figure 3.6 High CDC42 expression correlates with TNBC risks.**

**(A-C)** Gene expression profiles of human CDC42 and ACTR2 in a panel of breast cancer cell lines (from Human Protein Atlas). Statistical significance was assessed by the Student's t-test (*P < 0.05). **(D)** Kaplan–Meier survival plots of distant metastasis-free survival (DMFS) in breast cancer patients (all patients and TNBC subset) over a span of up to 180 months, based on CDC42 and ACTR2 expression. "HR" stands for "Hazard Ratio", and "log-rank P" refers to the p-value obtained from a log-rank test.

## 3.4 Discussion

Actin cytoskeleton reorganization regulates cell morphological changes, which are intimately linked to cancer cell migration, invasion, and metastasis (Olson et Sahai, 2009). The previous study from our lab revealed that OA treatment induced increased cell migration directionality and speed, as well as a more elongated and fibroblast-like shape in MDA-MB-231 cells (Lingrand *et al.*, 2020a). Here we report a novel alteration associated with cell migration induced by OA in TNBC cells. We observed rapid cell membrane ruffling of TNBC cell lines following OA treatment, with enhanced formation of Cdc42 dependent filopodia (Figs. 1 and 3). Aligning with our observations, filopodia-related regulatory mechanisms have been identified in some breast cancer cells. Filopodia and filopodia-like structures are not only prominent features of migrating cancer cells, but also associated with the degree of cancer cell malignancy (Jacquemet *et al.*, 2017). For instance, in MCF-7 cells, the oncoprotein HBXIP was found to enhance cell migration by increasing filopodia formation via MEKK2/ERK1/2/Capn4 signaling (Li, Y. *et al.*, 2014). In MDA-MB-231 cells, filopodia formation and cell migration was regulated by L-type calcium channels (Jacquemet *et al.*, 2016) and Cdc42 (Kiso *et al.*, 2018; Liu, L. *et al.*, 2019). Furthermore, similar Cdc42 dependent filopodia formation and cell migration observations were made in other cancer types, including colorectal (Aikemu *et al.*, 2021), ovarian (Horita, Kurosaki, Nakatake, Ito*, et al.*, 2019; Horita, Kurosaki, Nakatake, Kuwano*, et al.*, 2019), pancreatic (Yuan et Wei, 2021), and lung cancer (Pan *et al.*, 2011). These findings align with our observations in TNBC cell lines, highlighting the crucial roles of filopodia formation, especially through Cdc42 activity, in the context of cancer development and invasion.

The small GTPase Cdc42 is a member of the Rho family and a master regulator of the actin cytoskeleton, controlling cell motility and cell cycle progression (Murphy *et al.*, 2021). Our results showed that ML141, a highly specific Cdc42 inhibitor, efficiently abrogated OA-induced filopodia formation and cell migration in both MDA-MB-231 and MDA-MB-468 cells (Figs. 3 and 5). In

addition, OA treatment did not change the expression of Cdc42 (Fig. S3). While it induced a nucleus to cytoplasm translocation of Cdc42 (Fig. 3.2). This change in spatial distribution could facilitate its functional switch from cell cycle regulation in the nucleus to cytoskeleton regulation in the cytoplasmic region. Furthermore, as a small GTPase, Cdc42 is activated through the exchange of GDP for GTP. This reaction is mediated by guanine nucleotide exchange factors (GEFs), which catalyze the release of GDP and loading of GTP (Maldonado *et al.*, 2020). Most Rho-GEFs localize either in the cytoplasm or in the plasma membrane (PM), and only a few of them are detected in the nucleus (Dubash *et al.*, 2011). Therefore, the cytoplasmic distribution of Cdc42 (induced by OA treatment) increases its likeliness of being activated by GEFs. Although it is still unclear how OA activates Cdc42, it is well recognized that Cdc42 could be activated by a number of cell surface receptors, such as G protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and integrin receptors, which converge on Cdc42 by activating specific GEFs (Maldonado *et al.*, 2020). OA is known to be involved in activating GPR40/120 (Guo, Z. *et al.*, 2023; Liotti *et al.*, 2018; Marcial-Medina *et al.*, 2019), insulin receptor (a member of RTK family) (Tsuchiya *et al.*, 2014), and integrin receptor signaling (Guo, Z. *et al.*, 2023; Liu, Z., Xiao*, et al.*, 2013; Liu, Z. H. *et al.*, 2020) which, in turn, could potentially activate Cdc42 via downstream GEFs. Besides, lipid modifications play an important role in the regulation of Cdc42 activity (Nishimura et Linder, 2013; Wirth et Ponimaskin, 2022). The C-terminal region of Cdc42 contains a CAAX box which is a site for post-translational lipid modification, which regulates its localization and activity (Wirth et Ponimaskin, 2022). As a possible lipid moiety, OA might also directly influence the localization and activation of Cdc42 by changing its lipidation state.

Using bioinformatics tools, we found elevated expression of CDC42 and ACTR2 in breast cancer. In TNBC, however, high expression of CDC42 in the primary tumor was clearly correlated with cancer-related death. Interestingly, this association was even higher in TNBC patients (Fig. 3.6). Although relatively few Cdc42 oncogenic mutations have been reported in cancer (Murphy *et al.*, 2021; Stengel et Zheng, 2011), the overexpression of Cdc42 is observed in several types of cancers, such as breast (Bray *et al.*, 2013; Fritz, G. *et al.*, 2002; Fritz, G. *et al.*, 1999; Jiang, L. C. *et al.*, 2011), colorectal (Du, D. S. *et al.*, 2016), esophageal (Liu, Z. *et al.*, 2011), gastric (Du, D. S. *et al.*, 2016), lung (Chen, Q. Y. *et al.*, 2012; Liu, Y. *et al.*, 2009; Liu, Y. *et al.*, 2005), melanoma (Tucci *et al.*, 2007), ovarian (Bourguignon *et al.*, 2005; Guo, Y. *et al.*, 2015), pancreatic (Yang, D. *et al.*, 2017), and testicular cancer (Kamai *et al.*, 2004). Some studies also found the overexpression

of Cdc42 to be positively correlated with poor prognostic (Bourguignon *et al.*, 2005; Kamai *et al.*, 2004; Tucci *et al.*, 2007; Yang, D. *et al.*, 2017). This further supports Cdc42 as a potential therapeutic target in cancer treatment, especially in TNBC, which do not respond to most therapies.

Elevated expression and activity of PLD, especially PLD2, have been detected in various human cancer tissues and cells, including breast cancer (Bowling *et al.*, 2021; Brown, H. A. *et al.*, 2017; Henkels, Boivin*, et al.*, 2013). Our previous study also reported that PLD expression was associated with an increased proportion of metastasis-related deaths among TNBC patients. The previous study from our lab also showed that PLD activity was involved in OA induced MDA-MB-231 cell migration and invasion (Lingrand *et al.*, 2020a). Therefore, we further investigated if PLD was involved in OA-induced filopodia formation signaling. Here, we confirmed that OA could activate PLD activity in TNBC cell lines (Fig. 3.5A). Although we could not distinguish which isoform of PLD was activated due to our methodology. Several lines of evidence have suggested that PLD2 is the isoform stimulated by OA. For instance, Kim et al. reported that OA selectively stimulated the enzymatic activity of PLD2 but not of PLD1 *in vitro* (Kim, J. H. *et al.*, 1999). PLD activity was highly stimulated by OA in Jurkat T cells (only expressing PLD2) but not in HL-60 cells (only expressing PLD1)(Gibbs et Meier, 2000; Kasai *et al.*, 1998). In RBL-2H3 mast cells OA stimulated PLD activity only when PLD2, but not PLD1, is overexpressed (Sarri *et al.*, 2003). These all support the idea that OA could activate PLD2 in TNBC cells. Next, we further explored if the activation of PLD could trigger Cdc42 dependent filopodia formation and cell migration signaling. Our results showed that, much like OA, activation of PLD by PMA induced filopodia formation as well as cell migration in TNBC cells. This effect on migration was dependent upon Cdc42 activity (Fig.3.5G). In line with a role in cell migration, PLD2 was found to be frequently localized to the leading edge of motile cells in membrane ruffles (Colley *et al.*, 1997; O'Luanaigh *et al.*, 2002). Previous studies have shown that elevated expression of PLD2 substantially increased the length of cell protrusions while a catalytically inactive PLD2 mutant abolished them (Shen, Y. *et al.*, 2002). Therefore, we investigated the potential interaction between Cdc42 and PLD2. Our results revealed that Cdc42 and PLD2 were colocalized and OA treatment increased their degree of colocalization (Fig. 3.5C and D), suggesting a direct interaction. PLD2 possesses a unique GEF feature. It was reported that PLD2 potently stimulated GDP-GTP exchange on Rac2, a Rho family member involved in filopodia formation (Bischoff *et al.*, 2021; Mahankali, Peng, Henkels*, et al.*, 2011), just like Cdc42. There are two CRIB (Cdc42-and Rac-

interactive binding) motifs in and around the PH domain of PLD2 (Peng, H. J. *et al.*, 2011), raising the possibility that PLD2 could act as a GEF for Cdc42. However, further research is required to clarify the potential connection between PLD2 and Cdc42.

In addition to Cdc42, the Arp2/3 complex has been implicated in the formation of cell protrusions and in cell migration of motile cells. However, there remains some controversy whether it is indispensable in filopodia formation. In our study, two TNBC cell lines showed different responses to Arp2/3 complex activity. MDA-MB-468 cells showed no response to CK666, a Arp2/3 complex inhibitor, in OA-induced filopodia formation and migration (Figs. 3C and 4D). While in MDA-MB-231 cells, OA induced a nucleus to cytoplasm translocation of the Arp2/3 complex, and high concentration of CK666 inhibited OA-stimulated wound closure (Figs. 2D-F and 4C&D). These results support the involvement of the Arp2/3 complex in OA-induced filopodia formation and cell migration in MDA-MB-231 cells. Although, perhaps surprisingly, CK666 did not decrease the percentage of cells with filopodia (Fig. 3.3C). We assume this difference was caused by different filopodia formation mechanisms. There are two alternative models of filopodia initiation, the convergent elongation model, and the tip nucleation model. In the convergent elongation model, filopodia are emerging from a lamellipodial actin meshwork that is assembled through Arp2/3 complex-mediated nucleation (Yang, C. et Svitkina, 2011). In contrast, the tip nucleation model proposes that filopodia are able to self-assemble directly via the action of formins on the plasma membrane (Yang, C. et Svitkina, 2011), without the need for an Arp2/3 complex-dependent lamellipodial core. It is likely that both models of filopodia formation exist in MDA-MB-231 cells while the self-assembly model appears more dominant in MDA-MB-468 cells. In addition, lamellipodia provide the driving force for forward movement, while filopodia contribute to the sensing of the microenvironment, allowing the cell to navigate in a directed manner (Bischoff et Bogdan, 2021; Bischoff *et al.*, 2021; Caswell et Zech, 2018; Innocenti, 2018; Jacquemet *et al.*, 2015). The coordination and interplay between filopodia and lamellipodia during cell migration could contribute to a higher migration speed compared to single protrusion-based movement. This might partly account for why MDA-MB-231 cells demonstrated a higher basal migration speed, both with or without OA stimulation, compared with MDA-MB-468 cells (Fig. 3.4).

In summary, our study unveils a novel signaling pathway that orchestrates OA-induced migration in TNBC cells, highlighting the pivotal role of the PLD/CDC42 axis in facilitating

filopodia formation and subsequent cell motility. Our findings shed light on the intricate molecular mechanisms underlying TNBC metastasis, providing valuable insights for the development of innovative therapeutic strategies. Additional studies will be needed to explore the involvement of the Arp2/3 complex in the context of TNBC migration. Elucidating the role of Arp2/3 complex in filopodia signaling could also provide a more comprehensive understanding of actin dynamics during cell migration and invasion. Additionally, investigating the GEF functions of PLD2 and how they might contribute to the activation of CDC42 has the potential to offer valuable insights into the intricacies of this signaling pathway. These insights will bring us closer to the prospect of developing innovative and precise therapeutic strategies for combatting TNBC metastasis, addressing a critical aspect of cancer progression that has significant implications for patient outcomes and overall survival.

## 3.5 Materials and Methods

### 3.5.1 Materials

MDA-MB-231 cells were kind gifts from Dr. Jean-Jacques Lebrun (McGill University, Canada). MDA-MB-468 cells were kind gifts from Dr. Borhane Annabi (UQAM, Canada). PASS biosensor plasmids (GFP-PASS and RFP-PASS), kind gifts from Dr. David N. Brindley (University of Alberta, Canada), originated from Dr. Guanwei Du (The University of Texas Health Science Center at Houston, USA). Eagle's minimum essential medium (EMEM, #320-005-CL) and Dulbecco's modification eagle's medium (DMEM, #319-005-CL) were purchased from Wisent. PLD2 antibody (7E4D9, #MA5-31854), Cdc42 antibody (#PA1-092), Arp2 antibody (5H2L7, #703394), and Lipofectamine 3000 Transfection Reagent were purchased from Invitrogen Thermo Fisher Scientific. PLD2 antibody (E1Y9G, #13904), HRP-conjugated anti-rabbit IgG (#7074), Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate, #4412), and Anti-mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor 647 Conjugate, #4410) were purchased from Cell Signaling Technology. Bovine serum albumin (BSA)-conjugated oleic acid (OA; #O-3008), ML141 (SML0407), CK666 (SML0006), Phorbol 12-myristate 13-acetate (PMA, P8139) were purchased from Sigma-Aldrich.

### 3.5.2 **Cell culture and transfection**

MDA-MB-231 cells were cultured in EMEM. MDA-MB-468 cells were cultured in DMEM. Both culture media were supplemented with 10% fetal bovine serum (FBS, Gibco heat inactivated, Thermo Fisher Scientific), 500 U/mL penicillin and 500 µg/mL streptomycin (LT Gibco, #15070063). Cells were maintained in a 5% CO2 incubator at 37 °C. Cells used in these experiments were between passages 5 and 25. Adherent cells were detached using 0.25% Trypsin-EDTA (Gibco, 25200056). Cells were transfected with Lipofectamin 3000 following manufacturer's instructions.

For treatments of cells, BSA-conjugated OA was used at 50-100 µM with FA free BSA as control. Cells were also incubated with Cdc42 inhibitor ML141 and Arp2/3 complex inhibitor CK666 at 5-20 µM for 1 hour before treatment or overnight with PMA at 10 ng/mL.

### 3.5.3 **Immunofluorescence staining and F-actin staining**

MDA-MB-231 and MDA-MB-468 cells were seeded at a density of around 50%-70% confluency in 24-well plates on sterilized coverslips. After 24 h incubation and following treatments, cells were washed with ice cold phosphate-buffered saline (PBS) buffer, pH 7.4, fixed with 4% paraformaldehyde in PBS for 20 min and washed again. The fixed cells were permeabilized for 10 min with 0.1% Triton X-100 and blocked with 1% BSA for 1 h at room temperature. Cells were then incubated for 20 min with 50 µg/mL of Phalloidin-TRITC Reagent (P1951, Sigma-Aldrich) and 1 µg/mL of nuclear counterstain 4′,6-diamidino-2-phenylindole (DAPI) in PBS according to the manufacturer's instructions.For immunofluorescence staining, we followed the Immunofluorescence Protocols Guidelines from Thermo Fisher Scienfic. Breifly speaking, cells were incubated overnight at 4 °C with primary antibody. After washing with PBS, cells were then incubated in properly diluted secondary Alexa Fluor antibodies at dark for 1 h at room temperature. Lastly, the coverslips were placed cell side down onto a drop of mounting medium (90% glycerol in PBS) and sealed to the microscope slide using clear nail polish. The samples were stored at 4 ° before imaging.

### 3.5.4 **Confocal microscopy and image analysis**

Fluorescent images were obtained with a Nikon A1 plus inverted confocal microscope (63 × NA oil objective). Image processing, including Z-stacking, was performed using ImageJ Fiji software.

Cell membrane ruffling assay was performed by Ruffle Analysis Macro in ImageJ Fiji following instructions (Condon *et al.*, 2020). Cell numbers and nuclei mid-point offsets were manually corrected.

For filopodia quantification analysis, at least 60 random cells of each condition from 3 independent experiments were analysed. The FiloQuant plugin (Jacquemet *et al.*, 2017) for the ImageJ Fiji software was utilized to analyze filopodia number, density, and length. For MDA-MB-231 cells, FiloQuant single image analysis was used to detect and measure the length and the number of filopodia. For MDA-MB-468 cells, only cell edge regions without cell-cell contact were randomly chosen for this analysis. The cell edge length was manually corrected using the Free Hand Line tool and Multiple Point tool. Filopodia density was defined as a ratio of the number of detected filopodia to cell edge length.

Fluorescence intensity analyses were performed using a line-intensity histogram from a selected line spanning the cell using ImageJ Fiji, as modified from Lu et. al. (Lu, M. *et al.*, 2016).

Colocalization analyses were performed by Coloc2 plugin in Image J Fiji. Pearson's coefficient value was used as colocalization index.

### 3.5.5 **Wound healing assay**

MDA-MB-231 and MDA-MB-468 cells were seeded in 24-well plates at a density of $1 \times 10^5$ cells per well. Having reached around 80% confluency, cells were treated for 24 h with OA or PMA. For inhibitors analysis, cells were pretreated with inhibitors 4 h before OA or PMA treatment. The confluent cell monolayer was scratched with P-200 tips. The wound recovery was monitored under 5% $CO_2$ and at 37 °C. Images were acquired by a Nikon Eclipse Ti inverted microscope. Wound area at each acquisition was measured by Would Healing Size Tool Macro plugin in Image J Fiji (Suarez-Arnedo *et al.*, 2020). Wound closure corresponds to the shrinking wound surface area relative to the initial area.

### 3.5.6 **Cell viability assay**

MDA-MB-231 and MDA-MB-468 cell viability was evaluated with an MTT assay modified from the MTT Assay Protocal from Millipore Sigma. Cells were seeded at a density of about 80% confluency in 96 well plate. After the incubation with treatments, 10 µl of the MTT labeling reagent (final concentration 0.5 mg/ml, 475989, Sigma-Aldrich) was added to each well and incubated for 4 h in cell incubator. The formazan crystals were dissolved in lysis solution (10% NP-40, 10 mM HCl) overnight at 37 °C and absorbance was measured at 570 nm with correction at 690 nm with a BioTek Eon Microplate Spectrophotometer.

### 3.5.7 **Bioinformatic analyses**

Gene expression analysis of human CDC42 and ACTR2 across various human cancer types were performed using GEPIA (Gene Expression Profiling Interactive Analysis, http://gepia.cancer-pku.cn) (Li, C. *et al.*, 2021). CDC42 and ACTR2 gene expression in human breast cancer stages were performed using UALCAN (University of ALabama at Birmingham CANcer data analysis Portal, https://ualcan.path.uab.edu (Chandrashekar *et al.*, 2022)). Gene expression in breast cancer cell lines were based on the Human Protein Atlas database (https://www.proteinatlas.org ). Kaplan–Meier survival plots were generated using the Kaplan-Meier Plotter (https://kmplot.com/analysis/). Breast cancer patients were separated into 2 groups based on expression of ACTR2 (probe 200729_s_at) and CDC42 (probe 210232_at) in primary tumors over a period up to 180 months using "best cutoff" option.

### 3.5.8 **Western blotting**

MDA-MB-231/-468 cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma, #P8340; #P0044). After centrifugation, proteins were recovered in the supernatant. Protein concentration was determined by the Bradford protein assay (Bio-Rad, #5000006). Proteins were separated on SDS-PAGE transferred to PVDF membranes. Primary antibodies: PLD2 (1:1000; Cell Signaling, #13904) and Cdc42 (1:500; Invitrogen, #PA1-092) were used. HRP-conjugated anti-rabbit IgG (1:2000: Cell Signaling, #7074) was used as secondary antibody. Signals were revealed using the ECL substrate (Millipore, #WBKLS0100). To normalize and verify proteins amounts equality, membranes were finally stained with amido black solution

(0.25% amido black, 45% MeOH, 45% dd$H_2O$, 10% glacial HOAc) and de-stained with the same solution without dye.

### 3.5.9 **Statistical analyses**

Statistical analyses were carried out using GraphPad Prism version 8.0. The significance of differences between groups was tested using Student's t-test. Differences were considered significant when p values were $< 0.05$.

## 3.6 Supplementary Data

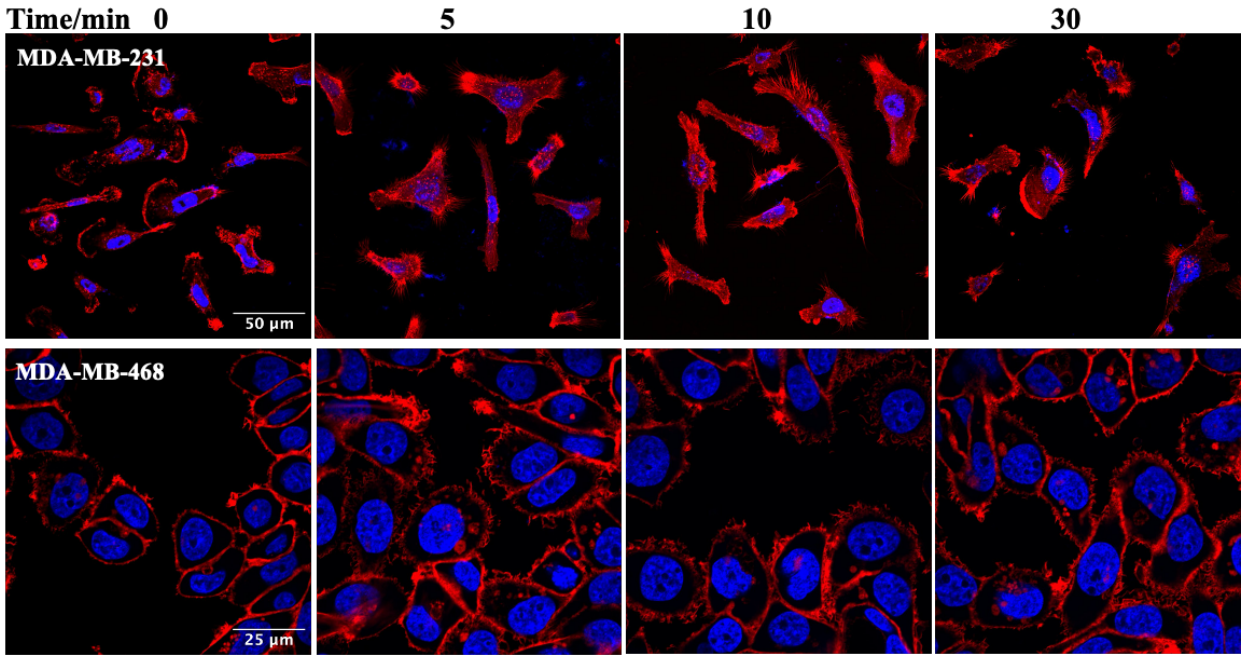


**Figure 3.7 S1 OA-induced cell protrusions in TNBC cells.**

Representative confocal microscopy images of MDA-MB-231 and MDA-MB-468 cells treated with BSA (Ctrl) or 100 µM OA for indicated times and then stained with DAPI (blue) and Phalloidin-TRITC (red). The scale bars shown in the first images apply uniformly to all images within their set.

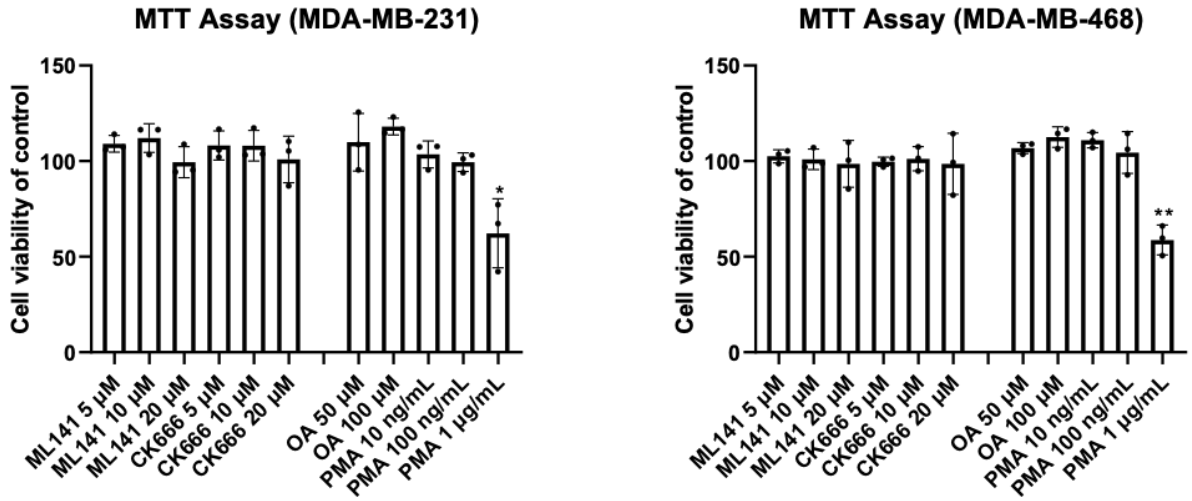

**Figure 3.8 S2 MTT assay of pharmacological inhibitors and lipid activators in TNBC cells.**

Cell viability of MDA-MB-231 and MDA-MB-468 cells treated with Cdc42 (ML141) and Arp2/3 complex (CK666) inhibitors, or OA/PMA for 24 h at indicated concentrations. MTT assay values were normalized to a control group (treated with DMSO/BSA; 100%). The average (± standard deviation) percentage was calculated from 3 independent experiments. Statistical significance was determined comparing the treatment groups to their control groups (BSA or DMSO) via the student's t-test (*P < 0.05, **P < 0.01).

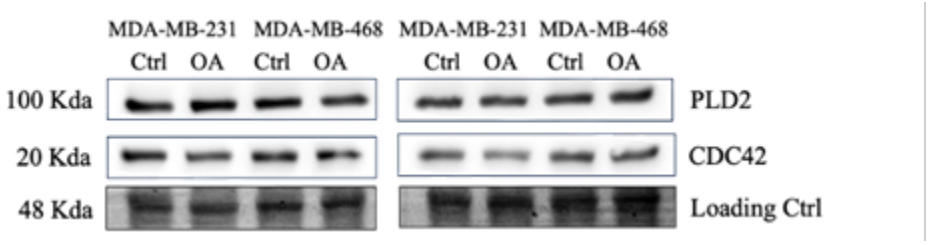


**Figure 3.9 S3 Effects of OA treatment on PLD2 and Cdc42 protein levels in TNBC cell lines.**

Protein expression of human PLD2 and Cdc42 in MDA-MB-231 and MDA-MB-468 cells treated with BSA (Ctrl) or 100 μM OA for 24 h. Loading control is provided as amido black.

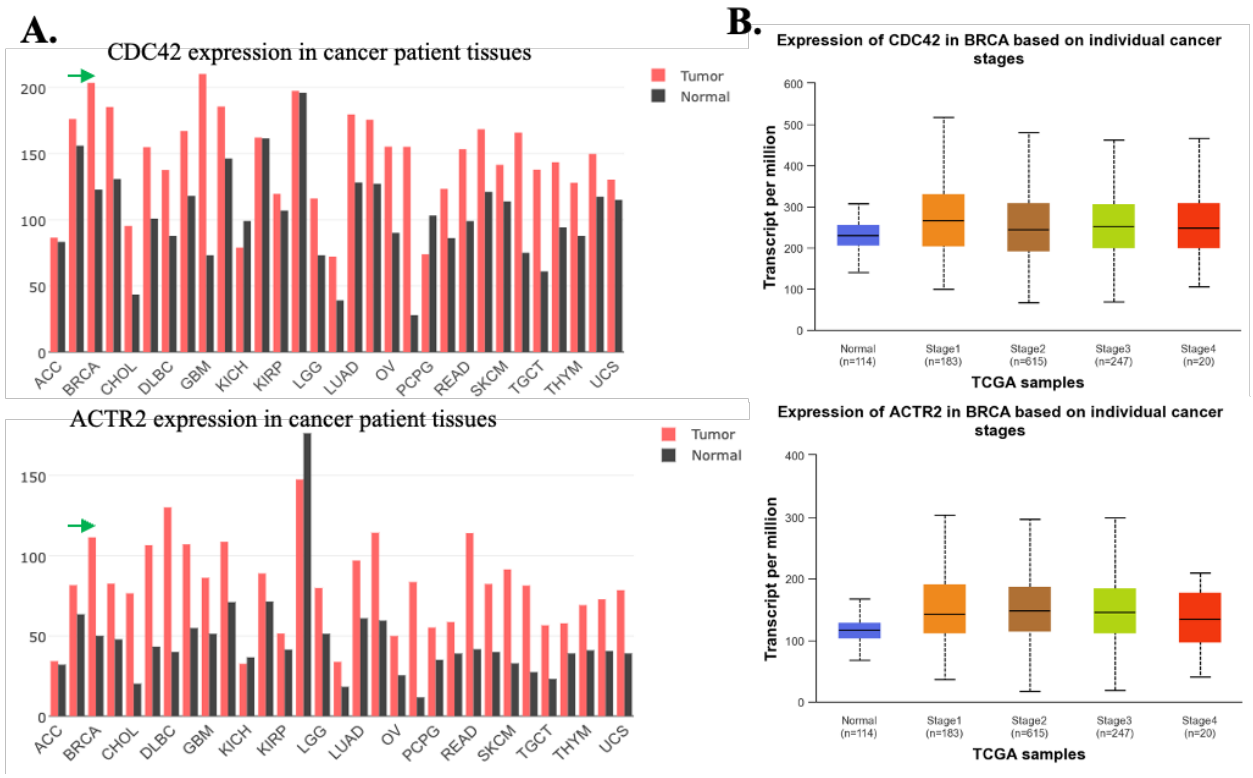


**Figure 3.10 S4 Expression profiling of CDC42 and ACTR2 in human cancers.**

**(A)** Gene expression profile of human CDC42 and ACTR2 (Arp2 gene) across tumor (red) and paired normal (black) tissues. Bar plots were generated by GEPIA**.** The x-axis displays an array of cancer types,

including ACC (adrenocortical carcinoma), BLCA (bladder urothelial carcinoma), BRCA (breast invasive carcinoma; highlighted with green arrows), CESC (cervical squamous cell carcinoma and endocervical adenocarcinoma), CHOL (cholangiocarcinoma), COAD (colon adenocarcinoma), DLBC (lymphoid neoplasm diffuse large b-cell lymphoma), ESCA (esophageal carcinoma), GBM (glioblastoma multiforme), HNSC (head and neck squamous cell carcinoma), KICH (kidney chromophobe), KIRC (kidney renal clear cell carcinoma), KIRP (kidney renal papillary cell carcinoma), LAML (acute myeloid leukemia), LGG (brain lower grade glioma), LIHC (liver hepatocellular carcinoma), LUAD (lung adenocarcinoma), LUSC (lung squamous cell carcinoma), MESO (mesothelioma), OV (ovarian serous cystadenocarcinoma), PAAD (pancreatic adenocarcinoma), PCPG (pheochromocytoma and paraganglioma), PRAD (prostate adenocarcinoma), READ (rectum adenocarcinoma), SARC (sarcoma), SKCM (skin cutaneous melanoma), STAD (stomach adenocarcinoma), TGCT (testicular germ cell tumors), THCA (thyroid carcinoma), THYM (thymoma), UCEC (uterine corpus endometrial carcinoma), UCS (uterine carcinosarcoma), UVM (uveal melanoma). **(B)** Gene expression of human CDC42 and ACTR2 across individual breast cancer stages. Bar plots were generated by UALAN.

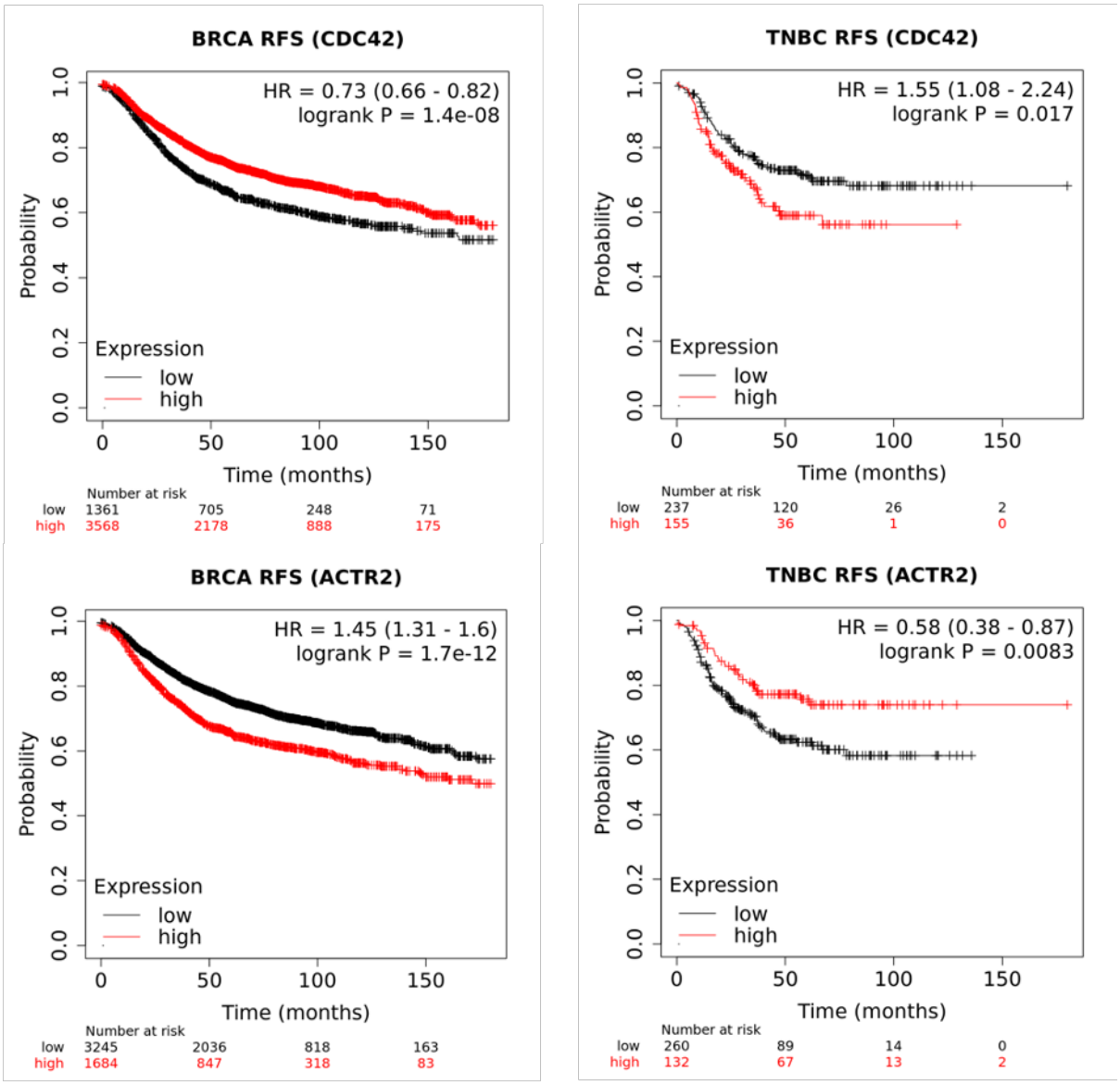

**Figure 3.11 S5 Kaplan–Meier survival plots of RFS in breast cancer patients.**

Kaplan–Meier survival plots of evaluation of relapse free survival (RFS) in breast cancer patients (all patients and TNBC subset) over a span of up to 180 months, based on CDC42 and ACTR2 expression. "HR" stands for "Hazard Ratio", and "log-rank P" refers to the p-value obtained from a log-rank test.

# CHAPITRE 4

# Article 2: Oleate Activates PLD2 Lipase and GEF Activity by Modulating Membrane Microdomain Dynamics via S-acylation

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Manuscript under preparation for *Journal of Lipid Research.* All co-authors have given their consent for this article to be used in this thesis.

## 4.1 Abstract

Phospholipase D2 (PLD2) plays critical roles in cellular signaling, membrane dynamics, and cancer progression. Oleate (OA) has been shown to activate PLD2 and promote triple-negative breast cancer (TNBC) cell migration, but the underlying molecular mechanisms remain poorly understood. Using confocal microscopy, lipid raft isolation, and acylation assays, we show that OA enhances PLD2 S-acylation at Cys223 and Cys224, disrupts its lipid raft localization, and increases its colocalization with $PIP_2$-enriched microdomains. Furthermore, we identify PLD2 as a guanine nucleotide exchange factor (GEF) for Cdc42, with its GEF activity regulated by OA-dependent S-acylation and lipid raft dynamics. Mutation of the acylation sites or disruption of lipid rafts abolishes PLD2-mediated Cdc42 activation and filopodia-like cell protrusion formation. These findings reveal a novel mechanism by which OA modulates PLD2 activity through S-acylation and membrane microdomain reorganization, providing new insights into the regulation of PLD2 in cancer cell migration and signaling.

Key words: oleate, PLD2, S-acylation, Cdc42, lipid raft, guanine nucleotide exchange factor

## 4.2 Introduction

PLD2 is a key enzyme in lipid signaling, catalyzing the hydrolysis of PC to produce PA, a lipid second messenger that plays essential roles in cell proliferation, survival, and migration (Brown, H. A. *et al.*, 2017; Bruntz *et al.*, 2014). PLD2 has been increasingly implicated in cancer progression, particularly in metastatic cancers such as TNBC, where it enhances cell motility and invasion (Henkels, Boivin*, et al.*, 2013; Henkels *et al.*, 2016). The previous studies from our lab have highlighted that PLD2 expression is associated with poor clinical outcomes among TNBC patients, and that PLD activity is involved in OA-induced TNBC cell migration and invasion by modulating cytoskeletal dynamics and cell membrane remodeling (Guo, Z. *et al.*, 2024; Lingrand *et al.*, 2020a). Unlike PLD1, which is predominantly found in intracellular compartments, PLD2 localizes mainly at the plasma membrane and exhibits constitutive catalytic activity (Bowling *et al.*, 2021; Jenkins et Frohman, 2005). Of note, PLD2 is inhibited when sequestered in lipid rafts and raft dissociation liberates PLD2, enabling its PC-hydrolyzing activities (Petersen *et al.*, 2016). Additionally, PLD2 is subject to multiple regulatory mechanisms, including phosphorylation, protein-protein interactions, and lipid modifications (Gomez-Cambronero, 2014; Gomez-

Cambronero et Carman, 2014). One of its most intriguing functions beyond its enzymatic role is its GEF activity, specifically activating small GTPases such as Rac2 and RhoA (Jeon *et al.*, 2011; Mahankali, Peng, Henkels*, et al.*, 2011). This unique dual functionality positions PLD2 as a key player in cytoskeletal reorganization and cancer metastasis (Bruntz *et al.*, 2014; Gomez-Cambronero, 2014).

OA, as a MUFA, is an important regulator of cancer cell migration and progression (Guo, Z. *et al.*, 2023). It is primarily synthesized by SCD1, an enzyme that converts SFAs into MUFAs. SCD1 is frequently upregulated in various cancers, including breast cancer, and has been linked to increased cell survival, chemoresistance, and metastatic potential (Guo, Z. *et al.*, 2023; Igal, 2016). OA exerts diverse effects on cancer cells, promoting membrane fluidity, modulating signaling pathways, and influencing lipid metabolism (Guo, Z. *et al.*, 2024). Recent findings indicate that OA directly activates PLD2, but the underlying molecular mechanisms remain largely unknown (Guo, Z. *et al.*, 2024; Kasai *et al.*, 1998; Sarri *et al.*, 2003).

Protein lipidation is a significant PTM that usually refers to various lipids or lipid-like group covalently attached to proteins (Wang, R. et Chen, 2022). Among various lipidation types, S-acylation and prenylation are particularly noteworthy, especially concerning cysteine residues (Kouba et Demaurex, 2024). S-acylation is a reversible PTM that involves the attachment of FAs, such as palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), and OA (C18:1), to cysteine residues via thioester bonds (Chamberlain et Shipston, 2015; Montigny *et al.*, 2014; Nuskova *et al.*, 2021; Schulte-Zweckel *et al.*, 2019). Traditionally referred to as palmitoylation, this process is now recognized as a more diverse lipidation mechanism involving various FAs that differentially regulate protein function, localization, and interactions bonds (Chamberlain et Shipston, 2015; Liang *et al.*, 2001; Montigny *et al.*, 2014; Nuskova *et al.*, 2021; Schulte-Zweckel *et al.*, 2019). Unlike other lipid modifications, S-acylation is dynamic, allowing proteins to cycle between membrane-bound and cytosolic states (Wang, R. et Chen, 2022). Different FAs confer distinct functional properties to proteins; for instance, palmitate tends to anchor proteins in lipid rafts, whereas OA and palmitoleate promote protein redistribution to non-raft domains (Nůsková *et al.*, 2022; Nuskova *et al.*, 2021). This suggests a complex regulatory network in which specific lipid modifications dictate protein behavior within cellular membranes. Beyond S-acylation, cysteine residues can undergo prenylation, another critical lipid modification. This modification entails the attachment of isoprenoid groups, such as farnesyl (C15) or geranylgeranyl (C20) moieties, to

cysteine residues near the C-terminus of proteins. Prenylation is critical for the membrane association and protein-protein interactions of various signaling proteins, including Rho family GTPase Cdc42, which is geranylgeranylated (Berndt *et al.*, 2011; Hancock *et al.*, 1990; Parker et Mattos, 2018).

Membrane microdomains, including lipid rafts and $PIP_2$ clusters (also referred as $PIP_2$ microdomains or $PIP_2$ rafts), are specialized regions of the plasma membrane that organize signaling molecules and regulate cellular functions (Mollinedo et Gajate, 2020). Lipid rafts are cholesterol- and sphingolipid-rich domains that serve as platforms for receptor clustering and intracellular signaling (Allen *et al.*, 2007). Proteins localized within lipid rafts often undergo lipid modifications, such as S-acylation, which influence their association with these domains (Levental *et al.*, 2010). Conversely, $PIP_2$ clusters contain high levels of $PI_{(4,5)}P_2$), a phospholipid that plays a critical role in actin remodeling and membrane trafficking (Janmey et Lindberg, 2004; Saarikangas *et al.*, 2010). The distribution of proteins and lipid microdomains are regulated by lipid composition, with SFAs (such as palmitate) stabilizing lipid raft and $PIP_2$ clusters by promoting membrane rigidity, while unsaturated FAs, such as OA, disrupt rafts via cis-double bonds, dispersing $PIP_2$ (Lingwood et Simons, 2010; Pike, 2006).The distribution of proteins and lipid microdomains are regulated by lipid composition, with SFAs (such as palmitate) stabilizing lipid raft and $PIP_2$ clusters by promoting membrane rigidity, while unsaturated FAs, such as OA, disrupt rafts via cis-double bonds, dispersing $PIP_2$ (Lingwood et Simons, 2010; Pike, 2006).

Although previous studies have reported that OA activates PLD2, the precise mechanisms remain unclear (Gibbs et Meier, 2000; Kasai *et al.*, 1998; Kim, J. H. *et al.*, 1999; Sarri *et al.*, 2003). Like double acylated PLD1, human PLD2 is predicted to be S-acylated at Cys223 and Cys224, but experimental validation of these sites and their functional significance has been lacking. Given the role of S-acylation in protein localization, it is essential to determine whether OA-dependent PLD2 activation involves S-acylation-mediated shuttling between membrane microdomains. Additionally, the functional implications of PLD2's GEF activity remain underexplored.

In this study, we demonstrate that OA induces PLD2 dissociation from lipid rafts and promotes its translocation to $PIP_2$ clusters, a process dependent on S-acylated Cys223 and Cys224. Furthermore, we identify PLD2 as a GEF for Cdc42, with its GEF activity regulated by OA-dependent acylation and lipid raft dynamics. These findings provide a molecular-level

understanding of how OA modulates PLD2 activity, highlighting potential therapeutic targets for SCD1- and PLD2-dependent TNBC and other metastatic cancers.

## 4.3 Results

### 4.3.1 OA strongly activates PLD in HEK293T and MDA-MB-231 cells

The previous findings from our lab demonstrated that PLD is involved in OA-induced TNBC cell migration and invasion (Guo, Z. *et al.*, 2024; Lingrand *et al.*, 2020a). While the mechanism of unsaturated FA stimulation of PLD2 is not fully understood, several lines of evidence have suggested PLD2 is the isoform stimulated by OA (Bruntz *et al.*, 2014; Gibbs et Meier, 2000; Kasai *et al.*, 1998; Kim, J. H. *et al.*, 1999; Sarri *et al.*, 2003). To investigate the effect of different FAs on PLD activation, we utilized PA sensors (PASS-RFP/GFP) to monitor PLD lipase-dependent PA recruitment to the plasma membrane (Lu, M. *et al.*, 2016). This method offers a highly visual approach to assessing PLD activation without the addition of exogenous lipids, which could otherwise confound results. We used two cell lines as models: HEK293T cells, which have low basal PLD2 expression but high transfection efficiency, served as an overexpression model, while MDA-MB-231 cells, which endogenously express high levels of PLD2 but have lower transfection efficiency, were used as a physiologically relevant model.

First, we examined the effects of 4 FAs linked to SCD1 activity (C16:0, C16:1, C18:0, C18:1). To block endogenous FA desaturation and elongation, cells were pretreated with SCD1 and ELOVL6 inhibitors. Confocal imaging revealed a striking increase in plasma membrane localization of PASS in response to OA (C18:1) treatment in both cell lines (Figure 4.1). Treatment with the lipid raft disruptor MβCD (100 μM) also resulted in increased PASS recruitment, confirming the implication of lipid rafts in PLD activation within our assay. Interestingly, palmitoleate (C16:1) induced moderate activation in HEK293T cells but failed to elicit a similar response in MDA-MB-231 cells. This suggests a cell-type-specific FA preference in PLD activation.

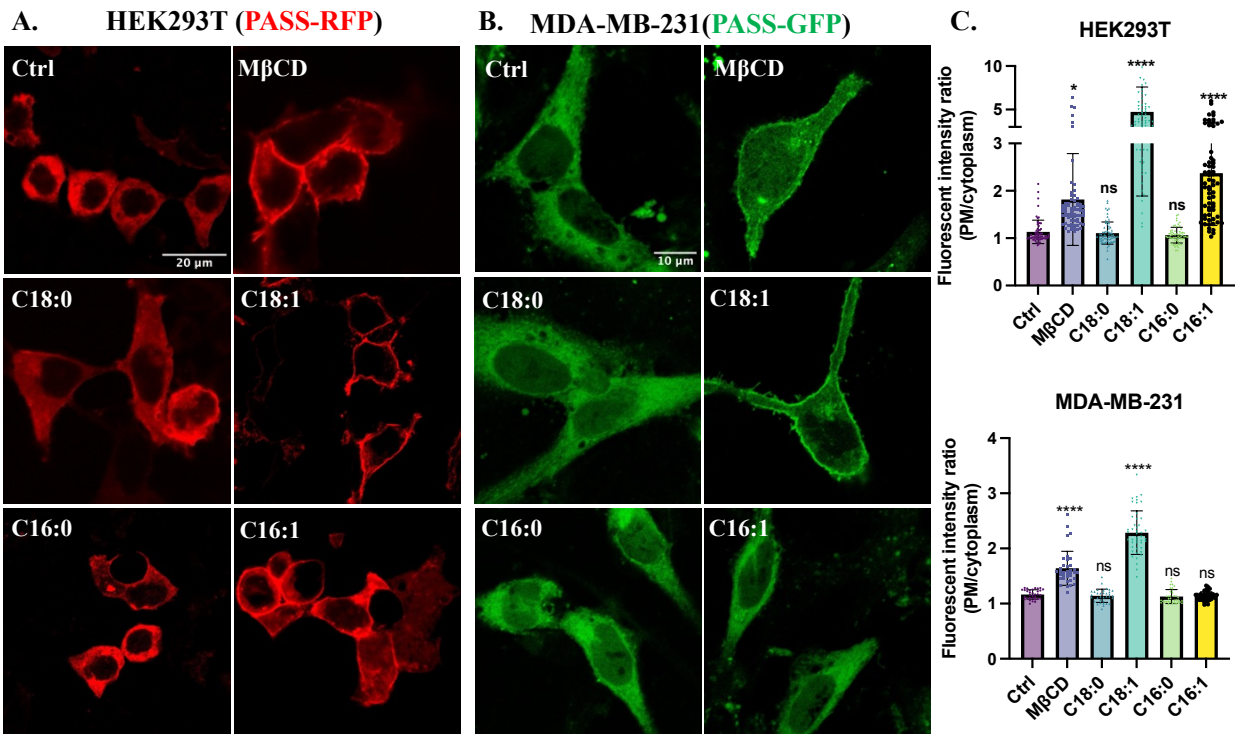


**Figure 4.1 OA induces PLD activation in HEK293T and MDA-MB-231 cells.**

(A, B) Representative confocal microscopy images of the PA sensor (PASS-RFP in HEK293T cells and PASS-GFP in MDA-MB-231 cells) in response to FA treatments. Cells were transiently transfected with PASS-RFP/GFP and starved with 1 μM SCD inhibitor A939572 and 1 μM ELOVL inhibitor ELOVL6-IN-4 (or 100 μM MβCD) for 3 h to prevent FA desaturation and elongation. For treatments, cells were incubated with 100 μM BSA-conjugated FAs (BSA for Ctrl) with the presence of inhibitors. Scale bars in the first panel apply to all images in the series. (C) Quantification of PASS-RFP/GFP fluorescence on the plasma membrane (PM) relative to the cytoplasm. The average (± standard deviation) ratio was calculated from three independent experiments (HEK293T, n≥60, MDA-MB-231, n≥33). Statistical significance was assessed using ordinary one-way ANOVA with multiple comparisons to the control group (Ctrl), with significance thresholds defined as follows: ns, not significant, $p \geq 0.05$, *$p < 0.05$, and ****$p < 0.0001$.

### 4.3.2 OA disrupts PLD2 localization to lipid rafts

Given the findings in the previous section (4.3.1), which demonstrated that OA and lipid rafts are involved in PLD activation, we hypothesized that OA might modulate PLD2 activity by altering its localization within membrane microdomains. To test this, we first examined the colocalization of PLD2 with a lipid raft marker, Caveolin-1, using confocal microscopy. Under control conditions, PLD2 and Caveolin-1 exhibited strong colocalization at the plasma membrane in both HEK293T and MDA-MB-231 cells (Figure 4.2A). However, OA treatment significantly disrupted this colocalization, as evidenced by a more punctate distribution of both proteins (Figure

4.2C) and a marked reduction of colocalization (Figure 4.2B). These findings demonstrate that OA disperses the distribution of PLD2 and lipid rafts, and also disrupting their association.

To further validate these observations, lipid raft fractions were isolated using sucrose density gradient ultracentrifugation. Western blot analysis confirmed that PLD2 was predominantly localized in lipid raft-enriched fractions (fractions 3–5) under control conditions in both HEK293T and MDA-MB-231 cells (Figure 4.2D and E). However, OA treatment significantly reduced the proportion of PLD2 in these raft fractions, consistent with the confocal microscopy results. Notably, among the FAs tested (C18:0, C18:1, C16:0, and C16:1), unsaturated FAs - OA (C18:1) and palmitoleate (C16:1) both induced a significant decrease in PLD2 localization within lipid rafts in HEK293T cells, while only OA alone had a pronounced effect in MDA-MB-231 cells (Figure 4.2D and E). These results align with the PLD activation data from 4.3.1, supporting the hypothesis that OA activates PLD2 by promoting its dissociation from lipid rafts.

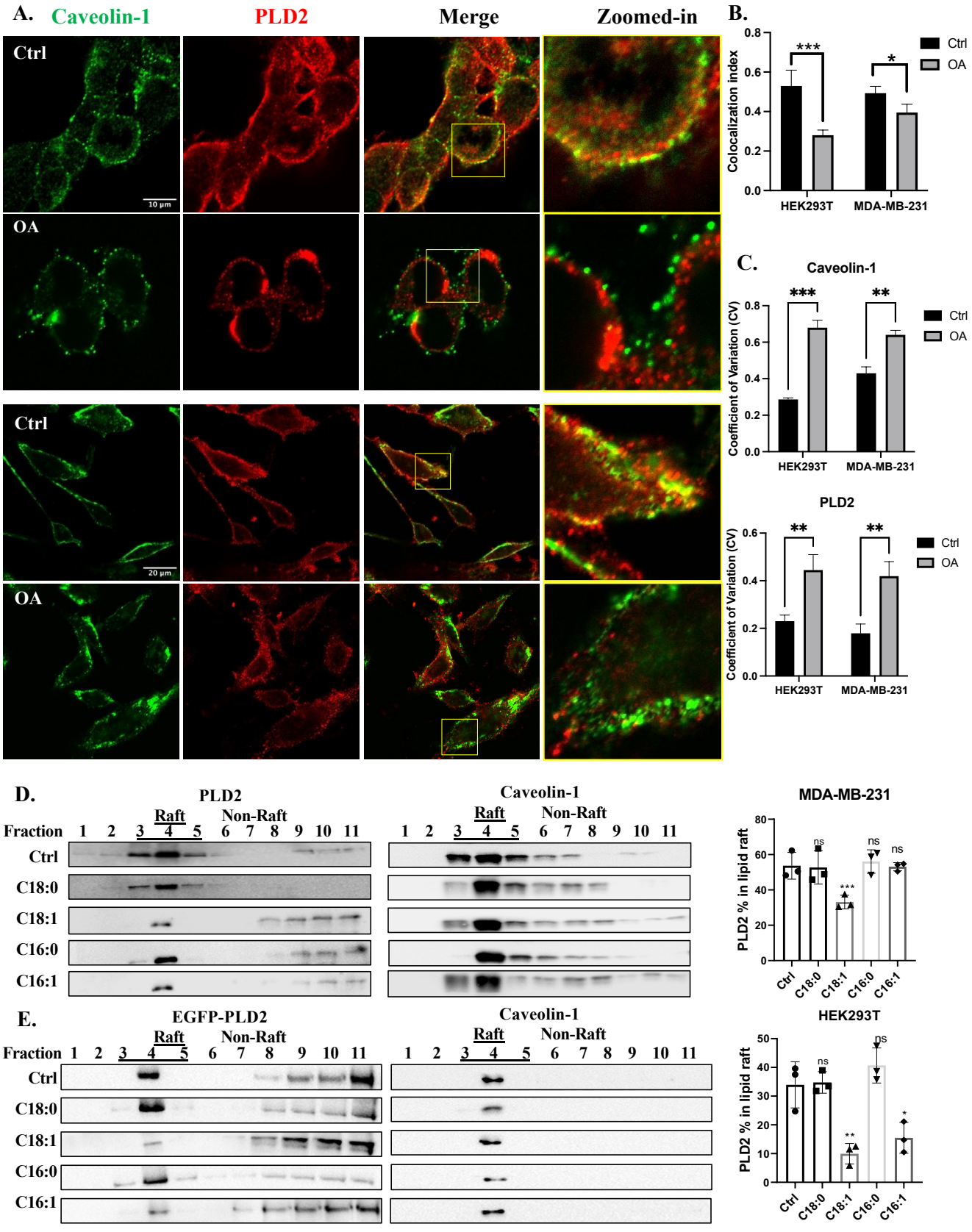
A.
Caveolin-1
PLD2
Merge
Zoomed-in
Ctrl
OA
10 μm
20 μm
B.
Colocalization index
Ctrl
OA
HEK293T
MDA-MB-231
C.
Caveolin-1
Coefficient of Variation (CV)
PLD2
D.
PLD2
Caveolin-1
Raft
Non-Raft
Fraction 1 2 3 4 5 6 7 8 9 10 11
Ctrl
C18:0
C18:1
C16:0
C16:1
MDA-MB-231
PLD2 % in lipid raft
E.
EGFP-PLD2
HEK293T

**Figure 4.2 OA disrupts the colocalization of PLD2 with lipid rafts.**

(A) Representative confocal microscopy images showing the colocalization of PLD2 (red) and Caveolin-1 (green) in HEK293T cells and MDA-MB-231 cells treated 3h with BSA (Ctrl) or 100 μM OA. Scale bars in the first panel apply to all images in the series. (B, C) Quantification of caveolin-1 and PLD2 colocalization (B) and distribution variability (C). Colocalization was assessed using Pearson's correlation coefficient. Distribution heterogeneity was measured by coefficient of variation (CV). Data are presented as mean ± SEM (n = 3 independent experiments). Statistical significance was assessed using unpaired two-tailed t-tests with multiple comparisons to the control group (Ctrl), with significance thresholds defined as follows: *p < 0.05), **p < 0.01, and *** p < 0.001. (D, E) Western blot analysis of lipid raft fractions isolated by sucrose density gradient ultracentrifugation from HEK293T cells expressing EGFP-PLD2 (D) and MDA-MB-231 cells (E). Fractions 3-5 are enriched in lipid rafts, as indicated by the predominant presence of Caveolin-1. Bar charts show the percentage of PLD2 localized in lipid raft fractions under treatments of BSA (Ctrl), C18:0, C18:1, C16:0, and C16:1. Cells were pretreated 3h with 1 μM SCD inhibitor A939572 and 1 μM ELOVL inhibitor ELOVL6-IN-4 to prevent FA desaturation or elongation. 10 μM Palm B was used to block deacylation in cell lysis buffer. The proportion of PLD2 in raft fractions was calculated as the ratio of the sum of PLD2 in raft fractions (fractions 3–5) to the sum of PLD2 in all 11 fractions. Data are presented as mean ± SEM (n = 3 independent experiments). ns, not significant, p ≥ 0.05, *p < 0.05,** p < 0.01, ***p < 0.001 by ordinary one-way ANOVA with multiple comparisons to the control group (Ctrl).

### 4.3.3 OA enhances recruitment of PLD2 to $PIP_2$ clusters

To further investigate the localization of PLD2 to membrane microdomains, we examined the colocalization of PLD2 with $PIP_2$ in HEK293T cells. Due to methodological limitations (both PLD2 and $PIP_2$ antibodies are derived from mouse), we utilized an EGFP-PLD2 overexpression system to visualize their colocalization. In untreated cells, $PIP_2$ was scattered on the membrane, and PLD2-WT showed partial colocalization with $PIP_2$ (Figure 4.3A and B). Treatment with 100 μM OA for 3 h significantly enhanced $PIP_2$ detection on the membrane and increased its colocalization with PLD2 (Figure 4.3A, B and C). In contrast, treatment with palmitate sequestered $PIP_2$ into fewer, more concentrated puncta on the membrane, reducing the overall $PIP_2$ detection area on the membrane and decreasing colocalization with PLD2 (Figure 4.3A, B, and C). These findings indicate that OA enhances $PIP_2$ cluster into more homogenous distribution on the membrane and promotes its colocalization with PLD2. While palmitate has the opposite effect, it sequesters $PIP_2$ to form larger clusters and reduces the colocalization with PLD2. This differential regulation of $PIP_2$ distribution by OA and palmitate aligns with their distinct roles in modulating PLD2 activity (Fig. 4.1A and C).

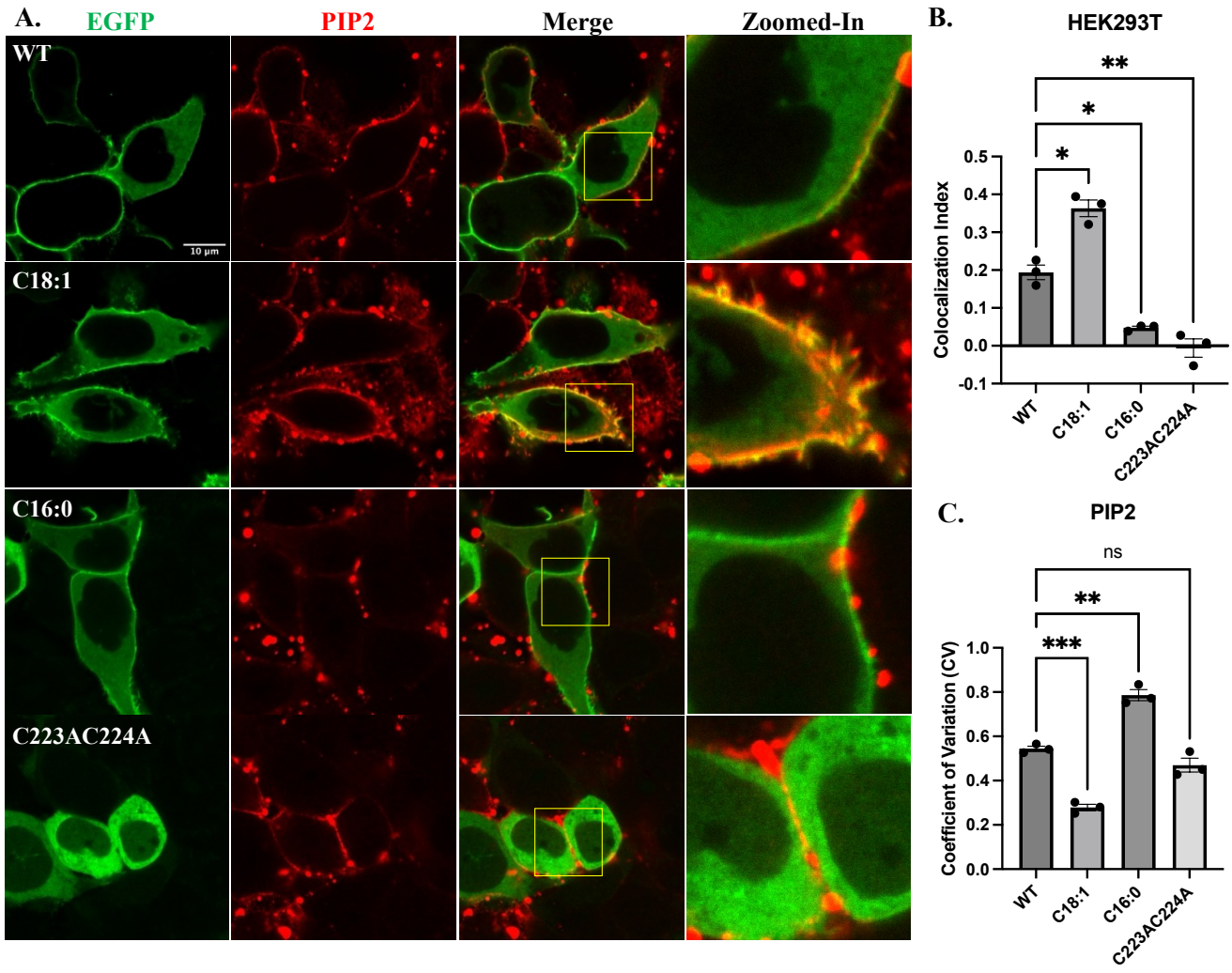


**Figure 4.3 Colocalization analysis of $PIP_2$ and PLD2 in HEK293T cells.**

(A) Confocal microscopy images of HEK293T cells overexpressing EGFP-PLD2-WT or EGFP-PLD2-C223AC224A. Cells were treated 3h with BSA (WT control), or 100 µM OA (C18:1) or palmitate (C16:0). Cells were stained with anti-$PIP_2$ antibody (red) to assess colocalization with EGFP-PLD2 (green). The scale bar in the first panel apply to all images in the series. (B, C) Quantification of $PIP_2$ and PLD2 colocalization (B) and $PIP_2$'s distribution variability (C). Colocalization was assessed using Pearson's correlation coefficient. Distribution heterogeneity was measured by coefficient of variation (CV). Data are presented as mean ± SEM (n = 3 independent experiments, n > 20 cells per experiment). Statistical significance was determined by one-way ANOVA with multiple comparisons test versus wild-type controls (WT): ns ($p \geq 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

### 4.3.4 PLD2 is S-acylated at Cys223 and Cys224

To further investigate the molecular mechanism underlying PLD2 regulation by OA, we explored whether PLD2 undergoes S-acylation, a post-translational modification that could influence its localization and activity. According to the SwissPalm database (Blanc *et al.*, 2019),

human PLD2 is predicted to be S-acylated at Cys223 and Cys224 (Fig. 4.4A), although experimental validation of these sites is lacking. To address this, we performed an acyl-PEG exchange assay, which replaces acyl groups with a PEG moiety, resulting in a detectable mass shift on western blots.

We first examined the acylation status of wild-type (WT) PLD2, single Cysteine mutants (C223A and C224A), and double Cysteine mutant (C223AC224A) expressed in HEK293T cells by an EGFP-C1 vector (as illustrated in Fig. 4.4B). A specific inhibitor of acyl-protein thioesterases (APTs), palmostatin B (Palm B) was supplemented in the cell lysis buffer to preserve the S-acylation. As shown in Figure 4.4C, WT-EGFP-PLD2 exhibited two mass-shifts above the original ~130 kDa band, indicating double S-acylation. Single mutations (C223A or C224A) resulted in one mass-shift band, while the double mutation (C223AC224A) completely abolished the shifting effects. These findings provide direct experimental evidence that PLD2 is S-acylated at Cys223 and Cys224.

Next, we investigated whether OA could serve as an acyl donor for PLD2 acylation. HEK293T cells expressing WT-EGFP-PLD2 were treated with BSA (Ctrl), palmitate (C16:0), or OA (C18:1) for 3h before the acyl-PEG exchange assay. Cells were also treated with ELOVL and SCD inhibitors to prevent modification of supplemented FAs. As shown in Figure 4.4D and E, both OA and palmitate treatments resulted in stronger mass-shift bands and weaker original bands compared to the control, indicating increased acylation of PLD2 following FAs treatments. This suggests that OA, like palmitate, can be incorporated into PLD2 via S-acylation.

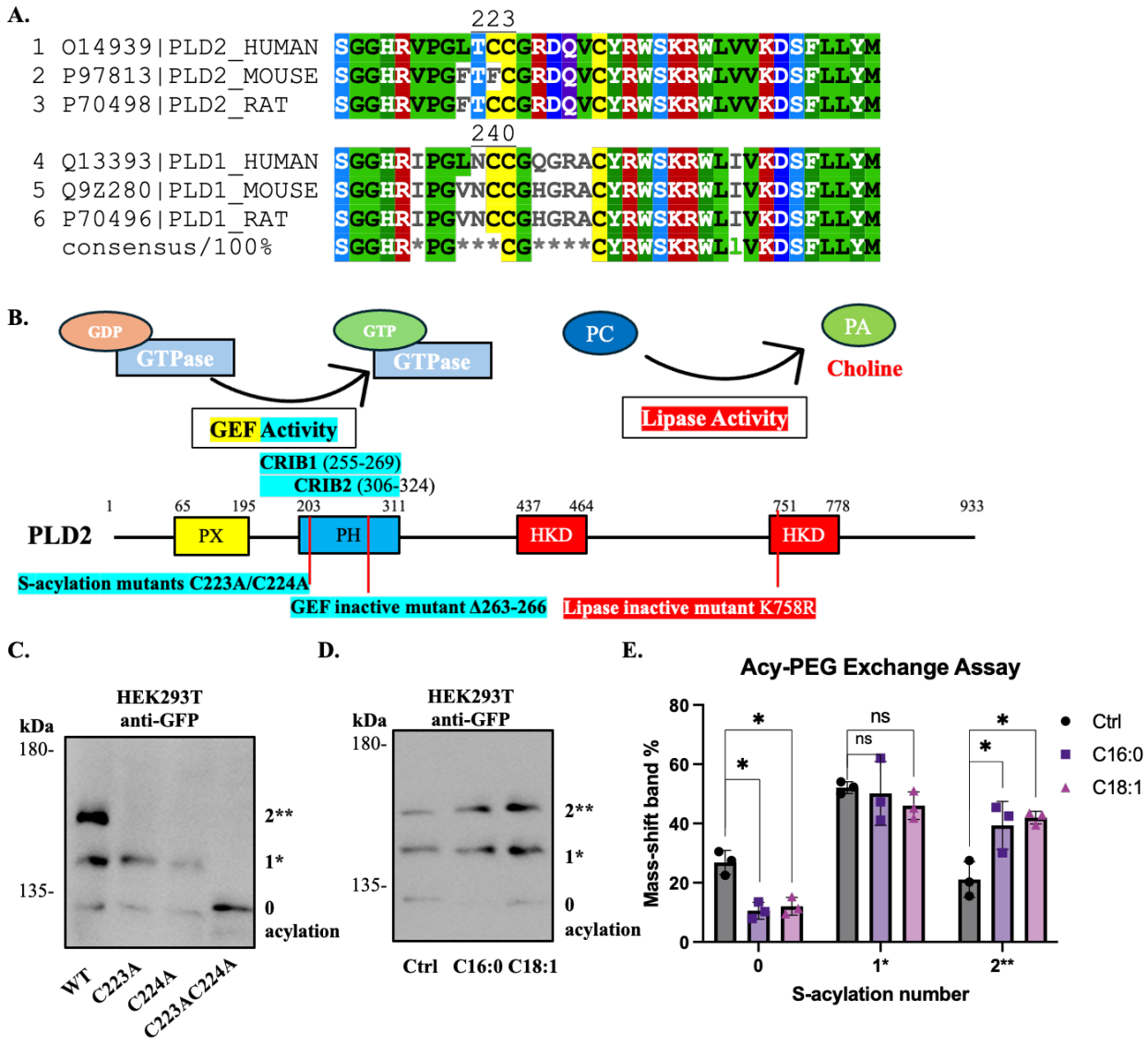


**Figure 4.4 Acyl-PEG exchange assay of PLD2 in HEK293T cells.**

(A) Partial amino acid sequence alignment of canonical PLD1 and PLD2 from *Homo sapiens* (Human), *Mus musculus* (Mouse), and *Rattus norvegicus* (Rat). Numbering indicates positions of predicted or verified S-acylated cysteines (based on SwissPalm database annotations). Conserved acylation sites are highlighted. (B) Schematic representation of the major structural domains of human PLD2, highlighting the mutants generated in this study: S-acylation-deficient mutant: PLD2-C223A/C224A, GEF-activity-deficient mutant: PLD2-Δ263–266 (impairs GTPase interaction), catalytically inactive mutant: PLD2-K758R (abolishes lipase activity). (C) Western blot analysis of acyl-PEG exchange assay in HEK293T cells transiently transfected with EGFP-PLD2, EGFP-PLD2-C223A, EGFP-PLD2-C224A, or EGFP-PLD2-C223AC224A. Mouse anti-GFP antibody was used to detect EGFP-PLD2. Mass-shift bands (above the original ~130 kDa band) were marked with *. (D) Acyl-PEG exchange assay in HEK293T cells expressing WT-EGFP-PLD2 treated with BSA (Ctrl), C16:0, or C18:1. (E) Quantitative analysis of mass-shift bands. Band intensities were quantified and expressed as percentage relative to total protein levels (each band/total bands ×100%).

Data represent mean ± SEM from three independent experiments. Statistical significance was determined by one-way ANOVA with multiple comparisons test versus controls (Ctrl): ns ($p \geq 0.05$), *$p < 0.05$.

### 4.3.5 **S-acylation of PLD2 optimizes membrane association and lipase activity**

To determine whether the acylation of PLD2 at Cys223 and Cys224 influences its lipase activity, we performed a PLD activation assay using a PA sensor (PASS-RFP) in HEK293T cells expressing WT-EGFP-PLD2 or its acylation site mutants (C223A, C224A, and C223AC224A). Confocal microscopy imaging revealed that WT-EGFP-PLD2 exhibited strong membrane localization (Figure 4.5A and B). In contrast, the double mutant (C223AC224A) impaired membrane targeting, while single mutants (C223A or C224A) retained membrane association, indicating that acylation at either Cys223 or Cys224 is sufficient for PLD2 membrane targeting (Figure 4.5A and B). In the RFP channel, which reflects PA production as the measure of PLD lipase activity, overexpression of all PLD2 constructs (WT and mutants) resulted in higher PA membrane recruitment compared to the vector control (Figure 4.5A and C). Therefore, PLD2 retains basal lipase activity even in the absence of acylation and membrane localization. However, the double mutant (C223AC224A) exhibited significantly lower activation compared to WT (Figure 4.5A and C). These results demonstrate that S-acylation at both Cys223 and Cys224 is essential for optimal PLD2 membrane localization and lipase activity.

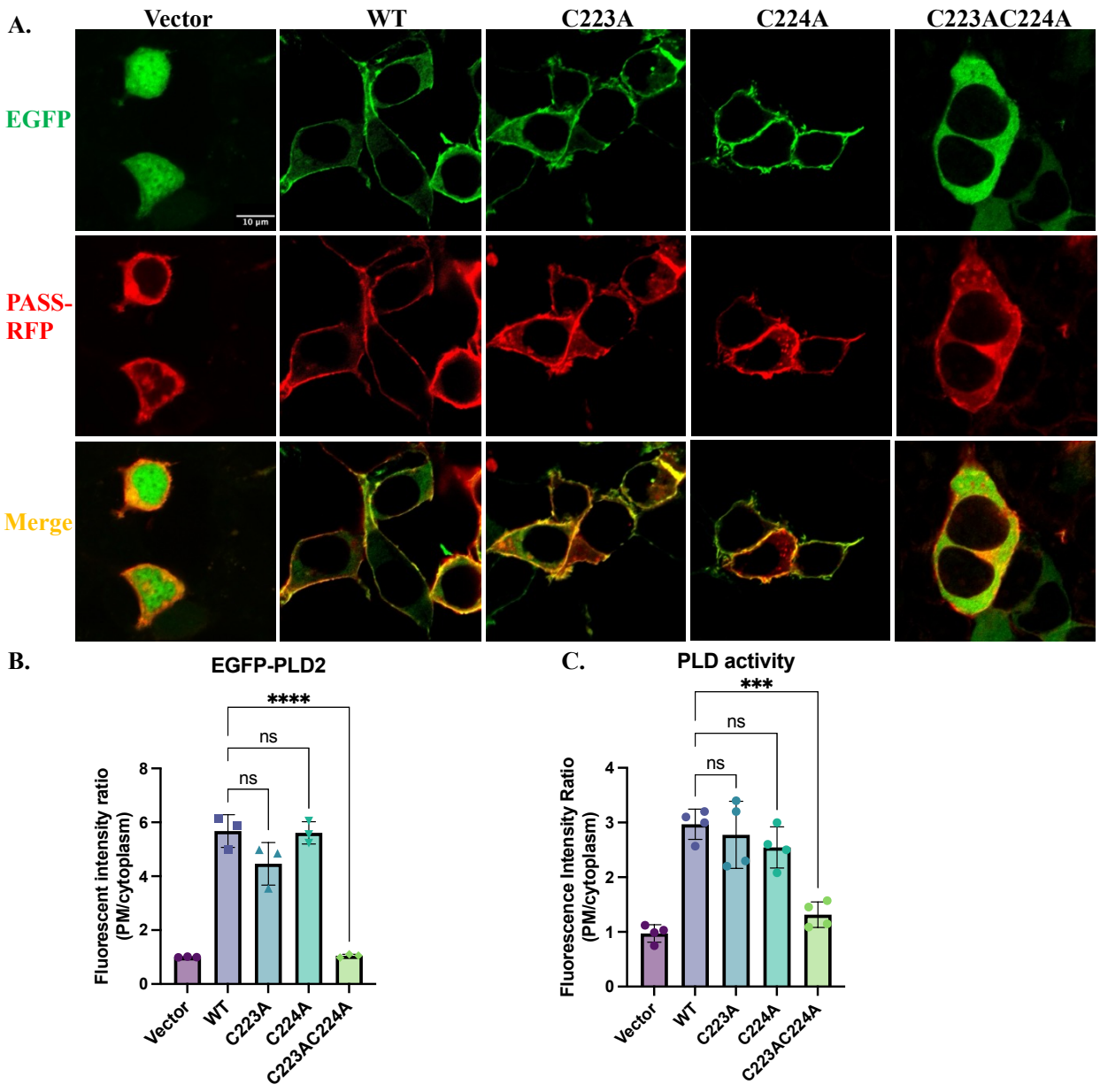


**Figure 4.5 S-acylation sites of PLD2 optimizes membrane localization and lipase activity.**

(A) Confocal microscopy images of HEK293T cells transiently co-transfected with PASS-RFP and EGFP-C1 (empty vector), EGFP-PLD2-WT, EGFP-PLD2-C223A, EGFP-PLD2-C224A, or EGFP-PLD2-C223AC224A. The GFP channel shows the localization of EGFP-PLD2 constructs, while the RFP channel shows PA production as a measure of PLD2 lipase activity. The merge channel combines both signals. The scale bar in the first panel apply to all images in the series. (B, C) Quantitative analysis of EGFP-PLD2 (B) and RFP-PASS (C) membrane/cytosolic distribution. Fluorescence intensity ratios (PM/cytoplasm) are presented as mean ± SD from three biological replicates (n > 20 cells per experiment). Statistical significance was determined by one-way ANOVA with multiple comparisons test versus wild-type controls (WT): ns ($p \geq 0.05$), ***$p < 0.001$,****$p < 0.0001$.

### 4.3.6 **The lipid raft targeting of PLD2 is regulated by S-acylation**

To further investigate the role of S-acylation in PLD2 membrane localization, we performed lipid raft isolation in HEK293T cells expressing EGFP-PLD2-WT or its S-acylation site mutants (C223A, C224A, and C223AC224A). Consistent with the findings presented above in Figure 4.5, WT and single mutant (C223A and C224A) constructs showed significant presence in lipid raft-enriched fractions (fractions 3–5) under basal conditions (Figure 4.6). In contrast, the double mutant (C223AC224A) exhibited a marked decrease in lipid raft localization and a corresponding increase in non-raft fractions, underscoring the importance of acylation at both Cys223 and Cys224 for PLD2 association with lipid rafts.

To examine whether OA treatment affects the lipid raft localization of PLD2 single mutants, transfected HEK293T cells expressing WT and single mutant (C223A and C224A) constructs were treated with 100 μM OA for 3h. OA treatment significantly reduced the presence of single mutants in lipid raft fractions (Figure 4.6), indicating that S-acylation at either Cys223 or Cys224 is sufficient to retain OA-mediated regulation of PLD2 localization. Since the double mutant (C223AC224A) has impaired association with lipid rafts, we tested whether palmitate treatment could restore its colocalization with lipid rafts. However, palmitate treatment failed to rescue the lipid raft association of the double mutant (Figure 4.6), confirming that Cys223 and Cys224 are the key sites required for PLD2 localization to lipid rafts.

In addition, we also analysed the impact of PLD2 acylation on its association with $PIP_2$ clusters. In HEK293T cells expressing the double S-acylation mutant (C223AC224A), PLD2-C223AC224A has impaired membrane localization, resulting in minimal colocalization with $PIP_2$ (Figure 4.3, bottom row). This further supports the critical role of acylation at Cys223 and Cys224 in anchoring PLD2 to the membrane and facilitating its interaction with $PIP_2$.

These results demonstrate that S-acylation at Cys223 and Cys224 is essential for PLD2 localization with lipid rafts and $PIP_2$ clusters. Collectively, these findings provide strong evidence that S-acylation at Cys223 and Cys224 is a critical determinant of PLD2 function, linking its membrane localization, regulation by FAs, and enzymatic activity.

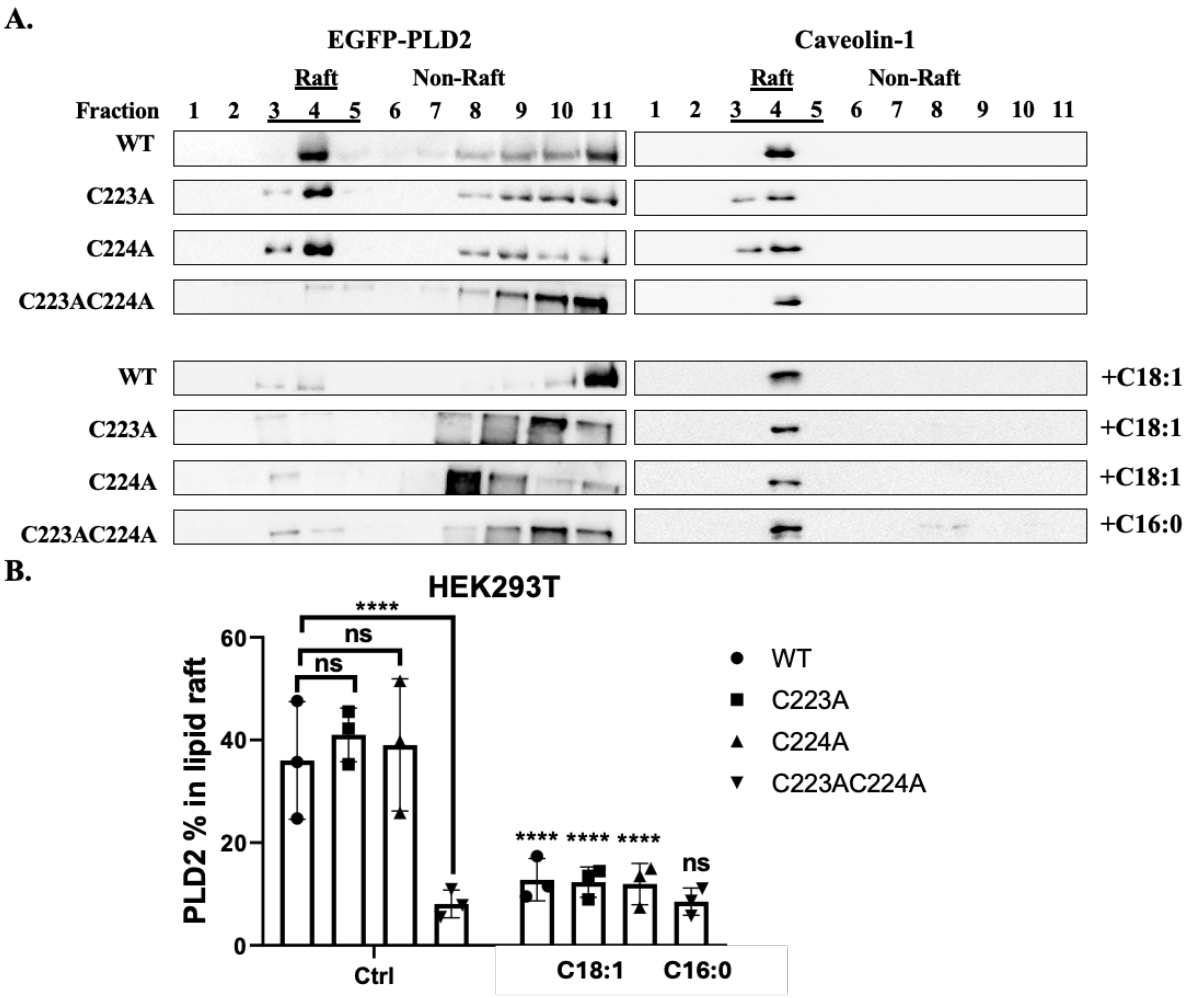


**Figure 4.6 The impact of PLD2 S-acylation site mutation on its targeting to lipid rafts.**

(A) Western blot analysis of lipid raft fractions isolated by sucrose density gradient ultracentrifugation from HEK293T cells expressing EGFP-PLD2-WT, EGFP-PLD2-C223A, EGFP-PLD2-C224A, or EGFP-PLD2-C223AC224A. Cells were treated with BSA to assess basal localization of PLD2 mutants, or treated with FAs for 3 h (100 μM OA for WT, C223A, C224A or 100 μM palmitate for C223AC224A). Cells were starved and pre-incubated with 1 μM SCD inhibitor A939572 and 1 μM ELOVL inhibitor ELOVL6-IN-4 for 3 h to prevent FA desaturation and elongation. Fractions 3–5 pinpoint lipid raft-enriched fractions, as indicated by the presence of Caveolin-1. (B) Quantification of the proportion of PLD2 in raft fractions (fractions 3–5) relative to the total PLD2 across all fractions. Data are presented as mean ± SEM (n = 3 independent experiments). For comparisons with wild-type controls (WT) without FA treatment, significance was determined by ordinary one-way ANOVA with Dunnett's multiple comparisons test. Fatty acid (FA)-treated groups were analyzed using unpaired two-tailed Student's t-tests with their corresponding control groups (Ctrl). Significance thresholds were defined as: ns (not significant), ****$p < 0.0001$.

### 4.3.7 **PLD2 functions as a GEF for Cdc42 activation**

Previous studies have identified PLD2 as a GEF for Rac2 and RhoA (Jeon *et al.*, 2011; Mahankali, Peng, Henkels*, et al.*, 2011), and our earlier work demonstrated that PLD2 is involved in OA-induced Cdc42-dependent filopodia formation (Guo, Z. *et al.*, 2024). To investigate whether PLD2 could also act as a GEF for the Cdc42 GTPase, we first examined the role of PLD2 in Cdc42 activation using filopodia formation assays with PLD2 plasmid constructs (as illustrated in Fig. 4.4B). In Figure 4.7A, confocal microscopy images of HEK293T cells overexpressing EGFP-PLD2-WT, lipase inactive EGFP-PLD2-K758R (Mahankali, Peng, Henkels*, et al.*, 2011) or GEF inactive EGFP-PLD2-ΔCRIB (deletion of aa 263-266 (Mahankali, Peng, Henkels*, et al.*, 2011)) revealed that WT and lipase inactive constructs induced significant filopodia-like cell protrusions, while the GEF inactive construct did not. This indicates that the GEF regulation domain, but not the lipase catalytic domain, is critical for PLD2-mediated filopodia formation.

To directly assess Cdc42 activation, we performed a pull-down assay to detect GTP-bound Cdc42 (active form). Overexpression of PLD2-WT and -K758R constructs in HEK293T cells significantly increased Cdc42-GTP levels, while the ΔCRIB construct showed a much weaker effect, confirming that PLD2 acts as a GEF for Cdc42 and that its lipase activity is dispensable for this function (Figure 4.7B). Next, we investigated whether S-acylation is required for PLD2's GEF activity. In HEK293T cells, OA treatment increased Cdc42-GTP levels in cells expressing PLD2-WT and PLD2-K758R but failed to do so in cells expressing the double S-acylation mutant C223AC224A (Figure 7B). This demonstrates that OA-dependent acylation is also required for PLD2's GEF activity. In MDA-MB-231 cells, treatment with OA or MβCD, a lipid raft disruptor, also increased Cdc42-GTP levels (Figure 4.7 B and C), suggesting that OA-dependent S-acylation and membrane microdomain translocation is at play in both cell types. These results establish PLD2 as a GEF for Cdc42 activation and reveal that OA-dependent S-acylation and membrane lipid microdomain localization are critical for regulating PLD2's GEF function.

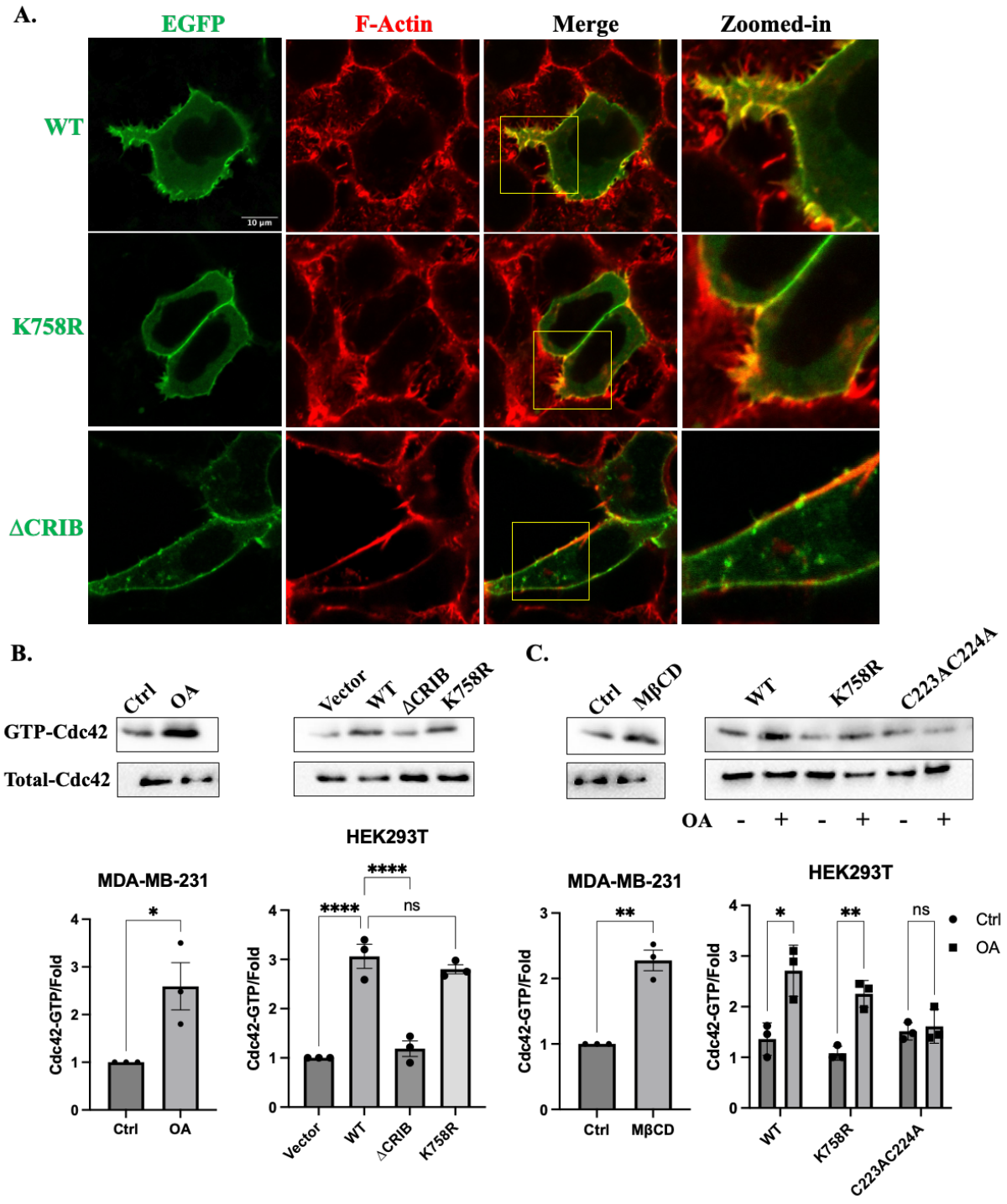


**Figure 4.7 PLD2 functions as a GEF for Cdc42 activation.**

(A) Confocal microscopy images of HEK293T cells overexpressing EGFP-PLD2-WT, EGFP-PLD2-K758R (lipase inactive), or EGFP-PLD2-ΔCRIB (GEF inactive). Cells were stained with Phalloidin-TRITC to visualize filopodia-like cell protrusions. The scale bar in the first panel apply to all images in the series. (B) Western blot analysis of Cdc42-GTP (active form) and total Cdc42 (input) in MDA-MB-231 cells treated 10 min with BSA (Ctrl) or 100 μM OA and in HEK293T cells transfected with EGFP-C1

(Vector), PLD2-WT, PLD2-ΔCRIB, or PLD2-K758R. (C) Western blot analysis of Cdc42-GTP and total Cdc42 in MDA-MB-231 cells treated 3 h with DMSO (Ctrl) or 100 μM MβCD, and in HEK293T cells transfected with PLD2-WT, PLD2-K758R, or PLD2-C223AC224A treated with BSA or 100 μM OA for 10 min. Data are presented as mean ± SEM (n = 3 independent experiments). For MDA-MB-231 groups, significance was determined by unpaired two-tailed Student's t-tests with their corresponding control groups. HEK293T groups were analyzed using ordinary one-way ANOVA with Dunnett's multiple comparisons test. Significance thresholds were defined as: ns (not significant), *p < 0.05, **p < 0.01 ****p < 0.0001.

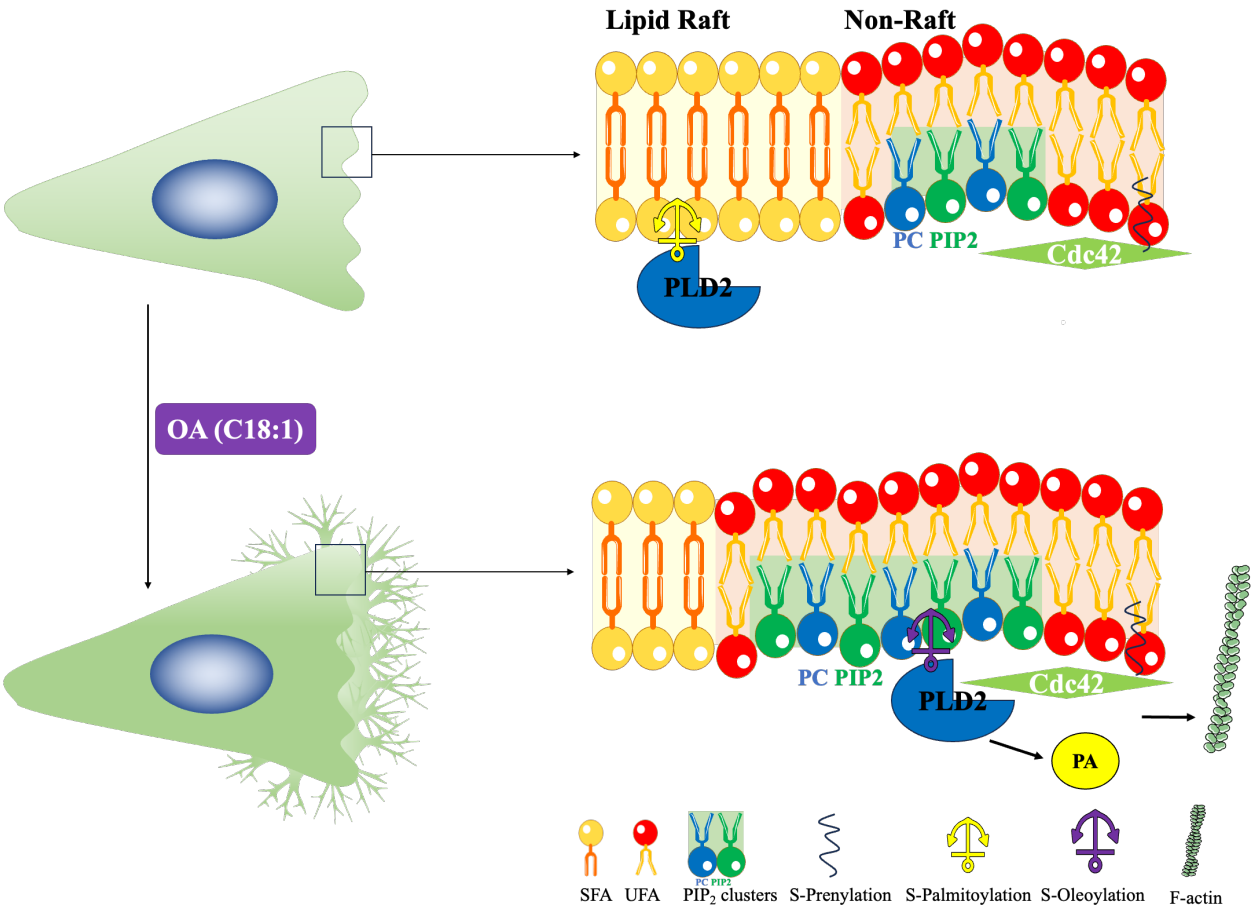


**Figure 4.8 Proposed mechanistic model for OA-activated PLD2 signaling.**

Under basal conditions, PLD2 is predominantly S-palmitoylated and localized to lipid rafts. Following SCD1 upregulation or OA treatment, palmitate at Cys223 and Cys224 is replaced by OA (S-oleoylation), triggering PLD2 translocation to $PIP_2$-enriched membrane domains. This redistribution enhances lipase-dependent phosphatidic acid (PA) production and GEF-mediated activation of Cdc42 signaling. Image is created by powerpoint.

## 4.4 Discussion

This study delineates a novel molecular mechanism by which OA activates PLD2 through S-acylation, regulating its lipase and GEF activities via membrane microdomain translocation dynamics. We demonstrate that OA induces PLD2 dissociation from lipid rafts and promotes its translocation to $PIP_2$-enriched microdomains, where it accesses its substrate and exerts enzymatic functions. This process is dependent on S-acylation at Cys223 and Cys224, as mutation of these sites abolishes PLD2 membrane localization and OA-induced activation. Interestingly, while single mutations at either Cys223 or Cys224 retained partial function, the double mutation resulted in a complete loss of activity, indicating a cooperative role of these residues in PLD2 regulation.

Our results show that palmitoleate (C16:1) induces moderate PLD activation and PLD2 association with lipid rafts in HEK293T cells but does not elicit the same response in MDA-MB-231 cells, suggesting a FA preference in S-acylation (Figs. 4.1 and 4.2). Preference for specific FAs in S-acylation depends on the cellular lipid pool as well as the expression/activity of acyltransferases and thioesterases (Nůsková *et al.*, 2022). For example, a research group used hydroxylamine probes to profile S-acylated FAs across various cell types and found that palmitate (C16:0), palmitoleate (C16:1), and OA, C18:1) were the most commonly incorporated FAs in HEK293T and HeLa cells. In contrast, in mouse neuroblastoma N2a cells, stearate (C18:0) was more prominent, while palmitoleate levels were significantly lower. Notably, the incorporation of unsaturated FAs into proteins for S-acylation correlated with SCD1 activity, suggesting a direct link between FA metabolism and lipid modification of proteins (Schulte-Zweckel *et al.*, 2019). The specificity of S-acylation also depends on the substrate preferences of zDHHC family acyltransferases. Some, like zDHHC3 and zDHHC7, show broad substrate specificity, while others, such as zDHHC9 and zDHHC17, exhibit highly selective activity (e.g., toward H-Ras and SNAP-25, respectively) (Fukata *et al.*, 2006). While HEK293T cells generally express most zDHHC isoforms at moderate and balanced levels (McClafferty et Shipston, 2019; Ocasio *et al.*, 2024), cancer cells—including breast cancer—often display skewed zDHHC expression patterns, with certain enzymes upregulated or downregulated. This imbalance can lead to biased S-acylation substrate selection and may impact the functional outcomes of lipid modifications (Bian *et al.*, 2024; Kwon, H. *et al.*, 2023; Liu, Z. *et al.*, 2020; Sharma *et al.*, 2017). Although data on zDHHC expression in MDA-MB-231 cells remain limited, studies have implicated zDHHC3, 5, 15, and 20

in cancer-related S-acylation processes (McClellan *et al.*, 2024; Ocasio *et al.*, 2024; Sharma *et al.*, 2017; Wang, J. *et al.*, 2025). Interestingly, even within the same zDHHC enzyme, substrate specificity can vary across cell types. For instance, zDHHC20 was found to be expressed in both HEK293T and MDA-MB-231 cells; however, it modified 231 substrates in HEK293T cells versus only 50 in MDA-MB-231, with just 30 shared between the two cell types (Ocasio *et al.*, 2024).

In addition, breast cancer cells are also characterized by elevated SCD1 and ELOVL6 activity, which contribute to a higher MUFA pool—especially OA—compared to non-cancerous cells (Feng *et al.*, 2016; G. S. Zakharova, 2019; Lingrand *et al.*, 2020a). In MDA-MB-231 cells, single-cell lipidomic analysis confirmed that OA is the dominant unsaturated FA, whereas C16:1 is present only at minor levels (Li, Z. *et al.*, 2021). These differences in lipid metabolism and S-acylation enzyme expression likely explain the cell-line-specific responses we observed, such as the weak PLD activation by palmitoleate in MDA-MB-231 cells compared to HEK293T. In contrast, OA consistently activated PLD2 in both cell lines, suggesting that OA acts as a widely utilized S-acylation lipid—similar to palmitate—but with distinct functional consequences. Specifically, OA's ability to disrupt PLD2's lipid raft association and enhance its colocalization with $PIP_2$-enriched microdomains appears to underlie its potent regulatory role in cancer cell signaling and migration.

Our findings provide direct evidence that the type of FA modification differentially regulates PLD2 localization and function through distinct membrane microdomains. Specifically, we observed that treatment with OA led to a more homogenous distribution of $PIP_2$ across the membrane and significantly enhanced the colocalization of PLD2 with $PIP_2$ clusters (Fig. 4.3). In contrast, palmitate treatment caused $PIP_2$ to condense into fewer, larger puncta and reduced PLD2 colocalization. These results suggest that OA promotes the redistribution of PLD2 away from lipid rafts and toward $PIP_2$ clusters, where its enzymatic activity is likely optimized. This is consistent with our caveolin-1 imaging data, which showed that OA disrupts PLD2 association with lipid rafts (Fig. 4.2), while palmitate reinforces it. Importantly, our observations align with previous studies demonstrating that palmitoylation tends to anchor proteins in cholesterol-rich lipid rafts, whereas unsaturated FAs like OA destabilize these domains and favor translocation to more dynamic, signaling-active regions such as $PIP_2$ clusters (Nuskova *et al.*, 2021; Petersen *et al.*, 2016). The OA-induced dissociation of PLD2 from lipid rafts and enhanced localization to $PIP_2$ microdomains supports a model in which oleoylation serves as a regulatory switch that redirects

PLD2 to microdomains of the membrane more conducive to its signaling role. Moreover, the fact that $PIP_2$ is typically excluded from rigid lipid rafts but becomes enriched in more fluid, unsaturated regions (McLaughlin et Murray, 2005; van den Bogaart *et al.*, 2011) further reinforces our interpretation that OA promotes a membrane environment favorable for PLD2 activation. Thus, our data provide mechanistic insight into how FA-driven lipid remodeling alters the spatial dynamics of PLD2, with functional consequences for downstream signaling.

OA has been specifically implicated in PLD2 activation (Gibbs et Meier, 2000; Kasai *et al.*, 1998; Kim, J. H. *et al.*, 1999; Sarri *et al.*, 2003). PLD1, the other major isoform of phospholipase D, also undergoes S-acylation, but its regulation differs from PLD2. PLD1 is primarily palmitoylated at two cysteine residues (Cys240 and Cys241, Fig. 4.4A), which facilitate its translocation from the cytosol to the membrane. Unlike PLD2, which is constitutively membrane-associated, PLD1 requires palmitoylation for membrane localization and enzymatic activation (Bowling *et al.*, 2021; Hodgkin *et al.*, 2000). This difference in regulation may reflect the distinct roles of palmitate in regulating PLD1 and PLD2 via S-acylation. Palmitoylation serves as an activation mechanism for PLD1 while it diminishes PLD2 activity by anchoring it in lipid rafts to isolate it from its substrate. Our findings align with previous studies showing that the type of FA incorporated into membrane and proteins during S-acylation impacts membrane microdomain localization and acylated protein functions (Nuskova *et al.*, 2021).

Our study provides the first direct experimental evidence that human PLD2 is S-acylated at Cys223 and Cys224, a modification essential for its membrane localization and function (Figs. 4.3, 4.5, and 4.6). Furthermore, our data indicate that OA enhances PLD2 acylation (Fig. 4), suggesting that OA serves as an acyl donor or indirectly promotes acylation. Given that acylation is reversible, PLD2 localization and activity may be dynamically regulated in response to metabolic cues. Interestingly, our results show single mutants (C223A or C224A) retaining function, while the double mutant (C223AC224A) loses membrane localization and lipid raft association (Fig.4.2). This suggests that acylation at either Cys223 or Cys224 is sufficient for PLD2 membrane targeting and function, but double acylation enhances stability and efficiency. This is consistent with studies on other S-acylated proteins, where multiple acylation sites increase membrane affinity and functional robustness. The biochemical basis for membrane binding is clear: two FAs are better than one (Resh, 1999; Shahinian et Silvius, 1995). For example, $G_{\alpha q}$ is doubly palmitoylated, and mutation of any single site reduces its membrane localization and function,

while double mutations abolish activity entirely (Wedegaertner *et al.*, 1993). Similarly, mutation of GAP43 has shown that double palmitoylation is required for its membrane association while single mutants only impair it (Liu, Y. *et al.*, 1993). In the case of PLD2, the conservation of Cys223 and Cys224 across species further highlights their functional significance. Rat PLD2, like human PLD2, has been shown to be S-acylated at Cys223 and Cys224, while mouse PLD2, which lacks Cys223, retains only one S-acylation site at Cys224. Despite this difference, mouse PLD2 remains functional, indicating that a single S-acylation site might be sufficient for membrane targeting and activity. This conservation across species indicates that S-acylation is a fundamental regulatory mechanism for PLD2.

Beyond its lipase activity, we identify for the first time PLD2 as a GEF for Cdc42, a key regulator of actin cytoskeleton remodeling and filopodia formation. The lipase activity of PLD2 is dispensable for its GEF function, as the lipase-inactive mutant (K758R) retains the ability to activate Cdc42 and induce filopodia formation. However, the GEF-inactive mutant (ΔCRIB) fails to activate Cdc42, underscoring the importance of the GEF function domain in cytoskeletal rearrangements. The CRIB (Cdc42/Rac Interactive Binding) domain is identified as a highly conserved protein domain that mediates interactions with the small GTPase Cdc42 and, to a lower extent, Rac) (Burbelo *et al.*, 1995; Morreale *et al.*, 2000). The discovery of 2 CRIB domains in PLD2's PX domain suggests that PLD2 plays a specialized role in Cdc42-dependent processes such as filopodia formation and cell migration, consistent with our previous findings in TNBC cells (Peng, H. J. *et al.*, 2011).

In MDA-MB-231 cells, disrupting lipid rafts using MβCD increased Cdc42 activation. In HEK293T cells, the C223AC224A mutant lost OA-induced Cdc42 activation. These results indicate that PLD2's GEF activity is regulated by OA-dependent S-acylation and lipid raft dynamics. Cdc42, which is S-prenylated and membrane-anchored, is known to be excluded from lipid rafts (del Pozo *et al.*, 2004; Fivaz *et al.*, 2002; Michaelson *et al.*, 2001). OA-induced S-oleoylation of PLD2 promotes its dissociation from lipid rafts, potentially facilitating interaction with prenylated Cdc42. Additionally, PA, the lipase product of PLD2, could further regulate the GEF activity by recruiting and stabilizing signaling complexes at the membrane (Mahankali *et al.*, 2013). Collectively, our results suggest that OA induced S-acylation of PLD2 has a dual regulation effect on both lipase and GEF activity.

Based on our findings, we propose a model in which OA-induced S-oleoylation of PLD2 promotes its dissociation from lipid rafts and translocation to $PIP_2$-enriched clusters (Fig. 7D). This redistribution enhances PLD2 accessibility to its substrate PC, facilitating PA production. Simultaneously, dissociation from lipid rafts facilitates PLD2 interaction with prenylated Cdc42 at the membrane, promoting GTP exchange and Cdc42 activation. Additionally, PA could also influence Cdc42 activation via interegulation with PLD2's GEF activity. This model provides a mechanistic link between OA-induced PLD2 activation, Cdc42-dependent cytoskeletal rearrangements, and cell migration.

Our findings provide a comprehensive understanding of how OA modulates PLD2 activity through S-acylation and membrane reorganization, offering new insights into the regulatory interplay between FA metabolism and membrane-associated signaling. Given that MUFAs such as OA are abundant in the tumor microenvironment, their role in regulating lipid-modifying enzymes like PLD2 may have significant implications for cancer progression. These findings advance our understanding of PLD2 regulation and suggest potential therapeutic targets for TNBC and other cancers. The differential effects of OA and palmitate on PLD2 activity and membrane dynamics suggest that FA metabolism plays a crucial role in modulating PLD2 function in cancer cells. Targeting PLD2 S-acylation or lipid raft dynamics might provide new therapeutic strategies for TNBC and other cancers where PLD2 and lipid signaling are dysregulated. Future studies should explore the role of specific acyltransferases and thioesterases in regulating PLD2 acylation and function. Additionally, the interplay between PLD2, Cdc42, and other small GTPases in cancer cell migration and invasion warrants further investigation. Understanding the molecular mechanisms underlying PLD2 regulation by FAs will provide new insights into lipid signaling and its role in cancer progression.

In conclusion, we propose a novel mechanism by which OA regulates PLD2 function by modulating its membrane localization. Through S-acylation, this mechanism links FA metabolism, lipid signaling, and cytoskeletal regulation. These findings highlight the intricate interplay between lipid modifications and signal transduction, opening new avenues for therapeutic interventions targeting PLD2 in cancer and beyond.

## 4.5 Materials and Methods

### 4.5.1 Cell lines and culture

MDA-MB-231 cells were a generous gift from Dr. Jean-Jacques Lebrun (McGill University, Canada), and HEK293T cells were provided by Dr. Benoît Barbeau (UQAM, Canada). Both cell lines were maintained at 37°C in a 5% $CO_2$ incubator. MDA-MB-231 cells were cultured in Eagle's Minimum Essential Medium (EMEM, Wisent #320-005-CL), while HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Wisent #319-005-CL), both supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 500 U/mL penicillin, and 500 µg/mL streptomycin (Gibco #15070063). Cells between passages 5 and 25 were used. Adherent cells were detached using 0.25% Trypsin-EDTA (Gibco #25200056).

### 4.5.2 Reagents and antibodies

Polyethylenimine (PEI) reagents were a kind gift from Dr. Nicola Pilon (UQAM, Canada). Methyl-β-cyclodextrin (MβCD, AC377110050) and Lipofectamine 3000 were purchased from Invitrogen Thermo Fisher Scientific (USA). Primary antibodies: anti-PLD2-7E4D9 (#MA5-31854, Invitrogen), anti-PLD2-E1Y9G (#13904, Cell Signaling Technology), anti-Caveolin-1 (#PA5-32297, Invitrogen), anti-$PIP_2$ (2C11, #ab11039, Abcam). Secondary antibodies: Alexa Fluor 488/647 conjugates (#4410/#4412, Cell Signaling Technology), HRP-conjugated anti-mouse/-rabbit IgG (#7076/#7074, Cell Signaling Technology). Additional reagents or kits included Cdc42 Activation Kit (#8819, Cell Signaling Technology), ChromoTek GFP-Trap Agarose (Proteintech #gta-20), Phalloidin-TRITC (Sigma #P1951), TCEP (Sigma #C4706), mPEG-Mal (Sigma #63187), and NEM (Sigma #E3876). Inhibitors: ELOVL6-IN-4 (MedChemExpress #HY-152947) and SCD1 inhibitor A939572 (Biofine #37062).

### 4.5.3 Cell transfection and treatments

MDA-MB-231 cells were transfected using Lipofectamine 3000, while HEK293T cells were transfected with PEI (1 µg/mL, PEI/DNA ratio 3:1). BSA-conjugated FAs were prepared at a 2:1 molar ratio (3.33 mM FA: 1.7 mM BSA) and 100 µM was used for treatment, with FA-free

BSA as control. Cells were also pre-treated with 1 μM SCD inhibitor A939572 and 1 μM ELOVL6 inhibitor ELOVL6-IN-4, or incubated with 100 μM MβCD overnight before treatment.

### 4.5.4 Plasmid construction and sequence analysis

The human PLD2 plasmid (EGFP-PLD2) was cloned from HepG2 cell cDNA into the EGFP-C1 vector. A reference plasmid was kindly provided by Dr. Min Do Sik (Yonsei University, South Korea). Site-directed mutagenesis generated PLD2 mutants (C223A, C224A, C223AC224A, K758R, and ΔCRIB). All constructs were sequence-verified. DNA sequence analysis was performed using ApE (A Plasmid Editor) software (v3.0.6) for sequence annotation, restriction mapping, and primer design. Protein sequence alignments were generated using Clustal Omega (EMBL-EBI) with default parameters. Homology percentages were calculated from pairwise alignments of conserved domains. Primers are shown in the table below.

**Table 4.1 Human PLD2 plasmid construction primers**

| Primer | Sequence |
|---|---|
| PLD2-WT-F | CCCAAGCTTGG**ATGACGGCGACCCCTGAGAG** |
| PLD2-WT-R | TCCCCGCGG**CTATGTCCACACTTCTAGGGGGATC** |
| PLD2-C223A-F | CCTCACC**GCC**TGTGGCCGAGACCAAGTTTG |
| PLD2-C223A-R | CCACA**GGC**GGTGAGGCCAGGAACACG |
| PLD2-C224A-F | CACCTGC**GCC**GGCCGAGACCAAGTTTGTTATCG |
| PLD2-C224A-R | CGGCC**GGC**GCAGGTGAGGCCAGGAAC |
| PLD2-C223AC224A-F | TCACC**GCCGCC**GGCCGAGACCAAGTTTGTTATCG |
| PLD2-C223AC224A-R | GGCC**GGCGGC**GGTGAGGCCAGGAACACG |
| PLD2-K758R-F | CCACAGC**AGG**GTGCTCATCGCAGATGACCG |
| PLD2-K758R-R | AGCAC**CCT**GCTGTGGATGTAGATGAGCTCCG |
| PLD2-ΔCRIB-F | TCTTTGAC[~~**CCTGGCTTT**~~**]**GAGGTGCAAGTGGGGAAAAGG |
| PLD2-ΔCRIB-R | GCACCTC[~~**AAAGCCAGG**~~**]**GTCAAAGAGCTGAACAAATGAGATG |

### 4.5.5 **Immunofluorescence and F-Actin staining**

Cells were seeded at 50-70% confluency on sterilized coverslips in 6-well plates. After 24h incubation and subsequent treatments, cells were fixed with 4% paraformaldehyde (PFA) for 15 min. For $PIP_2$ staining, fixation included 3% PFA + 0.1% glutaraldehyde followed by 0.1% NaBH4 quenching. Cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked with 3% BSA for 1h. F-actin and nuclei were stained with 50 µg/mL Phalloidin-TRITC and 1 µg/mL DAPI, respectively. Immunostaining followed standard protocols, with overnight incubation at 4°C in primary antibodies (for PLD2, 1:250, 7E4D9, Invitrogen) and 1h incubation with Alexa Fluor 488/647 secondary antibodies in the dark. Coverslips were mounted in 90% glycerol/PBS and sealed with clear nail polish.

### 4.5.6 **Confocal microscopy and image analysis**

Images were using a Nikon A1 Plus inverted confocal microscope (63× oil objective). Image processing was performed in ImageJ Fiji. PASS fluorescence intensity was analyzed using the line-intensity histogram function, as modified from Lu et. al. (Lu, M. *et al.*, 2016). Colocalization analysis was performed using the Coloc2 plugin, with Pearson's coefficient as a colocalization index. Membrane distribution analysis of PLD2, Caveolin-1, and PIP2 were performed by manually tracing the plasma membrane using ImageJ's freehand selection tool, with regions of interest saved in the ROI Manager. Fluorescence intensity measurements were used to calculate mean and standard deviation values, from which the coefficient of variation (CV = standard deviation/mean) was derived to quantify signal heterogeneity. Elevated CV values indicated scattered or clustered distributions, while lower CV values corresponded to homogeneous membrane localization patterns.

### 4.5.7 **Lipid raft isolation**

Lipid rafts were isolated using a detergent-free sodium carbonate method adapted from (Macdonald et Pike, 2005). Cells were lysed in 200 mM $Na_2CO_3$ (pH 11 in MBS buffer), sonicated (3 × 10s, 1500 Hz on ice), and centrifuged (17 000g, 10 min). Lysates were mixed with 90% sucrose and overlaid with 35% and 5% sucrose layers, followed by ultracentrifugation (200 000g,

20h, 4°C, SW41 Ti rotor, Beckman Instruments). 1 mL fractions were collected from top to bottom for Western blot analysis of caveolin-1 and PLD2.

### 4.5.8 **Acyl-PEG exchange assay**

S-acylation of PLD2 was assessed using a modified Acyl-PEG exchange assay based on (Percher *et al.*, 2017). Briefly, cells were lysed and proteins reduced with TCEP, followed by thiol blocking with 25 mM NEM. Hydroxylamine (1 M, pH 7.4) was treated to expose nascent thiols. Newly exposed thiols were labeled with 1 mM 5 kDa mPEG-Mal. Samples were analyzed by SDS-PAGE and Western blot.

### 4.5.9 **Active Cdc42 pull-down assay**

Cdc42 activation was measured using the Cdc42 Activation Assay Kit (Cell Signaling #8819) according to the manufacturer's instructions. Briefly, cells were lysed, and GTP-bound Cdc42 was affinity-precipitated using a PAK1 PBD-conjugated agarose bead slurry. Beads were washed, and bound proteins were eluted in SDS buffer. Samples were resolved by SDS-PAGE, and active Cdc42 (GTP-bound) was detected by Western blot using a Cdc42-specific antibody. Total Cdc42 levels were assessed from whole-cell lysates for normalization.

### 4.5.10 **Western blotting**

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma, #P8340; #P0044). After centrifugation, proteins were recovered in the supernatant. Protein concentration was determined by the Bradford protein assay (Bio-Rad, #5000006). Proteins were separated on SDS-PAGE transferred to PVDF membranes. Primary antibodies: PLD2 (1:500; Cell Signaling Technology, EY19G, #13904), Caveolin-1 (1:1000; Invitrogen, #PA5-32297), GFP (1:1000; Cell Signaling Technology, #2555), Cdc42 (1:167; Cell Signaling #8747) were used. HRP-conjugated anti-rabbit IgG (1:2000: Cell Signaling, #7074) and HRP-conjugated anti-mouse IgG (1:2000: Cell Signaling, #7076) were used as secondary antibody. Signals were revealed using the ECL substrate (Millipore, #WBKLS0100). Bands were quantified by Image J Fiji gel analyse tool.

### 4.5.11 **Statistical analyses**

Statistical analyses were carried out using GraphPad Prism version 9.0. The significance of differences between groups was tested using unpaired two-tailed Student's t-tests or ordinary one-way ANOVA with Dunnett's multiple comparisons test. Differences were considered significant when p values were $< 0.05$.

# CHAPITRE 5
# GENERAL DISCUSSION

TNBC is one of the most aggressive subtypes of breast cancer, with metastasis being the primary cause of poor patient prognosis. Cell migration is one of the fundamental behaviors contributing to the metastatic cascade, and our previous study identified SCD1 and its main product, OA, as key regulators of this process. Specifically, the recent study from our lab demonstrated that inhibiting SCD1 activity or directly treating cells with OA altered migration speed and direction, as well as cell morphology, implicating PLD signaling (Lingrand *et al.*, 2020a). However, the precise molecular mechanisms underlying these effects were not well understood. In this study, we reveal that OA treatment induces rapid cell membrane ruffling and filopodia-like protrusions in a Cdc42-dependent manner, with PLD playing a critical role. Further investigation into the molecular mechanisms uncovered that OA activates PLD2's lipase and guanine GEF activities through S-acylation, driving its translocation between membrane microdomains. These findings provide new insights into lipid-driven regulation of cytoskeletal dynamics and metastatic potential in TNBC.

## 5.1 OA Promotes TNBC Migration by Enhancing Filopodia Formation

The reorganization of the actin cytoskeleton is a fundamental driver of cancer cell motility, enabling morphological changes that facilitate migration, invasion, and metastasis (Olson et Sahai, 2009). Filopodia, thin, actin-rich membrane protrusions, play a crucial role in directional sensing and cell movement, particularly in highly invasive cancer types (Arjonen *et al.*, 2011; Bischoff et Bogdan, 2021; Bischoff *et al.*, 2021; Bray *et al.*, 2013; Gat *et al.*, 2020; Jacquemet *et al.*, 2015; Kishimoto *et al.*, 2020; Kiso *et al.*, 2018; Sakabe *et al.*, 2017). The previous study from our lab demonstrated that OA treatment enhances TNBC cell migration by increasing directionality and speed while inducing a more elongated, fibroblast-like morphology (Lingrand *et al.*, 2020a). Here, we expand on these findings by identifying a novel OA-driven cellular alteration—rapid membrane ruffling and the formation of Cdc42-dependent filopodia, which are critical for the migratory behavior of TNBC cells (Figs. 3.1 and 3.3).

The role of filopodia in cancer progression has been widely recognized, with accumulating evidence linking their presence to metastatic potential (Jacquemet *et al.*, 2017). In breast cancer,

filopodia formation is often associated with aggressive phenotypes. For instance, the oncoprotein HBXIP was shown to enhance filopodia formation via the MEKK2/ERK1/2/Capn4 signaling axis, promoting breast cancer cell migration (Li, Y. *et al.*, 2014). Similarly, in MDA-MB-231 cells, filopodia dynamics are regulated by L-type calcium channels and Cdc42 activity (Jacquemet *et al.*, 2016; Kiso *et al.*, 2018; Liu, L. *et al.*, 2019). Our findings align with these reports, reinforcing the idea that filopodia serve as functional structures that drive TNBC cell migration.

Beyond breast cancer, Cdc42-dependent filopodia formation has been observed in multiple malignancies, including colorectal (Aikemu *et al.*, 2021), ovarian (Horita, Kurosaki, Nakatake, Ito, *et al.*, 2019; Horita, Kurosaki, Nakatake, Kuwano, *et al.*, 2019), pancreatic (Yuan et Wei, 2021), and lung cancers (Pan *et al.*, 2011). This suggests that Cdc42-mediated cytoskeletal remodeling is a conserved mechanism across different cancer types. Given the elevated CDC42 expression in breast cancer and its correlation with poor patient prognosis, targeting filopodia formation through Cdc42 inhibition could present a potential therapeutic strategy for TNBC, a subtype that currently lacks effective targeted treatments.

Moreover, our findings reveal that OA-induced filopodia formation is not merely a structural adaptation but is closely tied to intracellular signaling pathways regulating cell motility. The dynamic interplay between OA, Cdc42 activation, and cytoskeletal remodeling suggests a lipid-driven mechanism that enhances TNBC metastatic potential. Further exploration of this axis may provide valuable insights into lipid-mediated cancer progression and uncover new avenues for therapeutic intervention.

## 5.2 Cdc42 is Crucial in OA-induced Filopodia Formation

The small GTPase Cdc42 is a member of the Rho family and plays a pivotal role in the actin cytoskeleton organization, controlling cell motility and cell cycle progression (Murphy *et al.*, 2021). Our results demonstrated that inhibition of Cdc42 using ML141, a highly specific Cdc42 inhibitor, efficiently abolished OA-induced filopodia formation and cell migration in both MDA-MB-231 and MDA-MB-468 cells (Figs. 3.3 and 3.5). Importantly, OA treatment did not alter Cdc42 expression levels (Fig. S3.3), but instead induced a translocation of Cdc42 outside the nucleus (Fig. 3.2), where it is functionally active in cytoskeletal remodeling. This shift in subcellular localization suggests that OA enhances the availability of Cdc42 for activation, likely

by GEFs, which catalyze the GDP-to-GTP exchange required for Cdc42 activation (Maldonado *et al.*, 2020). As most Rho-GEFs localize either in the cytoplasm or in the plasma membrane, and only a few of them are detected in the nucleus (Dubash *et al.*, 2011), therefore, the cytoplasmic distribution of Cdc42 induced by OA treatment increases its likeliness of being activated by GEFs.

Although the precise mechanism by which OA activates Cdc42 remains unclear, multiple lines of evidence suggest that Cdc42 activation is linked to lipid signaling and receptor activation. OA has been shown to activate GPCRs (e.g., GPR40/120), RTKs (e.g., insulin receptor), and integrins, all of which could trigger GEF-mediated activation of Cdc42 (Guo, Z. *et al.*, 2023; Liu, Z., Xiao*, et al.*, 2013; Liu, Z. H. *et al.*, 2020; Maldonado *et al.*, 2020; Tsuchiya *et al.*, 2014). Furthermore, lipid modifications, such as prenylation and acylation, play key roles in Cdc42 membrane localization and activity (Nishimura et Linder, 2013; Wirth et Ponimaskin, 2022). Given that OA is a lipid with known regulatory effects on membrane organization, it is possible that OA directly influences Cdc42 localization and activation through lipid modifications, an aspect that warrants further investigation. In fact, in our second research article (Chapter 4), we further explored this mechanism and demonstrated that OA activates Cdc42 via S-acylation–dependent regulation of PLD2 localization and GEF activity, revealing a direct molecular link between OA, PLD2, and Cdc42 signaling in cell migration.

In addition to Cdc42, the Arp2/3 complex is another key player in cell protrusions and migration. The Arp2/3 complex nucleates branched actin filaments, playing a significant role in lamellipodia formation and, by extension, cell motility. However, there has been some controversy over the essential role of Arp2/3 in filopodia formation. In our study, we observed differential responses to Arp2/3 inhibition between the two TNBC cell lines. Specifically, MDA-MB-468 cells showed no change in filopodia formation or migration upon treatment with CK666, an inhibitor of the Arp2/3 complex (Figs. 3.3C and 3.4D). In contrast, MDA-MB-231 cells exhibited nucleus-to-cytoplasm translocation of the Arp2/3 complex upon OA treatment, and CK666 effectively inhibited OA-stimulated wound closure (Figs. 3.2DEF and 3.4CD). These results indicate that the Arp2/3 complex is involved in OA-induced filopodia formation and migration in MDA-MB-231 cells, although the role of Arp2/3 in filopodia initiation may not be universally critical across different TNBC cell lines.

It is important to note that the discrepancy in Arp2/3 complex involvement between cell lines could be explained by the different mechanisms of filopodia formation. Filopodia can emerge through two potential models: the convergent elongation model, in which filopodia extend from an Arp2/3-mediated lamellipodial network, and the tip nucleation model, which proposes that filopodia form independently via formin-driven self-assembly at the plasma membrane without the need for Arp2/3 complex-dependent nucleation (Yang, C. et Svitkina, 2011). Our data suggest that while MDA-MB-231 cells likely utilize a combination of both models for filopodia formation, the self-assembly model may dominate in MDA-MB-468 cells. Additionally, filopodia, along with lamellipodia, are key to coordinated cell movement, where lamellipodia provide the forward propulsion, and filopodia sense the microenvironment, facilitating directed migration (Bischoff et Bogdan, 2021; Bischoff *et al.*, 2021; Caswell et Zech, 2018; Innocenti, 2018; Jacquemet *et al.*, 2015). This interplay between filopodia and lamellipodia could explain why MDA-MB-231 cells exhibit a higher basal migration speed compared to MDA-MB-468 cells, both with and without OA stimulation (Fig. 3.4).

Lastly, bioinformatics analysis further supports a role for Cdc42 in breast cancer progression, as we found elevated CDC42 and ACTR2 expression in breast cancer. Notably, in TNBC, high CDC42 expression in primary tumors correlates with increased cancer-related mortality (Fig. 3.6). While oncogenic mutations in Cdc42 are rare (Murphy *et al.*, 2021; Stengel et Zheng, 2011), its overexpression is associated with poor prognosis in multiple cancers, including breast (Bray *et al.*, 2013; Fritz, G. *et al.*, 2002; Fritz, G. *et al.*, 1999; Jiang, L. C. *et al.*, 2011), colorectal (Du, D. S. *et al.*, 2016), esophageal (Liu, Z. *et al.*, 2011), gastric (Du, D. S. *et al.*, 2016), lung (Chen, Q. Y. *et al.*, 2012; Liu, Y. *et al.*, 2009; Liu, Y. *et al.*, 2005), melanoma (Tucci *et al.*, 2007), ovarian (Bourguignon *et al.*, 2005; Guo, Y. *et al.*, 2015), pancreatic (Yang, D. *et al.*, 2017), and testicular cancer (Kamai *et al.*, 2004). Some studies also found the overexpression of Cdc42 to be positively correlated with poor prognostic (Bourguignon *et al.*, 2005; Kamai *et al.*, 2004; Tucci *et al.*, 2007; Yang, D. *et al.*, 2017). These findings position Cdc42 as a potential therapeutic target in TNBC, a cancer subtype with limited treatment options.

## 5.3 PLD2 is Involved in OA-induced Cdc42 Signaling

Elevated expression and activity of PLD, especially PLD2, have been detected in various human cancer tissues and cells, including breast cancer (Bowling *et al.*, 2021; Brown, H. A. *et al.*,

2017; Henkels, Boivin*, et al.*, 2013). The previous study from our lab has demonstrated that PLD2 expression correlates with an increased proportion of metastasis-related deaths in TNBC patients and that PLD activity is involved in OA-induced MDA-MB-231 cell migration and invasion (Lingrand *et al.*, 2020a). Given this association, we investigated whether PLD is involved in OA-induced filopodia formation signaling and cell migration.

Here, we confirmed that OA could activate PLD activity in TNBC (Fig. 3.5A) and HEK293T (Fig. 4.1) cell lines. Although our methodology did not allow us to distinguish between PLD isoforms, multiple lines of evidence suggest that PLD2 is the primary isoform activated by OA. Previous studies demonstrated that OA selectively stimulates PLD2 activity, but not PLD1, *in vitro* (Kim, J. H. *et al.*, 1999). Additionally, PLD activity was highly induced by OA in Jurkat T cells (which express only PLD2) but not in HL-60 cells (which express only PLD1) (Gibbs et Meier, 2000; Kasai *et al.*, 1998). Furthermore, in RBL-2H3 mast cells, OA only stimulated PLD activity when PLD2 was overexpressed, but not when PLD1 was overexpressed (Sarri *et al.*, 2003). These findings strongly support the idea that OA preferentially activates PLD2.

To further investigate whether PLD activation leads to Cdc42-dependent filopodia formation and migration, we tested PMA-induced PLD activation. Much like OA, PMA treatment induced both filopodia formation and cell migration in TNBC cells, and this effect was dependent on Cdc42 activity (Fig. 3.5G). In the HEK293T overexpression system, overexpression of PLD2 and catalytically inactive PLD2-K758R both induced filopodia-like cell protrusions but not GEF inactive mutant PLD2-ΔCRIB (Fig. 4.7), indicating the GEF function is indispensable in PLD2-induced cell protrusion formation. Consistently, PLD2 was frequently localized at the leading edge of migrating cells in membrane ruffles, supporting its role in cell motility (Colley *et al.*, 1997; O'Luanaigh *et al.*, 2002). Interestingly, previous report showed that elevated PLD2 expression in significantly increases the length of cell protrusions in v-Src-induced rat 3Y1 fibroblasts, while a catalytically inactive PLD2-K758R mutant abolishes them (Shen, Y. *et al.*, 2002). This discrepancy might be due to cell system differences as protrusion formation in 3Y1 cells was found to be microtubule-dependent.

We next examined the potential interaction between Cdc42 and PLD2. Colocalization analysis revealed that Cdc42 and PLD2 are spatially associated in TNBC cells, with OA treatment further enhancing their colocalization (Fig. 3.5C and D). These results suggest that PLD2 and

Cdc42 could interact directly. Given that PLD2 possesses a unique GEF feature, it is plausible that PLD2 may act as a GEF for Cdc42, akin to its previously reported role in stimulating GDP-GTP exchange on Rac2 and RhoA (Bischoff *et al.*, 2021; Mahankali, Peng, Henkels*, et al.*, 2011). There are two CRIB motifs in and around the PH domain of PLD2 (Peng, H. J. *et al.*, 2011), raising the possibility that PLD2 could act as a GEF for Cdc42. Therefore, we investigated whether PLD2 acts as a GEF for Cdc42. Our active Cdc42 pull-down results showed that OA treatment increased Cdc42-GTP levels in both HEK293T and MDA-MB-231 cells (Fig. 4.7). Furthermore, overexpression of PLD2-WT and PLD2-K758R in HEK293T cells significantly increased Cdc42-GTP levels, while PLD2-ΔCRIB showed a much weaker effect, confirming that PLD2 acts as a GEF for Cdc42 and that its lipase activity is dispensable for this function.

In addition to its role as a GEF, its enzymatic product PA is also recognized regulators of actin cytoskeleton dynamics. PLD-derived PA has been reported to directly interact with Arp2/3 complex to enhance actin filament branching, which supports protrusion formation and cell movement (Speranza *et al.*, 2014). In the context of our study, OA-induced activation of PLD2 likely contributes to Cdc42-dependent filopodia formation in TNBC cells through a dual mechanism: PLD2's GEF activity facilitates Cdc42 activation, while its lipase activity generates PA, which may enhance Arp2/3-mediated actin remodeling and interact with Cdc42. This coordinated signaling axis—linking Cdc42, PLD2, and the Arp2/3 complex—reinforces filopodia formation and supports OA-driven cell migration.

## 5.4 OA Activates PLD2 by S-acylation and Membrane Lipid Microdomain Modulation

As our results suggest that PLD2 acts as an upstream regulator of OA-induced Cdc42-dependent filopodia signaling, we next sought to investigate the molecular mechanism by which OA activates PLD2. Given the known role of S-acylation in regulating lipid enzyme activity and membrane dynamics, we hypothesized that OA modifies PLD2 via S-acylation, thereby influencing its localization and function.

Firstly, we investigated the impact of FAs on PLD activation. Our findings demonstrate that C16:1 induces moderate PLD activation and association with lipid rafts in HEK293T cells but does not elicit the same response in MDA-MB-231 cells, suggesting a FA preference in S-acylation

that is cell-type dependent (Fig. 4.1 and 4.2). The selection of specific FAs for S-acylation is influenced by the cellular lipid pool and the expression of acyltransferases (zDHHC enzymes) and thioesterases (APTs) (Nůsková *et al.*, 2022). This variation is well-documented in different cell types. For instance, a hydroxylamine probe study by the Triola group profiled S-acylated FAs in proteome of HEK293, HeLa, and N2a cells. It reveals that C16:0, C16:1, and C18:1 are the most abundant S-fatty acids. However, N2a cells exhibited higher levels of C18:0 and lower levels of C16:1. Further analysis with SCD1 inhibitor shows that the level of unsaturated S-fatty acids correlates with SCD1 activity (Schulte-Zweckel *et al.*, 2019). Additionally, zDHHC enzymes exhibit substrate specificity, influencing S-acylation patterns (Fukata *et al.*, 2006). For example, zDHHC-3 and -7 have broad substrate specificity, whereas zDHHC-9 and -17 show selectivity for specific proteins like H-Ras and SNAP-25 (Fukata *et al.*, 2006). While HEK293T cells generally express most zDHHC isoforms at moderate and balanced levels (McClafferty et Shipston, 2019; Ocasio *et al.*, 2024), cancer cells—including breast cancer—often display skewed zDHHC expression patterns, with certain enzymes upregulated or downregulated. This imbalance can lead to biased S-acylation substrate selection and may impact the functional outcomes of lipid modifications (Bian *et al.*, 2024; Kwon, H. *et al.*, 2023; Liu, Z. *et al.*, 2020; Sharma *et al.*, 2017). Although data on zDHHC expression in MDA-MB-231 cells remain limited, studies have implicated zDHHC3, 5, 15, and 20 in cancer-related S-acylation processes (McClellan *et al.*, 2024; Ocasio *et al.*, 2024; Sharma *et al.*, 2017; Wang, J. *et al.*, 2025). Interestingly, even within the same zDHHC enzyme, substrate specificity can vary across cell types. For instance, zDHHC20 was found to be expressed in both HEK293T and MDA-MB-231 cells. However, it modified 231 substrates in HEK293T cells versus only 50 in MDA-MB-231, with just 30 shared between the two cell types (Ocasio *et al.*, 2024).

Breast cancer cells are also characterized by elevated SCD1 and ELOVL6 activity, which contribute to a higher monounsaturated FA pool—especially OA—compared to non-cancerous cells (Feng *et al.*, 2016; G. S. Zakharova, 2019; Lingrand *et al.*, 2020a). In MDA-MB-231 cells, single-cell lipidomic analysis confirmed that OA is the dominant unsaturated FA, whereas C16:1 is present only at minor levels (Li, Z. *et al.*, 2021). These differences in lipid metabolism and S-acylation enzyme expression likely explain the cell-line-specific responses we observed, such as the weak PLD activation by palmitoleate in MDA-MB-231 cells compared to HEK293T. In contrast, OA consistently activated PLD2 in both cell lines, suggesting that OA acts as a widely

utilized S-acylation lipid—similar to palmitate—but with distinct functional consequences. Specifically, OA's ability to disrupt PLD2's lipid raft association and enhance its colocalization with $PIP_2$-enriched microdomains appears to underlie its potent regulatory role in cancer cell signaling and migration.

To further elucidate the role of S-acylation in PLD2 activation, we investigated how OA-induced oleoylation affects PLD2 localization, highlighting the contrasting effects of palmitoylation and oleoylation on PLD2 function. While palmitoylation anchors proteins within lipid rafts, oleoylation facilitates their translocation to $PIP_2$ clusters, enhancing enzymatic activity (Nuskova *et al.*, 2021; Petersen *et al.*, 2016). This aligns with our observations that OA enhances $PIP_2$ cluster formation and increases PLD2 colocalization with $PIP_2$, whereas palmitate sequesters $PIP_2$ into fewer puncta, reducing PLD2 activity (Fig. 4.3). Additionally, OA disrupts lipid rafts and decreases PLD2's association (Fig. 4.2). These results support the notion that OA-induced oleoylation promotes PLD2 translocation from lipid rafts to $PIP_2$ microdomains, optimizing its access to substrate and enhancing enzymatic efficiency.

Membrane domain stability is strongly influenced by FA composition. Lipid rafts are enriched in saturated FAs (e.g., palmitic acid) and cholesterol, which increase membrane rigidity and promote domain formation, whereas MUFAs (e.g., OA) disrupt lipid raft integrity by increasing membrane fluidity (Lingwood et Simons, 2010; Pike, 2006). Interestingly, $PIP_2$-enriched microdomains exhibit the opposite preference, favoring unsaturated FAs for their formation and function (McLaughlin et Murray, 2005; van den Bogaart *et al.*, 2011). This explains why OA promotes PLD2 translocation to $PIP_2$ domains, where it can exert its catalytic function more efficiently.

Furthermore, OA has been reported to selectively activate PLD2 over PLD1 as we have discussed above in Section 5.3 (Gibbs et Meier, 2000; Kasai *et al.*, 1998; Kim, J. H. *et al.*, 1999; Sarri *et al.*, 2003). Although PLD1 and PLD2 both undergo S-acylation, their regulatory mechanisms differ. PLD1 is primarily palmitoylated at Cys240 and Cys241, which is essential for its membrane localization and activation. In contrast, PLD2 is constitutively membrane-associated, and palmitoylation appears to suppress its activity by sequestering it in lipid rafts, isolating it from its substrate (Bowling *et al.*, 2021; Hodgkin *et al.*, 2000). This suggests that S-acylation with C16:0 acts as an activation mechanism for PLD1 but an inactivation mechanism for PLD2.

Together, these findings highlight the importance of S-acylation in regulating PLD2 activity and subcellular localization. The specific FA incorporated into proteins during S-acylation plays a critical role in defining membrane sublocalization and modulating acylated protein function. Our study provides further evidence that OA-induced oleoylation of PLD2 disrupts lipid raft association, promotes its translocation to $PIP_2$ clusters, and enhances its enzymatic activity, contributing to OA-driven TNBC migration.

## 5.5 C223 and C224 are the Key S-acylation Sites in OA-mediated PLD2 Regulation

Building on our findings that OA activates PLD2 through S-acylation and membrane lipid microdomain localization, we sought to identify the specific acylation sites that mediate this effect. We provide the first direct experimental evidence that human PLD2 is S-acylated at Cys223 and Cys224, a modification that is essential for its membrane localization and function (Fig. 4.3, 4.5, 4.6). Furthermore, our data indicate that OA enhances PLD2 acylation (Fig. 4.4), suggesting that OA serves as an acyl donor or indirectly promotes acyltransferase activity. Given that S-acylation is reversible, PLD2 localization and activity may be dynamically regulated in response to metabolic cues.

Interestingly, our results show that single mutants (C223A or C224A) retain PLD2 function, while the double mutant (C223AC224A) loses membrane localization and lipid raft association (Fig. 4.2). This suggests that acylation at either Cys223 or Cys224 is sufficient for PLD2 membrane targeting and function, but double acylation enhances stability and efficiency. This is consistent with studies on other S-acylated proteins, where multiple acylation sites increase membrane affinity and functional robustness. The biochemical basis for membrane binding is well established: two FAs provide stronger membrane anchorage than one (Resh, 1999; Shahinian et Silvius, 1995). For example, $G_{\alpha q}$ is doubly palmitoylated, and mutation of either acylation site reduces membrane localization and function, while double mutations completely abolish activity (Wedegaertner *et al.*, 1993). Similarly, GAP43 (neuromodulin) requires dual palmitoylation for proper membrane association, with Cys4 playing a particularly critical role (Liu, Y. *et al.*, 1993). In the case of PLD2, the conservation of Cys223 and Cys224 across species further underscores their functional significance. Rat PLD2, like human PLD2, is S-acylated at these residues, whereas mouse PLD2

lacks Cys223 but retains Cys224 as a single acylation site. Despite this difference, mouse PLD2 remains functional, suggesting that a single acylation site is sufficient for membrane targeting and activity. This evolutionary conservation highlights S-acylation as a fundamental regulatory mechanism for PLD2.

Beyond its role in lipase regulation, OA-induced membrane lipid microdomain modulation and PLD2 localization also play a crucial role in regulating PLD2's GEF activity. Our results show that disrupting lipid rafts using MβCD in MDA-MB-231 cells increased Cdc42 activation. Furthermore, in HEK293T cells, the C223AC224A mutant lost OA-induced Cdc42 activation (Fig. 4.7). These findings indicate that PLD2's GEF activity is regulated by OA-dependent S-acylation. Since Cdc42 is prenylated and membrane-anchored, it is known to be excluded from lipid rafts (del Pozo *et al.*, 2004; Fivaz *et al.*, 2002; Michaelson *et al.*, 2001). Our findings suggest that OA-induced oleoylation of PLD2 promotes its dissociation from lipid rafts, potentially facilitating its interaction with prenylated Cdc42. Additionally, PA—the lipase product of PLD2—may further regulate GEF activity by recruiting and stabilizing signaling complexes at the membrane (Mahankali *et al.*, 2013).

Collectively, our results suggest that OA-induced S-acylation of PLD2 exerts a dual regulatory effect, modulating both its lipase and GEF activities. This provides a mechanistic link between PLD2 activation, lipid microdomain remodeling, and Cdc42-dependent filopodia formation, further supporting the role of OA in promoting TNBC migration.

## 5.6 Proposed Model of OA-induced TNBC Cell Migration

Based on our findings, we propose a mechanistic model in which OA-induced S-acylation of PLD2 orchestrates a signaling cascade that enhances filopodia formation and promotes TNBC cell migration (Fig. 5.1). This model integrates PLD2's dual roles as a lipase and a GEF, linking OA metabolism to cytoskeletal reorganization and cancer cell motility.

1. OA-Induced PLD2 Oleoylation and Membrane Redistribution

Our results demonstrate that OA induces S-acylation of PLD2 at Cys223 and Cys224, triggering its dissociation from lipid rafts and translocation to $PIP_2$ clusters. This shift in localization is crucial for enhancing PLD2 enzymatic activity, as $PIP_2$ is a known cofactor for PLD2 function. Unlike palmitoylation, which anchors proteins within lipid rafts and restricts their

movement, oleoylation promotes membrane fluidity and facilitates PLD2 repositioning to signaling hotspots.

2. PLD2-Mediated PA Production and Cdc42 Activation

Once in $PIP_2$-enriched regions, PLD2 gains enhanced access to its substrate PC, leading to increased production of PA. PA is a bioactive lipid that serves as both a secondary messenger and a structural modulator of membrane curvature, further influencing cytoskeletal dynamics as well as through interact with Arp2/3 complex. At the same time, PLD2's dissociation from lipid rafts facilitates its interaction with prenylated Cdc42 at the membrane, allowing PLD2 to act as a GEF and catalyze GDP-GTP exchange on Cdc42. This step is critical for activating Cdc42 signaling, which directly regulates actin polymerization and filopodia formation.

Additionally, PA may further contribute to Cdc42 activation through direct interactions with signaling complexes, stabilizing GTP-bound Cdc42 and reinforcing its role in cytoskeletal remodeling. This highlights a synergistic interplay between PLD2's lipase activity and its GEF function, both of which are modulated by OA-induced S-acylation.

3. Downstream Effects: Filopodia Formation, Migration, and Metastasis

Once activated, Cdc42 orchestrates actin cytoskeletal remodeling through its downstream effectors, including the Arp2/3 complex and formins, leading to the formation of dynamic filopodia. These protrusions are essential for sensing the microenvironment and guiding directional migration, a hallmark of metastatic cancer cells. Our findings suggest that OA-stimulated filopodia formation enhances migration speed and directionality, which would allow TNBC cells to navigate through the tumor microenvironment more efficiently.

Furthermore, OA-induced disruption of lipid rafts may indirectly affect other signaling pathways further reinforcing the pro-migratory effects of OA. As a result, PLD2-mediated Cdc42 activation provides a mechanistic link between OA metabolism, cytoskeletal remodeling, and cancer cell motility, ultimately promoting TNBC metastasis.

In summary, our model suggests that OA functions as a key lipid signal that drives TNBC migration by activating PLD2 through S-acylation. This modification modulates PLD2's localization and function, allowing it to act as both a lipase and a GEF to facilitate Cdc42-dependent filopodia formation. Through this process, OA-induced PLD2 activation enhances cell migration

and contributes to the metastatic potential of TNBC cells. This model not only provides new insights into lipid-driven cell signaling but also highlights PLD2 as a potential therapeutic target in metastatic breast cancer.

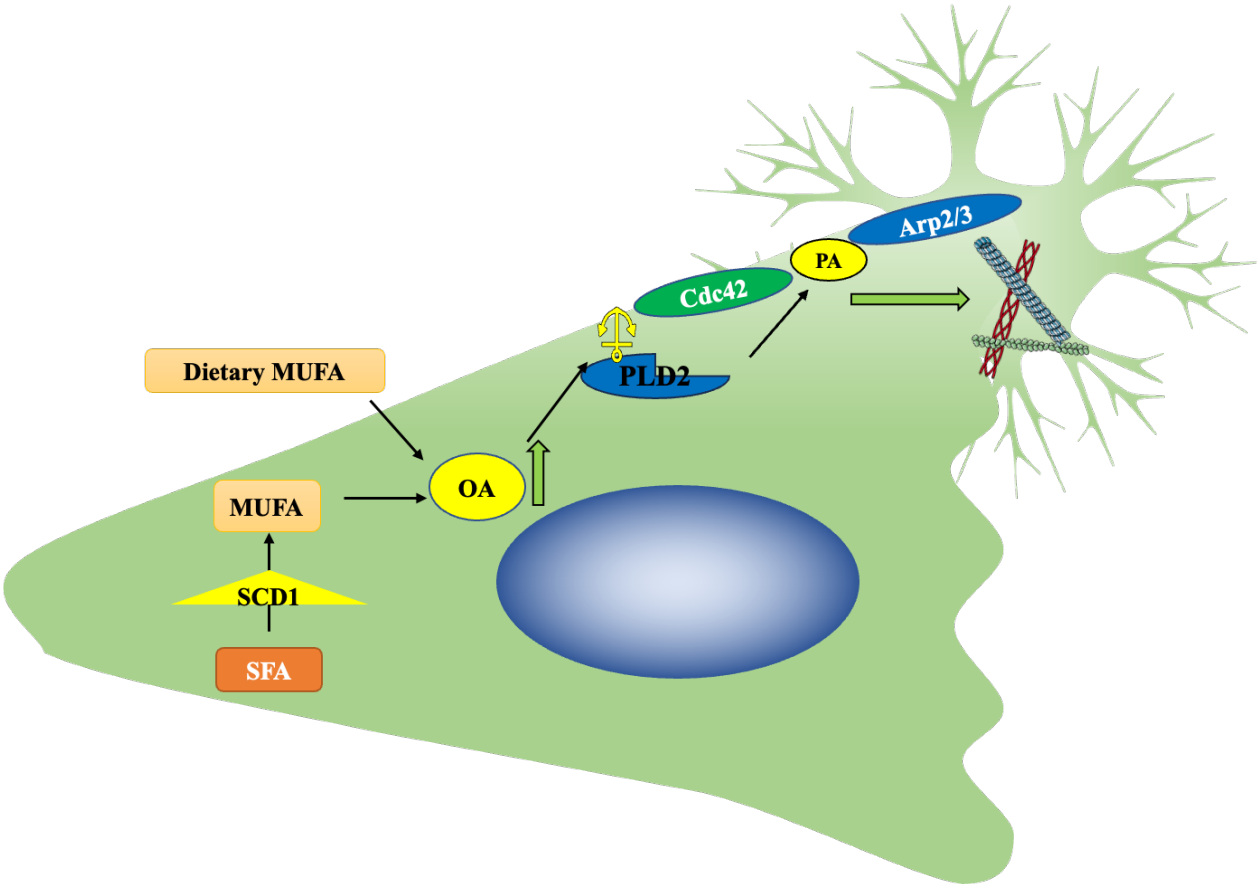


**Figure 5.1 Proposed molecular mechanism model of OA-induced TNBC cell migration.**

# CHAPTER 6: GENERAL CONCLUSION AND PERSPECTIVE

This thesis uncovers a novel molecular mechanism by which OA promotes cell migration, establishing a mechanistic link between FA metabolism, S-acylation, membrane signaling dynamics, and cytoskeletal remodeling. We demonstrate that OA activates PLD2 via S-acylation at cysteine residues C223 and C224, triggering its dissociation from lipid rafts and subsequent translocation to $PIP_2$ clusters. Within these specialized membrane compartments, PLD2 functions dually—as a phospholipase producing PA, and as GEF for the small GTPase Cdc42. These two activities converge to reorganize the actin cytoskeleton and promote filopodia formation, ultimately enhancing the migratory capacity of cells.

Our findings offer important insights into the spatial and functional regulation of PLD2 and provide a conceptual framework connecting lipid metabolism with cell motility. Notably, we show that acylation at both C223 and C224 is critical for PLD2's membrane localization and function. While single-site S-acylation preserves partial activity, the double mutant (C223AC224A) loses membrane association entirely and fails to trigger downstream signaling, underscoring the cooperative role of these S-acylation cysteine residues. Furthermore, OA alters membrane architecture by enhancing the formation of $PIP_2$ clusters, facilitating PLD2 interactions with its substrate PC and effector Cdc42, thereby promoting robust filopodia formation. Taken together, our data position PLD2 as a key integrator of lipid signaling and cytoskeletal dynamics in cell migration.

Despite these advances, several limitations of our study suggest important directions for future investigation. While our results indicate that PLD2 activates Cdc42 via its GEF domain, we did not directly demonstrate a physical interaction between the two proteins using techniques such as co-immunoprecipitation or proximity ligation assays. Additionally, although OA enhances PLD2 acylation, the specific FA species covalently attached to its cysteine residues remain unidentified. Mass spectrometry-based proteomics and lipidomic analysis of PLD2 with hydroxylamine cleavage, may clarify the lipid composition and heterogeneity of PLD2 S-acylation. Moreover, the identity of the acyltransferases responsible remains unknown. Determining whether specific zDHHC enzymes catalyze PLD2 oleoylation—and whether they exhibit substrate

preference for OA versus palmitate—would significantly deepen our understanding of lipid-protein regulation and identify novel targets for intervention.

Another open question concerns the coordination of PLD2's dual enzymatic activities. While our data show that the GEF function is retained in a lipase-deficient mutant, it remains unclear how these two functions are regulated in space and time. Does OA-induced relocalization to $PIP_2$ clusters favor one activity over the other? Could PA, the product of PLD2's lipase activity, feedback to modulate its own GEF activity? Similarly, $PIP_2$ may interact with PLD2's PH domain, possibly influencing its enzymatic output or recruitment of downstream effectors. These questions highlight the need for detailed structural and biochemical studies to dissect the molecular logic of $PLD_2$ activation, especially as a non-canonical GEF.

Beyond Cdc42, PLD2 is reported to regulate other small GTPases such as Rac2 or RhoA. Whether PLD2's substrate selectivity is determined by domain structure, membrane compartmentalization, or interaction with other GEFs remains to be defined. Exploring the full repertoire of PLD2-regulated GTPases and its interplay with other signaling networks will clarify its broader role in cytoskeletal remodeling. These studies could also explain how PLD2 modulates different types of membrane protrusions in diverse migratory contexts.

Our findings suggest several promising avenues for therapeutic development. Inhibiting PLD2 S-acylation—either by targeting specific zDHHC enzymes or using broad-spectrum S-acylation inhibitors—could block its membrane localization and OA-driven activation. This strategy offers a novel alternative to existing approaches that primarily target PLD2's lipase activity, which can be limited by compensatory signaling and metabolic redundancy. Additionally, disrupting membrane architecture (e.g., via cholesterol depletion or $PIP_2$ stabilization) could interfere with PLD2 recruitment and function. Targeting upstream regulators, such as SCD1—the primary enzyme responsible for OA synthesis in cancer cells—could also attenuate the pro-migratory lipid environment that activates PLD2.

Finally, although this study focused on filopodia-driven migration, future work should explore whether OA-activated PLD2 signaling contributes to other metastasis-related processes, such as EMT and invadopodia formation. These processes are crucial for TNBC invasiveness and may be similarly regulated by the SCD1–OA–PLD2–Cdc42 axis. Validating this pathway in *in*

*vivo* models and patient-derived samples will be essential to assess its physiological and clinical relevance.

In summary, this thesis establishes PLD2 as a central node at the intersection of lipid metabolism, membrane dynamics, and actin remodeling. By elucidating a previously unrecognized role for OA-driven S-acylation in PLD2 activation and membrane compartmentalization, our findings expand the current understanding of how FA metabolism promotes cancer progression and suggest new strategies for targeting lipid-mediated signaling in aggressive breast cancer.

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