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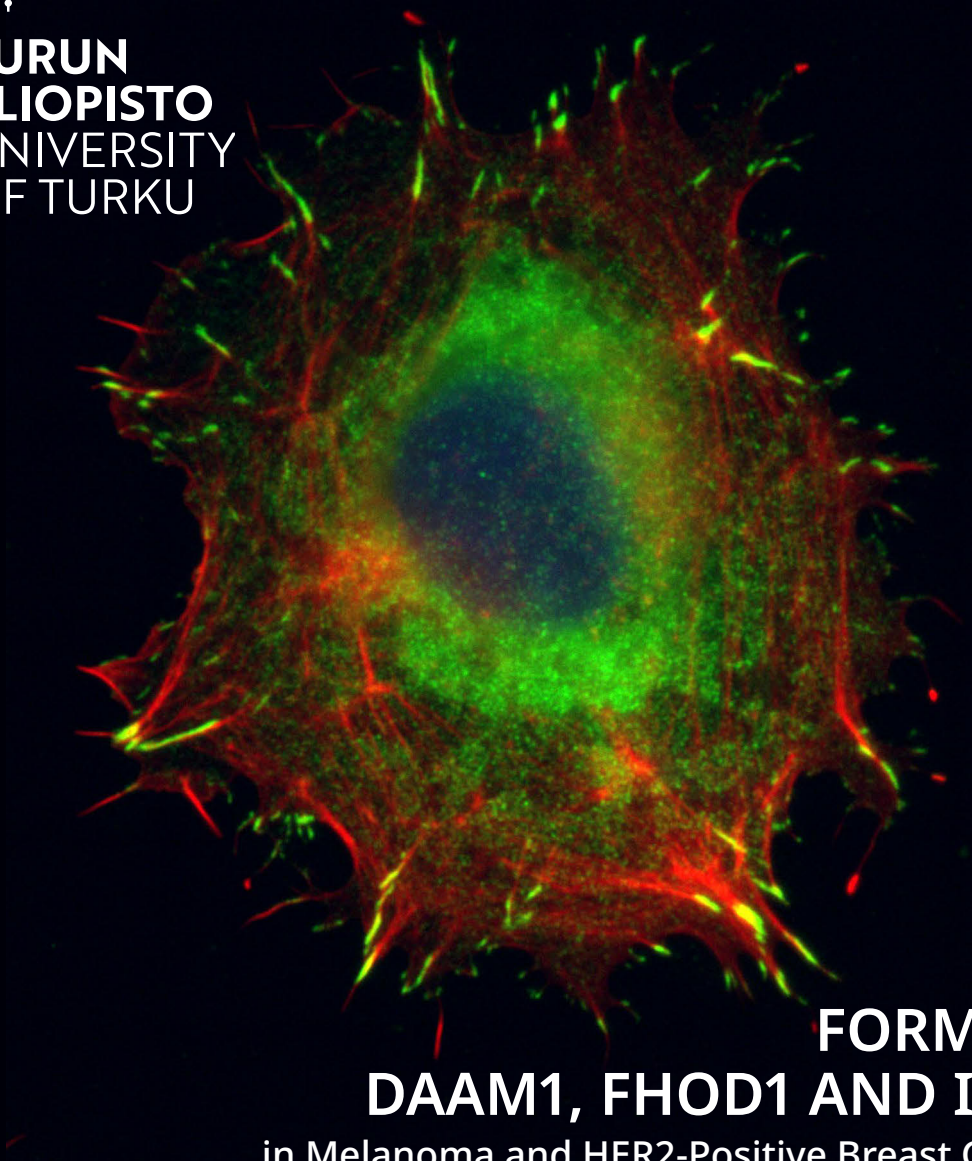

FORMINS DAAM1, FHOD1 AND INF2

in Melanoma and HER2-Positive Breast Cancer

Minna Peippo

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To my family

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Department of Pathology
MINNA PEIPPO: FORMINS DAAM1, FHOD1 and INF2 in Melanoma and HER2-Positive Breast Cancer
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ABSTRACT

Recent advances in various treatment modalities, including targeted therapies, combination therapies, and immunotherapies, have improved the prognosis for many types of cancer. However, cancer treatments often provide a good initial response but ultimately lose their efficacy during disease progression, or the tumour may not even initially respond to treatment. The reasons behind these are typically the high mutation burden of tumours or the acquisition of resistance to treatment by cancer cells. Further research is essential to better understand the mechanisms of acquired resistance and to elucidate the molecular events that lead to cancer invasion and metastatic dissemination. It is also important to identify new target molecules for cancer treatments and develop biomarkers for clinical use.

Specific actin structures and dynamics are tightly controlled and essential for most cellular functions and homeostasis. The formin protein family comprises 15 members, which are important regulators of the actin cytoskeleton and participate in various developmental and homeostatic cellular processes, including cell division, organelle trafficking, actin-dependent cell signalling, and cell migration. Altered functions of formins are linked to various cancers, but there is a need for cancer-specific studies.

This thesis analyses the expression of formins FHOD1, DAAM1, and INF2 along with their functional roles in two types of cancer: cutaneous melanoma and HER2-positive breast cancer. FHOD1 expression was mainly strong in cutaneous melanoma and cultured melanoma cells. The reduction of FHOD1 affected the ability of melanoma cells to attach, migrate, and proliferate, and an *in vivo* experiment showed attenuation of tumour growth. In HER2-positive breast cancer, the expression levels of DAAM1, FHOD1, and INF2 correlated with outcomes and were associated with HER2/ERBB2 expression. The results revealed FHOD1 and INF2 as downstream effectors of HER2 in the PI3K and MAPK pathways. Additionally, DAAM1 was shown to function downstream of HER2 but independently of the PI3K and MAPK pathways. As these formins are involved in regulating critical cancer-related pathways, they could be considered candidates for future cancer treatments.

KEYWORDS: Formin, DAAM1, FHOD1, INF2, melanoma, HER2-positive breast cancer

TURUN YLIOPISTO
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TIIVISTELMÄ

Syöpien hoidot ja ennusteet ovat merkittävästi parantuneet uusien täsmälääkkeiden, immunoterapian sekä eri hoitoyhdistelmien myötä. Syöpähoidot menettävät kuitenkin tehoaan sairauden edetessä ja osa kasvaimista ei reagoi lainkaan lääkehoitoon. Taustalla on usein kasvaimen suuri mutaatiokuorma ja syöpäsolujen kehittämä vastustuskyky lääkeaineelle. Lääkeresistenssien syntymekanismia ja syövän kehittymistä metastaattiseksi taudiksi on tutkittu jo vuosia, mutta lisää tutkimusta tarvitaan. Tavoitteena on myös löytää uusia kohdemolekyylejä lääkehoitoon sekä erilaisia merkkiaineita avuksi syöpädiagnostiikkaan.

Solun aktiinitukiranka ja sen tarkka säätely on välttämätöntä solujen normaalitoiminalle. Spesifiset aktiinirakenteet ja niiden toiminnallisuus on soluissa tarkasti säädelty ja yksi aktiinitukirangan säätelystä vastaava proteiiniperhe on formiinit, johon kuuluu 15 jäsentä. Formiinit osallistuvat useisiin solulle tärkeisiin toimintoihin kuten solujen jakautumiseen, soluelimien kuljetukseen, aktiinista riippuvaiseen signalointiin ja solujen liikkumiseen. Niillä on tutkimuksissa osoitettu olevan vaikutusta syövän kehittymiseen ja etenemiseen, mutta lisää tietoa tarvitaan syöpäkohtaisesti.

Väitöskirjatutkimukseni tavoite oli selvittää DAAM1-, FHOD1- ja INF2-formiinien ilmentymistä ja toiminnallisuutta kahdessa eri syöpätyypissä, ihon melanoomassa ja HER2-positiivisessa rintasyövässä. FHOD1-formiinin ilmentyminen oli pääosin korkeaa ihon melanoomassa ja melanoomasolulinjoissa. FHOD1:n ilmentymisen estolla oli selkeä vaikutus melanoomasolujen kiinnittymiseen, liikkumiseen ja jakautumiseen sekä *in vivo* kokeissa myös tuumorin kasvuun. HER2-positiivisessa rintasyövässä DAAM1-, FHOD1- ja INF2-formiinien ilmentymistasot korreloivat HER2/ERBB2 ilmentymisen kanssa sekä liittyivät syövän huonompaan ennusteeseen. FHOD1- ja INF2-proteiinien osoitettiin toimivan HER2-proteiinin alavirrassa PI3K- ja MAPK-signalointireiteillä. Samoin DAAM1 toimii HER2-proteiinin alavirrassa, mutta sitä ei kuitenkaan näytä säätelevän PI3K eikä MAPK signaloinnit. Tutkitut formiinit osallistuvat syöpien toiminnan kannalta olennaisten signalointireittien säätelyyn, joten tämän perusteella ne voisivat toimia kohdemolekyyleinä uusille syöpälääkkeille.

AVAINSANAT: Formiini, DAAM1, FHOD1, INF2, melanooma, HER2-positiivinen rintasyöpä

Table of Contents

Abbreviations

Ab	Antibody
ABP	Actin-binding protein
ADC	Antibody-drug conjugate
ADP	Adenosine diphosphate
Akt	Ak strain transforming (also known as PKB, protein kinase B)
ANOVA	Analysis of variance
APC	Adenomatous polyposis coli
ARP2/3	Actin-related protein 2/3
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRCA	Breast cancer susceptibility gene
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CAP	Cyclase-associated protein
CC	Coiled coil
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CNS	Central nervous system
CO_2	Carbon dioxide
CSC	Cancer stem cell
DAAM	Dishevelled associated activator of morphogenesis
DAB	3,3-diaminobenzene
DAD	Diaphanous autoregulation domain
DAPI	4,6-diamidino-2-phenyl-indole
DFNA1	Autosomal dominant non-syndromic hearing loss 1
DIAPH	Diaphanous-related formin
DID	Diaphanous inhibitory domain
DD	Dimerisation domain
DMEM	Dulbecco’s modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DRF	Diaphanous-related formin
Dvl	Dishevelled
ECM	Extracellular matrix
EGA	The European Genome-Phenome Archive
EGFR	Epidermal growth factor receptor
EMEM	Eagle's minimal essential medium
EMT	Epithelial-to-mesenchymal transition
ENA/VASP	Enabled/vasodilator-stimulated phosphoprotein
ER	Estrogen receptor
ERBB	ErbB-2 receptor tyrosine kinase
ERK	Extracellular signal-regulated kinase
F-actin	Filamentous actin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FH	Formin homology
FHDC1	FH2 domain-containing protein 1
FHOD	Formin homology 2 domain-containing protein
FHOS	Formin homologue overexpressed in spleen
FMN	Formin
FMNL	Formin-like protein
G-actin	Globular actin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBD	GTPase binding domain
GENT2	Gene expression database of normal and tumour tissues 2
GEO	Gene Expression Omnibus
GRID2IP	Glutamate receptor, ionotropic, delta 2-interacting protein (also known as Delphilin)
GTPase	Guanosine triphosphatase
HE	Hematoxylin-eosin
HEK	Human embryonic kidney
HER	Human epidermal growth factor receptor
HRG	Heregulin
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
INF	Inverted formin
KAc-actin	Lysine-acetylated actin
Ki67	Marker of proliferation Kiel 67
KM	Kaplan-Meier
MAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase

MEK	Mitogen-activated protein kinase kinase
MEM	Minimal essential media
MET	Mesenchymal-to-epithelial transition
MITF	Melanocyte-inducing transcription factor or Microphthalmia-associated transcription factor
miRNA	MicroRNA
MKL-1	Megakaryoblastic leukaemia 1 (also known as MRTF-A)
mRNA	messenger RNA
MRTF-A	Myocardin-related transcription factor A (also known as MKL-1)
NEAA	Non-essential amino acids
NF1	Neurofibromatosis type 1
NRAS	Neuroblastoma RAS proto-oncogene
PBS	Phosphate-buffered saline
PDZ	PSD95-DlgA-ZO-1 (Postsynaptic density protein - Drosophila disc large tumour suppressor - Zonula occludens-1 protein)
PFA	Paraformaldehyde
pH	Potential of hydrogen
PI3K	Phosphoinositide 3-kinase
Pen/Strep	Penicillin/Streptomycin
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
RAC1	Ras-related C3 botulinum toxin substrate 1
RAS	Rat sarcoma proto-oncogene
RFP	Red fluorescence protein
Rho	Ras homologous
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic Acid
ROCK	Rho-associated coiled-coil forming kinase
RPMI	Roswell Park Memorial Institute (medium)
SDS	Sodium dodecyl-sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SMIFH2	Small molecular inhibitor of formin homology 2 domains
SNP	Single-nucleotide polymorphism
SRE	Serum response element
STAT	Signal transducer and activator of transcription
TAN	Transmembrane actin-associated nuclear
TBST	Tris-buffered saline with 0,1% Tween20

TCGA	The Cancer Genome Atlas Program
TKI	Tyrosine kinase inhibitor
TMA	Tissue microarray
TME	Tumour microenvironment
TNBC	Triple-negative breast cancer
TP53	Tumour protein p53
UV	Ultraviolet
WASP	Wiskott-Aldrich syndrome protein
WH2	WASP-homology domain 2
WHIF1	WH2 domain containing formin 1
Wnt	Wingless/Integrated

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Peippo, M.*, Gardberg, M.*, Lamminen, T., Kaipio, K., Carpén, O. and Heuser, V.D. FHOD1 formin is upregulated in melanomas and modifies proliferation and tumor growth. *Exp Cell Res*, 2017; 350(1): 267-278.

II Peippo, M., Gardberg, M., Kronqvist, P., Carpén, O. and Heuser, V.D. Characterization of expression and function of the formins FHOD1, INF2 and DAAM1 in HER2-positive breast cancer. *J. Breast Cancer*, 2023; 26(6): 525-543.

* Authors have an equal contribution

1 Introduction

Cancer is a heterogeneous group of diseases in which functional changes in the genome have interfered with the normal regulation of cellular homeostasis. These changes include epigenetic modifications, mutations, and other genetic alterations in key genes that control cellular functions, such as cell growth, cell division, and cell metabolism.[1–3] Cancer development is a multiphase process during which cancer cells acquire characteristics that enable them to survive, adapt to their surrounding tissue, and sustain their proliferation.[4–6] Cancer initiation and progression are influenced not only by genetic variations but also by environmental factors. Cancer cells are surrounded by diverse non-malignant cell types embedded in the vascularised extracellular matrix (ECM). This normal tissue environment maintains homeostasis, a balance between cell proliferation and death. However, cancer cells disrupt this balance, transforming it into a tumour microenvironment (TME), a highly complex system in which various cell types communicate with the ECM and one another, thereby facilitating tumorigenesis.[7–10]

Cancer development begins with the initiation step, followed by progression, invasion, and metastasis. During these steps, the cancer cells must achieve a growth advantage to survive, proliferate more than the normal cells, form a primary tumour, and, over time, acquire the capability to invade and metastasise to nearby or distant organs.[11,12] During invasion and metastasis, cancer cells detach from each other and the ECM, migrate away from the primary tumour, invade the surrounding stroma and continue into the blood vessels. At the secondary site, cancer cells move from the bloodstream into the new organ parenchyma and adapt to the new microenvironment.[1,11]

The cancer burden has continuously increased, even though there has been enormous progress in the management and treatment of cancers. The challenges with the treatments seem to be the high mutation burden of tumours, cancer cells capability to evade the immune system, and the acquisition of mechanisms to resist the treatment. These may cause patients not to benefit from the therapy or relapse during treatment.[13–16] Current cancer research focuses on understanding the mechanisms leading to advanced metastasised disease and the mechanisms of resistance.

A critical aspect of cancer research is the regulation and functions of the actin cytoskeleton. Specific actin structures are necessary for cellular functions. For example, the cell cortex surrounding the entire cell provides support, focal adhesions attach cells to the ECM, and lamellipodia and filopodia are crucial for cell migration.[17–19] Actin nucleators and a diverse set of actin-binding proteins (ABPs) control the formation, architecture, and dynamics of filamentous actin (F-actin) by responding to cellular signals and other functions.[20–22] The abnormal expression of actin isoforms has been observed in various cancers, suggesting that the aberrant expression of actin subunits might promote proliferation, enhance migratory capability and increase chemoresistance.[23]

The members of the formin protein family regulate actin dynamics and higher-order structures.[24–26] In addition, most formins act as microtubule binding and bundling proteins, affecting microtubule organisation, subcellular localisation and dynamics.[27–30] With the ability to control both actin and microtubule dynamics, formins can function as regulators of the crosstalk between these two cytoskeleton networks and affect various developmental and homeostatic cellular processes such as cell division, membrane protrusions, cell migration, vesicle and organelle trafficking, endocytosis, cell junctions, actin-dependent cell signalling, gene transcription and genome integrity.[28,31–34] The aberrant function of formins can disrupt homeostatic cellular functions, leading to various diseases or influencing cancer development and progression.[35,36]

This thesis studies the roles of formins in melanoma and HER2-positive breast cancer. Treatments for these cancers have developed tremendously; however, some patients do not benefit from them at all or develop resistance during treatments. This thesis analysed the potential of formins to function as predictive or prognostic biomarkers. Additionally, we examined their functional roles to assess their contributions to cancer progression and their potential as new drug targets for cancer cells that have developed resistance to other treatments.

2 Review of the Literature

2.1 Cancer

Despite advances in cancer research, the global cancer burden continues to rise. Nearly 19.3 million new cancer cases and almost 10.0 million cancer deaths occurred in 2020 worldwide, and the burden is rapidly growing, with an estimated 28.4 million new cancer cases predicted to occur in 2040.[37]

Over the decades, cancer studies have revealed the disease's complexity and heterogeneity, enhancing our understanding of the mechanisms of cancer development and progression; however, further research is still needed. Cancer, a heterogeneous disease group, involves functional changes in the genome, including the accumulation of mutations, epigenetic modifications, and genetic alterations in key genes that control cell replication, growth, division, and metabolism.[1–3,38,39] These key genes responsible for tumorigenesis can be classified into three categories: oncogenes, tumour suppressor genes, and stability genes.[40] In normal cells, proto-oncogenes play a role as growth factors, transducers of cellular signals, and nuclear transcription factors controlling cell differentiation and proliferation. The gain-of-function mutations mainly create active oncogenes, and a mutation in a gene allele is sufficient to provide a selective growth advantage.[40–42] Tumour suppressor genes play a significant role in normal cell growth and differentiation, preventing abnormal cell division. Tumour suppressor genes can activate cell cycle arrest or apoptosis if the damaged deoxyribonucleic acid (DNA) cannot be repaired. The loss-of-function mutations need to inactivate both copies of the tumour suppressor gene before a cancer cell can proliferate and survive.[40,42,43] Stability genes, also known as caretakers or DNA repair genes, form the third class of cancer genes, which are responsible for repairing subtle errors that occur during normal DNA replication or are induced by exposure to mutagens. The primary role of these genes is to minimise genetic alterations; therefore, their inactivation leads to increased mutation rates in other genes.[40,44]

Cancer development is a multiphase process in which cancer cells must overcome the regulatory circuits that control normal cellular functions and homeostasis. Hanahan and Weinberg introduced and later updated the hallmarks of cancer, which comprise the capabilities that cancer cells need to acquire and the

events that assist cancer development. The capabilities required for cancer cell survival are sustaining proliferative signalling, evading growth suppressors, resisting cell death, deregulating cellular metabolism, avoiding immune destruction, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. The incidents associated with cancer development contain tumour-promoting inflammation, genome instability, and mutations. The new suggestions, as emerging hallmarks and enabling features, were introduced to include unlocking phenotypic plasticity, non-mutational epigenetic reprogramming, polymorphic microbiomes, and senescent cells.[4–6]

It is essential to bear in mind that, although genetic alterations are necessary for cancer initiation and progression, they are not sufficient alone; multiple interactions with surrounding tissues are also required.[45] Furthermore, tumours are more than mere masses of proliferating cancer cells. Tumours comprise cancer cells surrounded by a diverse array of non-malignant cell types embedded in a vascularised ECM.[7,8,45,46] The role of a normal tissue environment is to maintain homeostasis, simplified as preserving the balance between cell proliferation and cell death, thereby constraining the outgrowth of cancer.[47] The cancer cells modify the normal tissue environment containing diverse immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, and other tissue-specific cell types, turning it into a TME, which is a highly structured and complex system where all the different cell types communicate with the ECM and one another, assisting tumorigenesis. These assisting cells help the cancer cells acquire the capabilities necessary for survival and progression.[7,8,45] The ECM, a significant component of TME, consists of proteoglycans, glycoproteins, cytokines, enzymes, and growth factors. These molecules define and control the functions of the ECM to support and connect the cells, regulate cell signalling, separate tissues and organs, and act as a scaffold for migrating cells.[7,9,10]

2.1.1 Cancer Progression and Metastasis

Cancer development is a multi-step process comprising initiation, progression, invasion, and metastasis. During initiation, cancer cells acquire mutations or epigenetic changes that provide a growth advantage, allowing them to survive, proliferate more than their neighbours, and establish themselves as the dominant clone, ultimately forming the primary tumour.[1,12,48] Over time, these tumour cells acquire more advantageous characteristics and develop into malignant tumours, which have acquired the capability to invade and metastasise to nearby or distant organs. The most life-threatening features of cancer are invasion and metastasis, which influence the therapy response and the development of drug resistance, making them the main reason for around 90% of cancer-associated mortalities.[1,11]

Metastasis formation is highly ineffective, as tens of thousands of cancer cells may escape from the primary tumour into the circulation every day, with fewer than 0.01% of these invasive cancer cells surviving to form metastases.[49] Cancer cells face various challenges, including the sheer force of blood circulation, escape from immune cells, and adaptation to hostile environments. Cancer cells require assistance and support from surrounding cells and other factors within the TME throughout the entire metastatic cascade, which proceeds through a complex network of interactions between metastatic cells and TME components.[45,49]

The metastatic dissemination of cancer cells involves multiple steps (**Figure 1**), which can be simplified into two main phases. First, the cancer cells need to be translocated from the primary tumour to a distant organ, and the second phase is to colonise the translocated cells within the new organ.[11] During the first phase, cancer cells from the primary tumour need to invade the surrounding tissue and enter the microvasculature of lymph or blood (intravasation), survive, and move through the circulation to microvessels of distant tissue, and then exit from the vessels (extravasation). The second phase involves surviving in the new microenvironment of distant tissue. This process proceeds through adaptation into the new microenvironment to establish new cellular colonies by facilitating cell proliferation and forming a secondary tumour (colonisation).[11,45] Metastases that form through lymphatic intravasation differ due to structural differences in the vessels. The mechanism of the lymphatic route is incompletely understood, and the importance of forming distant metastases is a topic of debate.[45,50,51] Invasiveness requires cancer cells to detach from one another, and the ECM and disruptions of adhesions are promoted by an epithelial-to-mesenchymal transition (EMT) program.[52] The induction of EMT causes cancer cells to lose their epithelial features and acquire mesenchymal characteristics, resulting in the loss of intercellular junctions and epithelial polarity, which in turn leads to changes in the cytoskeletal arrangement and increased mobility of cancer cells. This phenotype switching enables the local migration of cancer cells away from the primary tumour.[45,52] With activated proteases, these cancer cells invade the basement membrane and the ECM into the surrounding stroma and then continue into blood vessels through the endothelial lining. EMT activation also inhibits apoptosis and increases the number of cancer cells with stem cell properties.[49,52] EMT is regulated by transcription factors, which can inhibit apoptosis by activating mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways.[46,52] Partial EMT forms the cancer stem cells (CSC) and can initiate tumour formation. CSC can self-renew or revert to a more epithelial phenotype through mesenchymal-to-epithelial transition (MET), a reverse EMT event essential for the colonisation step.[11,52,53]

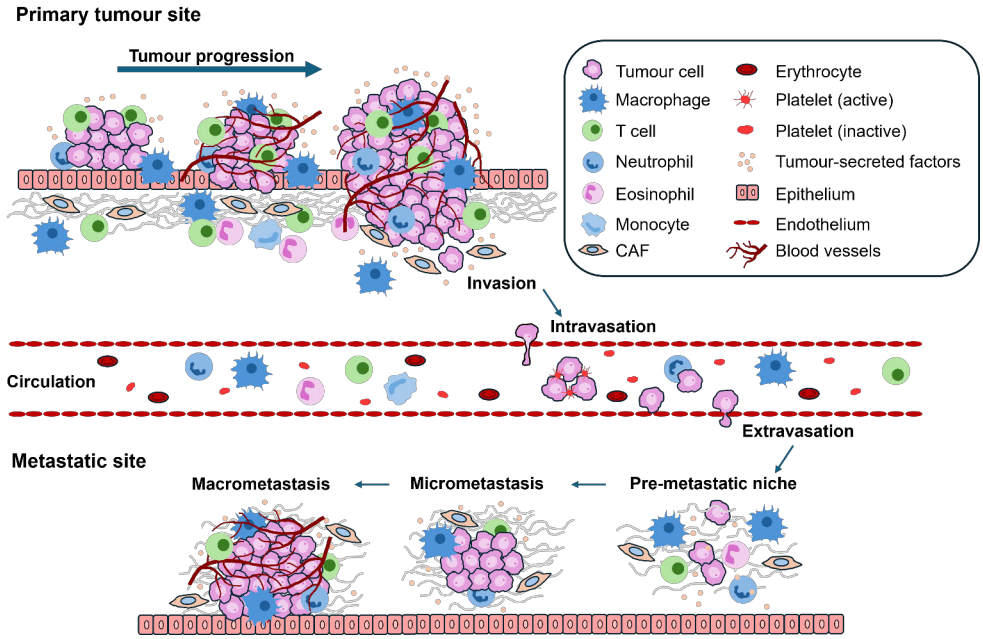

Figure 1. Cancer progression and metastasis model. Cancer cells form primary tumours and then progress into the invasive phase with the aid of tumour microenvironmental components. Invasive cancer cells invade the surrounding tissue (invasion), move into the blood circulation (intravasation), travel to distant organ sites, exit from the vessel (extravasation), begin to adapt to a new microenvironment (pre-metastatic niche) and establish new cellular colonies (micrometastasis). The final step is the formation of a secondary tumour (macrometastasis).

Single-cell migration and invasion can also proceed through amoeboid motility, where EMT is not readily detected, resembling the mechanism of leukocyte migration.[45,54] Cancer cells can also migrate and invade collectively by forming cohorts of cells as strands or clusters. These clusters contain cells that maintain cell-cell junctions and cells at the leading edge of the invasive masses, which express certain mesenchymal characteristics, such as the release of various proteases that degrade the ECM.[53,54] Cancer cells can also switch between forms of motility if one mode of migration is inhibited. For example, mesenchymal motility can be inhibited by blocking extracellular proteases; however, the overall motility is not reduced because cells switch to an amoeboid form of motility.[53,54]

The final phase of forming metastasis begins when cancer cells arrive at the secondary site, arrest, and attach to the endothelium of the secondary site's lumen with the aid of cell adhesion molecules and their ligands, as well as integrins and ECM components expressed by both the endothelial cells and the cancer cells. Attached cancer cells traverse endothelial cell junctions and possibly other vascular cell layers, as well as the ECM of the secondary site, gaining entry into the new organ

parenchyma with the assistance of proteases and degradative enzymes produced by both cancer cells and neighbouring non-cancerous cells.[45,53] In the metastatic site, during the colonisation, the cancer cells might exist in at least three alternative states: 1) as dormant cells, which are viable but non-proliferative; 2) as micrometastasis, which remain as small lesions because of a balance in proliferation and apoptosis, or 3) as actively growing macrometastasis.[11] The dormant period may be necessary for cancer cells to adapt to the new microenvironment. During this adaptation, the cancer cells must acquire the ability to respond to mitogenic stimulation from growth factors and cytokines present in the new microenvironment, which enables the formation of micrometastases.[11,49] To develop macrometastasis, cancer cells need to recruit an adequate blood supply to support the growth of the tumour.[11,45] The signalling pathways and mechanisms influencing colonisation require further study to understand the transitions from dormancy to micrometastasis and ultimately to macrometastasis.[11,45]

2.2 Actin

The actin protein family is one of the most conserved gene families in eukaryotes, and all eukaryotes have at least one gene for actin. Humans have six genes coding for α-, β-, and γ-actins. Three genes code for α-actin in muscle cells (skeletal α-actin, smooth muscle α-actin, cardiac muscle α-actin), one gene for β-actin in non-muscle cells, and two genes code for γ-actin: one in some smooth muscle cells and the other in almost all of the non-muscle cells.[55,56]

The basic unit of actin is a 42-kDa monomer, also called globular actin (G-actin).[57,58] Under physiological conditions, these monomers spontaneously polymerise into F-actin with a helical arrangement of subunits.[59] The polymerisation of actin monomers into actin filaments begins with the nucleation step, which contains two unfavourable phases: forming a dimer and adding a third subunit to form a trimer.[60] These dimers and trimers are highly unstable and are found at exceedingly low concentrations in polymerisation reactions.[61,62] These oligomers become more stable after the fourth subunit is added, forming the actin core. Then, filament elongation proceeds rapidly as a function of the concentration of available actin monomers.[63] The final step of polymerisation involves adenosine triphosphate (ATP) hydrolysis and phosphate release, creating adenosine diphosphate (ADP). The catalytic activity of free G-actin is low, but after polymerisation, the adenosine triphosphatase (ATPase) activity of actin increases, which can trigger the nucleotide hydrolysis and subsequent phosphate release.[64–66]

Actin filaments have barbed and pointed ends that have different polarities during polymerisation. Both ends can polymerise actin monomers; however, the barbed ends have faster polymerisation and depolymerisation rates than the pointed

ends.[67] The polymerisation of F-actin occurs primarily by adding ATP-bound G-actin to the dynamic barbed end rather than to the slow-growing pointed end. ADP-G-actin tends to dissociate at the pointed end.[68,69] This treadmilling process maintains the filament's constant length at a steady state by allowing monomers to flow through the filament from barbed ends to pointed ends.[70]

2.2.1 Structures and Functions of Actin

Actin filaments are flexible structures organised into various linear bundles, two-dimensional networks, and three-dimensional gels according to their functional role. They provide mechanical structures and rails for the intracellular transport of materials. Furthermore, actin filaments have a crucial role in cell motility, the movement of cells during development and cell cytokinesis during mitosis. Dozens of proteins, several signalling pathways, and the cellular environment control the functions of actin. During the assembly and disassembly of actin filaments, various proteins are needed to catalyse nucleotide exchange on actin monomers, initiate polymerisation, promote the dissociation of phosphate, cap the ends of polymers, cross-link the filaments to each other and other cellular components and sever the filaments.[17,19,20,22,71]

F-actins have two main architectures, branched and linear, and these have distinct nucleators (**Figure 2A**). The actin-related protein 2/3 (ARP2/3) complex promotes the formation of dense, branched F-actin networks. These are essential in generating the pushing forces needed in cell motility and endocytosis.[72,73] Formins and Enabled/vasodilator-stimulated phosphoprotein (ENA/VASP)-homology proteins facilitate the assembly of linear actin. These linear filaments can be bundled to create networks necessary for the cytokine contractile ring, membrane-protruding filopodia and polarisation of F-actin bundles.[24,26,74–77] The F-actin networks acquire specific architectures and dynamics according to their needs, and they are guided by a diverse set of specific ABPs, which in turn respond to cellular functions and signals. For example, profilin inhibits nucleation and elongation at the pointed ends while still allowing elongation at barbed ends, whereas cofilin is one of the proteins responsible for severing actin filaments.[17,20,22,78] Specific actin structures are distributed throughout the cell; the cell cortex surrounds the entire cell, providing structural support, while focal adhesions attach the cell to the extracellular matrix via stress fibres, whereas lamellipodia and filopodia are both crucial for cell migration (**Figure 2B**).[17,18,21,71,78]

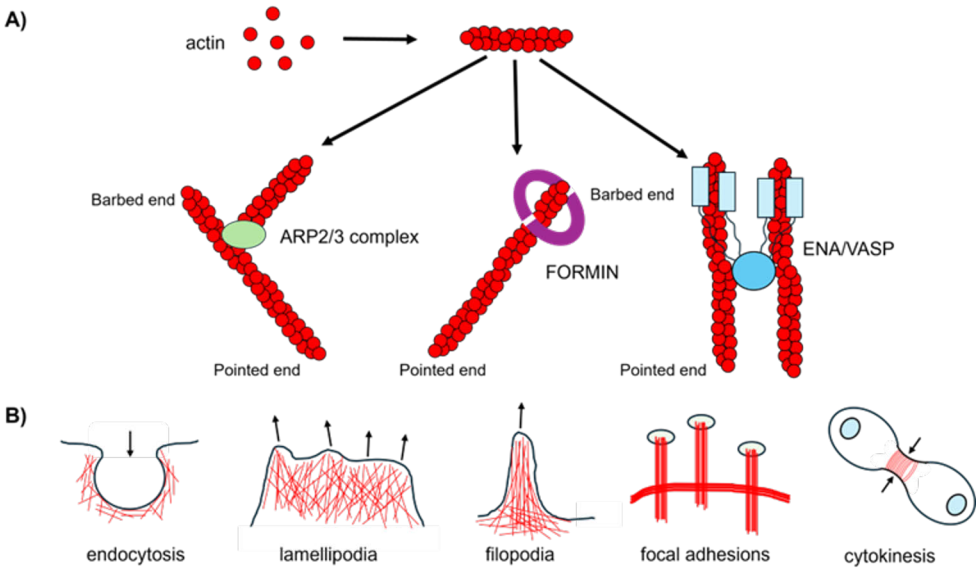

Figure 2. Actin filament nucleation and functions of actin networks. A) Two distinct forms of actin filaments, branched and linear, are primarily regulated by three major groups of actin nucleators: the ARP2/3 complex, formins, and ENA/VASP. B) Actin networks serve diverse functional roles in cells and contribute to various cellular structures.

2.3 Formins

As mentioned in the previous chapter, formins are an essential family of actin nucleator proteins, with fifteen members identified in humans (**Table 1**).[25,31,79] The defining feature of these proteins is the presence of the conserved formin homology (FH) domains, FH1 and FH2, which are necessary to control actin assembly.[24,80–87] The FH2 domain homodimerises, nucleates actin filaments and remains continuously attached to the elongating barbed end. Some formins also contain an FH2 domain with F-actin bundling activity.[24,83,85,88–92] FH1 is a proline-rich domain which captures actin monomers bound to profilin. The FH1 domain significantly enhances elongation rates by relocating actin subunits to the FH2-associated barbed end, but the enhancement effect varies between formins.[74,85,89,93–100] Beyond these conserved FH1 and FH2 domains, formins exhibit structural divergence, leading to variations in their ability to regulate actin dynamics and higher-order actin structures. These differences influence their capacity to nucleate and elongate unbranched actin, as well as to mediate actin capping, bundling, and severing features. Based on sequence similarities and domain architectures, formins can be classified into eight subfamilies (**Figure 3A**).[24,25,79]

Table 1. The fifteen members of the human formin protein family (alternative names presented in parentheses).

Formin	Full name	Subfamily
DAAM1	Dishevelled-associated activator of morphogenesis 1	DAAM
DAAM2	Dishevelled-associated activator of morphogenesis 2	DAAM
Delphilin (GRID2IP)	Delphilin	Delphilin
DIAPH1 (DRF1, Dia1)	Diaphanous-related formin-1	DIAPH
DIAPH2 (DRF2, Dia2)	Diaphanous-related formin-2	DIAPH
DIAPH3 (DRF3, Dia3)	Diaphanous-related formin-3	DIAPH
FHOD1 (FHOS)	Formin homology 2 domain-containing 1	FHOD
FHOD3 (FHOS2)	Formin homology 2 domain-containing 3	FHOD
FMN1	Formin-1	FMN
FMN2	Formin-2	FMN
FMNL1	Formin-like protein-1	FMNL
FMNL2	Formin-like protein-2	FMNL
FMNL3	Formin-like protein-3	FMNL
INF1 (FHDC1)	Inverted formin-1	INF1
INF 2 (WHIF1)	Inverted formin-2	INF2

Abbreviations: FHDC1 (FH2 domain-containing protein 1), FHOS (Formin homologue overexpressed in spleen), GRID2IP (Glutamate receptor, ionotropic, delta 2-interacting protein), WHIF1 (WH2 domain containing formin 1)

Four subfamilies contain autoinhibition domains and are collectively categorised as Diaphanous-related formins (DRFs), while the remaining formins are classified as non-DRFs. The DRFs possess a Diaphanous inhibitory domain (DID) at the N-terminus, which interacts with the Diaphanous autoregulatory domain (DAD) located at the C-terminus. This interaction maintains the DRFs in a closed, autoinhibited conformation. Autoinhibition must be relieved, allowing them to transition into an open conformation. This occurs, for example, through the binding of an activated Ras homologous (Rho) Guanosine triphosphatase (GTPase) to the GTPase-binding domain (GBD) (**Figure 3B**).[24,86,87,94,100–107] The Rho GTPase binding opens the autoinhibited conformation of DRFs, enabling dimerisation (**Figure 3C**), but additional signals are required for complete activation.[104–106,108–110] Not all DRFs require the binding of a GTPase to be activated. For example, FHOD1 is activated by Rho-associated coiled-coil forming kinase (ROCK), which phosphorylates serine and threonine residues in the C-terminal DAD.[111] In addition to autoinhibition, the regulation of formins can occur through various mechanisms, such as cellular localisation, the assistance of other proteins, or post-translational modifications, including phosphorylation, farnesylation, and myristoylation.[24,28,112]

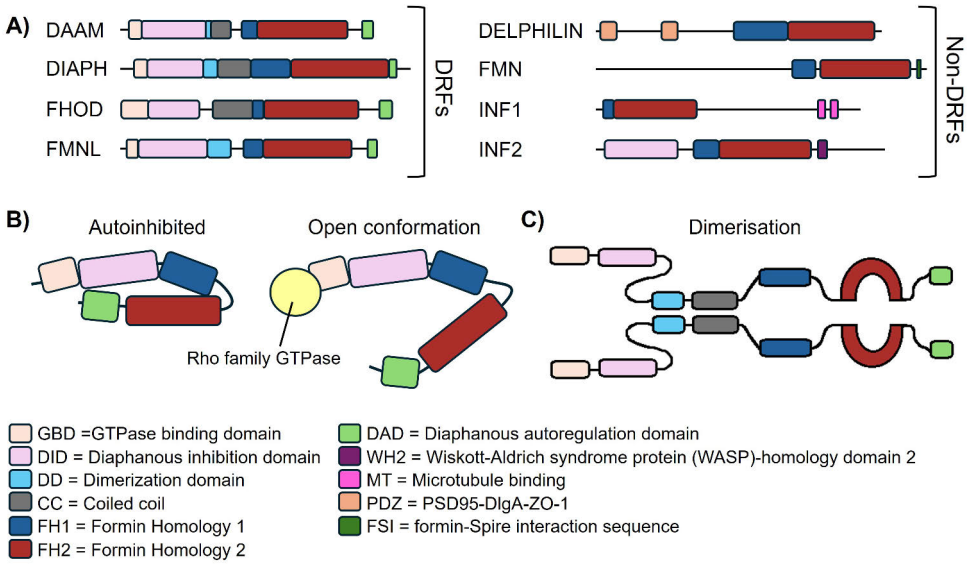

Figure 3. Graphic presentations of formin proteins. A) The members of the formin protein family can be divided into eight subfamilies according to domain structures. These subfamilies can be clustered into DRF and non-DRF groups depending on the existence of the autoinhibition domains. B) DRF formins have an autoinhibited and an open conformation. C) Model of formin protein dimerisation with DIAPH.

In addition to regulating actin filaments, most formins also influence the microtubule cytoskeleton by acting as microtubule-binding and bundling proteins, thereby affecting microtubule organisation, subcellular localisation, and dynamics.[28–30,113–122] The ability to control both actin and microtubule dynamics makes formins regulators of the crosstalk between the two cytoskeletal networks and thereby permits the participation in various developmental and homeostatic cellular processes such as cell division, membrane protrusions, cell migration, vesicle and organelle trafficking, endocytosis, cell junctions, actin-dependent cell signalling, gene transcription and genome integrity. The activities of formins are primarily associated with actin filaments and microtubule networks in the cell cytoplasm, but they also have functions in the nucleus and endoplasmic reticulum (**Figure 4**). Formins have different functional roles, varying subcellular locations, and partially cell-type-specific expressions.[24,28,31–34,123–127]

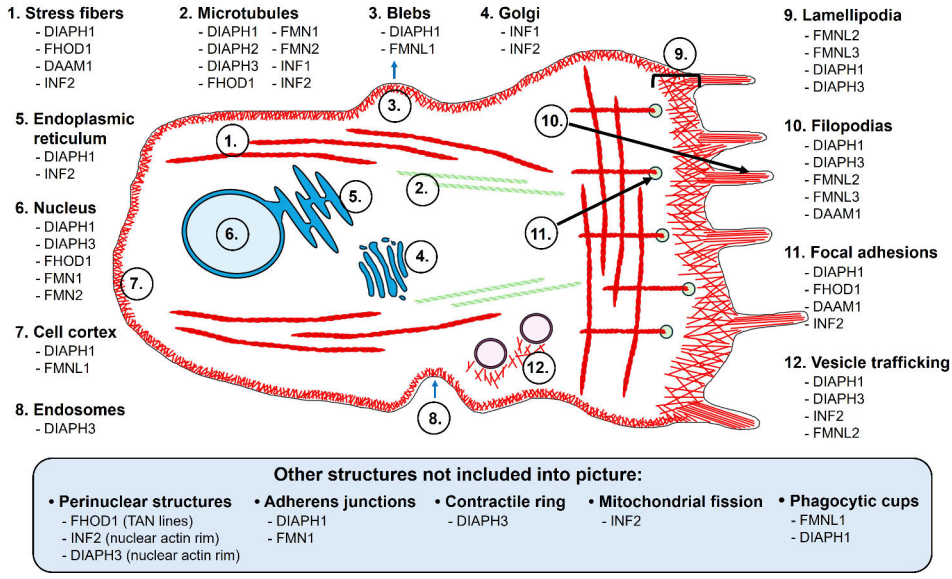

Figure 4. Formins have various roles in different cellular structures and functions. While their activities are generally related to actin filament and microtubule networks, they also function in the nucleus and endoplasmic reticulum. **Abbreviation:** TAN (Transmembrane actin-associated nuclear).

2.3.1 Formins in Human Diseases, Including Cancers

Formins are essential for many developmental and homeostatic cellular processes; therefore, it is not surprising that the improper functioning of formins can lead to disastrous effects, contributing to various diseases and cancers. Mutations, chromosomal rearrangements and copy number variations of formin genes can cause dysregulation of formins, potentially altering their expression levels or locations. The proteins can be mutated or incorrectly spliced, resulting in either constantly active or inactive formins. These aberrations give rise to developmental defects of the heart, nervous system, and kidneys, as well as ageing-related diseases, inherited human diseases, mental disorders, and cancers.[35,36]

The variation of diseases, which formins affect, is extensive, and the influence ranges from mild to severe. Some formins have been more extensively studied, but, above all, formins are associated with many diseases or syndromes. The DIAPH1 was the first formin gene found to be mutated in a hereditary disease called autosomal dominant non-syndromic hearing loss 1 (DFNA1).[128] Mainly, DIAPH1 but also some other formins (DIAPH3, FHOD3, INF2) are shown to be associated with deafness or hearing loss, either having a direct effect through mutations which cause changes in formin expression/activity or as a part of larger disease entity, for example patients with Charcot-Marie-Tooth disease combined to focal segmental

glomerulosclerosis experience hearing loss which could be result from INF2 mutations affecting peripheral nerve myelinisation.[36,129–133] Eleven of fifteen formins (excluding DIAPH2, FHOD3, FMNL1 and INF1) are connected to an aberrant brain or nervous system state, and the effects originate from formin gene deletion or different mutations affecting the formin expression or activity. This group contains extensive settings of diseases and dysfunctions such as defective neurogenesis, brain dysfunction, disorders of the central nervous system, Alzheimer's disease, Parkinson's disease, schizophrenia, depression, dementia, autism and mental disorders.[36,134–142] Heart and cardiovascular anomalies, such as cardiomyopathy, cardiac laminopathy, congenital heart defects, cardiac dysfunction, cardiovascular disease, cardiometabolic disease and hypertension-induced tunica media thickening, are also associated with deletions or mutations in six formins (DAAM1, DIAPH1, DIAPH2, FHOD1, FHOD3 and INF2), which affect the expression level or activity of the formins.[36,143–150] Additionally, at least five formins (DAAM1, DAAM2, DIAPH1, FHOD1, and FHOD3) have been implicated in diabetes or its complications. These formins exhibit single-nucleotide polymorphisms, differential methylation, or an impact on the signalling of diabetic neurovascular complications through their molecular binding partners.[36,151–154]

Cancers comprise a heterogeneous group of diseases, and formins have a role in various cancers, including breast cancers, colorectal cancers, endometrial cancers, ovarian cancers, prostate cancers, brain cancers, pancreatic cancers, lung cancers, gastric cancers, liver cancers, kidney cancers, skin cancers, and some other cancer types (**Table 2**).[36,155–187] Formins can influence cancer development or progression through expression level variation[36], alterations of methylation status[36], isoforms[36], mutations[36,160,165], deletions[36], genomic loss[36], single nucleotide polymorphism (SNP)[36,161,166,173,188] and by affecting gene expression[36,160,189]. The mechanisms by which formins contribute to cancer progression involve the induction of EMT[36], alterations of cell morphology[36], as well as changes in migration[36,156,167,169,190–194], proliferation[36,169,191,194–196], invasion[36,156,157,169,190–193,196–198], matrix degradation[36,198], mitochondrial dynamics[36,199] and microtubule organisation[36,157]. These listed activities allow formins to facilitate cancer development by influencing TME signalling networks and mediating extracellular vesicle trafficking.[36]

Formins are often upregulated in cancers, although downregulation has also been observed. The variation in expression levels of certain formins depends on the specific type of cancer. For instance, DIAPH3 is mainly overexpressed in cancers such as breast cancer, pancreatic cancer, hepatocellular carcinoma and osteosarcoma, while it is downregulated in triple-negative breast cancer.[36,158,182,196,200] Many studies have clarified the possibility of using formins as biomarkers, and the association of favourable or unfavourable prognoses can be connected to expression levels or gene variants. High expression of DIAPH1,

DAAM1, FHOD3 and INF1 transcripts is associated with a favourable prognosis, whereas DIAPH2, DAAM2, FMNL1, FMNL3 and FHOD1 are linked to poorer outcomes.[36] Specific FMN1 variants are linked to a higher risk of developing pancreatic and prostate cancer, whereas a particular DAAM2 variant is associated with a protective effect in lung cancer.[201–203]

Table 2. Formins are associated with various cancers.

	Brain	Breast	Colorectal	Gastric	Kidney	Liver	Lung	Ovarian	Pancreatic	Prostate	Skin	Other
DAAM1	X	X	X	X		X	X	X		X	X	X
DAAM2	X	X	X		X	X	X	X				X
Delphilin			X									X
DIAPH1	X	X	X		X			X	X	X	X	X
DIAPH2	X	X	X				X	X	X		X	X
DIAPH3	X	X	X	X		X	X	X	X	X	X	X
FHOD1	X	X	X	X			X	X		X	X	X
FHOD3	X	X					X	X		X	X	X
FMNL1	X	X		X	X		X	X		X	X	X
FMNL2		X	X	X		X					X	X
FMNL3	X	X	X	X				X	X	X	X	X
FMN1	X		X				X		X	X		
FMN2	X	X	X		X				X	X		X
INF1							X	X	X		X	
INF 2	X	X	X							X		X

This thesis focuses on formins in two distinct cancers: melanoma and HER2-positive breast cancer. Before our studies, a few publications had already explored the role of formins in melanoma, with the first study published in 2003. It demonstrated that the overexpression of FHOD1 enhances cell migration in an integrin-independent manner in the melanoma cell line WM35, which does not express detectable levels of endogenous FHOD1.[204] Subsequent melanoma studies have linked DIAPH1, DIAPH3, FMNL2, and FMNL3 to oncogenic pathways and cancer progression, demonstrating that formins affect melanoma cell proliferation, invasion, and migration.[159,205–208] Breast cancer and its relation to formins have been more extensively studied, with twelve out of fifteen formins being connected to various breast cancer subtypes.[164,165,169,188–190,192,193,197,198,206,208–227] However, none of the studies have focused explicitly on the HER2-positive subtype of breast cancer.

2.3.2 DAAM1

The dishevelled associated activator of morphogenesis (DAAM) subfamily of formins comprises two proteins, DAAM1 and DAAM2, which share a relatively low homology of approximately 60-68%.[228,229] DAAM proteins belong to the DRF group of formins and are autoinhibited through DID-DAD interaction. The activation of DAAMs proceeds through Dishevelled (Dvl) family members binding to the DAD domain or Rho GTPase binding to the GBD domain.[102,230–232] DAAM1 forms a dimer via its FH2 domain, then nucleates and elongates actin filaments. The actin assembly activity is weak due to the unique three-dimensional structure, which partially hides surfaces for barbed-end binding.[233,234] DAAM1 can also bundle F-actin, induce microtubule acetylation, and function as a signalling scaffold.[30,167,235,236] DAAM1 also has many functional roles in cells, including cytoskeletal rearrangement, stabilising epithelial junctions, and regulating filopodia formation and phagocytosis.[237–240]

DAAM1 is associated with various diseases through genetic variations (deletions, mutations, duplications), changes in expression levels, or alterations in activation.[36] Here, our focus is on cancer research, and DAAM1 is studied in several types of cancers (**Table 2**), including breast cancer[36,193,209,211,216–218,225–227], melanoma[241], glioblastoma[36,236], colorectal cancer[242,243], gastric cancer[194], prostate cancer[171], osteosarcoma[186,244], lung cancer[245], and ovarian cancer[179,246]. Mainly, DAAM1 seem to affect tumour initiation and progression as an activated or overexpressed member of different signalling cascades that trigger these features, for example, in melanoma[241], glioblastoma[236,247], colorectal cancer[242,243], gastric cancer[194], osteosarcoma[244] and lung cancer[245]. MicroRNAs (miRNAs) can also regulate the DAAM1-mediated metastatic features, as demonstrated in breast cancer[218], ovarian cancer[246] and prostate cancer[171]. DAAM1 overexpression has been shown to correlate with metastasis, and it predicts poor prognosis in breast cancer.[216] Functionally, DAAM1 appears to promote the migration and invasion of cancer cells in several types of cancers.[171,211,217,218,227,236,244,246] DAAM1's functions are reported to be mediated through various signalling pathways. The Wingless/Integrated (Wnt) signalling pathway is the most widely recognised, while signalling through the Erk/Akt pathway also plays a significant role.[194,227,236,241,244]

2.3.3 FHOD1

The family of formin homology 2 domain-containing proteins (FHOD) consists of two members, FHOD1 and FHOD3. These proteins belong to the DRF group of formins and undergo autoinhibition through DID-DAD interactions. However, the activation of FHODs differs from that of other DRFs because these proteins are primarily phosphorylated rather than activated by Rho GTPase binding.[111,248] Unlike

other DRFs, FHODs do not stimulate rapid actin polymerisation or filament elongation. For example, the polymerisation rate of FHOD1 is less than 5% of that of DIAPH1.[143,249] Instead, FHOD1 caps and bundles F-actin into stress fibres.[143,249,250]

FHOD1 was initially named FHOS (Formin homologue overexpressed in the spleen) because it was found as a formin abundantly expressed in the spleen.[251] Later studies have shown FHOD1 to be a ubiquitously expressed protein, with the highest expression levels in lung and skeletal muscle, in addition to the spleen.[251–253] Immunofluorescence stainings have shown that in cultured cells, FHOD1 colocalises with actin stress fibres, focal adhesions, the nucleus, and microtubules.[254–257] Studies have demonstrated that FHOD1's functional roles are connected to cell spreading, cell adhesion, cell migration, sarcomeric actin filament organisation, and nuclear movement in migrating cells.[204,255,257–259]

The expression levels and roles of FHOD1 have been studied in several cancers (**Table 2**). FHOD1 expression is upregulated in glioma[183,260], breast cancer[212], squamous cell carcinoma[261], and gastric cancer[191]. FHOD1 promotes cancer progression through cancer cell migration and invasion in various cancers.[191,204,212,260,261] Thus, FHOD1 downregulation can inhibit the metastatic potential, as shown with cell models in breast[212], lung[262], and gastric cancer[191] studies. FHOD1 also appears to participate in cancer cell-associated EMT in oral squamous cell carcinoma[261], breast cancer[189], and lung carcinomas[262].

2.3.4 INF2

Inverted formin 2 (INF2) was initially thought to belong to the inverted formin (INF) protein subfamily due to a misaligned reading frame, which missed the N-terminal domains preceding the FH2 domain, due to which it resembled INF1, the first member of the subfamily.[79] INF1 was identified as having an unusual domain structure, where typical N-terminal domains were missing, the FH domains were located near the N-terminus, and the domain structure appeared inverted compared to other formins.[79,263,264] However, later studies revealed that INF2 displays a more traditional organisation with N-terminal DID and DAD domains and C-terminal FH1 and FH2 domains, resembling those of other formins.[265] Although INF2 is partly similar to other formins, it also differs in lacking the GBD domain and containing the Wiskott-Aldrich syndrome protein (WASP)-homology 2 (WH2) domain.[265] As a result, it is also referred to as the WH2 domain containing formin 1 (WHIF1), and it should not be grouped with INF1 into a common subfamily, but rather, INF2 should be classified into its separate subfamily (**Figure 3A**).[25]

The regulation of INF2 appears more complex than other formins, as it lacks the GBD domain. The existing DID-DAD interaction is intrinsically weak, and it does

not inhibit actin polymerisation but prevents depolymerisation and severing.[265,266] In biochemical actin polymerisation assays, the purified INF2 was not autoinhibited but was constitutively active.[267] However, cellular studies have shown that INF2 activity is tightly regulated, suggesting the presence of cellular inhibitors that may utilise the interaction between the DID and DAD domains.[267,268] For example, the DAD domains of formins DIAPH1, DIAPH2 and DIAPH3 directly interact with the DID domain of INF2.[269] The DID-DAD interaction was also shown to be entirely relieved by binding monomeric actin into the DAD domain.[267] One research group presented a mechanism of "facilitated autoinhibition" where a complex consisting of cyclase-associated protein (CAP) bound to lysine-acetylated actin (KAc-actin) is required for INF2 inhibition. The CAP/KAc-actin complex might inhibit INF2 by forming a bridge between the DID and DAD domains of INF2. The deacetylation of actin in the CAP/KAc-actin complex activates INF2.[270,271]

As mentioned above, INF2 has dual activity in actin dynamics. It nucleates actin filaments, promotes elongation, and accelerates F-actin depolymerisation and filament severing.[265,266] INF2 binds to, stabilises, and bundles microtubules and regulates microtubules' acquisition of specific posttranslational modifications.[27,28,30,115,272,273] Furthermore, INF2 localises in stress fibres, focal adhesions, endoplasmic reticulum, Golgi and nuclear actin ring.[186,266,274–278] INF2 participates in the regulation of vesicular trafficking, mitochondrial fission and podosome formation.[268,279–285]

INF2 has been widely studied in kidney-related diseases, such as focal segmental glomerulosclerosis, Charcot-Marie-Tooth disease, and chronic kidney disease, with the primary mechanism being INF2 mutations.[36] Several studies have also clarified the involvement of INF2 mutations, expression level variation, and regulation of its activity in multiple types of cancer (**Table 2**). The regulation of cancer progression seems to be connected to INF2's role in mitochondrial fission, at least in osteosarcoma[280], prostate cancer[286], endometrial carcinoma[162,187], colorectal cancer[287], and thyroid cancer[288]. INF2 also regulates cell migration and invasion, as seen in glioblastoma[260] and breast cancer[212]. INF2 activation can also promote cancer cell apoptosis, as observed in thyroid[288] and colorectal[287] cancers.

2.4 Cutaneous Melanoma

Melanoma is an aggressive form of skin cancer arising from the melanocytes, which are melanin pigment-producing cells located in various anatomical sites, including the basal layer of the epidermis, the uvea of the eyes, the meninges, bones, the heart, and the inner ear. Genetic factors and exposure to ultraviolet (UV) radiation have prominent roles in melanoma development. Melanomas have a strong tendency to metastasise when diagnosed at a late stage; however, those diagnosed and treated at

an early stage have low mortality rates and may even be curable.[289–292] Overall, melanoma accounts for 1.7% of global cancer diagnoses, translating to nearly 325,000 new melanoma cases during the year 2020. During the same year, there were about 57,000 melanoma-related deaths.[37] While the incidence of melanoma continues to rise in developed countries, the overall mortality rates have decreased by nearly 18% from 2013 to 2016 due to the improvement in treatments, including targeted therapies and immunotherapy.[293]

Melanomas can be classified into cutaneous, mucosal, and uveal melanomas by their anatomical origin. Other factors, such as mutational signatures, sun exposure, and epidemiology, further divide them into nine groups.[294] Melanoma is among the cancers with the highest mutation burden. Its pathogenesis is also known as melanoma genesis because it is based on the acquisition of sequential alterations in specific genes and pathways controlling molecular or metabolic mechanisms and regulating crucial cellular functions.[295–297] Researchers have recognised specific key gene mutations that categorise cutaneous melanomas into four distinct subgroups: mutations in proto-oncogenes v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), Rat sarcoma (RAS), or Neurofibromatosis type 1 (NF1) and triple-negative melanomas (no BRAF mutation, no RAS mutation, no NF1 mutation).[298–300] NF1 mutations are also found in conjunction with BRAF and Neuroblastoma RAS (NRAS) mutations; thus, there is also a suggestion of only three groups: BRAF mutants, RAS mutants, and no mutations in either BRAF or RAS.[301]

All these central genes (BRAF, NRAS, and NF1) influence the activity of the MAPK pathway, with enhanced activation observed in 98% of melanomas, making it the most frequently mutated oncogenic pathway. The PI3K pathway is another crucial pathway in the development and progression of melanoma, influencing various cellular functions, including those of the MAPK pathway, such as cell proliferation and survival. Still, it also promotes metabolism, whereas the MAPK pathway is more active in processes such as proliferation and invasion. Different pathways may be activated by coexisting mutations (for example, mutated NRAS) or present a compensatory mechanism, where the blockage of one pathway causes the activation of the other pathway.[297,298,302–305] Primary mutations are insufficient to cause the development and progression of cutaneous melanoma; additional genetic alterations are necessary, affecting several tumour suppressor genes and/or oncogenes through deleterious mutations or structural changes. The list of significant, altered genes includes melanocyte-inducing transcription factor (MITF), Tumour protein p53 (TP53), Cyclin-dependent kinase inhibitor 2A (CDKN2A), Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Ras-related C3 botulinum toxin substrate 1 (RAC1). These genes play a role in cell proliferation, invasion, metastasis, migration, apoptosis, and angiogenesis, functioning through various pathways, mainly the MAPK and PI3K pathways.[299–301,306,307]

The management and treatment of metastatic melanoma have been revolutionised after the introduction of immunotherapies, targeted therapies, and combination therapies.[304,308] The challenges with the treatments are the high mutation burden of tumours, cancer cells' capability to evade the immune system, and the acquisition of mechanisms to resist the treatment.[308,309] Most patients relapse during cancer treatments due to acquired resistance, which might be caused by the reactivation of inhibited pathways or cancer cells succeeding in finding compensatory ways to function.[310,311] Inhibiting one target molecule, for example, mutated BRAF in the MAPK pathway, may prompt cancer cells to find an alternative route to bypass this inhibition and reactivate the pathway. Alternatively, it may also lead to the activation of the PI3K pathway.[304,310]

Current melanoma research continues to unravel the genetic variability of tumours, the mechanisms leading to advanced metastatic disease, and the factors contributing to relapse during treatment. Much of the research also focuses on identifying prognostic and predictive biomarkers that can be valuable for clinicians. Prognostic biomarkers help determine disease outcomes, identify patients at increased risk of metastasis, and aid in stratifying patients for optimal treatment strategies. Predictive biomarkers can be used to estimate treatment response, which is essential for implementing personalised therapy.[312–314]

2.5 HER2-Positive Breast Cancer

Breast cancer is the most common cancer in women worldwide, with over 2.2 million new cases reported in 2020. It is also the most fatal cancer, accounting for nearly 690,000 deaths among women in that year.[37] Breast cancer is a highly heterogeneous disease, both genetically and clinically. Most commonly, breast tumours are classified by immunohistochemical (IHC) stainings of the following receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). These stainings divide tumours into four subtypes: luminal A, luminal B, HER2-positive and triple-negative breast cancer (TNBC). These subtypes have different characteristics, prognoses, metastases, and treatment methods (**Table 3**). When considering cellular morphologies, tumour growth patterns, various architectures, and genetic variations, these four subtypes can be further divided into numerous subgroups, each exhibiting variability in treatments and prognosis.[315–320]

Table 3. The main characteristics of breast cancer subtypes.

	Luminal A	Luminal B	HER2	TNBC
Frequency	40-50%	15-20%	10-25%	15-30%
ER	Positive	Positive (≥20%)*	Negative or low positivity (<20% of the cases)	Negative
PR	Positive	Positive in some cases (<20%)	Positive in some cases	Negative
HER2	Negative	Negative	Positive	Negative
Ki67	Some cases (less than 20%)	High (greater than 20%)	Any**	Any**
Mutations	No	p53, BRCA2	p53	p53 and BRCA1
Histologic grade	Generally 1 or 2	Generally 3	Generally 3	Generally 3
Therapy	Hormonal	Hormonal/Chemo	Hormonal/Chemo/ Targeted drug	Chemo/ Experimental
Preferentially places to metastasise	Bone	Bone	Bone, liver, and 50% also to the brain and CNS	Brain, CNS, lung and liver
Prognosis	Good	Middle	Middle/Poor	Poor

*Generally, expression is lower relative to Luminal A. **More variation; any expression level from low to high is detected (usually >15% up to 30)

Abbreviations: BRCA (Breast cancer susceptibility gene), CNS (Central nervous system), ER (Estrogen receptor), HER2 (human epidermal growth factor receptor 2), Ki67 (Marker of proliferation Kiel 67), PR (Progesterone receptor), TNBC (Triple-negative breast cancer)

About 10% to 25% of breast cancers are diagnosed as HER2-positive tumours, which are aggressive and have an overall worse prognosis compared to luminal tumours. However, the overall outcome has significantly improved with the targeted therapy utilising drugs against highly expressed HER2. HER2-positivity means the tumour contains HER2 protein overexpression or gene amplification.[316,319,321,322] The positivity is defined as a 3+ score in IHC staining in ≥10% of tumour cells or a 2+ score with HER2 gene amplification verified with *in situ* hybridisation (ISH).[323] Targeted therapy trials have revealed a new subgroup of HER2-positive cancer called HER2-low positive, which comprises approximately 40-50% of breast cancer patients. These patients have an HER2 IHC score of 1+ or 2+ with a negative ISH result, and previously, these cases were diagnosed as HER2-negative. Patients with HER2-low breast cancer benefit from the treatment with a specific antibody-drug conjugate.[323–327]

HER2 (ERBB2 = erb-b2 receptor tyrosine kinase 2) is a transmembrane tyrosine kinase receptor that belongs to the epidermal growth factor receptor (EGFR) family (also called the ERBB or HER family).[328,329] All these family members have a common structure consisting of an extracellular ligand-binding domain, a single

membrane-spanning region and a cytoplasmic protein kinase domain. The dimerisation of these receptors stimulates the intrinsic tyrosine kinase activity, triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain and provides a docking site for signalling molecules involved in regulating different signalling cascades.[330–333] HER2 differs from other family members because it lacks known ligands that bind directly to it. Instead, it adopts a conformation resembling the ligand-activated state, which promotes dimerisation. HER2 signalling is initiated either through homodimerisation with itself or heterodimerisation with another family member (EGFR/HER1/ERBB1, HER3/ERBB3, or HER4/ERBB4).[331,334–337] The overexpression of HER2 leads to the spontaneous formation of homodimers or heterodimers, thereby inducing tumorigenesis through the activation of several downstream signalling pathways, including PI3K, MAPK, Signal transducer and activator of transcription (STAT), and SRC. The activation of these kinase cascades transmits signals from the receptor to the nucleus and alters the expression of various genes, which then regulate cell proliferation, survival, angiogenesis, and metastases.[338–341]

Patients with HER2-positive breast cancer are treated with chemotherapy and HER2-targeted therapies, which include monoclonal antibodies (MAb), tyrosine kinase inhibitors (TKI), and antibody-drug conjugates (ADC). These targeted therapies affect different regions of HER2. MAbs mainly inhibit receptor dimerisation through binding to the extracellular domain of HER2, while TKIs block the HER2-mediated signalling pathways by preventing the phosphorylation of the intracellular tyrosine kinase domains. ADCs consist of a monoclonal antibody that targets the cancer cell, coupled with a cytotoxic drug designed to destroy the cancer cell. The combinatorial and sequential use of different HER2-targeted therapies has improved the cure rates of early-stage disease and prolonged survival for patients with advanced-stage disease; however, these treatments still face challenges.[342–346] Some patients do not respond or develop acquired resistance during treatments. The reasons for the treatment resistance can be HER2 heterogeneity in tumours, the activation of alternative signalling pathways, or immune escape mechanisms.[347–352] For example, PI3K mutations or loss of PTEN functions can lead to the overactivation of the PI3K signalling pathway even when HER2 is inhibited upstream.[353,354] Considering that HER2 affects multiple signalling pathways and there is a high likelihood of activating mutations in the downstream signalling pathway from HER2, it is clear why the list of potential resistance mechanisms continues to expand.

Targeted and combinatorial therapies have improved clinical outcomes for patients with HER2-positive breast cancer. Many clinical trials have been conducted in the past and are ongoing to optimise treatment combinations for use in the first, second and third-line settings. To date, clinical trials have demonstrated improved

median overall survival, ranging from a few months to over 70 months, as well as median progression-free survival, which has extended from a few months to nearly 24 months.[345,355] However, metastatic HER2-positive breast cancer inevitably develops resistance, leading to disease progression, and approximately 50% of these patients will develop central nervous system metastasis.[346,356,357] Numerous ongoing studies are investigating the mechanisms of resistance, optimising the use of current treatments, and developing new therapies to overcome resistance.[345,346,351] Additionally, there is an urgent need for reliable biomarkers to predict treatment response or resistance, which would aid in the better selection of patients who may benefit from those therapies.[351,358]

3 Aims

The formin protein family members are essential cytoskeletal regulators, and their functions are linked to various malignancies. However, relatively little is known about their expression and functional relevance in individual cancers. My thesis focused on clarifying the roles and significance of formins in melanoma and HER2-positive breast cancer, particularly examining their involvement in the invasive migration of cancer cells and cancer progression, emphasising the formins that are most relevant to these two cancer types.

The specific aims of my dissertation were:

1. To analyse the expression levels of formin FHOD1 in cutaneous melanoma, benign nevi and melanoma cell lines
2. To reveal the functional role of formin FHOD1 in melanoma cells *in vitro* and *in vivo*.
3. To clarify the mechanism by which FHOD1 affects the cellular functions of melanoma cells.
4. To clarify the expression of DAAM1, FHOD1 and INF2 formins in HER2-positive breast cancer and to correlate their expression with clinicopathological parameters.
5. To study the regulatory and functional interplay between HER2 and formins DAAM1, FHOD1 and INF2 in HER2-positive breast cancer.
6. To explore the role of FHOD1 and INF2 in the regulation of HER2-mediated intracellular signaling

4 Materials and Methods

4.1 Cell Lines and Culture Conditions (I, II)

Table 4. Information about the cell lines and media used in studies.

Cell line	Origin	Genetic information (wild type, mutation or overexpression)	Growth medium	Article
Bowes	Cutaneous melanoma	BRAF wild type	EMEM + 10% FBS + 5 mM Ultraglutamine + 100 U/ml Pen/Strep	I
HEK-293	Human embryonic kidney cells		DMEM + 10% FBS + 5 mM Ultraglutamine + 100 U/ml Pen/Strep	II
Malme-3M	Metastatic melanoma	BRAF V600E	DMEM + 10% FBS + 5 mM Ultraglutamine + 1x NEAA + 100 U/ml Pen/Strep	I
MDA-MB-453	Metastatic breast cancer	Overexpression of FGF receptors	DMEM + 10% FBS + 5 mM Ultraglutamine + 100 U/ml Pen/Strep	II
SK-BR-3	Metastatic breast cancer	Overexpression of FGF receptors	DMEM + 10% FBS + 5 mM Ultraglutamine + 100 U/ml Pen/Strep	II
SK-MEL-28	Metastatic melanoma	BRAF V600E	MEM + 10% FBS + 5 mM Ultraglutamine + 100 U/ml Pen/Strep	I
WM164	Metastatic melanoma	BRAF V600E, BRAF V600D	RPMI 1640 + 10% FBS + 5 mM Ultraglutamine + 1x NEAA + 100 U/ml Pen/Strep	I
WM239	Metastatic melanoma	BRAF V600E, BRAF V600D	RPMI 1640 + 10% FBS + 5 mM Ultraglutamine + 1x NEAA + 100 U/ml Pen/Strep	I

Abbreviations: DMEM (Dulbecco's modified Eagle medium), EMEM (Eagle's minimal essential medium), FBS (fetal bovine serum), FGF (Fibroblast growth factor), MEM (Minimal essential media), NEAA (non-essential amino acids), Pen/Strep (penicillin-streptomycin), RPMI (Roswell Park Memorial Institute).

The cell lines and growth media used in the studies are listed in **Table 4**. All cells were grown at 37°C in a 5% Carbon dioxide (CO_2) incubator. The cell lines were periodically checked for mycoplasma contamination using the MycoAlert™ mycoplasma detection kit (Lonza).

4.2 Transfections (I, II)

Table 5. Details of reagents, siRNAs, shRNAs and vectors used in transfections.

Reagent	Specification	Manufacturer	Article
Dharmafect 4	Transfection reagent	Dharmacon Research	II
FuGENE6	Transfection reagent	Promega	II
Lipofectamine 2000	Transfection reagent	Life Technologies	I
Puromycin	1.0 µg/ml in selection medium, 0.5 µg/ml maintenance medium	Sigma	I
siRNA/shRNA			
DAAM1 siRNA	50nM SMARTpool small interfering RNA	Dharmacon Research	II
ERBB2 siRNA	50nM FlexiTube siRNA	Qiagen Sciences	II
FHOD1 siRNA	50nM SMARTpool small interfering RNA	Dharmacon Research	II
Human FHOD1 targeting shRNA FI351919 (shRNA 1)	pRFP-C-RS HuSH shRNA RFP vector	Origene	I
Human FHOD1 targeting shRNA FI351920 (shRNA 2)	pRFP-C-RS HuSH shRNA RFP vector	Origene	I
INF2 siRNA	50nM SMARTpool small interfering RNA	Dharmacon Research	II
Non-targeting pool siRNA	50nM SMARTpool small interfering RNA	Dharmacon Research	II
Scramble shRNA TR30015	pRFP-C-RS HuSH shRNA RFP vector	Origene	I
Vector			
pEGFP	Expression plasmid	Addgene	II
pERBB2-GFP	Expression plasmid	Addgene	II

Abbreviations: RFP (red fluorescence protein), shRNA (short hairpin RNA), siRNA (small interfering RNA)

4.2.1 Generation of Stable Knockdown Cell Lines (I)

Stable knockdown cell lines were established using short hairpin RNA (shRNA) technology. WM164 cells were transfected in a 24-well plate using Lipofectamine 2000

and pRFP-C-RS HuSH shRNA red fluorescence protein (RFP) vectors containing either the scrambled shRNA cassette TR30015 (scramble control), human FHOD1 targeting shRNAs FI351919 (CGCTGTGCCAAGGTGGACTTTGAACAGCT; shRNA 1) or FI351920 (CCGAGACAGAGAAGTTCTCAGGTGTGGCT; shRNA 2). The transfection medium was changed after 24 hours. The cells were kept in a selection medium containing 1.0 µg/ml puromycin for 2 weeks and then transferred to a 10 cm dish. Colonies expressing RFP were isolated, amplified and analysed for FHOD1 expression by western blotting. The FHOD1 shRNA clones were stable in culture and maintained a low FHOD1 level for at least 15 passages in medium containing 0.5 µg/ml puromycin. The used reagents are listed in **Table 5**.

4.2.2 Knockdown with Small Interfering RNAs (siRNA) (II)

Small interfering RNAs (siRNAs) were used to knock down proteins in the SK-BR-3 and MDA-MB-453 cell lines. The cells were transfected in suspension using Dharmafect 4 transfection reagent (Dharmacon Research) and siRNAs targeting ERBB2, FHOD1, INF2, DAAM1, and a non-targeting siRNA as a control. All proteins were knocked down separately, and some were also knocked down in combinations. The efficacy of the knockdowns was examined 48 hours after the transfection by immunoblotting. The reagents used in the experiments are listed in **Table 5**.

4.2.3 Transient Overexpression (II)

Human embryonic kidney cells (HEK-239) were plated in 6-well plates and transfected with 3 µg of pEGFP empty vector (Addgene) or pERBB2-GFP (Addgene) expression vector using FuGENE6 (Promega) transfection reagent according to the manufacturer's protocol. After 48 hours of transfection, the cells were starved by serum deprivation for 24 hours and then stimulated with a medium containing 50% serum for 30 minutes. The reagents used in the experiment are presented in the **Table 5**.

4.3 Western Blot Analysis (I, II)

Table 6. Details of antibodies used in Western blotting analyses.

Antibody	Dilution	Host and clonality	Manufacturer	Article
Akt	1:1000	Rabbit monoclonal Ab (clone11E7)	Cell Signaling Technology	II
α-tubulin	1:10 000	Mouse monoclonal Ab (clone B-5-1-2)	Sigma-Aldrich	I
DAAM1	1:1000	Rabbit polyclonal Ab	Proteintech	II
ERK1	1:1000	Rabbit polyclonal Ab	Santa Cruz Biotechnology	II
ERK2	1:1000	Rabbit polyclonal Ab	Santa Cruz Biotechnology	II
FHOD1	1:1000	Rabbit polyclonal Ab	Sigma-Aldrich	I, II
GAPDH	1:10 000	Rabbit polyclonal HRP-conjugated Ab	Abcam	II
GAPDH	1:5000	CoraLite® Plus 488-conjugated GAPDH mouse monoclonal Ab	Proteintech	II
HER2/ERBB2	1:1000	Rabbit polyclonal Ab	Proteintech	II
INF2	1:1000	Rabbit polyclonal Ab	Proteintech	II
pAkt	1:1000	Rabbit monoclonal Ab (clone D9E)	Cell Signaling Technology	II
p44/42 MAPK (Erk1/2)	1:1000	Rabbit polyclonal Ab	Cell Signaling Technology	II
HRP-conjugated secondary Ab	1:3000	Rabbit anti-Mouse	Agilent	II
HRP-conjugated secondary Ab	1:2500	Rabbit anti-Mouse	Dako	I
HRP-conjugated secondary Ab	1:2500	Swine anti-Rabbit	Dako	I
HRP-conjugated secondary Ab	1:3000	Swine anti-Rabbit	Agilent	II

Abbreviations: Ab (Antibody), Akt (Ak strain transforming), ERK (Extracellular signal-regulated kinase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), HRP (Horseradish peroxidase), MAPK (Mitogen-activated protein kinase)

Cells were harvested and lysed in a Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (1x Complete Mini) and phosphatase inhibitors (1x PhosSTOP). Insoluble cell debris was removed by centrifugation, and the protein concentration of the lysates was determined using the Bradford method (BioRad) before adding 5x Laemmli to the samples. Sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate equal amounts of total protein, and then the proteins were transferred to a nitrocellulose membrane (Whatman PROTRAN, PerkinElmer). The membranes were first blocked with 5% dry milk in TBST (Tris-buffered saline with 0.1% Tween 20) and then immunoblotted with different antibodies diluted in 5% bovine serum albumin (BSA) in TBST. The information on primary and secondary antibodies used in studies is presented in **Table 6**. The anti-α-tubulin antibody (Sigma-Aldrich), the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody conjugated to horseradish peroxidase (HRP) (Abcam) or CoraLite® Plus 488-conjugated GAPDH mouse monoclonal Ab (Proteintech) was used as a control for protein loading. The secondary antibodies were diluted in the blocking solution. Membranes were washed three times with TBST between each step.

4.4 Immunofluorescence Staining and Microscopy (I, II)

For immunofluorescence staining, the cells were plated on gelatin-precoated 13 mm coverslips and grown in a complete medium for 24 hours. Next, the cells were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature. The coverslips were washed with PBS and blocked with 3% BSA, 5% dry milk, and 0.5% Triton X-100 in PBS for 45-60 minutes. Then, the primary antibodies were incubated for one hour at room temperature. Next, the coverslips were incubated with Alexa Fluor 555, 568, or 488 secondary antibodies for 1 hour at room temperature, followed by washes with PBS. Alexa Fluor 488, 546, or 568-conjugated phalloidin was then incubated with secondary antibodies to visualise the actin filaments. The mounting media used contained 4′,6-diamidino-2-phenylindole (DAPI) for staining nuclei (ProLong Gold Antifade Mountant with DAPI, Life Technologies/Thermo Fisher). After each staining step, the cells were washed three times with PBS. Images were taken with an Olympus BX60 or a Nikon Eclipse Ni fluorescence microscope. All the antibodies and their corresponding dilutions are presented in **Table 7**.

The images were handled and analysed with ImageJ software versions 1.49b, 1.53t or 1.53a (http://rsbweb.nih.gov/ij/). Different fluorescence channels were merged in software, and the changes in morphology were quantified. The measurements for cell circularity were also conducted using ImageJ (Measure and Analyze Particles commands; circularity = 4π (area/perimeter^2)). A circularity value of 1.0 indicates a perfect circle. For actin filament quantification, the mean intensity of fluorescent phalloidin in cell cytoplasm was calculated for 100 cells per cell line in 8-bit pictures using "IntDen" (Integrated Density; the product of Area and Mean Gray Value).

Table 7. Details of reagents and antibodies used in immunofluorescence staining studies.

Antibody	Dilution	Host and clonality/Specification	Manufacturer	Article
Alexa Fluor 488	1:500	Goat anti-mouse IgG	Invitrogen	I
Alexa Fluor 488	1:500	Donkey anti-goat IgG	Invitrogen	I
Alexa Fluor 488 conjugated Phalloidin	1:100	Visualisation of actin	Invitrogen	I
Alexa Fluor 488 conjugated Phalloidin	1:500	Visualisation of actin	Invitrogen	II
Alexa Fluor 546 conjugated Phalloidin	1:100	Visualisation of actin	Invitrogen	I
Alexa Fluor 568	1:500	Goat anti-rabbit IgG	Invitrogen	I, II
Alexa Fluor 568 conjugated Phalloidin	1:500	Visualisation of actin	Invitrogen	II
Alexa Fluor 555	1:500	Donkey anti-mouse IgG	Invitrogen	II
DAAM1	1:100	Rabbit polyclonal Ab	Proteintech	II
FHOD1	1:200	Rabbit polyclonal Ab	Millipore	I
FHOD1	1:100	Rabbit polyclonal Ab	Sigma-Aldrich	II
HER2/ERBB2	1:100	Rabbit polyclonal Ab	Proteintech	II
INF2	1:100	Rabbit polyclonal Ab	Proteintech	II
Ki67	1:200	Mouse monoclonal Ab (clone MIB-1)	Dako	II
MKL-1 (MRTF-A)	1:300	Goat polyclonal Ab	Santa Cruz	I
Paxillin	1:100	Mouse monoclonal Ab (clone 349)	Transduction Laboratories	I
ProLong® Gold Antifade Mountant with DAPI		Mounting media containing DAPI for staining nuclei	Life Technologies	I
ProLong™ Gold Antifade Mountant with DAPI		Mounting media containing DAPI for staining nuclei	Thermo Fisher	II

Abbreviations: MKL-1 (Megakaryoblastic leukaemia 1), MRTF-A (Myocardin-related transcription factor A), DAPI (4´,6-diamidino-2-phenyl-indole)

4.4.1 Focal Adhesion Analysis (I)

Cells were grown on coverslips previously coated with 25 µg/ml fibronectin (Sigma-Aldrich). The cells were allowed to attach for 60 minutes, then fixed and stained as previously described. Focal adhesions were visualised with an anti-Paxillin antibody (staining protocol described previously), and images were captured using a 10X objective on an Olympus BX60 fluorescence microscope. The images (2040 × 1536 px) were analysed with ImageJ 1.49b software to quantify changes in the size and number of focal adhesions. The average area and number of focal adhesions per cell were quantified from binary images using the Analyze Particle tool in ImageJ. Particles larger than 0.2 μm^2 were counted as focal adhesions.

4.4.2 Nuclear Translocation of MKL-1 (I)

WM164 cell lines with and without knockdown of FHOD1 were plated on gelatin-coated coverslips and grown overnight in complete medium. The normal medium was replaced with a starvation medium for 72 hours, after which the cells were stimulated for 20 minutes with a medium containing 10% serum. After this, the cells were washed with PBS and fixed using 4% PFA for 10 minutes at room temperature. MKL-1 staining was performed as described previously using a goat polyclonal anti-MKL-1 (MRTF-a) antibody, followed by the secondary antibody AlexaFluor 488, together with Alexa Fluor 546-conjugated phalloidin to visualise filamentous actin. The mounting media with DAPI was used to visualise nuclei. Images were captured using an Olympus BX60 fluorescence microscope, and the images were analysed with ImageJ 1.49b software (http://rsbweb.nih.gov/ij/) to quantify MKL-1-positive nuclei by counting 1500 cells per cell line.

4.5 Functional Assays (I, II)

Table 8. Details of reagents used in functional cell experiments.

Reagent	Concentration	Specification	Manufacturer	Article
Agar	0.5 %	Prepared in complete medium	Sigma-Aldrich	I
Agarose (Low melting point)	0.3 %	Prepared in complete medium	Sigma-Aldrich	I
Bio-Rad Protein Assay Dye Reagent, Concentrate		Colourimetric assay for measuring total protein concentration based on the Bradford dye-binding method.	BioRad	I, II
10xComplete Mini	1x	Protease inhibitor cocktail	Roche	I
Crystal Violet		Staining solution (0,05% Crystal Violet, 1% Formaldehyde, 1% Methanol, PBS)	In-house prepared	I
Dimethyl sulfoxide (DMSO)		Negative control	Thermo Fisher Scientific	
Fibronectin	25 µg/ml	Extracellular matrix glycoprotein	Sigma-Aldrich	I
Gelatin	100 µg/ml	Cell-attachment substrate	Sigma-Aldrich	I, II
Heregulin β1 (HRG)	50 ng/ml	Growth factor	Peprotech	II
LY294002	10 µM	PI3 kinase inhibitor	Tocris Bioscience	II
Matrigel	100 µg/ml	Basement membrane matrix	Corning	I
Matrigel	8 mg/ml	Cell inoculations in mice	BD Biosciences	I
MycoAlertTM		Mycoplasma detection kit	Lonza	II
10xPhosStop	1x	Phosphatase inhibitor cocktail	Roche	I
Propidium Iodide	1:100	Cell viability assessment	Molecular Probes	I
U0126	10 µM	MEK 1/2 inhibitor	Cell Signaling Technology	II

Abbreviation: MEK (Mitogen-activated kinase kinase)

4.5.1 Migration and Invasion Assays (I, II)

The melanoma cells were grown overnight in 96-well ImageLock microplates (Essen BioScience) coated with Matrigel in a complete medium. Wounds were precisely made by the 96-pin Wound-Maker provided with the IncuCyte FLR (Essen Bioscience). After thoroughly washing the wounds with PBS to remove detached cells, they were covered with Matrigel (for invasion) or medium (for migration). After 30 minutes in the incubator, 100 µl of the completed medium was added, and the cells were placed in the IncuCyte FLR, which automatically acquired images at 2-hour intervals for 48 hours.

The breast cancer cells in suspension were transfected in 96-well ImageLock microplates. The transfection medium was replaced with a starvation medium after 48 hours, and the cells were grown overnight. Wounds were created with an IncuCyte S3 96-pin Wound-Maker (Essen Bioscience), and detached cells were washed away with PBS. Cells were covered either with pure starvation medium (100 µl) to lessen the proliferation effect on migration or with starvation medium supplemented with 50 ng/ml heregulin β-1 (HRG, Peprotech). The IncuCyte S3 machine automatically captured images of the wound at 12-hour intervals over 96 hours.

IncuCyte™ software was used to analyse wound closure kinetics, with the area under the curves used for comparison. Each sample was measured in triplicate, and these experiments were repeated at least three times.

4.5.2 Transwell Migration (I, II)

4.5.2.1 Boyden Chamber Assay (I)

Melanoma cells in 150 µl of medium without FBS were loaded into Boyden chambers (Millipore) and placed in 24-well plates containing 900 µl of complete medium with 10% FBS. Cells were allowed to migrate through porous (8 µm) membranes for 48 hours. Following the medium removal, the chambers and wells were washed once with PBS. Non-migrating cells were removed from the upper chamber using a cotton swab. In contrast, migrating cells adherent to the underside of the filter were fixed and stained with a Crystal Violet solution (0.05% Crystal Violet, 1% Formaldehyde, 1% methanol, PBS) for 10 minutes. The inserts were washed with water and allowed to dry overnight. The migrated cells were photographed at four random sites using light microscopy at a magnification of 100X. To quantify the number of migrated cells in another way, 1% Sodium dodecyl sulfate (SDS) solution (400 µl/well) was added to solubilise the stain from the cells. The plate was agitated on an orbital shaker for 30 minutes until the colour of the

membranes was uniform. The absorbance of each sample was measured at 570 nm with a Multiskan FC Machine (Thermo Scientific).

4.5.2.2 IncuCyte Chemotaxis Assay (II)

Transwell migration experiments were conducted using the IncuCyte Chemotaxis Assay with 8 µm pore membranes. Cells were trypsinised 48 hours after the siRNA transfections and suspended in a starvation medium. Next, the cells were plated onto IncuCyte Clearview 96-well plates (5 x 10^6 cells/well in 60 µl) in the upper chamber. A medium supplemented with 10% FBS (200 µl) was placed in the lower chamber to create a base for studying direct migration towards a chemoattractant. The plates were placed in an IncuCyte S3, and images were captured automatically at 24-hour intervals over 96 hours. The IncuCyte™ software was used to normalise the confluence area of cells on the membrane's bottom surface against the initial confluence on the top surface. Each sample was assayed in duplicate, and the experiment was repeated three times.

4.5.3 Cell Spreading Assay (I)

Cells were seeded in a 96-well plate precoated with fibronectin (25 µg/ml) and allowed to attach for 60 minutes before fixation. Fixed cells were stained with Crystal Violet solution (0.05% Crystal Violet, 1% Formaldehyde, 1% methanol, PBS) for 10 minutes at room temperature and washed five times with distilled water. The images were taken using a 100X objective of a light microscope (Olympus IX70). The images (1280x1024 pixels) were analysed using ImageJ 1.49b software, and the percentage of cells with spread morphology and cell area was calculated.

4.5.4 Colony Formation Assay (I)

Cells were mixed in 500 µl of complete medium containing 0.3% agarose (Sigma-Aldrich) and plated in 12-well plates (4 wells per cell line per experiment) that had been previously coated with 0.5% agar (Sigma-Aldrich) in the same medium. Cells were allowed to grow for 2 weeks. Eight random pictures (1280 × 1024 pixels) were taken from each well using the 4X objective of a light microscope (Olympus IX70). The percentage of colonies (containing more than 30 cells) with a diameter greater than 100 µm was calculated using ImageJ 1.49b software.

4.6 Cell Cycle Analysis (I)

Cells in 6-well plates were maintained in a serum-free medium for 48 hours and then trypsinised and resuspended in PBS. Cell viability was analysed using Propidium Iodide (Molecular Probes) according to the manufacturer's instructions, followed by fluorescence-activated cell sorting with a FACS Fortessa (BD Biosciences). Data obtained from the cell cycle distributions were analysed with FlowJo flow cytometry analysis software (Ashland).

4.7 Proliferation (I, II)

Melanoma cells were plated in 96-well plates and placed in an IncuCyte FLR, allowing them to grow for 72 hours. Proliferation was monitored by analysing the automatically acquired phase-contrast images taken at 2-hour intervals and the occupied area (% confluence) of cell images over time using IncuCyte^{TM} software.

The breast cancer cells were trypsinised 48 hours after the transfection with siRNAs and plated in 96-well plates at 5-10% confluence in a standard growth medium. Cell proliferation analysis was conducted for 144 hours, with measurements taken at 24-hour intervals using IncuCyte S3. The proliferation was quantified by assessing the confluence area normalised with the first scan (timepoint 0). Each sample was measured in six wells of a 96-well plate, and the experiments were repeated at least three times.

4.8 Mouse Experiments (I)

4.8.1 Mouse Model/Inoculations (I)

All animal experiments and handling were performed in accordance with the Finnish Animal Experiment Board and the institutional animal care policies of Turku University, Central Animal Laboratory. These fully meet the requirements defined in the U.S. National Institutes of Health guidelines on animal experimentation. Adult female athymic nude Foxn1nu mice (Harlan Laboratories) were housed with free access to irradiated food (SDS RM3 (E) soy-free, irradiated 25 kGy, Special Diet Service) and sterilised water under controlled conditions of light (12 h light/dark cycle) and temperature. Three mice per cell line were subcutaneously inoculated in both flanks with 1×10^6 cells in 75 µl of RPMI medium mixed with 75 µl of Matrigel (BD Biosciences). Mice were weighed weekly, and tumour growth was measured using callipers to determine the volume ($[\pi \times \text{length} \times \text{width}^2]/6$) and by optical imaging (IVIS Spectrum, PerkinElmer). The RFP fluorescence images were acquired using 570 nm excitation and 620 nm emission filters. When the tumour size

of at least one mouse/ group reached the level of predetermined maximal tumour size (17 mm length as determined in the animal experiment license), all mice in the study group were euthanised under isoflurane anaesthesia by blood collection through heart puncture and neck dislocation. Tumours and organs that showed fluorescence in IVIS imaging were fixed in 10% formalin for 24 hours and then paraffin-embedded for histological and immunohistochemical analysis.

4.8.2 The Mouse Xenograft (I)

The mouse melanoma xenografts were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. For immunohistochemistry, slides were sectioned at 3 µm, stained for Ki-67 and cleaved caspase 3 using a Ventana Discovery XT autostainer device (Ventana Medical Systems). After a standard pretreatment with Cell Conditioning Solution CC1 (Ventana), the slides were incubated with a Ki-67 polyclonal antibody (1:1000, Chemicon International) for 36 minutes or Cleaved Caspase 3 (D3E9) (1:100, Cell Signalling Technology) for 40 minutes, respectively. OmniMap anti-Rb HRP using ChromoMap DAB, both from Ventana, were used for detection. FHOD1 immunohistochemistry was performed using a LabVision Autostainer device with a BrightVision Poly-HRP-anti-Rabbit IgG detection kit, according to the manufacturer's protocol (Immunologic). The slides were pressure-cooked for 2 min for antigen retrieval. The primary antibody against FHOD1 (1:150, Sigma-Aldrich) was incubated for 1 hour, and the secondary antibody for 30 minutes. Diaminobenzene (DAB) was used as chromogene. The tumour histology was evaluated by microscopy of Hematoxylin-eosin (HE) stainings. For analysis of cell diameter and Ki-67 staining, four photomicrographs were taken at 400x magnification from different tumours in each treatment group. The diameter of at least 50 cells was measured from each micrograph.

4.9 Tissue Samples and Immunohistochemistry (I, II)

All patient tissue materials were prepared according to basic clinical histopathology laboratory practice: fixed in buffered formalin (pH 7.0) and embedded in paraffin. The tissue microarrays (TMAs) were prepared by punching the paraffin block of each tumour using either a 1.0 mm or 1.5 mm diameter cylinder. Paraffin-embedded tissues and TMAs were sectioned at 3,0-3,5 µm. Immunostainings were performed using a Ventana Discovery XT autostainer device (Ventana Medical Systems) or a Labvision autostainer (Thermo Fisher Scientific). The Ventana Discovery XT autostainer utilised OmniMap anti-Rb HRP (Ventana) with ChromoMap DAB (Ventana) as the detection system, while the LabVision Autostainer employed a

BrightVision poly-HRP-anti-Rabbit/Mouse IgG detection kit with DAB as the chromogen for detection. The antibodies and dilutions used for staining are listed in **Table 9**.

Table 9. Details of reagents and antibodies used in immunohistochemistry.

Antibody	Dilution	Host and clonality /Specification	Manufacturer	Article
Cleaved Caspase 3	1:100	Rabbit monoclonal Ab (clone D3E9)	Cell Signaling Technology	I
DAAM1	1:200	Rabbit polyclonal Ab	Proteintech	II
FHOD1	1:150	Rabbit polyclonal Ab	Sigma-Aldrich	I
FHOD1	1:500	Rabbit polyclonal Ab	Sigma-Aldrich	II
INF2	1:500	Rabbit polyclonal Ab	Proteintech	II
Ki67	1:1000	Rabbit polyclonal Ab	Chemicon International	I
OmniMap anti-Rb HRP using ChromoMap DAB		Detection system (DAB)	Ventana, Roche	I
BrightVision Poly-HRP-anti-Rabbit IgG detection		Detection kit	Immunologic	I
BrightVision two components detection system Goat Anti-Mouse/Rabbit IgG HRP		Detection system (DAB)	Immunologic, WellMed Company	II

4.9.1 Melanoma and Nevi (I)

The study of FHOD1 expression in nevi and melanoma included eight benign nevi, 10 melanomas and three paired metastatic melanomas. These samples were collected from the tissue archive of the Department of Pathology at Turku University Hospital with the approval of the Joint Committee on Ethics of the University of Turku and Turku University Hospital. These samples were prepared as 3µm sections and stained using the Labvision Autostainer, as described earlier for mouse xenografts, and the staining intensity was evaluated by microscopy. Antibodies and the dilutions are listed in **Table 9**.

4.9.2 Breast Cancer TMA (II)

The study included 70 HER2-positive breast cancer patients diagnosed and treated at Turku University Hospital, Turku, Finland, between 2008 and 2013. TMA with patient samples and clinical follow-up information from pathology reports and patient files of all patients were obtained from Auria Biobank under Decision AB20-2144 and research permission T208_2020 from the Hospital District of Southwest

Finland. The follow-up data included the established prognostic parameters of clinical breast cancer treatment, that is, HER2-oncogene status, tumour size, proliferation marker Ki-67, estrogen and progesterone receptor positivity, axillary lymph node status, histological grade, and intrinsic classification. The range of follow-up was from 5 years 4 months to 12 years (mean, 9 years 6 months).

Immunohistochemistry was performed on TMAs comprising tissue samples from each patient's centre and the invasive border of the tumour. TMA sections (3.5 µm) were stained with FHOD1 (Sigma-Aldrich), INF2 (Proteintech), and DAAM1 (Proteintech) using the streptavidin-peroxidase method on the Labvision autostainer. The antigen retrieval method for INF2 and DAAM1 was microwaving with Tris-EDTA pH 9.0 (Genmed). With the FHOD1 antibody, antigen retrieval was performed using a pressure cooker (Decloaking chamber, Biocare Medical) in Citrate Buffer pH 6.0 (Genmed). Incubations at each step were performed at room temperature, with the following incubation times: primary antibodies, 60 minutes; secondary antibodies, 30 minutes; DAB, 10 minutes; and Mayer's hematoxylin, 1 minute. The stained slides were scanned with NanoZoomer S60 (Hamamatsu Photonics) and viewed with NDP.view2 Image viewing software (Hamamatsu). The antibodies and the dilutions used in stainings are presented in **Table 9**.

4.10 In Silico Data Mining (II)

Our studies utilised data from two publicly available databases: Gene Expression Database of Normal and Tumour Tissues (GENT2) and Kaplan-Meier Plotter (KM Plotter). GENT2 (http://gent2.appex.kr) utilises expression data from Gene Expression Omnibus (GEO).[359] KM Plotter (https://kmplot.com/analysis/) contains data downloaded from GEO, the European Genome-Phenome Archive (EGA), and the Cancer Genome Atlas Program (TCGA).[360] The prognostic significance of formins (for which there is no data for GRID2IP) on messenger RNA (mRNA) expression levels in HER2-positive breast cancer was validated using the KM Plotter database analysis. GENT2 was used to investigate the correlations between the 14 formins and ERBB2 mRNA expression in 230 HER2-positive breast tumour subtypes.

4.11 Statistical Analysis (I, II)

In the first publication, all cell experiments were repeated at least three times unless otherwise specified. One-way ANOVA (Analysis of Variance) was performed using Tukey's multiple comparison procedure to identify the differences between cell lines, and error bars represent the standard error of the mean (SEM).

KM Plotter provided the Kaplan-Meier curves and p-values in the second publication. The Spearman correlation test investigated correlations between ERBB2 and formin mRNA expressions. To test the differences in clinicopathological parameters (age, tumour size, Ki-67, ER, and PR) and changes in migration and proliferation, we used the Student *t*-test. Histological grade, formin stainings, and lymph node metastasis frequencies were analysed using the *Chi*-square test. The IBM SPSS Statistics 26.0 program was utilised for statistical calculations. All the *in vitro* experiments were repeated at least three times. Two-tailed p values ≤ 0.05 were considered significant.

5 Results

5.1 FHOD1 Expression in Melanoma (I)

First, we analysed the FHOD1 expression level in 8 benign melanocytic nevi and 10 primary melanomas, of which three also had metastasis. Immunohistochemical staining revealed that FHOD1 expression was very low or absent in all benign nevi (I: Figure 1A, upper panel), whereas clear upregulation, ranging from moderate to strong, was observed in most primary melanomas and metastatic samples (I: Figure 1A, lower panel).

The next step was to study the expression of FHOD1 in several melanoma cell lines by western blotting. FHOD1 was expressed in all cell lines used in our studies, but the expression levels varied. We found high FHOD1 expression in SK-MEL-28, WM164, and WM239, moderate expression in Bowes, and low expression in Malme-3M (I: Figure 1B). By using immunofluorescence double staining with FHOD1 antibody and phalloidin, we observed that FHOD1 is localised in the cytoplasm of WM164 cells, with the staining appearing mainly as small dots but also along actin filaments (I: Figure 1C).

5.2 FHOD1 and Morphology of Melanoma Cells (I)

To study the function of FHOD1 in the WM164 melanoma cell line, we performed a stable knockdown of FHOD1 using shRNA. We chose to continue into further experiments with two clones, which had 80% (clone named as FHOD1 20%) and 95% (clone named as FHOD1 <5%) reduction of FHOD1 protein expression compared with the parental or scrambled control (clone named as FHOD1 100%) cell lines (I: Figure 2A).

The reduction of FHOD1 led to changes in cellular morphology. Fluorescent phalloidin staining revealed that melanoma cells with reduced levels of FHOD1 had 10-25% fewer actin filaments compared to control cells (I: Figure 2C, upper panel). The FHOD1 <5% cells seemed less polarised and larger, with a rounder shape. The total area and circularity of cells were quantified from the cell outline pictures (I: Figure 2C, lower panel), and these were significantly increased when FHOD1 <5% cells were compared to FHOD1 20% and FHOD1 100% cells (I: Figure 2B).

5.3 FHOD1 Affects Migration, Spreading and Adhesion of Melanoma Cells (I)

A wound-healing migration assay was used to analyse the capability of the cells to migrate after FHOD1 knockdown. The reduced cell lines, FHOD1 20% and FHOD1 <5%, exhibited significantly slower wound closure compared to the control cell line, FHOD1 100% ($p \leq 0.001$ and $p \leq 0.01$, ANOVA) (I: Figure 3A). Next, we added a Madrigel plug to challenge the cells' invasion ability in the wound-healing assay. With the FHOD1-depleted cell lines, invasion was significantly reduced ($p \leq 0.001$) (I: Figure 3B). Furthermore, migration was studied with a transwell migration assay using Boyden chambers and a serum gradient to challenge cells to migrate as single cells (I: Figure 3C). Similarly, the FHOD1 knockdown cell lines, FHOD1 20% and FHOD1 <5%, migrated significantly less than the control cell line, FHOD1 100% ($p \leq 0.01$, ANOVA) (I: Figure 3D).

Increased migration and invasion are often linked to cancer-associated epithelial-to-mesenchymal transition (EMT). To investigate this, we analysed the expression of well-established EMT markers using a Western blot. No alterations were detected in the expression levels of the markers (vimentin, slug, snail, E-cadherin, N-cadherin, and β-catenin), indicating that these mesenchymal transcription factors were expressed equally in both knockdown and control cell lines (data not shown).

Reduced migration may relate to changes in the adhesive properties of the cells. *In vitro*, cell adhesion begins with cell spreading. To test this, we performed a cell-spreading assay by culturing FHOD1-depleted and control cells in fibronectin-coated wells, followed by fixation and staining (I: Figure 4A). We calculated the percentage of cells exhibiting spread morphology (I: Figure 4B) and measured their cell areas (I: Figure 4C). A higher percentage of spread cells was observed in the FHOD1-depleted groups, which also displayed a significantly larger cell area than the control cells. The difference was statistically significant with FHOD1 <5% versus control ($p \leq 0.05$, ANOVA). These results support the assumption that adhesive structures might be altered. Therefore, the next logical step was to visualise and evaluate focal adhesions. FHOD1-depleted and control cells were stained for the focal adhesion protein paxillin (I: Figure 4D). There was no difference in the number of focal adhesions in these cells (I: Figure 4E); however, FHOD1 knockdown cells exhibited significantly smaller focal adhesions ($p \leq 0.001$, ANOVA) (I: Figure 4F).

5.4 The Colony Formation and Proliferation of Melanoma Cells are Reduced After FHOD1 Knockdown (I)

In a colony-forming assay, cells were seeded with 0.3% agarose and grown for two weeks before quantifying colonies with a diameter greater than 100 μm (I: Figure

5A). The percentage of these colonies was significantly lower in FHOD1 <5% cells as compared to FHOD1 100% and FHOD1 20% cells ($p \leq 0.01$ and $p \leq 0.05$, ANOVA). The percentage of large colonies in FHOD1 20% cells did not differ significantly from that of the control cells (I: Figure 5B).

Proliferation was examined using IncuCyte™ live cell imaging by analysing the area occupied by the cells (% confluence). FHOD1 knockdown significantly reduced proliferation ($p \leq 0.01$, ANOVA) (I: Figure 5C). To determine whether this reduction was due to increased apoptosis, we assessed cleaved caspase-3 levels by Western blot. However, no increase in cleaved caspase 3 was detected after the FHOD1 knockdown (data not shown). Therefore, the reduction in proliferation and colony formation does not appear to be caused by increased apoptosis. Instead, cell cycle analysis provided a mechanistic explanation for the reduced proliferation. Flow cytometry analysis revealed that FHOD1-knockdown cells were significantly arrested in the G0/G1 phase ($p \leq 0.05$, ANOVA) of the cell cycle (I: Figure 5D).

5.5 Melanoma Tumour Growth was Reduced with Knockdown Cells *in vivo* (I)

The relevance of the previous *in vitro* FHOD1 knockdown results was analysed *in vivo* using a tumour formation experiment. FHOD1-depleted and control cells were inoculated subcutaneously in both flanks of athymic mice. Measurements followed up on the tumour growth with callipers. The growth rate of tumours varied between the groups; therefore, the mouse groups were sacrificed at diverse time points. The mice from different groups were imaged on day 14 (I: Figure 6A). The tumours originating from the FHOD1 100% cell line grew faster than the FHOD1-depleted groups, and these mice were sacrificed due to the large tumour size already on day 14 (I: Figure 6B). At this time point, the tumour size in the FHOD1 20% group was significantly smaller than in the control group ($p \leq 0.01$, ANOVA) (I: Figure 6C). A statistically significant difference ($p \leq 0.01$) was also detected after combining the FHOD1 20% and FHOD1 <5% results and then comparing them with the control using a *t*-test. The size limit of tumours with depleted cell lines were reached in 30 days with FHOD1 20% cells and in 22 days with FHOD1 <5% cells (I: Figure 6B).

The histology of mouse tumours varied slightly between the groups expressing differing levels of FHOD1 (I: Figure 6D, top panel). A diffuse growth pattern was observed in FHOD1 100% cells, whereas tumours originating from FHOD1 20% and FHOD1 <5% cells exhibited a somewhat lobular architecture with strands of connective tissue present within the tumour. The edges of the tumours were morphologically invasive across all groups, and the quantification of tumour cell size showed no differences between the groups. FHOD1 expression levels in the tumours were verified using immunohistochemistry (I: Figure 6D, middle panel). The

staining was intense and diffusely cytoplasmic in FHOD1 100% tumours; a minor diffuse expression was noted in FHOD1 20% cells, and small islands of distinct FHOD1-positive cells were scattered in both FHOD1 20% and more frequently in FHOD1 <5% tumours. The tumours were stained with cleaved caspase 3, which revealed no changes among the groups (data not shown). A similar result was observed in the *in vitro* cell line experiment. Thus, the downregulation of FHOD1 does not affect tumour growth through apoptosis. The Ki-67 staining, however, indicated a noticeable reduction in proliferation due to the reduction of FHOD1 (I: Figures 6D, bottom panel, and 6E). The decreased proliferation rate likely accounts for the slower growth of FHOD1-depleted tumours and reflects the cell cycle arrest observed *in vitro*. We wondered if the cell cycle arrest could be linked to the Serum Response Element (SRE) and whether FHOD1 knockdown would diminish the serum response, thereby affecting transcription from the SRE. To clarify this, we quantified the nuclear translocation of the SRE co-activator MKL-1 from the cytoplasm after serum treatment in control and FHOD1 knockdown cells. FHOD1 knockdown significantly reduced the number of MKL-1-positive nuclei compared to control cells, suggesting that FHOD1 influences transcription from the SRE (I: Figure 7).

5.6 FHOD1, INF2 and DAAM1 Expression in HER2-Positive Breast Cancer and Correlations with Markers (II)

First, we utilised a publicly available database and Kaplan-Meier overall survival analysis to study the association between formin mRNA expression and outcomes of HER2-positive breast cancer. The analysis contained 14 formins; data for GRID2IP encoding Delphilin was unavailable. It revealed that increased mRNA levels of FHOD1 (HR = 1.63, $p = 0.012$), INF2 (HR = 1.62, $p = 0.0079$) and DAAM1 (HR = 1.56, $p = 0.02$) were associated with an unfavourable prognosis (II: Figure 1A). The same formins were discovered to have a significant positive correlation with ERBB2 mRNA expression: FHOD1 (R = 0.161, $p = 0.015$), INF2 (R = 0.248, $p < 0.001$), and DAAM1 (R = 0.195, $p = 0.003$) (II: Figure 1B). The Kaplan-Meier plots and correlations for all formins (no data for GRID2IP) are shown in the Supplementary material (II: Supplement Figure 1 and 2).

We continued our studies by validating the expression of FHOD1, INF2, and DAAM1 at the protein level using immunohistochemistry on TMAs comprising samples from 70 HER2-positive breast cancer patients. The clinicopathological attributes of the study cohort and detailed sample information are indicated in the publication (II: Table 1). We combined our results from the central and invasive border sample analyses, as the staining intensities were comparable. The samples

were scored as negative, low, moderate, or high according to the prevalent cytoplasmic staining intensity (II: Figure 2). However, for subsequent analysis, we decided to group the intensities into broader categories: FHOD1 into negative/low and moderate/strong groups, INF2 into negative and low/moderate (no notable high intensity observed), and DAAM1 into negative/low and moderate/high. FHOD1 and INF2 exhibited more homogeneous staining distribution than DAAM1. The DAAM1 staining also revealed intensely staining cytoplasmic granules in 34 tumours, and in six cases, a dual staining pattern with both cytoplasmic and membranous locations was observed (II: Supplement Figure 3).

The expression results were analysed alongside clinicopathological features to reveal potential correlations (II: Table 2). A significant difference was noted between the average patient age and INF2-negative tumours compared to INF2 low/moderate tumours ($p = 0.004$). FHOD1 moderate to high staining was correlated with a lower percentage of ER ($p = 0.041$) and PR ($p = 0.014$). All FHOD1 moderate/high samples were classified as grade 3. With the grade 3 tumours, significantly higher values were observed for the percentage of Ki-67 ($p < 0.0001$), and lower values for ER ($p < 0.0001$) and PR ($p < 0.002$) compared to grade 1 or 2 tumours. No significant differences in formin expressions related to lymph node metastases were observed.

5.7 FHOD1, INF2 and DAAM1 in HER2/ERBB2-Amplified Breast Cancer Cell Lines (II)

The roles of FHOD1, INF2, and DAAM1 were studied in two HER2-amplified breast cancer cell lines, MDA-MB-453 and SK-BR-3. Double immunofluorescence staining with phalloidin and anti-FHOD1 (II: Figure 3, upper panel), INF2 (II: Figure 3, middle panel), or DAAM1 (II: Figure 3, lower panel) was used to analyse the subcellular localisation of these three formins in the selected cell lines. Staining for FHOD1, INF2, and DAAM1 was localised in the cytoplasm, primarily appearing as small dots in lamellipodia and along the actin filaments. Next, we aimed to assess the functional roles of FHOD1, INF2, and DAAM1, both independently and in combination, in MDA-MB-453 and SK-BR-3 cells using formin-specific or non-targeting control siRNAs. The effectiveness of knockdown was evaluated using immunoblotting 48 hours after transfection (II: Figure 4A). The knockdown of formins did not alter the morphology of the cells (II: Figure 4B). We conducted a wound-healing assay to assess the effect of knockdown on cell motility, using a starvation medium to minimise the influence of proliferation. MDA-MB-453 and SK-BR-3 cells are slow-moving; therefore, we also tested the addition of heregulin β1 (HRG) to the starvation medium to enhance migration (II: Figure 5A). The following siRNAs with combinations caused a delay in migration in MDA-MB-453 cells under both starvation and HRG conditions: FHOD1 ($p \leq 0.05$ and $p \leq 0.01$),

FHOD1+INF2 ($p \leq 0.05$ and $p \leq 0.05$), FHOD1+DAAM1 ($p \leq 0.05$ and $p \leq 0.05$), and FHOD1+INF2+DAAM1 ($p \leq 0.05$ and $p \leq 0.05$). Similarly, significant effects of siRNA treatment were observed for FHOD1, INF2, DAAM1, and their combinations in SK-BR-3 cells under starvation medium (FHOD1, INF2, DAAM1, INF2+DAAM1, FHOD1+INF2+DAAM1: $p \leq 0.05$; FHOD1+DAAM1: $p \leq 0.01$) and HRG-supplemented medium (FHOD1, DAAM1, FHOD1+INF2: $p \leq 0.01$; INF2, INF2+DAAM1: $p \leq 0.05$; FHOD1+INF2+DAAM1: $p \leq 0.05$; FHOD1+DAAM1: $p \leq 0.01$; FHOD1+INF2+DAAM1: $p \leq 0.001$).

The migration sensitivity was also analysed using a transwell migration assay (II: Figure 5B). The simultaneous reduction of all three formins in MDA-MB-453 cells decreased migration at 72 hours ($p \leq 0.05$), whereas individual and other combined siRNAs impaired migration at 96 hours (all $p \leq 0.05$). In SK-BR-3 cells, impaired cell migration was noted after 48 hours of treatment with DAAM1 siRNA combined with FHOD1 ($p \leq 0.01$), INF2 ($p \leq 0.001$), or both ($p \leq 0.001$). All individual siRNAs and their various combinations showed diminished migration after 72 hours (FHOD1, $p \leq 0.05$; INF2 and DAAM1, $p \leq 0.05$; FHOD1+DAAM1, $p \leq 0.01$; INF2+DAAM1 and FHOD1+INF2+DAAM1, $p \leq 0.001$). By 96 hours, significant effects were observed for INF2, DAAM1, FHOD1+DAAM1, and INF2+DAAM1 ($p \leq 0.05$), and for FHOD1+INF2 and FHOD1+INF2+DAAM1 ($p \leq 0.01$).

Proliferation was studied by quantifying the cell area over a 144-hour period. A significant reduction ($p \leq 0.05$) in proliferation was observed only when all three formins (FHOD1, INF2, and DAAM1) were concurrently knocked down from MDA-MB-453 cells (II: Figure 5C).

5.8 The Effect of HER2/ERBB2 Knockdown on FHOD1, INF2 and DAAM1 in HER2/ERBB2-Amplified Breast Cancer Cell Lines (II)

HER2-specific antibody was used in immunostainings to reveal the protein expression and localisation in MDA-MB-453 and SK-BR-3 cells. HER2 was localised in the nucleus and cytoplasm, concentrating on the actin-rich cellular protrusions (II: Figure 6A). ERBB2-targeting siRNA was used to silence HER2 and explore the effect of downstream pathways on FHOD1, INF2 and DAAM1. The knockdown was verified by Western blot in both MDA-MB-453 and SK-BR-3 cell lines (II: Figure 6B).

We decided to investigate the dependency of formin expression on individual MAPK or PI3K signalling pathways by exposing the MDA-MB-453 and SK-BR-3 cell lines to the MEK 1/2 inhibitor UO126, the PI3K inhibitor LY294002, or the

vehicle DMSO (control). The inhibition of pathways was confirmed by Western blot analysis of Akt and MAPK phosphorylation states (II: Figure 6B).

FHOD1, INF2, and DAAM1 levels were reduced in MDA-MB-453 cells after HER2/ERBB2 knockdown; similarly, the levels of phosphorylated MAPK (pMAPK) decreased. The inhibition of PI3K or MAPK resulted in effects similar to those observed with HER2/ERBB2 knockdown, suggesting that ERBB2 exerts a regulatory influence on FHOD1 and INF2 expression via the Akt/MAPK pathway in this cell line. DAAM1 expression was also affected by ERBB2 knockdown; however, no comparable effect was noted with the individual inhibition of the PI3K or MAPK pathways. ERBB2 knockdown also decreased FHOD1 and INF2 levels and reduced Akt and MAPK phosphorylations in SK-BR-3 cells; however, the inhibition of either PI3K or MAPK did not affect formin expression. The DAAM1 did not have as unambiguous a result as the other two formins, indicating that its regulation may be affected by different pathways. The exogenous ERBB2 overexpression did not modify the expression of FHOD1, INF2, or DAAM1 in HEK-239 cells (II: Supplement Figure 4).

6 Discussion

6.1 FHOD1 in Melanoma (I)

The introduction of immunotherapies, targeted therapies, and combination therapies has revolutionised the treatment and management of metastatic melanoma.[304,308] However, treatments confront complications due to the high mutation burden of tumours and the capacity of cancer cells to evade the immune system and evolve mechanisms to resist treatments.[308,309] More basic research is needed to uncover the mechanisms behind melanoma progression, including those that regulate melanoma cell growth, migration and invasion. Our studies specifically focused on clarifying the expression and functional role of FHOD1 in melanoma.

We showed that FHOD1 expression was increased when comparing human melanomas with benign *nevi* samples. FHOD1 is primarily expressed in cells of mesenchymal origin, such as endothelial cells and smooth muscle cells, with no detectable expression in cells of epithelial origin.[261] Melanocytes originate from the neural crest. Neural crest cells delaminate from the neural tube through EMT, and these migratory cells then move to the dermis and epidermis, where they can differentiate into melanocytes.[361] Melanocytes are challenging to distinguish from other cells of the skin without special stainings; however, it is feasible to say that FHOD1 is not ordinarily present in melanocytes, as our group's earlier publication showed that no FHOD1 could be detected on normal stratified squamous epithelium.[261] Thus, the expression of FHOD1 in melanomas and benign *nevi* differs from that in normal skin. Other studies have also demonstrated increased FHOD1 expression in malignancies such as squamous cell carcinoma, basal-like breast cancer, and gastric cancer, further highlighting its potential role in cancer progression.[191,212,261]

The FHOD1 expression levels varied from moderate to strong in melanoma samples, and a similar variation, ranging from low to high, was observed in the tested melanoma cell lines. This suggests a potential correlation between FHOD1 expression and melanoma progression, justifying further investigations into its role in melanoma tumorigenesis. Knockdown of FHOD1 resulted in noticeable morphological changes and functional alterations in melanoma cell cultures. Cells with the most efficient FHOD1 knockdown appeared more rounded, exhibited a

greater surface area, and had less prominent actin filaments. Functional experiments demonstrated that reducing FHOD1 significantly decreased migration in wound healing, invasion, and Boyden chamber transwell migration experiments. Other studies conducted on breast cancer and squamous cell carcinoma support these findings.[189,212,261] Therefore, the shared function of FHOD1 across these different cancers appears to be the maintenance of an elongated, actin fibre-rich, and migratory phenotype. The migration of melanoma cells with downregulated FHOD1 levels was markedly reduced in the Boyden chamber experiment compared to the results from the wound healing assay. These two experiments elucidate different modes of migration; wound healing tests a more collective migration mode, while the Boyden chamber challenges cells to migrate individually through membrane pores. Thus, our results indicate that FHOD1 knockdown substantially influences migration, where the adhesion and detachment of individual cells are crucial. Previous studies support this observation by demonstrating that FHOD1 is involved in adhesion; for instance, exogenously expressed FHOD1 accumulates into integrin clusters that subsequently mature into focal adhesions. Furthermore, studies on FHOD1 knockdown have revealed impaired cell spreading and focal adhesion formation.[250,255,257,362] Our results also confirmed that FHOD1 affects focal adhesion; melanoma cells with reduced FHOD1 levels exhibited noticeably smaller focal adhesions than control cells. These smaller focal adhesions may account for the reduced migration and a more spread morphology. The size of focal adhesions has been shown to predict cell migration. Specifically, this biphasic relationship between focal adhesion size and cell speed indicates that initially, an increase in focal adhesion size enhances cell migration speed until an optimal size is reached, beyond which cell speed declines.[363]

FHOD1 knockdown also affects proliferation and the cell cycle. Proliferation was lower in the FHOD1-knockdown cells; however, this decrease was not associated with apoptosis but rather with cell cycle arrest. FHOD1 is known to activate SRE-sensitive gene expression.[189,364,365] We found that FHOD1 knockdown reduced the nuclear translocation of MKL-1 after serum stimulation, indicating that SRE co-activation was also reduced. Our results align with previous data, and we propose a model in which FHOD1 knockdown reduces transcription from the SRE, leading to sequential cell cycle arrest and decreased proliferation. SRE has been shown to control the transcription of focal adhesion genes, at least in NIH-3T3 fibroblasts.[366] One possibility is that FHOD1 influences the transcription of focal adhesion genes through SRE, resulting in smaller focal adhesions in FHOD1-knockdown melanoma cells.

We also studied the effect of FHOD1 knockdown on tumorigenesis *in vivo*. Tumorigenesis was not abrogated in mice, but the FHOD1-knockdown melanoma cells formed smaller tumours with reduced proliferation. However, the marginals of

tumours still contained histologically infiltrative cells, suggesting that FHOD1 is not indispensable for the invasion of melanoma cells *in vivo*.

To recapitulate our main results, FHOD1 is a protein commonly upregulated in melanoma. Its role in melanoma cell biology is versatile, affecting adhesion, migration, and tumour growth. However, FHOD1 might not be essential for maintaining the invasive capacity of cancer cells *in vivo*. Modern treatments have increased the life expectancy in metastatic melanoma, but at the advanced stage, the cancer is still an incurable disease.[308,367] Current research is increasingly focusing on understanding the mechanisms of melanoma progression and how melanoma cells develop resistance to treatments. Additionally, new predictive and prognostic biomarkers are necessary to determine disease outcomes, identify patients at high risk for metastasis, estimate treatment response, and aid in finding the optimal treatment.[312,314,368–370] The actin cytoskeleton and the modulating proteins, including formins, could provide new potential as drug targets in addition to current treatment modalities. Therefore, future studies should elucidate the mechanisms of FHOD1 upregulation and activation during melanoma progression to determine whether it can be utilised as a biomarker or a potential drug target.

6.2 DAAM1, FHOD1 and INF2 in HER2-Positive Breast Cancer (II)

The overall outcome for HER2-positive breast cancer patients has significantly improved due to targeted therapy utilising drugs against highly expressed HER2. However, some patients do not respond to treatment or develop resistance during treatment.[342,343,371] Ongoing research focuses on uncovering the resistance mechanism, developing new treatments and finding new biomarkers to predict response or resistance to treatment.[345,346,358,372,373] Our study aimed to clarify the role of formin proteins in HER2-positive breast cancer. Some formins have been shown to have a significant role in cancer progression, and their functions are connected to the same signalling pathways as HER2. For example, DAAM1 overexpression promotes malignant activity by regulating the ERK and AKT pathways in gastric cancer cells.[194] Additionally, silencing DIAPH3 in human carcinoma cells, among other features, induced hyperactivation of the EGFR/MEK/ERK signalling pathway[374], and FHOD1 has been shown to interact with components of the ERK/MAPK pathway in HEK293T cells[375].

We utilised publicly available databases and identified three specific formins, DAAM1, FHOD1, and INF2, that correlate with outcomes in HER2-positive breast cancer, and are also associated with ERBB2 in this context. It should be noted that this type of mRNA quantification is primarily based on averaging across all cell types present in the tissue, which may overlook intratumoral heterogeneity.

Therefore, we aimed to ascertain whether a comparable correlation could be established at the protein level, specifically within cancer cells, using IHC. Unfortunately, possibly due to the limited sample size, we were unable to confirm such a correlation. However, we noticed a connection between low ER and PR percentages and elevated tumour grade. This aligns with the outcomes of an earlier study where the absence of receptor expression correlated with a higher histological grade.[376] By further analysing the patient samples and clinicopathological features, we noticed a trend wherein all the samples with moderate or high expression of FHOD1 were characterised as histological grade 3. We also identified a negative correlation between elevated FHOD1 expression and reduced ER and PR percentages. This suggests a potential link between the upregulation of FHOD1 and tumour dedifferentiation. Cell dedifferentiation has been presumed to coincide with EMT.[377–379] Overexpression of FHOD1 is also observed during EMT, accompanied by the upregulation of mesenchymal markers and the downregulation of epithelial markers.[261,380] We used two HER2-amplified cell lines and knocked down formins with siRNAs to study their functional role. The cell lines exhibited slightly different behaviour in experiments, as the MDA-MB-453 cells showed a slower migration profile than the SK-BR-3 cells. The knockdown of DAAM1, FHOD1, or INF2, either individually or in combination, decelerated the migration of SK-BR-3 cells, especially when all three formins were knocked down simultaneously. In MDA-MB-453 cells, only the knockdown of FHOD1, DAAM1, or their combination caused a notable delay in migration. The results from the transwell migration assay supported the wound healing results, showing enhanced outcomes with the combination of siRNAs targeting the specific formins. These results align with earlier *in vitro* studies, which have utilised breast cancer cell lines and the knockdown of formins, including FHOD1, INF2, FMNL2, and DAAM2.[169,192,212]. Interestingly, in SK-BR-3 cells, the DAAM1 and INF2 siRNAs caused a significant reduction in migration, as observed in both wound healing and transwell migration assays. In contrast, a similar effect was not seen in MDA-MB-453 cells. This highlights the cell-specific nature of formins. The knockdown of formins slightly decreased proliferation only in MDA-MB-453 cells when all three formins were knocked down concurrently. Therefore, these formins appear to have a more significant impact on the migration of HER2-amplified cancer cells than on their proliferation.

The positive correlation observed between ERBB2 mRNA levels and those of DAAM1, FHOD1, and INF2 in HER2-positive breast cancer samples prompted us to further investigate HER2/ERBB2 downstream signalling and its role in regulating formins *in vitro*. The knockdown of HER2/ERBB2 with siRNA caused a reduction in the expression of FHOD1 and INF2 in both cell lines. The HER2/ERBB2 knockdown reduced the phosphorylation of AKT and MAPK in the SK-BR-3 cell line, whereas only the MAPK pathway was affected in MDA-MB-453 cells. Hence,

the ERBB2-mediated signalling cascades influence the modulation of FHOD1 and INF2 expression, a conclusion supported by earlier studies. In one study, trastuzumab was used to inhibit HER2 activation in BT-474 cells, resulting in a reduction in FHOD1 mRNA levels and downregulation of the Ki-67 proliferation marker, which supports our results. However, in the same study, the inhibition of HER2 increased the INF2 mRNA level, which is contrary to our findings.[381] In another study, protein levels of INF2 reduced significantly when SK-BR-3 cells were treated with lapatinib, an inhibitor targeting ERBB1/EGFR and HER2 phosphorylation.[382] Thus, these data support our conclusions: INF2 and FHOD1 expressions are affected by ERBB2 knockdown, and this is accompanied by a reduction in Akt and MAPK phosphorylation in SK-BR-3 cells; in contrast, only MAPK phosphorylation is affected in MDA-MB-453 cells. Our findings also align with our group's previous results, demonstrating that the PI3K-Akt and RAS-MAPK pathways regulate formin expression in squamous cell carcinoma and melanoma.[205,261]

Our inhibitor experiments with PI3K and MEK inhibitors revealed distinct regulatory mechanisms in the two cell lines used. The inhibition of PI3K or MAPK replicated the reduction of FHOD1 and INF2 observed after ERBB2 knockdown in MDA-MB-453 cells. However, in SK-BR-3 cells, neither Akt nor MAPK inhibition could induce changes in the expression of FHOD1 and INF2. Previously, it has been suggested that combining PI3K or MAPK pathway inhibition with anti-HER2 therapy could enhance the treatment of patients with HER2-positive breast and gastric cancers.[383,384] Results related to the connection between DAAM1 expression and HER2/ERBB2 knockdown were not particularly convincing. Although DAAM1 expression was slightly decreased due to ERBB2 knockdown in MDA-MB-453 cells, accompanied by reduced levels of phosphorylated MAPK, we could not confirm this relationship with MAPK-specific inhibitors. Therefore, DAAM1 appears to be regulated by an additional pathway downstream of HER2/ERBB2. The differential regulation of DAAM1 compared to FHOD1 and INF2 may arise from differences in their activation. FHOD1 is activated by ROCK phosphorylating its serine and threonine residues in the DAD domain, and INF2 activity is tightly controlled by cellular inhibitors utilising the DID-DAD interaction, with the exact mechanism remaining unknown.[111,267,268] In contrast, DAAM1 is activated by binding Dvl to the DAD domain, which further mediates RhoA activation in the Wnt signalling pathway.[102,231,232]

Although HER2-positive breast cancer patients initially respond to trastuzumab-based therapies, resistance often evolves. The key factors behind acquired resistance to trastuzumab appear to be the activation of the PI3K pathway and alterations in the downstream components of the PI3K/Akt signalling pathway.[351,385,386] To overcome resistance, the search for alternative therapeutic targets related to HER2 is underway.

Our work concludes that FHOD1 and INF2 function as downstream effectors of the HER2/Akt and HER2/MAPK pathways. These two formins are potential candidates for future therapeutic drug targets. A formin inhibitor, which prevents formin-mediated actin assembly, was published in 2009 and named SMIFH2 (small molecular inhibitor of formin homology 2 domains).[387] Later studies have confirmed that SMIFH2 disrupts specific actin structures regulated by formins, leading to antiproliferative effects by affecting cell division and motility.[156,388–390] However, the challenge with SMIFH2 usage is its nonspecific nature, as it not only inhibits formins but also certain classes of myosins.[391–393] Thus, research into more specific antiformin drugs continues.

6.3 Overview and Future Studies

My thesis research is based on patient tumour samples, basic cell experiments, and publicly available mRNA databases. We could clearly show that formins FHOD1, INF2, and DAAM1 are upregulated in cancerous tissue samples. However, the protein expression levels exhibit high variability. Similar protein variations were detected in cancer cell lines used in these studies, and all these variations, together with slightly differently behaving cancer cell lines from the same origin, are clear indications of the intratumoral heterogeneity shown in various studies.[345,394,395]

The functional role of FHOD1 was studied in melanoma and HER2-positive breast cancer. The knockdown of FHOD1 reduced migration in human melanoma and HER2-positive breast cancer cell lines; however, the reduction in proliferation in HER2-amplified cancer cells was achieved only when DAAM1 and INF2 were also knocked down. PI3K inhibition downregulated FHOD1 expression in both cancer types. However, MAPK inhibition did not affect FHOD1 expression in melanoma cells. In the case of breast cancer cells, MAPK inhibition reduced FHOD1 expression in MDA-MB-453 cells; however, in SK-BR-3 cells, neither pathway inhibition alone altered FHOD1 expression level. Double inhibition might have a better impact, but the combination becomes too toxic for *in vitro* experiments. The functional roles of DAAM1 and INF2 were studied only in HER2-positive breast cancer, where their knockdown reduced the migration of SK-BR-3 cells and the simultaneous knockdown of all three formins caused even more reduced migration. However, a notably reduced migration of MDA-MB-453 cells was observed only when DAAM1 and FHOD1 were knocked down individually or in combination. INF2 expression decreased in both cell lines after PI3K and MAPK inhibition, as with FHOD1. However, DAAM1 expression was not clearly affected by the inhibitors. In general, these results support the regulatory differences between cancers and between different cell lines of the same origin.

Although our results were straightforward and showed the knockdown of formins to reduce migration and invasion, slightly affecting the proliferation of cancer cells, these results should be verified with experiments that better mimic the tumour microenvironment. Our cell experiments, such as migration and proliferation, were conducted on plastic plates and glass slides supplemented with agarose, gelatin, fibronectin, or Matrigel, which provide a simplified 2D or 3D environment. In the melanoma study, we inoculated melanoma cells into mice with Matrigel supplement to see differences in tumour growth *in vivo*. However, the tumour microenvironment is a vastly more complicated system than just one supplement added; it comprises different cell types that communicate with the ECM and with one another. We also need to bear in mind the complexity of ECM in tissues and organs *in vivo*, which is characterised by a versatile assortment of biochemical and biophysical features, including ligand composition and density, elasticity, stiffness and a diverse set of topological cues. The composition and features of ECM are different in tumours and normal tissues due to the actions of cancer cells and other components of the tumour microenvironment.[396–399] Researchers continue to challenge themselves to generate 3D matrix models that more accurately mimic specific *in vivo* microenvironments. Studies have, for example, evaluated the effects of matrix compositions and different matrix stiffness on cancer cell progression.[400–404] One promising model system for cancer researchers is the development of cancer organoids, which are more representative models of tumours.[405–408]

Cancer research is extremely challenging because signalling pathways are interconnected, forming complex networks. Different regulators of cellular functions influence each other, and one must also consider the impact of the surrounding environment, which adds another layer of complexity to understanding and effectively treating cancer. We must begin with a simple system and then progress to more complex ones. Our next steps would be to determine which pathways regulate DAAM1 in HER2-positive breast cancer, test our results in more complex environments, and investigate whether these formins also affect other factors that assist tumour progression. For example, a recent study demonstrated that tumour invasiveness is regulated by the concerted function of formins, the ARP2/3 complex, and adenomatous polyposis coli (APC).[409]

7 Summary/Conclusions

The main conclusions of this thesis are:

1. FHOD1 formin is upregulated in cutaneous melanomas compared to benign nevi.
2. In cultured melanoma cells, reduced FHOD1 expression alters cell adhesion, migration, and proliferation and attenuates tumour growth *in vivo*.
3. FHOD1 affects transcription from the SRE in melanoma cells.
4. In HER2-positive breast cancer, high DAAM1, FHOD1 and INF2 mRNA levels correlates with reduced overall survival in HER2-positive breast cancer. A correlation exists between ERBB2 and formins DAAM1, FHOD1 and INF2 transcript levels.
5. FHOD1 and INF2 act as downstream effectors of the HER2/Akt and HER2/MAPK pathways.
6. DAAM1 functions downstream of HER2, but it is regulated by a different pathway than the PI3K or MAPK pathways.

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