Turun yliopisto
University of Turku

FORMIN PROTEINS IN NORMAL TISSUES AND CANCER

Maria Gardberg

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1163 | Medica - Odontologica | Turku 2015

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ISBN 978-951-29-6044-6 (PRINT)
ISBN 978-951-29-6045-3 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2015

To my family

ABSTRACT

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Formin proteins in normal tissues and cancer

Department of Pathology, University of Turku, Turku, Finland (2015)

The actin cytoskeleton is a dynamic structure that determines cell shape. Actin turnover is mandatory for migration in normal and malignant cells. In epithelial cancers invasion is frequently accompanied by epithelial to mesenchymal transition (EMT). In EMT, cancer cells acquire a migratory phenotype through transcriptional reprogramming. EMT requires substantial re-organization of actin. During the past decade, new actin regulating proteins have been discovered. Among these are members of the formin family.

To study formin expression in tissues and cells, antibodies for detection of formin proteins FMNL1 (Formin-like protein 1), FMNL2 (Formin-like protein 2) and FHOD1 (Formin homology 2 domain containing protein 1) were used. The expression of formins was characterized in normal tissues and selected cancers using immunohistochemistry. The functional roles of formins were studied in cancer cell lines.

We found that FMNL2 is widely expressed. It is a filopodial component in cultured melanoma cells. In clinical melanoma, FMNL2 expression has prognostic significance. FHOD1 is a formin expressed in mesenchymal cell types. FHOD1 expression is increased in oral squamous cell carcinoma (SCC) EMT. Importantly, FHOD1 participates in invasion of cultured oral SCC cells. FMNL1 expression is low in normal epithelia, but high in leukocytes and smooth muscle cells. Expression of FMNL1 can be found in carcinoma; we detected FMNL1 expressing cells in basal type of breast cancer.

Our results indicate that formins are differentially expressed in normal tissues and that their expression may shift in cancer. Functionally FMNL2 and FHOD1 participate in processes related to cancer progression. Studying formins is increasingly important since they are potential drug targets.

Key words: formin, actin cytoskeleton, immunohistochemistry, EMT, melanoma, breast cancer, squamous cell carcinoma

TIIVISTELMÄ

Maria Gardberg

Formiinit terveissä kudoksissa ja syövässä

Patologian oppiaine, Biolääketieteen laitos, Turun Yliopisto, Turku (2015)

Solujen aktiinitukiranka on dynaaminen rakenne, joka määrää solujen muodon. Solujen liikkuminen ja syöpäsolujen invaasio edellyttää huomattavaa aktiinitukirangan muokkausta. Epiteeliperäisten syöpäsolujen invaasiokykyyn liittyy monesti epiteeli-mesenkymaalinen transitio (EMT). EMT on käsitteenä tunnettu yksilön kehitysbiologiasta, jossa se on välttämätön kehon osien ja kudosten muovautumisessa. Kehityksellistä prosessia muistuttavalla tavalla syöpäsolujen EMT:ssa transkriptio muuttuu, minkä seurauksena syöpäsolut irtoavat ympäristöstään, muuttuvat pitkänomaisiksi ja tehokkaasti liikkuviksi. Muutokset edellyttävät tehokasta aktiinisäikeiden muokkausta.

Yksi aktiinitukirankaa muokkaavien proteiinien perheitä ovat formiinit. Ne ovat viimeisen vuosikymmenen aikana olleet aktiivisen tutkimuksen kohteena. Formiinien keskeinen toiminto on aktiinisäikeiden muodostaminen.

Tutkiaksemme formiinien ilmentymistä kudoksissa, varmistimme kolmen formiinin vasta-aineen luotettavan toimivuuden. Vasta-aineet oli valmistettu formiinien FMNL1 (Formin-like protein 1), FMNL2 (Formin-like protein 2) ja FHOD1 (Formin homology 2 domain containing protein 1) immunohistokemialliseen tunnistamiseen. Näiden vasta-aineiden avulla kartoitimme formiinien ilmentymisprofiilit normaaleissa kudoksissa, ja jatkoimme tutkimalla niiden ilmentymistä syöpäkudoksissa ja funktiota viljellyissä syöpäsoluissa.

Osoitimme että FMNL2 ilmentyminen on laajaa normaalikudoksissa. FMNL2 paikantuu solukalvon ulokkeisiin viljellyissä melanoomasoluissa, ja sillä on ennusteellinen merkitys kliinisessä melanoomassa. FHOD1 puolestaan ilmentyy mesenkymaalisissa kudoskomponenteissa. FHOD1 ilmentyminen nousee suun levyepiteelikarsinooman EMT:ssa. Saatoimme solukokein osoittaa, että FHOD1 osallistuu EMT:hen liitettyihin toimintoihin. Lopuksi tutkimme FMNL1 ilmentymistä, ja totesimme sen lähes rajoittuvan valkosoluihin ja sileälihassoluihin. Terveissä epiteeleissä FMNL1 ilmentyminen on vähäinen, mutta saattaa lisääntyä basaalisessa rintasyövässä.

Tuloksemme osoittavat että formiinit ilmentyvät toisistaan poikkeavalla profiililla normaaleissa kudoksissa. Lisäksi niiden ilmentyminen voi muuttua syövässä. Viljellyissä syöpäsoluissa formiinit osallistuvat syövän progressioon eli invaasiokykyä ja metastasointia edistäviin toimintoihin. Formiinien tutkiminen syövässä on entistäkin tärkeämpää, koska niille on jo löytynyt pienimolekyläärinen inhibiittori. Formiinien toiminnan inhibointi saattaa tulevaisuudessa kehittyä osaksi syövän lääkkeellistä hoitoa.

Avainsanat: formiini, aktiinitukiranka, immunohistokemia, EMT, melanooma, rintasyöpä, levyepiteelisyöpä

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
ANOVA	analysis of variance
ATCC	American Type Culture Collection
Arp2/3	actin-related protein 2/3
BSA	bovine serum albumin
CAM	cell adhesion molecule
CI	confidence interval
DFNA	Deafness, Autosomal Dominant
DMEM	Dulbecco's Modified Eagle's Medium
DRF	Diaphanous-related Formin
ECM	extracellular matrix
EMT	epithelial-mesenchymal transition
FCS	fetal calf serum
FFPE	formalin-fixed paraffin embedded
FHOD1	Formin homology 2 domain containing protein 1
FMNL1	Formin-like protein 1
FMNL2	Formin-like protein 2
FMNL3	Formin-like protein 3
F-actin	filamentous actin
G-actin	globular actin
GFP	green fluorescent protein
GTPase	guanosine triphosphatase
HPA	Human Protein Atlas
IHC	immunohistochemistry
kDa	kiloDalton
LDH	lactate dehydrogenase
MAL	megakaryocytic acute leukemia protein
MAPK	mitogen-activated protein kinase
MET	mesenchymal-epithelial transition
miRNA	micro ribonucleic acid
mRNA	messenger ribonucleic acid

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PI3-kinase	phosphoinositol-3-kinase
PrEST	Protein epitope signature tag
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
ROCK	Rho-associated protein kinase
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SRF	serum response factor
TBS	Tris-buffered saline
TGF-β	transforming growth factor β
TIL	tumour-infiltrating lymphocyte
TNM	Tumour, Node, Metastasis
VASP	Vasodilator-stimulated phosphoprotein

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, which are referred to in the text by the corresponding Roman numerals, I-IV. In addition, some unpublished results are included.

I. Gardberg M, Kaipio K, Talvinen K, Iljin K, Kampf C, Uhlen M, Carpén O (2010): Characterization of Diaphanous-related formin FMNL2 in human tissues. *BMC Cell Biology* 11:55

II. Gardberg M, Kaipio K, Lehtinen L, Mikkonen P, Heuser VD, Talvinen K, Iljin K, Kampf C, Uhlen M, Grénman R, Koivisto M, Carpén O (2013): FHOD1, a formin upregulated in epithelial-mesenchymal transition, participates in cancer cell migration and invasion. *PLoS One* 2013 8:e74923

III. Gardberg M, Heuser VD, Iljin K, Kampf C, Uhlen M, Carpén O. Characterization of leukocyte formin FMNL1 expression in human tissues. *J Histochem Cytochem* 2014 62:460-470.

IV. Gardberg M, Heuser VD, Koskivuo IO, Koivisto M, Carpén O. FMNL2/FMNL3 formin family members and melanoma: prognostic association and role in cellular characteristics. *Submitted*

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1. INTRODUCTION

Cancer cells evolve from normal cells in a process which is associated with a series of genetic alterations. These genetic alterations are thought to be instrumental for the transformation of cells from benign to malignant. Malignant cells have the capacity to both invade and metastasize. During transformation, dramatic changes in cell morphology usually take place. Cells lose their polarization and tightly controlled morphology. They gain invasive motility by altering the structure that gives cells their shape and mediates their adhesion to adjacent structures – the actin cytoskeleton. Dynamic reorganization of the cytoskeleton is required for formation of protrusions that enhance migration and invasion. Actin filaments further provide a scaffold for motor proteins that create the contractile force for invasive movement.

The filaments of the actin cytoskeleton are formed from monomers, as guided by several actin-polymerizing protein**s** with divergent subcellular location and functional characteristics. For instance, the Arp2/3 complex is active in branched actin filament networks. The formins are another, more recently discovered protein family associated with modulating the actin cytoskeleton. Through formin activity, straight unbranched actin filaments or actin filament bundles are efficiently formed. There are fifteen mammalian formins, many of which have been functionally characterized *in vitro*. Among them, differences in actin nucleating, polymerizing, bundling and even severing activity have been reported. However, the expression of individual formin proteins in human tissues and in cancer is largely unexplored.

2. REVIEW OF THE LITERATURE

2.1. Cancer invasion and metastasis

The multiple and divergent steps of cancer development are conventionally described as a process where six essential tissue maintenance systems are broken down. These maintenance systems are regulatory circuits that control proliferation and tissue homeostasis. Cancer cells must be self-sufficient in growth signals and insensitive to anti-growth signals. These properties give them limitless replicative potential. They are capable of evading apoptosis, they sustain angiogenesis and are able to invade and metastasize (Hanahan, Weinberg 2000). In addition, recently acknowledged concepts in cancer biology are the importance of evading the immune system and reprogramming of energy metabolism (Hanahan, Weinberg 2011).

In a localized tumour the first five capacities have been acquired. Such lesions are usually curable by surgery. The sixth acquired capability, the ability to invade and metastasize, is responsible for a vast majority of cancer related deaths. Tumour cells can escape the parent tissue by suppressing cell adhesion molecules (CAMs), which maintain cell-cell contacts and mediate anti-growth signals. The most widely observed CAM altered in cancer involves the loss of E-cadherin, a homotypic cell-cell interaction molecule expressed in epithelial cells. E-cadherin is crucial for the maintenance of adherens junctions that form epithelial sheets (Canel et al. 2013). Along with the loss of cell-cell contacts, a shift in integrin expression from one type to another can favor adhesion to other stromal components than the ones present in intact basal lamina.

A second group of molecules that can facilitate cell movement through the extracellular matrix (ECM) in surrounding tissue barriers are proteases. Protease activity around invading cells can either be increased by tumour cells themselves or surrounding inflammatory cells. The proteolytic breakdown of tissue barriers such as basal lamina, stroma, lymphatic and blood vessel walls promotes invasive migration and subsequent colonization of distant tissue sites, i.e., metastasis (Murphy, Nagase, 2008, Friedl, Alexander 2011).

Progression of a tumour from local to metastasized involves two steps: firstly, spread of tumour cells (this involves invasive migration, proteolysis of ECM, spread through lymphatic vessels or blood vessels and subsequent extravasation) and secondly, colonization of a faraway metastatic site. This process requires adaptation to a completely new environment. Such adaptation is possible due to that malignant cells can evolve through mutation and clonal selection. Clonal selection of cancer cells best adapted for growth in a new environment is essential in development of metastasis. Further complexity to tumour biology is due to the plasticity of tumour cells: cancer cells are able to utilize differentiation programs designed for developmental processes and regeneration in order to shift between migratory and tumour forming adherent state (Klein 2013).

2.2. Epithelial to mesenchymal transition (EMT)

Epithelium is a basic tissue type, which covers surfaces and cavities in the human body and forms glands in different organs. Developmentally epithelia evolve from either the ectoderm or endoderm, which are the inner and outer layers of the very early three-layered embryo. The ectoderm and endoderm are separated by the mesoderm.

Epithelia specialize to have protective, secretory and absorptive functions depending on their anatomical localization. Accordingly, they have great morphological diversity. At the cellular level they still have many common structural properties. Epithelia are composed of cobblestone-like sheets of polygonal cells, which have abundant cell-to-cell adhesions. This architecture leaves little or no space between cells. Another common property is that epithelial cells are polarized; the deepest layer of cells lies on a basement membrane. Epithelia are generally devoid of vasculature. This means that they are dependent on diffusion from tissues underneath for nutrients.

Mesenchymal tissues, on the other hand, are developmentally derived from the middle layer of the early embryo, the mesoderm. The embryonic mesoderm consists of an abundant ECM with spindle-like bipolar cells. The cells are loosely arranged and separate from each other. In contrast to epithelial cells, the mesenchymal cells are highly motile. During organogenesis, these cells migrate and differentiate to form the lymphatic and circulatory systems as well as connective tissues such as bone, cartilage and muscle.

Epithelial-mesenchymal transition (EMT) is a fundamental process originally recognized in developmental biology. Cells undergoing EMT disconnect from each other and remodel into bipolar cells of mesenchymal morphology. The altered cells are highly motile. During development, this transformation is necessary for embryo- and organogenesis, when cells migrate and differentiate in a distant site. After EMT, cells can obtain their original epithelial properties by going through a reverse transition, mesenchymal to epithelial transition (MET) (Kalluri, Weinberg 2009).

EMT also occurs in situations unrelated to embryology. EMT can be a physiological response to tissue injury, for instance in keratinocytes migrating to heal a wound. It is further seen in pathological conditions, such as fibrotizing diseases, and in carcinoma, where it is seen in invasion and metastasis (Thiery et al. 2009).

The molecular machineries behind EMT are diverse. However, one common event triggered by intrinsic or external cues initiates the EMT process. This common event is the repression of CDH1, the E-cadherin gene, and the disassembly of adherens junctions and other intercellular adhesions. E-cadherin loss is mostly preceded by the activation of one or several transcription factors including Snail1, Snail2, ZEB1, ZEB2, or Twist. These transcription factors bind to the E-cadherin promoter and repress transcription, either directly or indirectly (Qin et al. 2012, Zheng, Kang 2013). The subsequent loss of E-cadherin, together with appearance of mesenchymal markers such as vimentin, N-cadherin and fibronectin are considered as the molecular markers of EMT. In cancer, EMT as a phenotypic or molecular

event may be partial and reversible, histopathologically observable at the invasive edge of tumours (De Craene, Berx 2013).

2.3. The actin cytoskeleton

The actin cytoskeleton is a fine-structured cellular network of filaments composed of actin polymers. Actin polymers, in turn, are formed from actin subunits called globular actin or G-actin. The actin cytoskeleton has many tasks. It gives cells structural support, mediates adhesion and forms the skeleton of cellular protrusions that are necessary for migration. Actin filament formation occurs spontaneously in the presence of G-actin. The formation of a new actin filament begins with two or three actin monomers forming an actin nucleus. This step is kinetically unfavorable. The nucleus is unstable, and quickly dissolves without the presence of actin nucleating factors. Actin nucleation factors shift this balance by bringing together actin monomers and stabilizing the actin nucleus. Once the rate-limiting nucleation has occurred, filament elongation can take place spontaneously. The actin filament is a polarized structure, with a pointed and barbed end (Figure 1 A).

Once an actin nucleus has formed, actin monomers are added quickly at the barbed end, but slowly at the pointed end. Thus, the rate of spontaneous elongation is limited by the availability of free actin monomers. In cells, most of the monomeric actin pool is bound to profilin, an actin binding molecule that inhibits spontaneous actin nucleation and elongation. Profilin-bound actin monomers are utilized by nucleation and elongation factors, which shifts the balance toward nucleation/elongation factor controlled actin assembly. Promoting further complexity to the actin cytoskeleton modulating protein machinery are molecules that are able to terminate assembly. Capping proteins can bind to the barbed end of actin filaments, preventing elongation factors from attachment. Once actin filaments are formed, they form higher-order structures by a process of bundling. This step is promoted by actin cross-linking proteins that bind to several actin filaments. Disassembly of actin filaments is crucial for efficient remodeling and recycling of actin monomers. This step is promoted by another group of actin modulating proteins, the actin severing proteins (Insall, Machesky 2009, Nurnberg, Kitzing & Grosse 2011) (Figure 1 B).

In cell migration, localized polymerization of actin filaments is required for cell shape changes and for creation of a scaffold for motor proteins to attach to. Furthermore, actin filaments participate in adhesion through a multitude of membrane-associated proteins. By influencing adhesion and migration through actin structures, actin modulating proteins form a molecular basis for tumour dissemination (Pollard, Cooper 2009). The actin cytoskeleton basically has two architectural forms: it forms either branched filament networks or long unbranched filaments. Instrumental for the formation of the branched network is the actin nucleating Arp2/3 complex. It is needed for formation of new actin filaments that branch from existing filaments. Polymerization and growth of long unbranched actin filaments, which can be crosslinked together to actin bundles and stress fibers, is mediated by several actin elongation factors. The most abundant of these are the formin family of proteins

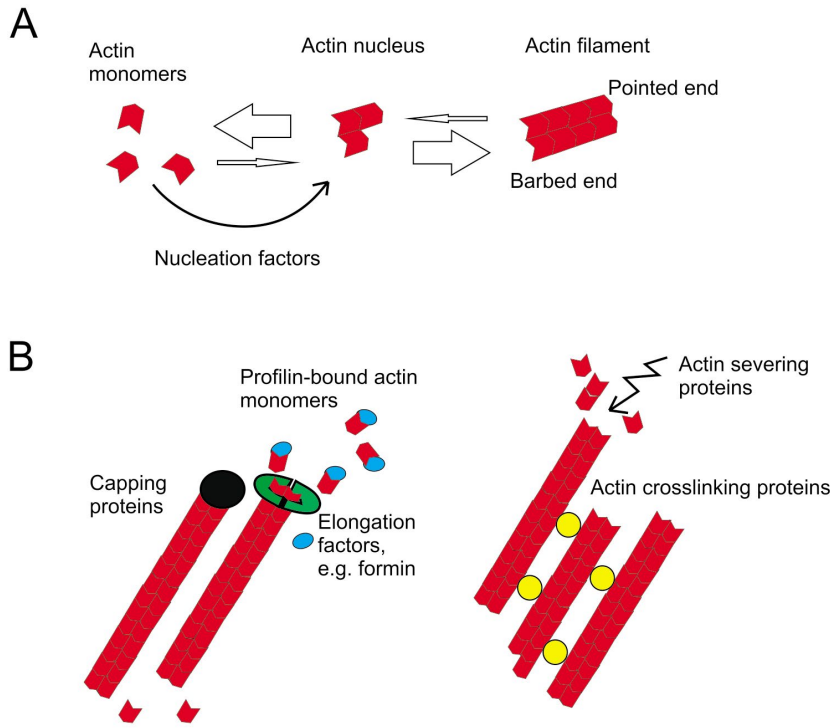

Figure 1. Actin filament initiation, elongation and modification by different types of modulating proteins. A) Dimerization and trimerization is unfavorable, unless nucleating factors are present. Subsequent addition of actin monomers is more favorable. Actin filaments are polarized structures, with a barbed and pointed end. Polymerization is more rapid at the barbed end. B) Barbed end elongation can be stopped by capping proteins, or alternatively be facilitated by engagement of elongation factors, such as formins. Elongation factors utilize profilin-bound actin monomers for filament elongation. Actin crosslinking proteins bind to actin filaments whereby actin bundles are formed. Disassembly of filaments is enhanced by severing proteins.

(discussed in detail below). A unique property of formins is the ability to both nucleate and elongate actin filaments. Formins elongate actin filaments at their barbed ends. Elongation by formins is very efficient, since the formins stay attached to the filament, recruiting and adding actin monomers, while it is elongating. Not only do formins promote filament elongation but also prevent binding by other capping or severing molecules (Randall, Ehler 2014).

A migrating cell has many actin-based, membrane-associated structures. These structures, their principal actin morphology and associated actin modulating proteins are schematically presented in Figure 2. A characteristic structure in migrating cells is the leading edge, a three-dimensional cellular protrusion at the front of a migrating cell. In most cultured cell types the leading edge is called a lamellipodium, referring to its veil-like structure. Other actin-based membrane structures are filopodia, invadopodia and blebs, designations based on their distinctive and characteristic shapes. Cell adhesion is further associated with focal adhesions, which are anchoring, multi-protein structures that mediate force generated

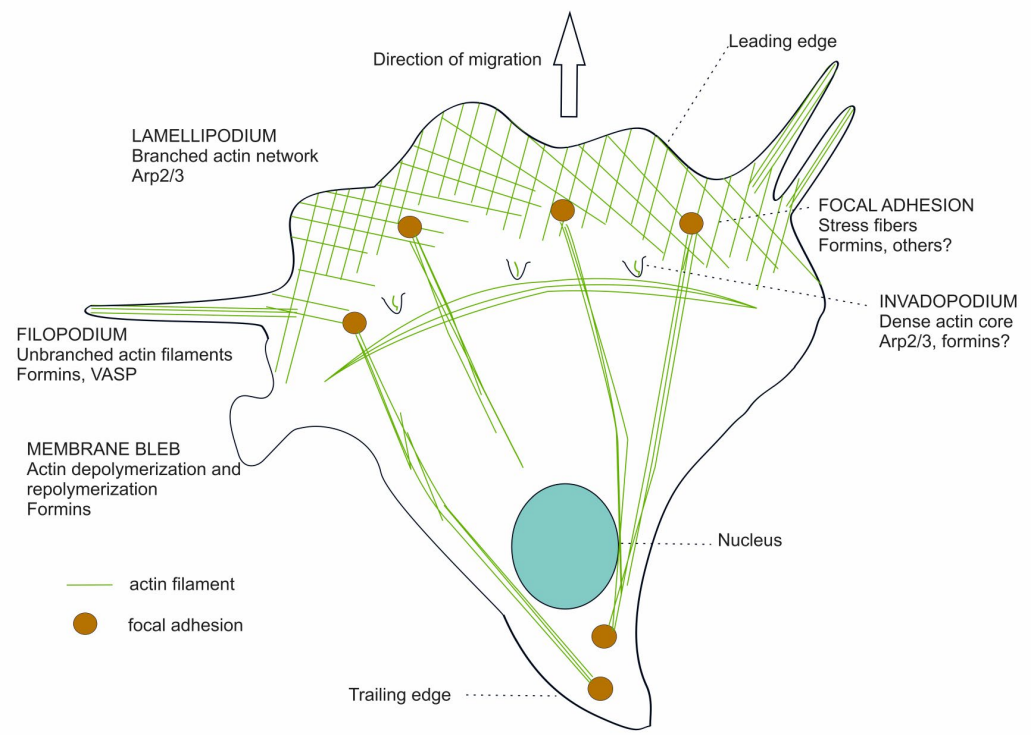

Figure 2. Typical structures encountered in a migrating cell. The predominant actin filament morphology or modulation is mentioned, as well as the principal nucleation factor associated with the structure.

by contractile stress fibers against the growth or culture substratum. These structures are involved in migration and invasion and their distict phenotype varies according to cell type and surrounding environment. The principal coordinators of these cell protrusions are the Rho GTPases. They are small signaling molecules that activate different actin modulating proteins in a spatially controlled manner. There are 20 mammalian Rho GTPases. In their activated state they orchestrate the formation of different cellular structures. (Bravo-Cordero, Hodgson & Condeelis 2012). They bind to specific downstream effectors, and function as switches that trigger actin polymerization in defined cell compartments, enabling the formation of secondary structures discussed in detail below. Rho GTPases Rac1 and Cdc42 for instance induce formation of lamellipodia by activating the Arp2/3 complex and formation of filopodia by activating formins, respectively. RhoA, on the other hand, contributes to formation of blebs (Ridley 2006, Fackler and Grosse 2008). Upregulation of individual Rho GTPases have been reported in many forms of cancer (Vega, Ridley 2008).

Cancer cell invasion involves detachment from its original environment, migratory activity and invasion. In a simplified model, cancer cell invasion occurs in three morphological and molecular modes depending on the cell type and tumour environment. These include ameboid, mesenchymal and collective modes of invasion. Such characterization is, however too constrictive since cancer cells are very adaptive. This property commonly recognized as

plasticity enables switching from one mode to another quickly depending on the tumour environment. The simplified model does however have the advantage of describing morphologically and mechanistically different ways of cancer cell dissemination.

The ameboid form of motility refers to tumour cells that have a roundish morphology and, relying on actomyosin contractility, crawl and squeeze through the surrounding extracellular matrix. Examples of cell types that are traditionally considered to utilize ameboid migration are normal leukocytes and malignant melanoma cells. Ameboid motility produces high velocity, and does not principally require proteolytic activity (Nurnberg, Kitzing & Grosse 2011).

Adaption of migratory behavior characterized as mesenchymal migration refers to individually moving spindle-shaped cells that move directionally and continuously degrade the extracellular matrix at their leading edge. The forward push of the edge is created by the elongation of actin filaments against the leading edge. New contacts form with the extracellular matrix at the leading edge, followed by detachment from its anchoring point and tail retraction. In two-dimensional cell culture, cells exhibiting mesenchymal migration form lamellipodia in the direction of movement. Degradation of the ECM occurs at small yet essential actin-rich protrusions at the ventral surface of cells. In these protrusions, called invadopodia, proteolytic enzymes are secreted and activated.

Collective mode of cancer invasion denotes migration of a group of cells that maintain their cell-to-cell contacts. This form of invasion has been observed in many cancers. In these instances there is not always EMT in separate cells of the group. Invasive capacity is dependent on degradation of the ECM. It is achieved either by the cancer cells at the invading front of the cell group, or by cancer associated fibroblasts (Gaggioli 2008).

Many cell types, regardless of mode of invasion, additionally form actin-rich protrusions called filopodia. Filopodia are highly dynamic needle-like protrusions of the plasma membrane that probe the environment for cues such as the presence of neighboring cells, and sense matrix composition and chemotactic stimuli (Arjonen, Kaukonen & Ivaska 2011). They also harbor integrins in distinct matrix contact sites called focal complexes. They are adhesion sites that gradually mature into focal adhesions. Focal adhesions are large molecular complexes that link cells to the extracellular matrix, mediating both mechanical force and signaling events (Schafer et al. 2010). Filopodia contain long parallel actin filaments. Several formins participate in the formation of filopodia, and are accordingly encountered at their tips (Mellor 2010).

2.4. Formins

2.4.1. The formin protein family

Formins are a family of proteins conserved in all eukaryotic organisms. They are large multi-domain proteins defined by the presence of a highly conserved formin homology 2 (FH2) domain. This domain mediates dimerization of formins and their binding to actin filaments.

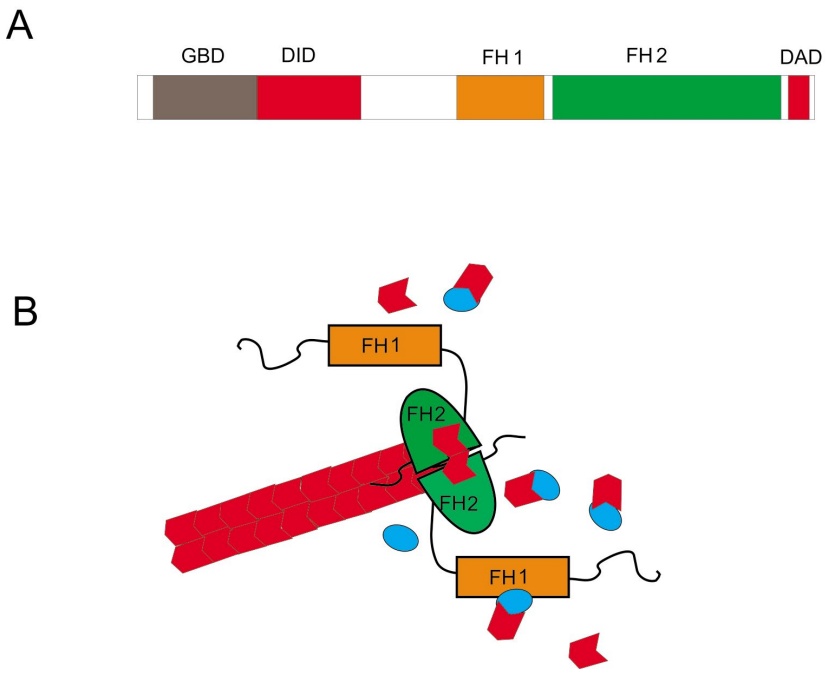

Figure 3. Schematic representation of a formin molecule and formin-mediated actin filament assembly. (A) The domain arrangement that characterizes DRF family monomers. GBD, GTPase binding domain; DID, diaphanous inhibitory domain; FH1, formin homology domain 1; FH2, formin homology domain 2; DAD, diaphanous autoregulatory domain. (B) The FH1 domains are thought to reel in actin via their interaction with profilin, while the FH2 domains are responsible for creating doughnut shaped homodimers in which actin nucleation can occur. Actin monomers are shown in red, profilin in blue, the FH1 domain in orange and the FH2 domain in green. Modified from Randall and Ehler 2014.

It is instrumental in actin filament elongation, and protects them from capping proteins. The FH2 domain of some formins is further capable of nucleating formation of new actin filaments from G-actin. Located N-terminal to the FH2 domain there is a formin homology 1 (FH1) domain in almost all formins. It mediates the recruitment of profilin-bound actin monomers for the growing actin filament (Higgs 2005). Adjacent to and downstream of the FH1 and FH2 domains, there is less homology of formins, and it seems that these more divergent areas are responsible for subcellular targeting.

Besides FH1 and FH2 domains, formins also contain various types of regulatory domains important in conformational autoinhibition and activation. Based on their presence or absence formins can be divided into two subgroups. They are diaphanous related formins (DRFs) and the non-DRFs. The most studied group is the DRFs, which are direct effectors of Rho GTPases. They contain several regulatory domains: a GTPase binding domain (GBD) and a diaphanous inhibitory domain (DID) close to the N-terminus, and a small diaphanous autoregulatory domain (DAD) near the C-terminus. DAD and DID can interact with each other and mask the FH2 domain, leading to an inactive state of the DRF. In most cases, this prevents actin nucleation and elongation. Binding of a specific Rho GTPase to the GBD

leads to release of this autoinhibition. This involves opening up the ring-like inhibitory conformation and uncovering of the functional FH2 domain.

The domain structure of DRFs and their mechanism of actin filament elongation are illustrated in Figure 3. The regulation of non-DRFs, which lack identifiable N-terminal GBD and DID domains, is less well characterized (Goode, Eck 2007).

There are fifteen formin genes in mammals (Higgs, Peterson 2005). Several of them give rise to multiple splice variants (Table 1). This large number suggests expression in different cell types and involvement in divergent functions. In fact, studies on mammalian cells have shown that in various cell types several formins are co-expressed. Individual formins seem to have different subcellular locations and have functions that even counteract each other. Functions of formins include formation of many cellular protrusions, stress fiber formation, migration, adhesion and development (Faix, Grosse 2006). Several formins have also been shown to influence transcription. The formin-induced polymerization or stabilization of actin fibers leads to a decrease of cytoplasmic G-actin, which in turn releases MAL for translocation to the nucleus. MAL is a co-activator of the transcription factor SRF which, in turn, activates the transcription of many cytoskeleton-associated genes. Such transcriptional regulation has been shown for formins FHOD1 and DRF1 (Westendorf 2001a, Copeland, Treisman 2002).

Table 1. Human DRF- and non-DRF formins and their genes.

	Formin/alias	Full name	Gene
DRFs	DAAM1	Dishevelled associated activator of morphogenesis 1	DAAM1
	DAAM2	Dishevelled associated activator of morphogenesis 2	DAAM2
	DRF1/DIAPH1	Diaphanous-related formin-1	DIAPH1
	DRF2/DIAPH2	Diaphanous-related formin-2	DIAPH2
	DRF3/DIAPH3	Diaphanous-related formin-3	DIAPH3
	FHOD1/FHOS	FH1/FH2 domain-containing protein 1	FHOD1
	FHOD3	FH1/FH2 domain-containing protein 3	FHOD3
	FMNL1/FRL1	Formin-like protein 1	FMNL1
	FMNL2/FRL3/FHOD2	Formin-like protein 2	FMNL2
	FMNL3/FRL2	Formin-like protein 3	FMNL3
non-DRFs	Delphilin	Delphilin	GRID2IP
	Formin-1	Formin-1	FMN1
	Formin-2	Formin-2	FMN2
	Inverted formin-1	Inverted formin-1	INF1
	Inverted formin-2	Inverted formin-2	INF2

Recent findings indicate that formins are present and involved in actin filament formation also in cell nuclei. The SRF-mediated effect on transcription may partly be due to a decrease in nuclear G-actin caused by formin activity (Baarlink, Wang & Grosse 2013).

Several formins have another common function, which is regulation of microtubules in both interphase and mitotic cells: they are capable of interacting with microtubules, probably through mechanisms that are independent of actin dynamics. The effect of formins on

microtubules is mainly stabilization, although also more specific roles have been found, such as participation in centrosome polarization in T lymphocytes (Bartolini, Gundersen 2009). Cellular processes that have been linked to formin activity are summarized in Table 2.

Table 2. Basic cellular processes that are regulated by formins and examples of implicated formins.

Process	Formin
Cell division	DRF2, FMN2
Migration	FMNL2, FHOD1
Adhesion	FMN1
Phagocytosis	FMNL1, DRF1
Transcription	FHOD1, DRF1
Microtubule stabilization	INF1, DRF1, DRF3

2.4.2. Formins in human disease

Since the discovery of formins, several hereditary or congenital diseases have been mapped to formin genes. Furthermore, some of formins have been implicated in malignancy. Formins with demonstrated or suggested disease associations are presented in Table 3.

The first hereditary disease discovered to be associated with a mutation in a formin gene was autosomal non-syndromic deafness (DFNA1). DFNA1 is inherited as autosomal dominant, and leads to progressive hearing loss after the age of 10, and bilateral deafness by the age of 30. DFNA1 families have a frameshift mutation in the DIAPH1 gene, which is suggested to interfere with the maintenance of the cytoskeleton of the inner ear hair cells (Lynch et al. 1997). Soon after the discovery of the DIAPH1 mutation in DFNA1, another possible formin-related disease was discovered: a family with premature ovarian failure (POF) was found to carry translocation that disrupts the DIAPH2 gene (Bione et al. 1998).

In families with a kidney disease, focal segmental glomerulosclerosis (FSGS), the affected family members have been found to carry dominantly inherited mutations in the INF2 gene (Brown et al. 2010, Boyer et al. 2011a). INF2 mutations can also cause a phenotype with FSGS combined with Charcot-Marie-Tooth neuropathy, a syndrome with chronic peripheral motor and sensory neuropathy (Boyer et al. 2011b).

A 3.9-Mb deletion in the long arm of chromosome 2 which includes the FMNL2 gene is linked to short stature, mental retardation and precocious puberty, suggesting important roles for development (Lybaek et al. 2009). Although the evidence of causal effect in this case is lacking (several other genes are present in the deletion as well as additional chromosomal abnormalities), two mouse formin knock-out models (FHOD3 -/- and DAAM1 -/-) have resulted in an embryonic lethal phenotype with heart defects (Kan-O et al. 2012, Li et al. 2011). These findings, along with other mouse formin knock-outs with milder phenotypes, indicate that formins are essential in development (Liu et al. 2010).

Much less is known about formins in tumorigenesis and in cancer progression. The profound change of cytoskeletal architecture in malignant cells compared to their normal counterparts

suggests that altered expression and activation of formins may play a role in these processes. During recent years, a few studies have searched for and found formin involvement in malignancy. For instance, FMNL1 expression is increased in lymphoid malignancies (Favaro et al. 2003, Favaro et al. 2006). This has encouraged the study of FMNL1 functions in hematological cells, unraveling diverse specific functions that are discussed below.

Another type of cancer that is of interest in respect to roles of formins is colorectal cancer (CRC). In clinical cancer samples, high FMNL2 expression has been found and seems to correlate with metastasis and occurrence of EMT (Zhu, Liang & Ding 2008b, Li et al. 2010). On the other hand, in hepatocellular carcinoma low FMNL2 expression has been found to correlate with worse outcome of the patients (Liang et al. 2011). Such opposite results could point to tissue-specific or overlapping functions. The expression levels of other formins were not investigated in these studies.

A third member of the formin family that has been linked to cancer is FHOD1. It has been found to mediate EMT-morphology, and to enhance migration and invasion in breast cancer cells (Jurmeister et al. 2012).

Table 3. Human diseases in which formin mutations or altered formin expression have been described.

Disease	Formin
Autosomal non-syndromic deafness	DIAPH1
Premature ovarian failure	DIAPH2
Focal segmental glomerulosclerosis	INF2
Charcot-Marie-Tooth neuropathy	INF2
Developmental disease	FMNL2
Cancer	FMNL1, FMNL2, FHOD1

In this study, we focused on four DRFs: FMNL1, FMNL2, FMNL3 and FHOD1. This choice was made partly because of their functions based on cellular studies. In addition, prior immunohistochemical stainings revealed interesting immunohistochemical profiles in normal tissues and pilot stainings in cancer. These formins have a similar domain organization (Figure 4). They have an N-terminal GBD, followed by DID, FH1 and FH2 domains, and a DAD at the C-terminus (Randall, Ehler 2014).

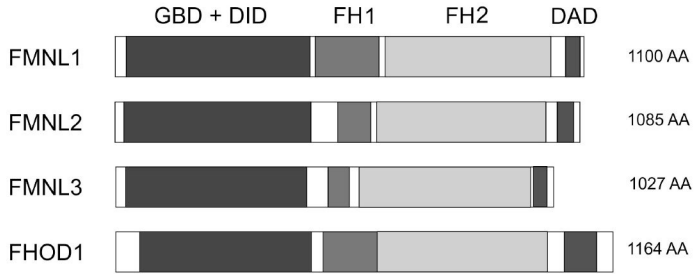

Figure 4. Domain structure of formins FMNL1, FMNL2, FMNL3 and FHOD1. Details in text.

2.4.3. FMNL1

FMNL1 is also called “human leukocyte formin”, due to its restricted expression pattern in hematopoietic and lymphoid tissues such as thymus, spleen and bone marrow (Favaro et al. 2003, Han et al. 2009, Krainer et al. 2013). FMNL1 is overexpressed in human hematological malignancies, more specifically in non-Hodgkin lymphoma and in leukemic cell lines (Favaro et al. 2006, Schuster et al. 2007, Favaro et al. 2013).

There are three FMNL1 isoforms, with C-terminal differences. Of these, isoform 3 (also known as FMNL1γ) has retention of an intron. In this isoform there is no autoinhibition. Myristoylation of the N-terminus is seen in all FMNL1 isoforms and is associated with membrane localization (Han et al. 2009). FMNL1 isoforms 1 and 2 are autoinhibited by interaction between DAD and DID domains. How this autoinhibition is released remains somewhat controversial. FMNL1 interaction with the Rho GTPase Rac1 has been reported, as well as activation by Cdc42 (Yayoshi-Yamamoto, Taniuchi & Watanabe 2000, Gomez et al. 2007, Seth, Otomo & Rosen 2006). Among formins, FMNL1 has weak actin nucleating activity. Its main function seems to be elongation of actin fibers, and it is also capable of bundling actin filaments (Harris et al. 2006).

Recently, many immunologic functions have been attributed to FMNL1. In macrophages, important components of innate immunity, FMNL1 is important for survival and migration (Yayoshi-Yamamoto, Taniuchi & Watanabe 2000). It is also essential for the formation of podosomes, protrusive structures that mediate adhesion in macrophages, and is therefore necessary for migration (Mersich et al. 2010). Further relevance for innate immunity comes from studies in which different forms of phagocytosis have been found to be compromised by silencing FMNL1 (Seth, Otomo & Rosen 2006, Naj et al. 2013). FMNL1 is essential for fundamental cellular processes also in T-lymphocytes. Upon encountering an antigen presenting cell, T-cells form an immunologic synapse in which the actin dynamics is controlled by the Arp2/3 complex. Then, for T-cell receptor-mediated signaling, and to effect cellular cytotoxicity, the microtubule organizing center (MTOC) together with the cytolytic secretory machinery must be redirected to synapse proximity. For this, as well as cytolytic activity of T-cells, the presence of FMNL1 as well as two other formins, DRF1 and INF2 are required (Gomez et al. 2007, Andres-Delgado et al. 2012).

FMNL1 is also required for myofibrillogenesis and cardiac repair in mouse cardiomyocytes (Rosado et al. 2014). The expression of FMNL1 in the developing or adult human heart has not been studied.

Interestingly, FMNL1 is highly expressed in many epithelial cancer cell lines (Colon-Franco, Gomez & Billadeau 2011). This observation suggests that it might be upregulated during malignant transformation. The relevance of FMNL1 for macrophage and leukemia cell adhesion and motility gives clues for potential tasks in carcinoma cells (Favaro et al. 2013, Yayoshi-Yamamoto, Taniuchi & Watanabe 2000). However, detailed studies of FMNL1 in carcinoma cells are lacking. Studies on FMNL1 involvement in clinical cancer have been held back by the lack of antibodies that could be used in formalin-fixed paraffin embedded (FFPE) material.

2.4.4. FMNL2

FMNL2 is one of the more extensively studied DRFs. It has been called a homeostatic formin, due to its ubiquitous expression pattern (Krainer et al. 2013). FMNL2 occurs as two isoforms, A and B, due to alternative splicing. They have minor differences in their C-terminal regions (Katoh, Katoh 2003). FMNL2 is, as most DRFs, subject to negative autoregulation. FMNL2 activation has initially been attributed to the Rho GTPase RhoC (Kitzing et al. 2010). Later studies however, have pointed toward activation by Rho GTPase Cdc42 with participation of N-myristoylation (Moriya et al. 2012). In lamellipodia, FMNL2 is associated with rapidly elongating actin filament branches presumably generated by the Arp2/3 complex. In line with this, FMNL2 knockdown reduces the rate of lamellipodial protrusion (Block et al. 2012).

Formins are generally thought to form homodimers. FMNL2 and the closely related formin FMNL3 can, however, also form heterodimers, as shown in studies in which truncated and full length FMNL2 have been expressed in cultured cells (Vaillant et al. 2008). There is to date no evidence of heterodimerization of endogenous formins, but this finding raises new questions about the regulation of formin activity, since their activity may be dependent on the presence of other formins.

There are previous studies on the presence and role of FMNL2 in clinical cancer. Increased expression of FMNL2 has been seen in colorectal carcinoma and also in their lymph node metastases. Higher levels of expression have been found in metastases than in primary tumours, suggesting that FMNL2 could contribute to dissemination of cancer cells (Liang et al. 2013). The expression of FMNL2 in colorectal cancer tissue correlates with the expression of EMT-markers. Moreover, in vitro studies show that FMNL2 is necessary for TGF-β-induced EMT in CRC cells (Li et al. 2010). Overexpression of constitutively active FMNL2 shows, that FMNL2 is a positive regulator of both cell motility and metastasis in CRC (Zhu et al. 2011). Thus, FMNL2 seems to participate in cellular functions that are linked to cancer progression.

The level of FMNL2 in CRC is higher than in normal colon. The mechanisms that underlie the increased of FMNL2 expression in the malignant tissue are not well known. One possibility could be the downregulation of microRNAs (miRNAs) that target FMNL2 mRNA. This could lead to an increase FMNL2 translation. MiRNAs are small non-coding single strand RNAs with an approximate length of 22 nucleotides. MiRNAs bind to mRNA with complementary sites. Binding initiates the breakdown of the mRNA that leads to inhibition of translation. MiRNAs are in many cases tumour suppressive. Dysregulation of miRNA expression is a common finding in cancer (Suzuki et al. 2013). In fact, FMNL2 has been found to be a target for miRNA-137, which is expressed in normal colorectal mucosa and is downregulated in CRC. In CRC tissues expression of miRNA-137 inversely correlates with the expression of FMNL2 (Liang et al. 2013).

Expression of FMNL2 has also been found in other types of cancer cells, confirming that FMNL2 participates in cancer cell migration in a wider context. In two separate studies FMNL2 siRNA knockdown has been found to reduce invasion of melanoma cells (Kitzing

et al. 2010, Block et al. 2012). These results motivate further studies of FMNL2 expression in melanoma. To date, there are no publications on the expression of any formin in clinical cases of melanoma.

2.4.5. FMNL3

FMNL3 shares extensive homology with FMNL1 and FMNL2 both in structure and biochemical function. It appears to have only weak actin nucleating capacity, but effectively elongates and bundles actin filaments (Kitzing et al. 2010, Vaillant et al. 2008). There have not been FMNL3 antibodies suitable for immunocytochemistry. Thus, transfection with tagged FMNL3 DNA constructs has been the favourite strategy to investigate the cellular roles for FMNL3. Transfected cells expressing FMNL3 form filopodia (Harris et al. 2010). FMNL3 itself is, like FMNL2, targeted to filopodia (Block et al. 2012, Harris et al. 2010). In early studies, FMNL3 full length constructs and FMNL3 constructs lacking autoregulatory and other domains had similar effects, which led to the assumption that FMNL3 is constitutively active (Vaillant et al. 2008). More recent experiments have indicated, however, that this may be an artefact due to an N-terminal tag that might interfere with autoregulation or N-myristoylation. In control experiments employing C-terminally, instead of N-terminally tagged FMNL3, the full length protein induced less filopodia than the FMNL3 constructs lacking autoregulatory domains (Harris et al. 2010). Recently, FMNL3 has been recognized as a downstream target of the RhoGTPase RhoC (Vega et al. 2011). The recognition of a formin as a specific effector of RhoC is of interest, since RhoC is essential for the development of metastasis (Hakem et al. 2005). RhoC has further been implicated in the progression of clinical melanoma, as the expression of RhoC correlates with metastasis and poor prognosis in primary cutaneous melanoma (Boone et al. 2009).

A few studies on cultured cell lines have suggested involvement of FMNL3 in cancer. In a study in which human melanoma cells from mouse xenografts were sorted in differentiated, less motile and undifferentiated more motile groups, increased FMNL3 expression was found among the undifferentiated motile cells (Pinner et al. 2009). Knockdown of FMNL3 reduces migration and invasion in neuroblastoma and prostate cancer cells (Vega et al. 2011, Lynch et al. 2013). An interesting finding in neuroblastoma cells is that miRNA-335, a potent suppressor of invasion and metastasis, directly targets FMNL3 along with ROCK1, MAPK1 and LRG1, which are key players in pathways up-regulated in cancer. MiRNA-335 is suppressed in neuroblastomas with N-myc amplification, presumably due to the binding of N-myc near the miRNA-335 transcriptional start site (Lynch et al. 2013). Future studies are needed to determine if FMNL3 expression is increased in other malignancies with N-myc amplification or overexpression.

2.4.6. FHOD1

FHOD1 was originally identified as a formin highly expressed in the spleen, in vascular cells and in many cancer cell lines (Westendorf, Mernaugh & Hiebert 1999, Wang et al. 2004). Overexpression of full length FHOD1 induces an elongated phenotype and enhances

migration in fibroblasts and melanoma cells. Cells that have been transfected with a truncation mutant FHOD1 that lacks an autoinhibitory domain display prominent stress fibers (Koka et al. 2003).

Biochemically, FHOD1 has turned out to be an outlier among formins, since it lacks both actin nucleation and fiber elongation capacity that otherwise characterizes the formin family. FHOD1 inhibits polymerization actin and simultaneously binds to the side of another filament. Thus, it has the role of an efficient capping and bundling protein of existing actin filaments (Schonichen et al. 2013). FHOD1 also has a unique property in its mechanism of release from conformational autoinhibition. FHOD1 has the typical domain structure of DRFs, including the presence of a GBD. However, it is not activated by Rho GTPase binding. FHOD1 is activated by the RhoA effector Rho-associated kinase (ROCK), which phosphorylates three tyrosine residues in C-terminal region of FHOD1 (Takeya et al. 2008).

Through its effect on the cytoskeleton, FHOD1 influences transcription of other cytoskeleton-associated genes. As FHOD1 caps and stabilizes existing actin filaments, the cellular G-actin pool decreases. This leads to nuclear translocation of MAL and activation of SRF. SRF activates transcription of other cytoskeleton associated genes (Westendorf 2001). FHOD1 also has the ability to modify microtubules, a property first associated with FHOD1 and later found to be a common feature in formins (Gasteier et al. 2005, Thurston et al. 2012).

Recent work has shown that in mouse fibroblasts transfected with a FHOD1 construct, FHOD1 accumulates to integrin clusters that subsequently mature to focal adhesions during cell adhesion and spreading. This accumulation is dependent of Src. Knockdown of FHOD1 impairs cell spreading and focal adhesion formation, as well as migration (Iskratsch et al. 2013) Similar adhesion-related results have been reported in human osteosarcoma cells, in which FHOD1 has been shown to stimulate stress fiber assembly, as well as participate in the formation of focal adhesions (Schulze et al. 2014). The involvement of FHOD1 in ventral stress fiber and focal adhesion formation may partly explain how FHOD1 participates in migration.

MiR200 is a family of miRNAs, which is associated with an epithelial phenotype in cells. Mir200c, a member of this family, directly targets FHOD1 mRNA, reducing FHOD1 expression. Silencing mir200c in human breast cancer cells leads to increased FHOD1 expression, mesenchymal morphology and increased invasiveness. In line with this finding, breast cancer samples have an inverse correlation between FHOD1 and miR200c expression. This finding suggests that FHOD1 expression is regulated by miR200c also *in vivo* (Jurmeister et al. 2012). Interestingly, miR200c silencing mediates EMT or progression in other malignancies as well, such as head and neck squamous cell carcinoma and melanoma (Chen et al. 2013, Liu et al. 2012). However, the direct role of FHOD1 expression in this event has so far only been investigated in breast cancer (Liu et al. 2012, Lo et al. 2011).

2.5. Prognostic clinicopathological parameters in cutaneous melanoma

The incidence of cutaneous melanoma is rising worldwide. In 2012, 230 000 new melanoma cases were diagnosed. The yearly mortality rate is 55 000 (WHO International Agency of Cancer Research, globocan.iarc.fr).

Localized cutaneous melanoma is primarily treated by surgery. For most patients surgery with sufficient margins is curative. However, melanoma may metastasize also in cases where the primary tumour has been small and radically excised. After a period of remission that can last even up to decades, recurrence can occur locally in skin, in lymph nodes or at distant sites, such as liver, lung or brain. Once distant metastases have developed, few treatment options are available and the prognosis is poor. Chemotherapy is of limited effect.

The molecular mechanisms underlying cancerous transformation in melanocytes have been studied intensively. One of the key events is activation of the MAPK signalling pathway. An important member of this pathway is BRAF. Half of primary melanomas harbour an activating BRAF mutation that leads to over-activation of the MAPK pathway. Its presence is a basis of a treatment in which the patient is given vemurafenib, a small molecule inhibitor of BRAF (Chapman et al. 2011). Despite the initial success in shrinking the tumour, patients eventually and almost invariably develop resistance to the drug. Development of resistance could possibly be prevented, by targeting several MAPK signalling molecules from the start; clinical trials combining vemurafenib with MEK inhibitors such as cobimetinib are ongoing (Akinleye et al. 2013).

Clinical prognostic factors that should be considered in the treatment of melanoma are location and stage of the tumour, and age and gender of the patient (Balch et al. 2009, Mandala et al. 2009, Green et al. 2012). Furthermore, several histopathological parameters correlate with outcome. In localized melanoma, these are tumour thickness, presence of ulceration, and mitotic count. The presence of ulceration has predictive value also in melanomas with nodal involvement. Absence of tumour-infiltrating lymphocytes (TILs) predicts sentinel node positivity, *i.e.* presence of melanoma cells in the first lymph node receiving drainage from the tumour area (Balch et al. 2009, Mandala et al. 2009). Recently, in a retrospective study, it has been found that BRAF mutation status may be an additional prognostic factor in localized melanoma (Nagore et al. 2014).

The most powerful predictor of survival in melanoma is clinical stage. The current staging strategy of melanoma is the “2009 Melanoma Staging and Classification” by the American Joint Committee on Cancer (AJCC). The staging is developed based on reports from the literature and multivariate analysis of data from more than 38,000 patients. This system divides melanomas into four clinical stages: stages I and II include localized melanomas, stage III includes cases with regional lymph node metastasis, and stage IV includes those with distant metastasis. Within the stages there are substages that are based on the Tumour, Node, Metastasis (TNM) classification (Table 4). The TNM classification in turn is based on the clinicopathological parameters that have been identified as independent prognostic factors. The histological parameters are thickness of the tumour, presence of ulceration, and mitosis count (Balch et al. 2009).

Table 4. The prognostic factors of melanoma, TNM-staging and the anatomic stage grouping of melanoma. Modified from Balch et al 2009.

T	Thickness	Ulceration/mitoses	Stage
Tis	*In situ melanoma*		0
T1	≤ 1 mm	a: without ulceration, mitoses < 1/mm^2 b: with ulceration or mitoses ≥ 1/mm^2	IA IB
T2	1,01-2 mm	a: without ulceration b: with ulceration	IB IIA
T3	2,01-4 mm	a: without ulceration b: with ulceration	IIA IIB
T4	>4 mm	a: without ulceration b: with ulceration	IIB IIC
N	**No. of metastatic nodes**	**Tumour burden in metastatic node**	**Stage**
N0	0		
N1	1	a: micrometastasis b: macrometastasis	IIIA IIIB
N2	2-3	a: micrometastasis b: macrometastasis c: *in transit* metastases/satellites without metastatic nodes	IIIA/B IIIB/C IIIB
N3	≥ 4 or ≥1 if *in transit* metastases or satellites are present		IIIC
M	**Site**	**Serum LDH**	**Stage**
M0	No distant metastasis	NA	
M1	Distant skin, subcutaneous or nodal metastases	a:normal	IV
M1	Lung metastases	b: normal	IV
M1	All other distant metastases Any distant metastasis	c: normal c: elevated	IV

In the 2009 revision of the previous AJCC classification scheme, the anatomical depth of the tumour (Clark level) was left out since it turned out not to be an independent prognostic factor after mitotic rate was introduced in the classification of T1 melanomas. Recognition of serum LDH level as independent prognostic factor among patients with distant metastasis is another novelty in the 2009 classification. This is taken into account in the TNM classification. However, as the overall prognosis in all patients with distant metastasis remains poor, stage IV is not substaged.

Staging is useful and practical for grouping patients for prognostic and therapeutic purposes. However, based on the available criteria it does not allow prediction which of the patients with a clinically localized melanoma will develop metastasis (stages I-II). Along with the unforeseen emergence of resistance to targeted therapy in BRAF mutated melanomas, the nature of melanoma has led to an active search for additional biomarkers to reliably predict the behaviour and outcome of the therapy. Potential biomarkers could serve several functions in diagnostics and treatment of melanoma: they could be useful in differential diagnosis between nevi and melanomas, recognizing high-risk patients, and development of molecular therapy (Kashani-Sabet 2014).

Gene expression profiling in a large set of melanomas has shown that there are distinct differences in transcription between those with metastasis and without metastasis after four years follow-up (Winnepenninckx et al. 2006). Further studies are needed to find the most useful markers for clinical prognostication.

A study on invasive melanomas has identified EMT-markers N-cadherin and osteopontin as determinants for metastasis (Alonso et al. 2007). The role of EMT in melanoma is debated since melanocytes do not belong to an epithelial lineage. However, they express E-cadherin, which plays a role for contacts with keratinocytes in the basal layer of the epidermis. A recent study has shown that EMT transcription factors are differentially expressed during the course of melanoma. In melanomas, the expression or Snail2 and ZEB2 is reduced together with increased expression of TWIST1 and ZEB1 in correlation with the depth of invasion. These changes are accompanied by a decreased expression of E-cadherin. Similar EMT transcription factor switching occurs when comparing primary melanomas to their metastases (Caramel et al. 2013). The clinical significance of these alterations is currently not known.

3. AIMS OF THE STUDY

Biochemical and functional roles of the formin family of actin polymerization-associated proteins have been extensively characterized. Surprisingly, however, their differential expression and expression levels in tissues and in different cell-types are largely unknown. In this study, we set out to map the expression of formins in normal tissues and in various cancers. This was done in collaboration with the Human Protein Atlas (HPA), which has as one of its aims to produce immunohistochemistry-quality antibodies towards the major isoforms of all proteins encoded in the human genome. Initially the antibodies were at our exclusive use, but were later made available to the public. The specific aims of this study were, by using these novel antibodies:

1. To elucidate the expression pattern of individual formins in normal tissues.
2. To elucidate the subcellular location of formins in various types of cultured cells.
3. To compare expression of formins in normal and malignant tissues.
4. To evaluate whether differential expression of formins in various cell types and in normal versus malignant cells could be utilized as a biomarker for diagnostic or prognostic purposes.
5. To investigate roles of formins in maintenance of cellular morphology, as components of migration machinery, and as elements of invasion and ECM degradation in cultured cells.

4. MATERIALS AND METHODS

The study protocols regarding the procurement and handling of clinical specimens were evaluated and approved by the Joint Committee on Ethics of the University of Turku and Turku University Hospital. For the collection of normal tissue specimens and for establishing cell lines UT-SCC-43A and UT-SCC-43B written consent from the donor was also obtained.

4.1. Cell lines and cell culture (I-IV)

Table 5. Cell lines, respective growth media and origin.

Cell line	Growth medium	Origin	Source (in case of cell lines unavailable from public sources)
A431	DMEM	Squamous cell carcinoma	
Bowes	DMEM	Melanoma	
HaCat	DMEM	Human keratinocyte	
HEK 293T	DMEM	Human embryonic kidney cells	
HMEC	EBM2	Human dermal microvascular endothelial cells	
HUVEC	EBM2	Human umbilical vein endothelial cells	
IGROV1	DMEM	Ovarian cancer	
Jurkat	RPMI 1640	T-cell lymphoma	
K562	RPMI 1640	Erytroleukemia	
MCF-7	DMEM	Breast carcinoma	
MDA-MB-231	DMEM	Breast carcinoma, basal type	
MKN-1	RPMI 1640	Gastric carcinoma	
SK-Mel-28	MEM	Melanoma	
TIME	EBM2	Telomerase-immortalized human microvascular endothelium cell line	
U138MG	DMEM	Glioblastoma	
UT-SCC-43A	DMEM	Oral squamous cell carcinoma (Haikonen et al. 2003)	Prof Reidar Grénman, University of Turku, Turku, Finland
UT-SCC-43B	DMEM	Oral squamous cell carcinoma recidive (Haikonen et al. 2003)	Prof Reidar Grénman, University of Turku, Turku, Finland
43SNA	DMEM	UT-SCC-43A transfected with murine Snail (Takkunen et al. 2006)	PhD Minna Takkunen, University of Helsinki, Helsinki, Finland
WM164	RPMI 1640	Melanoma metastasis	
WM239	RPMI 1640	Melanoma metastasis	

DMEM and MEM culture media were from Invitrogen (Carlsbad, CA), RPMI 1640 from Gibco-BRL (UK) and EBM-2 from Lonza (Basel, Switzerland). All growth media were supplemented with 10 % fetal calf serum (FCS, Gibco), 5 mM UltraGlutamine (Gibco) and antibiotics. The culture media for UT-SCC-43A, UT-SCC-43B and 43SNA were further supplemented with 0.1 mM Non-Essential Amino Acid Solution(NEAA, Gibco). Genetically engineered 43SNA cells were selected with G418 (Sigma, St Louis, MO). The culture media for endothelial cells were supplemented with SingleQuots Kits (Lonza).

For experiments probing for the signaling pathways, the following inhibitors and agents were used: PI3 kinase inhibitor LY294002 (Tocris Bioscience, Bristol, UK) and MEK 1/2 inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA). The efficacy of pathway inhibition was checked by immunoblotting with antibodies to p-Akt and Akt (PI3K signaling) and p-ERK 1/2 and ERK 2 (MAPK signaling) (see table 7 for antibody manufacturers).

4.2. Melanoma patients and tissue samples (IV)

At Turku University Hospital, clinical and histological data of cutaneous melanoma cases have been available in a database dating back to 1990. The patients have given written consent for their data being included. The database also has authorization from The Joint Committee on Ethics of the University of Turku and Turku University Hospital.

The data from the melanoma database was utilized to study the presence and level of FMNL2 expression in melanomas and their relation to clinical course and prognosis. For that purpose, 175 consecutive archived tissue samples and follow-up data of patients with primary invasive cutaneous melanoma AJCC stages I-II operated at the Turku University Hospital 1990-2005 were collected. Clinical stages I and II represent localized melanomas of any thickness without clinical nodal involvement or distant metastases (Balch et al. 2009). The time period was chosen to allow a sufficient follow-up period. At Turku University Hospital, the practice of sentinel node biopsy was initiated in 2001. The cases with detected micrometastases in sentinel nodes were included (postoperative AJCC stage III with nodal involvement, n=11). In cases with occurrence of metastasis during follow-up, also metastatic tissue was included in immunochistochemical analysis in case it was available. This amounted to 34 metastasis samples.

The clinicopathological parameters relevant for prognostication of melanoma (i.e. age, gender, anatomic location, Breslow thickness, Clark level, ulceration, mitosis count, TILs) were recorded. Evaluation of the histological variables was done by at least two pathologists. All patients had undergone wide local excision with histologically confirmed tumour-free margins. During the follow-up, suspected metastases were further investigated and mostly treated by surgery, chemotherapy, interferon or radiotherapy. The follow-up was at the Department of Oncology and Radiotherapy at Turku University Hospital. The most recent follow-up information on the patients was updated from electronic medical records in June 2012. The final follow-up date of each patient was defined as the date of the most recent hospital call or the date of death. The cause and

time of death were obtained from patient records, autopsy reports or from the Statistics Finland's Archive of Death Certificates.

4.3. Other tissues (I-III)

Tissue samples for the study of formin expression in normal tissues were collected prospectively from surgical specimens sent fresh (no fixation) to the Department of Pathology at the Turku University Central Hospital for diagnostic purposes. Within one hour from surgical removal, macroscopically normal tissue areas were sampled. The following tissues were included: adrenal gland, bone marrow, breast, colon, duodenum, oesophagus, kidney, liver, lung, lymph node, mouth, ovary, pancreas, parathyroid gland, parotid gland, peripheral nerve, placenta, prostate, skeletal muscle, skin, small intestine, spleen, stomach, testis, thyroid gland, urinary bladder and uterus.

Specimens from central nervous tissue and cardiac muscle specimens were collected from autopsies. In the laboratory, the specimens were formalin fixed for 24 hours, dehydrated, paraffin-embedded and sectioned.

Paraffin-embedded oral squamous cell cancer samples, triple negative (basal type) breast cancer specimens and melanoma samples were collected from specimens archived at the Department of Pathology at Turku University Hospital.

4.4. Immunohistochemistry, immunocytochemistry and microscopy (I-IV)

Immunohistochemical staining of tissue sections was performed using the peroxidase method, using a LabVision Autostainer device (LabVision/Thermo Fisher Scientific, Cheshire, UK). For antigen retrieval, the slides were treated with citrate buffer (pH 6.0). Endogenous peroxidase was quenched with 3 % H_2O_2 for 10 min. After a 30-min incubation with normal nonimmune serum at 37° C, the sections were incubated at 4° C for 60 min with affinity-purified rabbit anti-formin antibody (Table 6). After washing with PBS, the slides were incubated with BrightVision Poly-HRP-Anti-mouse/rabbit rat IgG (Immunologic, Duiven, Netherlands) for 30 min, rinsed with PBS, followed by 3-3'-diaminobenzidine (Dako, Glostrup, Denmark) for 10 min. The reaction was stopped by rinsing with PBS. Last, the slides were counterstained with Hematoxylin. Individual tissues and cell types were evaluated for negative and positive staining reaction by scoring the staining intensity from 0 (no staining) to +++ (strong staining). Staining intensity in melanomas was evaluated using a scheme where the basal layer of skin keratinocytes served as internal reference. Negative staining was noted as 0, weak staining as 1, moderate staining similar to the basal keratinocytes as 2, strong staining as 3.

Table 6. Formin antibodies used for immunohistochemical stanings. The antibodies were initially provided to us by the HPA project. The same antibodies were later made available to the public from Sigma-Aldrich.

Specificity	Catalogue number	Manufacturer	Dilution
FMNL1	HPA008129	Sigma-Aldrich Corporation, St Louis, MO, USA	1:75
FMNL2	HPA005464	Sigma-Aldrich	1:250 (I) or 1:500 (IV)
FHOD1	HPA024468	Sigma-Aldrich	1:150

Immunohistochemical double-staining employing antibodies to FMNL1 and CD68 was performed by using a Benchmark XT autostainer device (Roche Tissue Diagnostics/Ventana Medical Systems, Tucson, AZ, USA). Antibodies to CD68 (clone PG-M1), obtained from Dako, were used to identify macrophages. Pretreatment was extended cell conditioning solution (CC1), antibody incubations were done at 37°C. Incubation time with anti-FMNL1 was 44 min, followed by denaturation incubation 95°C for 4 min and anti-CD68 for 32 min, followed by an Amplification Kit and counterstaining with hematoxylin for 4 min and bluing reagent for 4 min. For detection and visualization of the bound antibodies, *Ultra*View Universal DAB Detection Kit and *Ultra*View Universal Alkaline Phosphatase Red Detection Kit were used (Roche Tissue Diagnostics/Ventana).

For immunocytochemistry of cultured cells, the cells were cultured on glass coverslips. In some experiments, the coverslips were precoated with gelatin. The cells were fixed at room temperature (RT) with fresh 4 % paraformaldehyde for 15 min. The cells were permeabilized in cold acetone for 5 min. Nonspecific binding was blocked by incubating in 1 % bovine serum albumin (BSA) in PBS for 15 min. Alternatively, permeabilization and blocking were done simultaneously with 3 % BSA, 5 % dry milk, 0.5 % triton X-100 in PBS for 45 minutes. Primary antibodies were applied for one hour (Table 7). Next, incubation with secondary antibodies at 1:500 dilution was performed for one hour. Either Alexa Fluor 546 goat anti-mouse IgG or Alexa Fluor 568 goat anti-rabbit IgG (Molecular probes, Eugene, OR) was used as a secondary antibody. Filamentous actin was visualized by incubation with Alexa Fluor 488-conjugated phalloidin (1:50; Invitrogen) for 1 hour. After each staining step, the cells were washed with PBS three times. The mounting media contained 4',6-diamidino-2-phenylindole (DAPI) for staining nuclei (Vector laboratories, Burlingame, CA).

The sections and stainings were analyzed and images were taken with a Zeiss LSM 510 Meta confocal microscope (I, II; Carl Zeiss, Göttingen, Germany), an Olympus BX60 fluorescence microscope (III, IV; Olympus microscopes, Essex, UK) and with a Zeiss LSM780 confocal microscope (III; Carl Zeiss). The images were analyzed with ImageJ software for quantification of changes in intensity of fluorescent phalloidin staining and changes in cell area and shape.

Table 7. Primary antibodies and dilutions used in immunocytochemistry (ICC) and/or western blotting (WB).

Specificity	Host, clonality	Catalogue nr	Manufacturer	Dilution ICC	Dilution WB
FMNL1	Rabbit polyclonal	HPA008129	Sigma-Aldrich	1:200	1:1000
FMNL2	Rabbit polyclonal	HPA005464	Sigma-Aldrich	1:150-200	1:1000-2000
FHOD1	Rabbit polyclonal	HPA024468	Sigma-Aldrich	1:250	1:2000
FHOD1	Rabbit polyclonal	ABS53	Millipore, Bedford, MA	1:250	1:2000
FMNL2+3	Mouse monoclonal	ab56963	Abcam, Cambridge, MA	1:200	1:1000
α-tubulin	Mouse monoclonal	13-8000	Invitrogen		1:20000
E-cadherin	Mouse monoclonal	610405	BD Biosciences, San Jose, CA		1:2000
N-cadherin	Mouse monoclonal	610921	BD Biosciences		1:2000
Cortactin (p80/85)	Mouse monoclonal	05-180	Millipore	1:200-300	
Ki-67 clone MIB-1	Mouse monoclonal	M7240	Dako	1:100	
CD138 clone B-A38	Mouse monoclonal		Ventana Medical Systems, Tucson, Arizona, USA		
ERK2	Rabbit polyclonal		Santa Cruz Biotechnology, Santa Cruz, CA		1:1000
Phospho-p44/p43 ERK ½	Rabbit polyclonal		Cell Signaling Technology		1:1000
Phospho-Akt	Rabbit monoclonal		Cell Signaling Technology		1:1000
Akt	Rabbit monoclonal		Cell Singaling Technology		1:1000

4.5. Transfections

4.5.1. Small interfering RNAs (I-IV)

For post-transcriptional transient silencing of the expression of FMNL2, FMNL3 and FHOD1 in cells *SMART*pool siRNAs (Dharmacon Research, Boulder, CA) were used. Non-targeting Pool siRNA (Dharmacon) was used as a control. Transfection of the siRNAs to cultured cells was done by using a Dharmafect 1 or Dharmafect 4 transfection reagent, according to manufacturer's instructions. Silencing efficacy was determined by analyzing lysates of the transfected cells by western blotting 72 hours after transfection.

4.5.2. Transfection of FMNL1 constructs (III)

The pCMV-sport6 vector containing the C-terminal half of human FMNL1 (BC021906.1, denoted FMNL1') was obtained from the Genome Biology Unit, Institute of Biotechnology,

University of Helsinki, Finland. FMNL1' was subcloned into the pEGFP-C1 vector (BD Biosciences Clontech, Palo Alto, CA) which encodes a green fluorescent protein (GFP) tag. Cultured cells were transfected with the recombinant pEGFP-FMNL1', with an empty pEGFP-C1 control vector (Clontech) and recombinant pCMV-sport6-FMNL1' using Lipofectamine 2000 (Invitrogen). The cells were fixed 48 h after transfection for immunocytochemical staining and/or immunofluorescence microscopy.

4.6. Western blot analysis

Cells were harvested and lysed in in RIPA buffer supplemented with protease inhibitors. Samples were normalized for protein concentration and equal amounts of material in Laemmli sample buffer were subjected to SDS-PAGE, transferred to nitrocellulose filters and immunoblotted with a diluted primary antibody in BSA/TBS/Tween 0,1% 60 min, followed by secondary antibody, HRP-conjugated swine anti-rabbit (Dako). Bound proteins were detected by enhanced chemiluminescence. Equal protein loading was checked using tubulin as a reference.

4.7. Northern blot analysis (I, II)

Total RNA was from cultured cells was extracted by using Trizol Reagent (Invitrogen), according to manufacturer's instructions. The concentration of RNA was determined spectrophotometrically and the quality analyzed by evaluation of the integrity of 28S and 18S ribosomal RNAs upon agarose gel electrophoresis followed by ethidium bromide staining. Northern blotting, hybridization and the detection of the hybridized probe were performed according to DIG Application Manual for Filter Hybridization (Roche Applied Science, Mannheim, Germany) with some modifications. Briefly, 1.0 μg of the purified total RNA was size-fractionated on a denaturing 1.3% agarose/formaldehyde gel which was stained with ethidium bromide. RNA was then transferred to a positively charged nylon membrane (Roche Applied Science) by capillary transfer. Before transfer the gel RNA was treated with 50 mM NaOH. The membrane was UV-crosslinked (UV Stratalinker™ 2400, Stratagene, LaJolla, CA) and quantity of transferred RNA samples was monitored by staining with ethidium bromide. After prehybridization for one hour in SDS hybridization buffer [50 % deionized formamide, 5xSSC, 50 mM sodium phosphate pH 7, 7 % SDS, 0.1 % N-lauroylsarcosine, 2 % Blocking Reagent (Roche Applied Science)] at 50 °C, the membrane was hybridized in the same solution overnight at 50 °C with a denaturated DIG-11-UTP labelled human FMNL2 or FHOD1 RNA probe (DIG RNA Labelling Kit SP6/T7, Roche Applied Science). The probes were selected to match the sequences corresponding to the peptides used for production of the HPA FMNL2 and FHOD1 antibodies (FMNL2: NCBI RNA RefSeq clone GenBank: NM_052905.3, bases 1544 – 1871, FHOD1: NCBI RNA RefSeq clone GenBank NM_013241.2, bases 1517-1810). The final washing conditions were 0.5 x SSC, 0.1 % SDS at 65°C for two times 15 min. The hybridized probe was visualized with alkaline

phosphatase labelled Anti-Digoxigenin Fab Fragments (Roche Applied Science) and CDP-*Star* chemiluminescent substrate (Roche Applied Science).

4.8. Bioinformatics (I-III)

The GeneSapiens database (www.genesapiens.org) was utilized to study the FMNL1, FMNL2, FMNL3 and FHOD1 mRNA expression across all normal and neoplastic human tissues (Kilpinen et al. 2008). The samples included in this database have been analyzed on the Affymetrix platform and due to unique normalization and data quality verifications, gene expression profiles collected from different studies can be combined to generate an overview of the expression profile in human tissues. The database was used to compare mRNA expression with immunohistochemical staining patterns for validation of antibodies, and for searching potential upregulation of formin transcription in various forms of cancer.

4.9. mRNA microarray (II)

For gene expression profiling in cell lines UT-SCC-43A, UT-SCC-43B and 43SNA the Illumina HumanHT-12 v4 Expression BeadChip available at the Finnish Microarray and Sequencing Centre, Turku Center for Biotechnology was used. For that purpose total RNA was extracted from cultured cells using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. It was then processed to cDNA by using cDNA synthesis kit (Applied Biosystems, Foster City, CA). The array-based data on cell lines has been loaded online to ArrayExpress where it is accessible to the public (accession number E-MTAB-1420).

4.10. Quantitative reverse transcription polymerase chain reaction

Gene expression analysis by mRNA microarray analysis provided information on formin mRNA expression in various cell types (above). Based on this data, TaqMan qRT-PCR performed by using an Applied Biosystems 7900HT instrument (Finnish Microarray and Sequencing Centre) was used to specifically determine the level of expression of the following formins: DIAPH1, FHOD1, FHOD3, and FMNL3. Probes and primers were from Oligomer, Helsinki, Finland. Quantitation was carried out with RQ manager 1.2 software using the ΔΔCT method (Applied Biosystems). Three replicate samples were studied for detection of target mRNA expression. β-actin was used as an endogenous control. The quantities were expressed as an n-fold difference in cell lines UT-SCC-43B and 43SNA relative to the UT-SCC-43A cell line. The results are presented as means ±SD. Statistical analyses were performed using Student's *t*-test and Pearson's linear association test, unless otherwise indicated. Gene specific primers were: DIAPH1 (forward 5'-cagtcaggggcagcattc-3' , reverse 3'-cactgttcttggacaccttgg-5'), FHOD1 (forward 5'- cctcagctgacacctccag-3', reverse 3'-cagcgcaacctgcttctc-5'), FHOD3 (forward 5'-ggccaggttggaaaggtc-3', reverse 3'-tctgctgccagtgactcttg-5'), FMNL3 (forward 5'-ccatcgaggacatcatcaca-3', reverse 3'-ccgagagggtctcagtgg-5').

4.11. Functional assays (II-IV)

The effect of silencing the expression of formins by siRNA transfection on organization of cellular F-actin was studied by using Alexa 488-conjugated phalloidin staining to visualize F-actin. Intensity of staining in FHOD1 silenced and control UT-SCC-43B cells by using confocal images of 20 cells for each experiment. Mean fluorescent intensities were measured from the cell cytoplasm. Image J 1.42q (NIH, USA) software was used for image analyses.

The effect of siRNA-mediated silencing of formins on wound healing was studied by using cells cultured on gelatine-coated 24-well-plates. A wound was created by manually scraping the monolayer of cells with a 10 µl pipette tip. The plates were washed with PBS, and a filtered complete growth medium was added. Phase contrast images were taken at 10 min intervals. ImageJ software was used for measuring the wound area at 1 h intervals for 24 hours.

Alternatively, cell migration was studied by growing cells in a poly-L-lysine (Sigma-Aldrich) coated 96-well ImageLock microplate (Essen BioScience, Ann Arbor, MI) in a standard CO_2 incubator. Wounds were made by using the 96-pin Wound Maker tool. After washing with PBS to remove the detached cells, the cells were kept in complete medium placed in the IncuCyte FLR Microscope system (Essen BioScience). The images were acquired automatically from the incubator at 1 h intervals. The kinetics of the wound closure was analyzed by IncuCyte™ software.

Also invasive properties of cells were studied by using the IncuCyte™ real-time imaging system. Cells were allowed to attach to 96-well ImageLock plates, in this case coated with 50 µl 10% Growth Factor Reduced Matrigel (BD Biosciences). A wound was scratched across each well with the Wound Maker (Essen BioScience) and the growth media was removed. The cells were then covered with 50 µl 25% Matrigel in normal growth medium. Incubation at 37˚C for 2-3 hours followed to allow gelling, after which 100 µl of growth medium was carefully added to each well. The rate of invasion (wound closure through the matrix) was monitored hourly for 72 hours by using Incucyte imaging software. Invasion efficiency was determined as percentage of the relative wound confluence compared to respective negative control (regarded as 100%).

Another migratory response assay was Transwell migration Assay. For that, 900 µl of culture medium containing 10 % FBS was added in wells of a 24 well plate. A Boyden chamber (Millipore) was placed in each well. The upper chambers were loaded with 200 µl of a suspension of cells. The cells were allowed to migrate for 48 h. Non-migrating cells were removed from the upper chamber with a cotton swab. Migrating cells adherent to the underside of the filters were fixed with 4% paraformaldehyde, and stained with 0.5% crystal violet (Thermo Fisher Scientific, Waltham, MA) solution for 10 min. Next, the filters were washed with PBS and allowed to dry overnight. The migrated cells were photographed at 4 random sites under light microscopy at a magnification of 100X. In an alternative strategy to determine the amount of cells on the filter, 1% SDS (400 µl/well) was added to lyse the crystal violet stained cells. The plate was agitated on an orbital shaker for 30 min until

the colour of the solution was uniform. The absorbance of each sample was measured photometrically at 570 nm on a Multiskan FC Machine (Thermo Fisher Scientific). The experiment was repeated three times.

The effect of FHOD1 silencing on extracellular matrix (ECM) degradation and invadopodia formation was studied by, first, transfecting the cells with non-coding siRNA and FHOD1 siRNA as described above, and then plating them on 8-well glass slides pre-coated with Cy3-labeled gelatine according to manufacturer's instructions (QCM Gelatine Invadopodia Assay (Red), Millipore). After incubation for 24 h cells were fixed with 4 % paraformaldehyde and incubated for immunofluorescence microscopy with anti-cortactin or fluorescent-labelled phalloidin. Degradation of ECM was visible as dark foci devoid of fluorescence. Cavities produced by cells were photographed and resorption areas per cell (px) were measured by using ImageJ software. For each group, the mean extent of degradation by 100 cells from eight wells was compared.

To evaluate the extent of invadopodia formation, cells were screened for actin-rich comet- or ring-like protrusions with positive cortactin staining at the ventral surface (Takkunen et al. 2010). The percentage of cells containing invadopodia was counted from 10 different fields in each group.

4.12. Statistical analysis (II-IV)

For the analysis of the results of all cellular experiments, intergroup differences were tested for significance using Student's *t*-test, ANOVA or repeated measures ANOVA test was used.

In studies employing clinical and experimental data on melanomas, the distribution of categorical variables was given as frequencies and percent, and, in case of continuous variables, as means. Also ranges of values were used. Cox's regression analysis was used to estimate the effect of studied variables on putative prognostic factors for disease-free survival and recurrence-free survival. Disease-free survival was from the date of operation to the date of recurrence or till the end of follow up. If an explanatory variable was statistically significant in univariate analysis and unrelated to other explanatory variables, it was included in multivariate analysis. The results of Cox's regression analyses were given as hazard ratios (HR) with 95 % confidence intervals (95 % CI). The cumulative percentages for survival were estimated using Kaplan-Meier technique and differences between FMNL2 expression groups were tested using log-rank –test.

P-values less than 0.05 were considered as statistically significant in all studies. Statistical analyses were carried out using SAS system for Windows, Version 9.2 or 9.3 (SAS Institute Inc., Cary, NC, USA).

5. RESULTS

5.1. Expression of formins FMNL1, FMNL2, and FHOD1 in human tissues (I-III)

5.1.1. mRNA profiles (I-III)

We first wanted to learn about the presence and expression levels of FMNL1, FMNL2 and FHOD1 mRNA as measured by mRNA detection in human tissues. This data was later to be compared with the immunohistochemical results. The mRNA expression levels were obtained from the GeneSapiens database (Kilpinen et al. 2008). The results are presented in Figure 3 of Study III (FMNL1), Figure 2 of Study I (FMNL2) and Supplemental Figure 2 of Study II (FHOD1).

To summarize, we found that only FMNL2 mRNA is very highly expressed in tissues from the central and peripheral nerve systems. In contrast, the levels of FMNL1 and FHOD1 mRNA in these tissues are very low. In hematopoietic and lymphatic tissues, the most highly expressed formin is FMNL1. FMNL2 and FHOD1 mRNA is expressed in these tissues as well. Especially high expression of FHOD1 mRNA is seen in the spleen. In mesenchymal tissues such as skeletal muscle, blood vessels and adipose tissue, FHOD1 mRNA is highly expressed, while FMNL1 mRNA amounts are negligible. These tissues also express FMNL2, in low to moderate quantities. In epithelial tissues, the most widely expressed formin mRNA is FMNL2, with levels low to moderate. Some epithelial tissues express FHOD1 mRNA as well (e.g. ovary). FMNL1 mRNA is low or absent from epithelial tissues.

5.1.2. Validation of antibodies to formins (I-III)

The FMNL1, FMNL2 and FHOD1 rabbit anti-human polyclonal monospecific antibodies were produced and provided to us by the Swedish Human Protein Atlas (HPA), in a project with emphasis on generating antibodies for immunohistochemistry. Amino acid sequences specific for FMNL1 (Uniprot O95466, amino acids 880-991), FMNL2 (Uniprot Q96PY5, amino-acids 393-516) and FHOD1 (Uniprot Q94613, amino acids 469-583) were chosen for recombinant protein production. The recombinant proteins, called Protein Epitope Signature Tags (PrESTs), were used for immunization. Rabbit polyclonal antibodies were affinity purified and initially validated by an antigen array of 384 different PrESTs. In this assay, the antibodies only interacted with their designated antigen.

For further validation of the FMNL1 antibody, in study III, we performed Western blotting, cell transfections and immunofluorescence microscopy. The results from these experiments are presented in study III, Figure 2.

The predicted molecular masses for FMNL1 isoforms 1 and 2 is 122 kDa and for isoform 3 124 kDa. Upon blotting the antibody detected a single band with a 15 % higher molecular weight than expected in several of the tested cell lines (see Table 9). Next, to rule out the

possibility of cross-reaction with the two closely related formins, FMNL2 and FMNL3, we performed western blotting with an antibody that detects FMNL2 and FMNL3 but not FMNL1 (Block et al. 2012). For this, we chose the melanoma cell line SK-Mel-28. In this cell line, no reactivity was seen with the FMNL1 antibody. After reprobing the same membrane with the FMNL2 and FMNL3 antibody, bands corresponding to FMNL2 (150 kDa) and FMNL3 (130 kDa) were detected. Further attesting to the specificity of the antibody, we saw that the same 150kDa and 130 kDa bands were only weakly detected or not seen at all on blots of cells treated with corresponding siRNAs. These findings firmly show that the FMNL1 antibody does not cross-react with FMNL2 or FMNL3. For further validation of FMNL1 specificity, cells were transfected with FMNL1 coding DNA constructs. This experiment was conducted in cells that do not express detectable native FMNL1 in western blotting. The cell lines were transfected with the following constructs: the C-terminal half of FMNL1 isoform 1 (FMNL1') and green fluorescent protein (GFP)-tagged FMNL1'. After transfection, cell lysates blotted with the FMNL1 antibody. Single bands of 100 and 130 kDa were decorated with the antibody, respectively, indicating that the antibody specifically detects FMNL1. FMNL1' in transfected cell lines was visualized by immunofluorescence staining by using the FMNL1-antibody and compared to the localization of GFP-tagged FMNL1'. Immunostaining and green fluorescence emitted from the GFP-tagged FMNL1' displayed similar distributions, further attesting to the high degree of specificity of FMNL1 the antibody.

FMNL2 antibody validation was done for study I, using western blotting, peptide competition, siRNA transfection and northern blotting (Figure 1 in study I). Based on the sequence, the predicted molecular mass of FMNL2 is 124 kDa. The FMNL2 antibody detected a single band slightly higher (21 %) than the expected size in several human cell lines. The specificity of the antibody was tested by a peptide competition assay. A GST-FMNL2 fusion protein was incubated with the antibody prior to western blotting. This resulted in loss of the single band in all three tested cell lines, whereas preincubation with GST only did not affect the reactivity. As a further test, FMNL2 expressing cell lines were transfected with FMNL2-specific or control siRNA. In western blotting, markedly reduced or undetectable staining was seen in treated cell lines, whereas control siRNA had no effect on antibody reactivity. Last, northern blotting was performed in several cell lines and compared to the western blotting results. A major 5.6 kb transcript was found in cell lines matching the expression profile from western blotting.

The FHOD1 antibody was validated by using western blotting, peptide competition, siRNA treatment and northern blotting (Supplemental Figure S1 in Study II). FHOD1 has a predicted molecular weight of 127 kDa. In western blotting, a single band slightly (14 %) larger than predicted was detected with the FHOD1 antibody in several endothelial and cancer cell lines. Reactivity was blocked in a peptide competition with the antigenic GST-FHOD1 fragment. Specificity was further confirmed by transfecting MDA-MB-231 breast cancer cells with FHOD1 siRNA or with non-targeting control siRNA. After FHOD1 siRNA treatment, western blotting showed that the staining intensity with the FHOD1 antibody was significantly reduced as compared to control cells. Further assurance of specific protein detection was provided by northern blotting of cell lines. Northern blotting showed a 4.0 kb band, indicating FHOD1 mRNA presence in the same cell lines where the antibody had detected FHOD1.

5.1.3. Expression of FMNL1, FMNL2 and FHOD1 in normal tissues as determined by immunohistochemistry (I-III)

Expression patterns of FMNL1, FMNL2 and FHOD1 proteins in normal human tissues were studied by immunohistochemistry. For that purpose we used 26-28 different human tissues that were deemed morphologically normal by conventional hematoxylin and eosin staining. Representative photomicrographs from the stainings are presented in Figure 4 of Study III for FMNL1, Figure 3 of Study I for FMNL2, and Figure 2 of Study II for FHOD1. A summary of the staining results is presented in Table 8.

None of the tissues we studied was completely devoid of formin staining. However, within the tissues, we found cell types that did not express detectable levels of any of the three studied formins. These cells were adipocytes, erythropoietic cells in the bone marrow, submucosal fibroblasts in the urinary bladder and mesangial as well as stromal cells of the kidney. All these cell types are specialized cells of mesenchymal lineage.

Many tissues parenchymal cells only expressed one formin were also found. In all these, FMNL2 was the only detected formin. These tissues were brain and peripheral nerve and several epithelial tissues (liver, pancreas, parathyroid gland, parotid gland and skin).

Moderate to strong staining of two formins was a common finding. Within these tissues, however, the cell type expressing the individual formin varied. For instance in prostate, strong FMNL1 staining was seen in smooth muscle cells only, while moderate FMNL2 staining was restricted to the epithelium. Tissues including cell types with moderate to strong staining with all three formin antibodies were lung (due to alveolar macrophages), and tissues from the hematopoietic and lymphatic system.

Table 8. Expression of formins in different tissues and cell types, as studied by immunohistochemistry. *0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining. [a]: weaker staining towards surface. [b]: strong staining in plasma cells.

Tissue	Cell type/localization	FMNL1 staining*	FMNL2 staining*	FHOD1 staining*
Adrenal gland	cortical cells	0	3	1
	medullary cells	0	3	1
Bone marrow	erythropoietic cells	0	0	0
	myelopoietic cells	3	2	1
	megakaryocytes	0	3	1
Breast	ductal epithelium	1	3	1
	lobular epithelium	1	3	1
	myoepithelium	3	2	1
Brain	glial cells	0	3	0
	neurons	0	3	0
Colon	epithelium	0	3	1
	stromal cells	0	1	0
	stromal lymphocytes	3	2	1[b]
Duodenum	epithelium	0	3	1
	stromal cells	0	1	0
	stromal lymphocytes	3	2	1[b]
	smooth muscle cells	3	1	0

Tissue	Cell type/localization	FMNL1 staining*	FMNL2 staining*	FHOD1 staining*
Esophagus	squamous epithelium	1[a]	1	0
Heart	cardiomyocytes	0	N.A.	N.A.
Kidney	mesangial cells	0	0	0
	endothelium	0	0	1
	podocytes	0	3	1
	tubular epithelium	0	2	0
	stromal cells	0	0	0
Liver	hepatocytes	0	1	0
	biliary epithelium	0	1	0
	Kuppfer cells	2	3	1
Lung	pneumocytes	0	1	1
	alveolar macrophages	3	3	2
Lymph node	germinal center lymphocytes	2	3	1
	other lymphocytes	3	3	1[b]
Mouth	squamous epithelium	N.A.	N.A.	0
Ovary	stromal cells	0	2	1
	granulosa cells	0	3	1
	follicles	0	2	1
Pancreas	exocrine epithelium	0	1	0
	ductal epithelium	0	1	0
	neuroendocrine cells	0	3	0
Parathyroid gland	chief cells	0	3	0
	oxyphil cells	0	1	0
	adipocytes	0	0	0
Parotid gland	acinar cells	0	2	0
	ductal cells	0	2	0
Peripheral nerve	Schwann cells	0	2	0
Placenta	trophoblasts	1	3	0
	stromal cells	0	1	0
	endothelium	0	0	2
Prostate	epithelium	0	2	0
	smooth muscle cells	3	1	0
Skeletal muscle	myocytes	1	1	1
Skin	keratinocytes	0	1	0
Small intestine	epithelium	0	3	1
Spleen	white pulp cells	3	3	0
	red pulp cells	3	3	0
	endothelium	0	0	3
Stomach	epithelium	1	3	1
	stromal cells	0	1	0
	smooth muscle cells	3	1	0
Testis	spermatocytes	0	3	0
	Leydig cells	0	1	1
	Sertoli cells	0	3	0
Thyroid gland	follicular epithelium	0	3	1
Urinary bladder	urothelial cells	0	2	1
	submucosal fibroblasts	0	0	0
Uterus	endometrial epithelium	0	3	1
	endometrial stromal cells	0	1	0
	myometrium smooth muscle cells	3	2	0

5.2. Expression of formins in cultured cell lines (I-IV)

As a starting point for cellular studies, the expression FMNL1, FMNL2, FMNL3 and FHOD1 was studied by western blotting in selected cell lines. The results are summarized in Table 9.

Table 9. Detection of formins FMNL1, FMNL2 , FMNL3 and FHOD1 in human cell lines as studied by western blotting. A band corresponding to the predicted size (± 20 %) of the target protein is indicated as +. No bands detected is indicated by -. Empty cells indicate that western blotting was not performed as a part of this study.

Cell line	FMNL1	FMNL2	FMNL3	FHOD1
Bowes melanoma	-	+	+	+
SK-Mel-28 melanoma	-	+	+	
WM164 melanoma	-	+	+	+
WM239 melanoma		+	+	
CCD18 fibroblast	+			
Hey ovarian cancer	+			
IGROV-1 ovarian cancer	-			
Ovcar-4 ovarian cancer	+	-	+	-
HEK 293T human embryonic kidney	-			
MDA-MB-231 breast cancer	+	+	+	+
MCF-7 breast cancer		+		
Jurkat T-cell lymphoma	+	-		+
K562 erythroleukemia		-		
HaCat Human keratinocyte		+		
A431 Squamous cell carcinoma		+		+
UT-SCC-43A Squamous cell carcinoma, epithelial	+	+		-
UT-SCC-43B Squamous cell carcinoma, mesenchymal	+	+		+
43A-SNA Squamous cell carcinoma, mesenchymal	+			+
MKN-1 gastric cancer		+		
U138 glioblastoma		+		+
HMEC Human dermal microvascular endothelial cells		+		+
HUVEC Human umbilical vein endothelial cells				+
TIME Telomerase-immortalized human microvascular endothelium cell line		+		+

5.2.1. FMNL1 (III)

FMNL1 expression was detected by western blotting in cancer cell lines derived from hematological malignancies and in some carcinoma cell lines (ovarian cancer cell lines and breast cancer of basal type). Examples of cell lines that did not express FMNL1 are melanoma cell lines and human embryonic kidney cells. The subcellular localization of FMNL1 in cell lines was studied by immunocytochemistry. FMNL1 was detected as granules in the cytoplasm of cancer cells and fibroblasts. In basal breast cancer cells (MDA-MB-231), FMNL1 was further seen at the tips of filopodia.

5.2.2. FMNL2 and FMNL3 (I, IV)

Western blotting showed that FMNL2 is widely expressed in human cell lines. All studied cell lines, including a variety of different malignant cell lines, expressed FMNL2. Of note, both FMNL2 and the closely related formin FMNL3 were co-expressed in all the melanoma cell lines. No FMNL2 was seen in cell lines from haematological malignancies. In immunocytochemical stainings, FMNL2 was mostly detected as cytoplasmic granules and as perinuclear accumulation consistent with the Golgi apparatus. In some cells also nuclear staining was seen. Furthermore, in all studied human melanoma cell lines, FMNL2 co-localized with F-actin to the tips of filopodia (Figure 3 in Study IV). We do not have at our disposal an antibody that would specifically detect FMNL3 in immunocytochemistry. However, we repeated the stainings with an antibody that detects both FMNL2 and FMNL3. The stainings results were identical with the FMNL2 stainings, which suggests that FMNL3 localization at least partially overlaps with FMNL2 localization. This finding is in line with the proposed function of FMNL2 and FMNL3 in the formation of actin filaments in filopodia.

5.2.3. FHOD1 (II)

In Western blotting, a single band of expected size was seen in several human endothelial and cancer cell lines when probed with the FHOD1 antibody. A wide expression across cell types is in line with earlier findings demonstrating FHOD1 expression in most immortalized cell lines (Gill et al. 2004).

UT-SCC-43A is an oral SCC cell line from primary tumour, and UT-SCC-43B is a line from the same tumour recurring after surgery and radiotherapy. The recurrent tumour has undergone a spontaneous EMT, as demonstrated by morphology and several EMT-markers in the UT-SCC-43B cell line (Takkunen et al. 2006). Another EMT cell line, 43A-SNA, has been established by transfecting the epithelial UT-SCC-43A cell line with Snail to induce EMT. Of the three cell lines, FHOD1 expression was seen in both EMT-cell lines UT-SCC-43B and 43A-SNA but not in the epithelial line UT-SCC-43A (Figure 3 B in Study II). This is particularly interesting since it suggests that FHOD1 upregulation could be functionally associated with EMT.

5.2.4. Formins in EMT (II)

Expression of FHOD1 in oral SCC that had undergone EMT encouraged us to study the transcriptome of the cell lines. We especially wanted to explore the possibility that formins play a role in cancer-associated EMT. Transcriptome analysis revealed that UT-SCC-43B and 43A-SNA have 35 up-regulated genes (logFC ≥2) and 153 down-regulated genes (logFC ≤ -2) in common. EMT-markers such as N-cadherin, vimentin and collagens were up-regulated, whereas epithelial markers such as E-cadherin, keratins, integrins and laminins were down-regulated, consistent with what is known from both phenotypic and mechanistic studies on EMT (Figure 1 A and B in Study II).

In the array 13 formin family members were included (DAAM1, DAAM2, DIAPH1, DIAPH2, DIAPH3, FHOD1, FHOD3, FMN1, FMN2, FMNL1, FMNL2, FMNL3, INF2). Of these two were

consistently up-regulated and four were down-regulated in both UT-SCC-43B and 43A-SNA. A most prominent change in expression level was seen with FHOD1. It was increased 2.3-fold in UT-SCC-43B and 3.2-fold in 43A-SNA as compared to UT-SCC-43A (Figure 1 C in Study II). The significant transcriptional differences were further verified by qRT-PCR, which confirmed the upregulation of formins FHOD1 and FMNL3 and downregulation of FHOD3 and DIAPH1 in both UT-SCC43B and 43A-SNA cells (Figure 1 D in Study II).

5.3. Functional studies

5.3.1. FMNL2 and FMNL3 (IV)

Next we set to explore the potential role of FMNL2 in the morphology of melanoma cells and in the formation of filopodia. First, we silenced FMNL2 in SK-Mel-28 cells using FMNL2 siRNAs. SK-Mel-28 is derived from a primary cutaneous melanoma. Morphologically, SK-Mel-28 is a cell line with dendritic-like processes and abundant filopodia. It harbours a BRAF V600E mutation. Efficacy of silencing was verified by western blot analysis.

FMNL2 knockdown had only a minor effect on the cell shape and filopodia in these cells. Well-structured filopodia were still abundant. However, a slight increase in irregular cellular protrusions was seen. One possible cause for the stability of the filopodia in FMNL2 knockdown cells could be the upregulation of the closely related FMNL3. To test for this possibility we looked at morphological alterations after depleting both filopodial formins FMNL2 and FMNL3, separately and simultaneously. Efficient knockdown to a nearly undetectable level was verified by western blotting. Knockdown of FMNL2 or FMNL3 individually did not influence the expression of the other, suggesting that their expression is autonomous. We saw a slight alteration of cell morphology in formin knockdown cells (Figure 4 in Study IV). Club-shaped or curved protrusions, located at the lateral periphery of cells, especially in lamellipodia were seen. They were enriched in filamentous actin and cortactin. Cells with such anomalous protrusions were more frequent in all siRNA treated groups when compared to control cells. Interestingly, the co-depleted cells did not contain more of these altered protrusions compared with single knockdowns. Proliferation was evaluated by ki-67 staining, which was not significantly altered.

Cellular protrusions are considered to be associated with directional migration. To see whether FMNL2 and/or FMNL3 knockdown has any effect on directional migration, we subjected SK-Mel-28 cells treated with FMNL2 and/or FMNL3 siRNAs to a wound healing experiment. Surprisingly, in all groups the wounds filled within 24 hours with a similar reduction of the wound area as a function of time, suggesting that FMNL2 and FMNL3 are redundant for migration under these conditions (Figure 5 A in Study IV).

It is well known that wound healing in cell culture does not reproduce the conditions that cell face *in vivo* when moving invasively through a three-dimensional environment. To simulate that situation, a Boyden chamber assay was performed. Indeed, in this migration assay, both FMNL3 and FMNL2/3 co-depleted cells, showed a significantly reduced capacity to

transmigrate the membrane. Although FMNL2 silenced cells also migrated less than control cells, this reduction was not statistically significant. Co-silencing of FMNL2 and FMNL3 did not have an additive effect when compared to FMNL3 silenced cells (Figure 5 B in Study IV).

Signalling pathways commonly activated in melanomas include the Mitogen-activated protein kinase (MAPK/ERK1/2) pathway and the Phosphatidylinositol-3-Kinase (PI3K) pathway. In order to investigate whether FMNL2 or FMNL3 expression is dependent on activation of these signalling pathways, SK-Mel-28 cells were treated with inhibitors of MEK1/2 (UO126) and PI3K (LY294002). Effective inhibition of the pathways was confirmed by western blotting MAPK2 + pMAPK1/2 or Akt + pAkt, respectively. FMNL3 expression was undetectable and FMNL2 expression was clearly reduced after the inhibition of either pathway (Figure 6 in Study IV). This suggests that both FMNL2 and FMNL3 function downstream of both melanoma-associated signalling pathways.

5.3.2. FHOD1 (II)

FHOD1 was the most upregulated formin in cell lines that represented oral SCC with EMT. This led us to explore the role of FHOD1 in EMT-associated cellular processes. The cell lines used were UT-SCC-43A and UT-SCC-43B, the former representing a primary oral SCC with epithelial phenotype, the latter a recurrent oral SCC with EMT. The two cell lines were morphologically distinct. UT-SCC-43A cells were organised as sheets of cells with clear cell-to-cell contacts and scarce stress fibers. In contrast, UT-SCC-43B cells did not form epithelial sheets. They were elongated, and had many filopodia and abundant stress fibers. In immunocytochemical stainings, FHOD1 was detectable only in UT-SCC-43B cells. The distribution of FHOD1 in UT-SCC-43B cells was in part punctuate and cytoplasmic, but it also clearly localised to stress fibers (Figure 3 C in Study II).

Signalling pathways commonly activated in clinical oral SCC undergoing EMT include PI3K- and MAPK/ERK1/2 pathways (Krisanaprakornkit, Iamaroon 2012). To investigate whether the enhanced expression of FHOD1 seen in our cells was dependent on either of these signalling pathways, the cells were treated with PI3K and MEK1/2 inhibitors (UO126 and LY294002, respectively). Inhibition of MEK1/2 did not influence FHOD1 expression, although the pathway was active and efficiently inhibited by the drug. In contrast, inhibition of PI3K was accompanied by substantially reduced FHOD1 expression (Figure 3 D in Study II). This strongly suggests that FHOD1 expression in UT-SCC-43B is dependent on active PI3K signalling.

The functional role of FHOD1 in the EMT cell line UT-SCC-43B was studied by RNA interference. Transfection of FHOD1 siRNA into UT-SCC-43B cells was accompanied by a significant decrease in FHOD1 expression. Expression of E-cadherin and N-cadherin were unaffected. The morphology of the cells was clearly altered. The knock-down cells were less elongated than control cells and actin stress fibers were reduced. This suggests that FHOD1 has a role in the maintenance of mesenchymal morphology. Quantitative analysis of cytoplasmic fluorescent phalloidin staining confirmed the reduced F-actin content in cells treated with FHOD1 siRNA ($p < 0.0001$) (Figure 4 in Study II).

Next we wanted to see whether the decrease in actin fibers, seen in FHOD1 depleted UT-SCC-43B cells, is in any way associated with changes in cell motility. This was studied in a wound healing assay. We found that FHOD1 depleted cells migrated slower, because they failed to close the wound in 24 hours. This was in contrast to control cells, which closed the wound within 18 hours (Figure 5 A in Study II). Lamellipodia were seen in both siRNA treated and control cells, but the active forward movement of the cell body was reduced in siRNA treated cells. The difference in wound healing efficacy was measured at one-hour intervals. It was statistically significant at every time point (Figure 5 B in Study II). We further tested the influence of FHOD1 knockdown on invasion through a three-dimensional matrix using the IncuCyte live cell imaging system with Matrigel as matrix. In this assay, FHOD1-depleted cells migrated less efficiently than control cells, indicating that FHOD1 is relevant not only for migration but also for invasive capacity of cancer cells (Figure 5 C in Study II).

Invasiveness is linked not only to alterations in the cytoskeleton, but also to extracellular matrix material degradation at the tips of invadopodia. To assess whether expression of FHOD1 is associated with this directed proteolytic capacity, we conducted a fluorescent gelatine degradation experiment. UT-SCC-43B cells were transiently silenced for FHOD1 expression. FHOD1 silenced cells degraded significantly smaller areas of Cy3 labelled gelatine than control cells (Figure 6 A and B in Study II). We also evaluated the proportion of cells with invadopodia. Invadopodia-containing cells were significantly fewer in FHOD1 depleted cells than in cells transfected with non-targeting siRNA (Figure 6 D in Study II).

5.4. Formins in cancer (II-IV)

Alongside with cellular experiments described above, the FMNL1, FMNL2 and FHOD1 antibodies developed for immunohistochemistry gave us the opportunity to investigate the expression of these formins in clinical cancer.

5.4.1. FMNL1 in basal type breast cancer (III)

Breast cancer of the basal type differs in expression from conventional breast cancer: it does not express estrogen or progesterone receptors, nor does it express human epidermal growth receptor 2 (HER-2). It usually affects younger women than ductal and lobular breast cancer. The basal type of breast cancer is a relatively rare subgroup. It has a more aggressive course than the conventional breast cancer types (Dent et al. 2007).

In the study of FMNL1 expression in normal tissues, FMNL1 expression was high only in cell types of hematopoietic lineage and smooth muscle cells. In epithelia, FMNL1 expression was low or absent. In an mRNA database search, a high FMNL1 expression was seen in a set of outliers among the generally low expressing breast cancer tissue samples. The database also contains mRNA data on commonly used cell lines. Among human breast cancer cell lines only MDA-MB-231, which is derived from a basal type breast cancer, expressed high levels of FMNL1 mRNA. We decided to investigate FMNL1 in MDA-MB-231 cells and basal

type breast cancer samples by using immunocytochemistry and immunohistochemistry. In immunofluorescent stainings of MDA-MB-231 cells, FMNL1 was detected in cytoplasmic granules and at filopodial tips (Figure 5 A in Study III). In immunohistochemical staining of eight cases of basal breast cancer, all tumours contained FMNL1 positive cells. In three of the cases, FMNL1 expression was restricted to inflammatory cells. In five samples, additional positive FMNL1 staining was detected in carcinoma cells, mostly near the infiltrative margin of an otherwise negatively staining tumour Figure 5 B in Study III). Next, we wanted to rule out the possibility of interpreting tumour infiltrating macrophages as FMNL1-positive cancer cells. Therefore we further double-stained the positive samples with anti-FMNL1 and a macrophage-specific marker, anti-CD68. In these stainings, CD68-negative and FMNL1 positive cancer cells were detected (Figure 5 C in study III).

5.4.2. FMNL2 expression in primary and metastatic melanoma (IV)

The motility of melanoma cells in a three-dimensional matrix can occur as ameboid single cells (Sanz-Moreno et al 2008). We wanted to evaluate FMNL2 expression in clinical melanoma by performing immunohistochemistry. This was based on our results suggesting that FMNL2 participates in melanoma cell migration as single cells. We also wanted to study melanoma metastases in our analysis, since FMNL2 expression has been shown to correlate with metastasis in colorectal carcinoma (Zhu, Liang & Ding 2008).

The selection of patients with melanoma of clinical stages I-II as well as follow-up has been described in the material and methods section. Of the selected 175 cases, FMNL2 staining could not be performed in six cases, since the melanoma tissue had been spent for diagnostic purposes. 169 cases were stained with the FMNL2 antibody and analysed. The cohort included 82 women and 87 men. The mean age at diagnosis was 63. The mean follow-up was 7.6 years (range: 0-21.9). During the follow-up, 46 patients (27.2 %) had a recurrence and 40 patients (23.6 %) died of melanoma, 54 patients (32.0 %) died of other disease. 75 patients (44.4 %) were alive at the end of the follow-up. Clinical parameters as well as the histologically evaluated parameters Clark level, presence of ulceration, level of dermal mitoses and amount of lymphocytic infiltration were recorded. The clinicopathological characteristics as well as the outcome of the patients are summarized in Table 1 of Study IV.

Expression of FMNL2 was seen in all melanomas as studied by IHC. Within the samples, keratinocytes, lymphocytes and endothelial cells were used as positive internal controls, areas where these were negative were left unevaluated. The staining intensity of keratinocytes was used as an internal reference and noted as moderate staining. Intensity of the staining for FMNL2 varied between the studied tumours, with cytoplasmic staining in a wide majority of cases. The staining intensity was weak in 54 cases, moderate in 69 cases, and strong in 46 cases. Examples of different staining intensities are shown in Figure 1 in Study IV.

Kaplan-Meyer analysis of recurrence-free and melanoma specific survival was performed. The outcome was significantly different in groups displaying different levels of FMNL2 expression. Recurrence-free survival was significantly shorter in the high FMNL2 expression

group than in the low FMNL2 expression group. In a similar analysis of melanoma specific survival, both moderate and high FMNL2 expressing groups had a significantly worse outcome than the low FMNL2 expressing group (Figure 2 in Study IV).

To explore the possible prognostic role of FMNL2 expression, univariate and multivariate analysis of recurrence free and disease specific survival was conducted. In the univariate analysis of recurrence free survival, the outcome was highly significantly associated with FMNL2 expression. Other statistically significant parameters turned out to be Breslow thickness, AJCC stage, Clark level, presence of ulceration, TILs, and dermal mitosis count ≥1/mm² (Table 2 in Study IV). In the univariate analysis of melanoma specific survival, the result was similar. Outcome was highly associated with FMNL2 expression level. Other statistically significant parameters were Breslow thickness, AJCC stage, Clark level and dermal mitosis count ≥1/mm² (Table 3 in Study IV).

FMNL2 expression level and the histopathological prognostic markers of melanoma Breslow thickness, presence of ulceration, dermal mitosis level were further subjected to multivariate analysis of recurrence free and melanoma specific survival. Of these, FMNL2 expression level and Breslow thickness came out as independent significant prognostic factors as both to recurrence free and melanoma-specific survival.

Also the staining intensity for FMNL2 in 34 metastases was compared to the primary tumours of the same patient. No significant difference was found. This suggests that FMNL2 upregulation is an early event in melanoma. Eight samples representing metastatic melanoma were tested for BRAF mutation status and stained with the FMNL2 antibody. Four of the samples had a BRAF V600E mutation and four were BRAF wild type. No correlation between BRAF mutation status and FMNL2 staining could be seen.

5.4.3. FHOD1 in oral squamous cell carcinoma (II)

Immunohistochemical results and the mRNA profiling suggested that FHOD1 is only minimally expressed in epithelial cells. On the other hand, the transcriptomic analysis and GeneSapiens bioinformatics survey of carcinomas indicated moderate FHOD1 mRNA levels in several forms of cancer. These findings, together with the enhanced expression of FHOD1 in our oral SCC EMT cells, led us to investigate whether the upregulation of FHOD1 occurs in clinical oral SCC. We randomly chose ten oral SCC specimens for FHOD1 staining. We found no FHOD1 expression in the non-neoplastic stratified squamous epithelium of oral mucosa in any of the cases. On the other hand, from moderate to strong FHOD1 staining was consistently seen in the invasive SCC cells with a mesenchymal spindle-shaped morphology (Figure 3 A in Study II). Interestingly, in the well-differentiated areas of invasive cancer, FHOD1 immunoreactivity was mild or absent. These results show that while the bulk of the tumour that consists of cellular areas with epithelial differentiation expresses little FHOD1, there is a high expression in the invasive front.

6. DISCUSSION

6.1. Formin expression is tissue and cell type specific

During the past decade, the structural and functional properties of individual mammalian formins have been intensively studied (Schönichen and Geyer 2010). Of special interest has been the observation that the activation of many formins is Rho GTPase-dependent and that formins in their activated state participate in cellular mechanisms related to shape, adhesion and migration. There is compelling evidence that Rho GTPases are involved in cancer-related cellular properties such as uncontrolled proliferation, loss of polarity and increased migration. Mouse models have further shown that they are involved in tumorigenesis and in particular, in metastasis (Karlsson et al. 2009). The discovery of formins as downstream effectors of the Rho GTPases is a significant addition to our knowledge of cancer biology (Young, Copeland 2010). The autoinhibition and activation mechanisms of diaphanous related formins have unraveled several potential drug targets. New formin-targeting drugs are the small-molecule intramimics of formin autoinhibition that disrupt the DID-DAD binding and activate DRFs (Lash et al. 2013), and the small molecule inhibitor of formin-mediated actin assembly (Rizvi et al. 2009). Although these activators and the inhibitor are nonspecific, it is possible that further drug development could lead to discovery of activators/inhibitors of individual formins. Specific modulators of formin activity could perhaps be utilized as part cancer adjuvant therapy.

A major problem in this field of research has been the incomplete knowledge of formin expression in normal and pathologic tissue. Without such knowledge, it is impossible to draw clinical conclusions from *in vitro* studies, or conclude whether formins could serve as biomarkers or drug targets. The major hurdle in gaining this basic information on formins has been the lack of well-characterized antibodies suitable for immunohistochemistry. In order to gain this elemental and especially for translational research efforts essential data we set out to explore the expression of different formins in normal human tissues. To reach this goal, we collaborated with the Human Protein Atlas project, by thoroughly validating antibodies provided by them and using the antibodies for FFPE tissue analysis. The validation process included cellular knockdown experiments, transfections with formin expression constructs and northern blotting, to confidently show that the antibodies are truly specific for formins that are by amino acid sequence closely related. So far, we have validated three antibodies (FMNL1, FMNL2 and FHOD1) and have characterized their expression in human tissues extensively.

The antibody to FMNL1 (HPA008129) detects all three isoforms of FMNL1. Overall, in normal tissues, FMNL1 exhibited an expression profile that was more restricted than the other studied formins. FMNL1 was not detected in nervous system parenchymal cells. It was only rarely expressed in epithelia. Strong expression of FMNL1 was found in the bone

marrow and in lymphatic tissues. The cell types expressing FMNL1 were myelopoietic cells, lymphocytes and macrophages. Such an expression profile is supported by earlier studies on FMNL1 mRNA or protein in different tissues (Favaro et al. 2003, Han et al. 2009, Krainer et al. 2013). A novel finding in our study was that two non-hematologic cell types express high levels of FMNL1: these were smooth muscle cells and myoepithelial cells. This addition to earlier knowledge is highly relevant, since it may attenuate the value of FMNL1 as a potential therapeutic target. FMNL1 has previously been proposed as a target for immunotherapy in hematologic malignancy (Schuster et al. 2007).

The antibody to FMNL2 (HPA005464) recognizes both known isoforms of FMNL2. For FMNL2 the expression profile in normal tissues was found to be widest when compared with the other studied formins. In contrast to FMNL1 (and FHOD1), FMNL2 is strongly expressed in epithelia and the nervous system. Similar to FMNL1, FMNL2 shows a moderate or strong expression in bone marrow, lymphatic tissues and macrophages.

The FHOD1 gene has multiple transcripts, of which two are known to be protein coding. The FHOD1 antibody (HPA 024468) used in this study was raised to detect both isoforms. By using this antibody, we found FHOD1 to be expressed especially in endothelial cells. No or only minor expression was seen in epithelial cells and in nervous system. No detectable FHOD1 expression was seen in squamous cells in epithelia in skin, mouth and esophagus. Thus, in contrast to FMNL1 and FMNL2 that are predominantly seen in epithelial and cells and leukocytes, respectively, FHOD1 seems to be a mesenchymal formin.

These immunohistochemical results represent the first comprehensive study on the distribution of formin proteins in different cell types in different organs. Such data is important for research on both physiological and disease-associated roles of formins, not to mention exploring the possibility of utilizing formins as drug targets. Furthermore, this data can serve as a starting point for discovering altered formin expression in disease. For instance FMNL1 and FHOD1 have mostly been studied in immunologic and endothelial processes *in vitro*, respectively (Yayoshi-Yamamoto, Taniuchi & Watanabe 2000, Gomez et al. 2007, Seth, Otomo & Rosen 2006, Naj et al. 2013). The expression of FMNL1 in lymphatic tissues and FHOD1 in endothelium supports the idea that these are cell types where FMNL1 and FHOD1 have key physiological roles.

6.2. Formin expression is altered in cancers

Based on our observation on the differential expression of formins in normal tissues and on their biochemical properties, it could be expected that their expression level is modified in malignancy. Our effort to test whether such altered expression occurs was based on two strategies. First, we used the expression profile of formins in normal tissues and compared them to results from the GeneSapiens database search on mRNA levels in cancers. Second, we studied formin protein expression and its localization in cell lines. Cancers with altered expression level, or immunocytochemical staining results suggestive of relevant functions, were chosen for further immunohistochemical and cellular studies.

Our immunohistochemical studies showed that FMNL1 is highly expressed in lymphoid tissues and in bone marrow. As to the expression of FMNL1 in cancers and cancer cell lines, a high level of expression is further seen in non-Hodgkin lymphoma and also in leukemic cell lines (Favaro et al. 2006, Schuster et al. 2007, Favaro et al. 2013). In our studies, by using mRNA profiling, we made some novel observations, in particular concerning breast cancer. Among studied cell lines, FMNL1 expression was high in a breast cancer cell line of basal type. When we further tested cancer tissues, we found that although most breast cancers express little or no FMNL1, a minority of breast cancers clearly do express FMNL1. We considered the possibility that this minority included basal type breast cancers. We felt encouraged to study the expression of FMNL1 in this aggressive subtype. It was found that FMNL1 was expressed in a subpopulation of malignant cells in over half of basal breast cancer samples. The expression was best seen near the infiltrating edge of the tumours, which suggests a role in invasion. This is the first time increased expression of FMNL1 protein has been shown in clinical carcinoma.

Silencing FMNL2 in melanoma cells reduces invasive migration (Kitzing et al. 2010, Block et al. 2012). Based on these observations we set out to elucidate whether clinical melanomas express FMNL2, and whether the expression level has impact on prognosis. The level of FMNL2 expression was evaluated in a large number of early stage primary melanomas and their possible metastases from the follow-up period. The patient data included a long follow-up. The mean follow-up time was 7.6 years (range 0-21.9 years). All relevant patient data was included and analysed.

We found that FMNL2 is consistently expressed in melanoma. Moreover, a high expression level in the primary tumour correlates with a poor outcome (measured as recurrence free survival and disease specific survival). Interestingly, when compared with the parent tumour, there was no increase in FMNL2 expression in metastases that occurred during the follow-up. This strongly suggests that the primary melanomas with high FMNL2 expression are destined to a more aggressive course. Such melanomas are more likely to be disseminated outside the primary tumour in a clinically silent way already at the time of diagnosis. The fact that FMNL2 expression is high in the primary tumours and not further upregulated in recurrence during follow-up also indicates that increase of FMNL2 expression is an early event in tumour cells, not an event related to clinical progression.

We also explored the possibility of using expression of FMNL2 as a biomarker for diagnostic or prognostic purposes in melanoma. Unfortunately, a widely expressed protein with staining in keratinocytes, nevus cells and melanoma cells cannot be considered as a diagnostic marker. It could, however be used as a prognostic tool to evaluate the risk of developing melanoma metastasis.

A characteristic feature of cancer-associated EMT is profound rearrangement of the actin cytoskeleton. It is characterized by the loss of cell-cell adhesions, a spindle-like cell shape and the appearance of actin-filament based stress fibers. With this change, cancer cells achieve a migratory and invasive phenotype for crossing tissue barriers and thereby reaching

blood and lymphatic vessels. The actin modulators responsible for these cytoskeletal rearrangements are largely unknown.

We explored the role of formins in EMT. For that purpose, we used a cellular model for EMT. FHOD1 appeared to be the most upregulated formin in oral SCC EMT model cell lines UT-SCC-43B and 43A-SNA when compared to the epithelial oral SCC cell line UT-SCC-43A. In both cell lines, a rise in FHOD1 expression was notable both at transcriptional and protein level. In functional studies, we found that FHOD1 participates in actin organisation, cell migration and the ability to degrade the extracellular matrix, all of which are considered to be morphologic and functional hallmarks of EMT. The results from our cellular model instigated the analysis of FHOD1 expression in invasive oral SCC specimens. By microscopy, we detected areas of moderate/high FHOD1 expression in every case of oral SCC (n=8). We found no detectable FHOD1 expression in normal oral mucosa. Within the tumours, the highest expression of FHOD1 was seen at the invasive front, where cancer-related EMT is thought to occur *in vivo*. Here, FHOD1 expression was increased in cells with an EMT-like morphology, *i.e.* in elongated spindle-shaped tumour cells.

Despite ample evidence that formins participate in EMT *in vitro*, there have been few reports concerning formins and EMT in clinical cancer. Previously one study has demonstrated an association between FMNL2 and colorectal cancer EMT (Li et al. 2010). In this sense our observations are significant. We show for the first time that the expression of another formin, FHOD1, is associated with cancer EMT.

6.3. Distinct cellular localizations suggest different functional roles of formins

Studies on white blood cells and hematological malignant cell lines have suggested cell type dependent functions for FMNL1. FMNL1 has been detected in the cytoplasm and in diverse polarized membrane processes. In macrophages, FMNL1 has been detected in podosomes, pseudopods and phagocytic cups (Yayoshi-Yamamoto, Taniuchi & Watanabe 2000, Seth, Otomo & Rosen 2006, Mersich et al. 2010, Naj et al. 2013). In its autoinhibited state FMNL1 is cytoplasmic, membrane localization being achieved by activation or mutations that render FMNL1 constitutively active. The targeting of FMNL1 to the membrane is furthermore dependent on N-myristoylation (Han et al. 2009).

In breast cancer cell line MDA-MB-231, we saw FMNL1 in small granules within the cytoplasm. In addition, it was present as small dots at the tips of filopodia. This is interesting for the following reasons. First, filopodia are regarded as exploratory sensors at the leading edge, which are needed for directional movement. Second, filopodia are assemblies of parallel long actin filaments (Ridley 2011). Given the role of FMNL1 as a potent elongator of actin filaments, it is likely that activated FMNL1 participates in filopodia assembly.

Also the localization of FMNL1 in podosomes is intriguing. FMNL1 regulates the motility of macrophages through podosomes, and also promotes transendothelial migration in

leukemia cells (Favaro et al. 2013, Yayoshi-Yamamoto, Taniuchi & Watanabe 2000). We found that FMNL1 is upregulated in basal breast cancers and that it is localized at filopodial tips in cultured basal breast cancer cells. Thus, it is imaginable that basal breast cancer cells could utilize similar migration mechanisms as macrophages by expressing FMNL1. Determining the role of FMNL1 in adhesion and migration of basal breast cancer cells is a challenge for future studies.

Similar to FMNL1, we have found that FMNL2 is a component of filopodia in cancer cells, *i.e.* in several melanoma cell lines. Another thought-provoking site where FMNL2 accumulates is the lamellipodium, where we detected FMNL2 in migrating glioma cells. Invasion is based on the migratory activity of cells. Cell migration, in turn, depends on substantial actin treadmilling that leads to extension of the plasma membrane at the leading edge. FMNL2, when activated, is known to potently enhance the formation of the unbranched actin filaments required for the plasma membrane extension (Block et al 2012).

Due to the lack of specific antibodies we have been unable to determine the exact location of FMNL3 separately from the closely related FMNL2. Staining with an antibody that detects both FMNL3 and FMNL2 shows a pattern suggesting an overlapping distribution. Thus, it can be assumed that both FMNL3 and FMNL2 are involved in the assembly or maintenance of filopodia and migration.

In vitro overexpression studies have introduced the possibility of formin heterodimerization (Vaillant et al. 2008). In that perspective it is highly interesting that knocking down FMNL2 and FMNL3 separately had similar effects on melanoma cell morphology and migration. Furthermore, there was no additive effect from co-silencing of both. Such a result could best be explained by assuming direct interaction between these two formins by the way of heterodimerization. Another possible scenario is that both are involved in different steps of the same process, e.g. initiation of filopodia formation and subsequent filopodial elongation. Further studies await the availability of a specific FMNL3 antibody.

To summarize, we have shown that all FMNLs studied here (FMNL1, FMNL2 and FMNL3), are localized in filopodia but in a cell type dependent manner. We also showed that silencing FMNL2 and FMNL3 in melanoma cells led to alterations in cellular protrusions. In migration studies, silencing did not reduce wound healing capacity, but did significantly reduce migration through holes in a membrane. Thus, based on what is known about the functional features of filopodia, FMNLs can be proposed to be involved in directional sensing and migration of cells.

FHOD1 has a very different cellular localization. It can be seen as minute granules in the cytoplasm and along filamentous actin in stress fibers.

FHOD1 is activated by Rho-associated kinase serine/threonine kinase (ROCK), through phosphorylation of three C-terminal serine/threonine residues. This releases FHOD1 from autoinhibition (Takeya et al. 2008). ROCK, in turn, is activated by the RhoGTPase RhoA. Activation of the Rho-ROCK cascade triggers stress fiber formation in cells. Our observation

on the localization of FHOD1 along stress fibers is nicely attuned with this functional arrangement. In support of functional involvement of FHOD1 in this network, we found a reduced number of actin stress fibers associated with loss of elongated cell shape typical of mesenchymal cells in FHOD1-depleted cells. This acquisition of a more epithelial phenotype and reduced invasive capacity was not associated with altered cadherin expression during the timeframe of the study. This suggests that the effects of FHOD1 on the actin cytoskeleton are likely to be direct, not mediated by E-cadherin loss. Very similar results have been shown after FHOD1 knockdown in breast cancer cells by EMT-repressing microRNA 200c (Jurmeister et al. 2012).

We found that knockdown of FHOD1 reduced the number of cells with matrix-degrading invadopodia. However, we were not able to detect FHOD1 in the invadopodia of control cells. This effect is therefore likely to be indirect. Indeed, three other formins, DRF1, DRF2 and DRF3 are required for invadopodia formation in breast cancer cells, without direct detectable presence in these structures (Lizarraga et al. 2009).

6.4. Formin involvement in different steps of cancer progression

Formins have a distinct expression in various cell types. Thus, in order to learn about the role of formins in carcinogenesis, it is important that it is studied in a cell specific context.

The role of formins in malignant transformation remains elusive. This is partly due to limited data on the expression of formins in various types of normal and malignant tissues. We have in this study validated and used antibodies formin expression profiles in normal tissues in order to fill in this gap in knowledge. Furthermore, we studied a large number of human tumours to elucidate the expression patterns of formins in cancer. Finding altered formin expression in primary tumours is a good starting point for identifiying formin roles in malignant transformation. By these means, we have gained increasing evidence of formin contributions to biochemical and functional processes that are linked to cancer progression.

The expression of formins FMNL1, FMNL2 and FHOD1 was studied in normal and transformed cells, healthy tissues and cancer tissues. We also conducted functional experiments focusing especially on cellular activities that are considered to be crucial in cancer progression.

An important step for cancer progression is acquisition of invasive capacity. EMT is an alteration of cellular phenotype, frequently seen in epithelial cancers during invasion. It leads to dissociation of cell-to-cell contacts and an elongated mesenchymal type of morphology. This change is reversible, through MET. We studied involvement of formins in cancer associated EMT. We discovered that in oral SCC, FHOD1 is the most upregulated formin. This expression is dependent on PI3-kinase signaling, which is one of the main pathways activated in EMT. We found that the morphological hallmarks of EMT are mediated by FHOD1. It is also associated efficient proteolysis and migration, as well as invasion. In FHOD1 knockdown these activities were reduced *in vitro*. In MET-EMT, cells acquire a morphology of epithelial or mesenchymal cells depending on whether they are adherent or in migration.

Our results showed that FHOD1 expression is associated with the mesenchymal phenotype. With loss of FHOD1, the epithelial-like morphology is restored. This raises an intriguing scenario where FHOD1 expression rises with EMT to facilitate invasion, to again go down with MET, when a metastatic tumour mass is formed. FHOD1 expression could be seen at the invasive edge of all studied cases of clinical oral SCC, we believe the elevated level of FHOD1 has true relevance *in vivo*. Affirmatively, it has also been found that that the level of FHOD1 is regulated by miRNAs controlling EMT, and that reduced FHOD1 expression leads to reduced invasiveness in breast cancer cells as well (Jurmeister et al. 2012). In our pilot study looking for FHOD1 expression in breast cancer, we found FHOD1 expression at the invasive edge in a minority of cases. All in all, FHOD1 may very well be one of the players in breast cancer EMT and invasion, but apparently not as crucial as in oral SCC. Clearly, FHOD1 expression is associated with proteolysis in oral SCC EMT cells. Another mechanism, by which FHOD1 knockdown could reduce invasion, is via integrins. Integrin mediated focal adhesion maturation is inhibited by FHOD1 loss, at least in mouse fibroblasts (Iskratsch et al. 2013). In order to delineate the roles of proteolysis and integrin-based adhesion in inferiorly migrating FHOD1-depleted cells, a stable FHOD1 knockdown oral SCC cells would be needed. To our disappointment, we have so far been unable to conduct such as study, since stable FHOD1 knockdown has led to cell death in our EMT cell line.

Melanoma cells can utilize the ameboid form of migration for invasion. This makes it possible for them to invade and squeeze through the ECM without protein degradation. We studied formin involvement in ameboid migration. We found that FMNL2 (as well as FMNL3) is associated with migration in cultured melanoma cells that are unable to degrade gelatin. We noticed that FMNL2 and/or FMNL3 silencing did not reduce migration in wound healing experiments. We also studied migration in Boyden chambers, where migration was reduced by FMNL3 or FMNL2 and FMNL3 co-silencing. Migration in Boyden chambers requires that the cells identify pores in a membrane and as single cells squeeze to the other side. The exact mechanism by which FMNL2 and FMNL3 silencing reduces this sort of migration is still unexplained. It is possible that a subtle disruption of filopodia affects the ability of cells to identify the pores or form focal complexes for adhesion. Albeit these mechanistic details are unclear, we feel safe to say that also FMNL2 and FMNL3 play roles in melanoma cell migration. Consistent results on FMNL2 as a positive regulator of cell motility have been reported from a study on colorectal cancer cells (Zhu et al. 2011).

Metastasis is the main cause of death among cancer patients. The ability to metastasize involves many steps after invasion through basement membranes and/or ECM. Melanoma is one of the cancer forms in which the outcome is hard to predict – metastases can develop years, even decades after a seemingly radical excision of the primary tumour. In our study on early stage melanomas, we found that the level of FMNL2 expression in the primary tumour correlated with poor outcome, both when measured as recurrence free survival (most recurrences were metastases) and melanoma specific survival. We did not, however, see a difference in staining results of primary melanomas compared to paired metastases that had developed during follow up. Treatment failure in clinically early stage melanomas is due to the subclinical dissemination of tumour cells outside the primary tumour. This

suggests that high FMNL2 expression in primary melanoma could mean early widespread clinically silent tumour cells or metastasis that only later become evident when proliferation results in detectable tumours. Again, the exact mechanisms for such pro-metastatic functions remain unclear. *In vivo* studies on colorectal carcinoma have actually shown that FMNL2 indeed participates in the development of metastasis (Zhu et al. 2011).

Steps between migration and metastasis that need further investigation for formin involvement are the adhesion of cancer cells to endothelium of lymphatic or blood vessels, as well as intravasation and extravasation of cancer cells. In leukemia cells, FMNL1 silencing reduces transendothelial migration (Favaro et al. 2013). This finding is exciting in showing that a formin participates in one of the more complicated steps of cancer progression, crossing cellular barriers.

7. CONCLUSIONS

During the final stages of cancer development, tumour cells obtain the capacity to invade and metastasize. While a localized tumour in many cases can be surgically removed, metastasis remains the main cause of treatment failure and mortality in cancer patients. Detailed knowledge of mechanisms underlying invasion, migration and metastasis is prerequisite for the development of new targeted drugs and clinical tools for prognostic or predictive use. In carcinomas, one of the mechanisms that lead to invasion is EMT. The actin modulators involved in this transition have not been clear. Widespread EMT in head and neck cancers is a negative prognostic factor that predicts treatment failure. Therefore, all invasive cancer do not undergo EMT, it is undoubtedly an important field for research in cancer biology.

Based on our studies on formin involvement in EMT we recognize FHOD1 as the major formin upregulated in the process of EMT in oral SCC. FHOD1 regulates the alteration of cytoskeletal and functional properties for EMT; stress-fiber rich phenotype, efficient migration, proteolysis and invadopodia formation. The cellular functions or structures associated with FHOD1 and other formins we have studied are summarized in Figure 5. Importantly, the increase of FHOD1 expression is seen in clinical cancers, in which EMT contributes to dissemination of tumours and subsequent treatment failure. In this setting, the activation cascade of FHOD1 could serve as a potential drug target. Whether increased expression of FHOD1 occurs in SCC in other locations, or other types of cancer in general, is an important question that remains to be addressed.

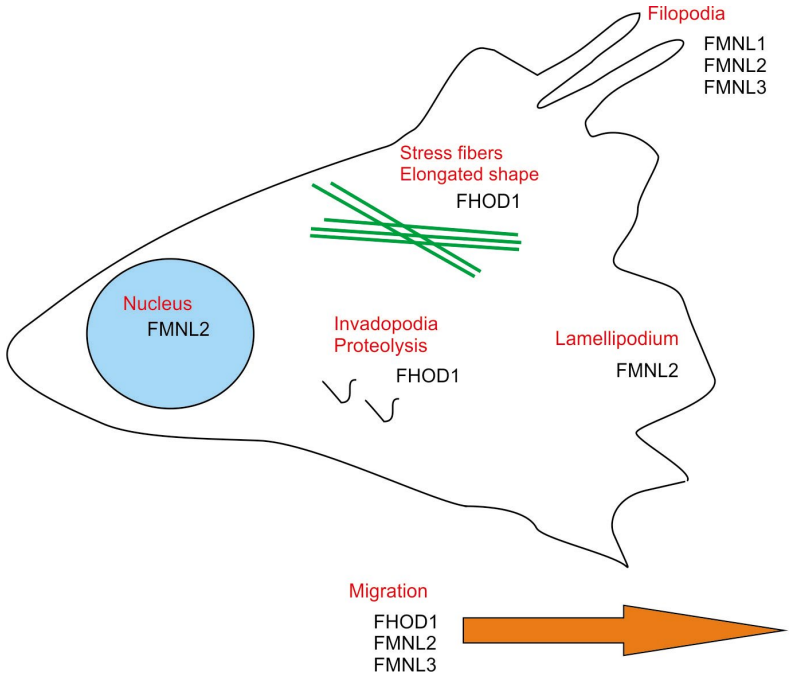

Figure 5. Schematic view of principal cellular findings in this study. Cellular structures with formin enrichment or functions with formin involvement are depicted in red.

In our studies on FMNL1, we found that it indeed is expressed in leukocytes, as suggested by the literature. However, it is also expressed in smooth muscle and myoepithelial cells. The functional roles of attributed to FMNL1 come from studies on immune cells and involve diverse processes such as phagocytic cup formation, podosome formation, and adhesion. What these processes have in common is that membrane protrusion is a central occurrence. We found that FMNL1 expression can be detected in clinical basal type breast cancer. Cellular studies placed this formin at filopodial tips of a basal breast cancer cell line. This location points toward involvement in either adhesion or migration. Unraveling the functional role of FMNL1 neo-expression in carcinoma will be the challenge of studies to come.

The principal finding from our research on FMNL2 expression is that it is an independent prognostic marker in localized melanoma. Cellular studies showed that FMNL2 is filopodial in melanoma cells. Inhibition of both PI3K- and MAPK-pathways reduces FMNL2 expression. As these are two of the most up-regulated signalling pathways in melanoma, it is possible that the FMNL2 level mirrors the activity of cancer related pathways. Although FMNL2 silencing did not cause dramatic morphological alteration, migration was clearly reduced. Further research will be needed to clearly define whether the prognostic impact of FMNL2 relates to a direct mechanism linked to actin assembly, or whether it merely is a surrogate marker for dysregulated cellular signalling.

ACKNOWLEDGEMENTS

This study was conducted at the Department of Pathology, University of Turku, the Turku University Hospital and the Medicity Research Laboratory, Turku, Finland during the years 2006-2015.

I wish to express my deepest gratitude to my supervisor Professor Olli Carpén, who has been my mentor in research and clinical pathology. His open-minded ideas, broad understanding of science and devotion to cancer research made this work a brilliant experience. Without his encouragement, this study, conducted while I was a part-time clinical pathologist and part-time researcher, would not have been possible.

Docent Maria Vartiainen from Helsinki University and Professor Veli-Pekka Lehto from Helsinki University Hospital are warmly acknowledged for their careful review and useful comments that substantially improved the quality of my thesis.

I wish to warmly thank all my co-authors both in the Carpén group and elsewhere for participating in this work. I especially want to thank Katja Kaipio and Vanina D. Heuser for guiding me, teaching me exciting methods and for helping with cellular experiments and imaging. Not forgetting your friendship and incredible sense of humor both in the cell laboratory and outside it. I wish to thank Piia Mikkonen and Laura Lehtinen for their skillful input in cellular experiments, Professor Reidar Grenman for the fantastic cell lines it has been a privilege to work with. My sincere thanks go to Ilkka Koskivuo for contributing with clinical data on melanoma patients and helpful comments on the manuscript, and Mari Koivisto for expert help with statistical analyses.

I would like to thank my colleagues at the Department of Pathology for support, memorable conversations and needless to say, friendship through the years, and all those who contributed to this thesis. I especially want to thank Jaakko Liippo for adding a professional touch to images, Sinikka Collanus for immunohistochemical stainings of tissues, and Paula Merilahti for organizing practical matters and helping with experiments at the lab.

I also take this opportunity to thank my treasured friends, with a special mention to Mirva, Nina and Maija, for the many times we have shared the joys and sorrows of life. I thank my dearest Jouni for encouragement and patience at times I have been preoccupied with this study.

I want to express gratefulness and love to my wonderful family and extended family. I thank my dear parents and my brothers, for your love and support. I am deeply thankful to my precious daughters Alma and Helga for all the happiness you have brought to my life.

This study was financially supported by The National School of Clinical Science, the Juselius foundation, the Medical Society of Finland, the Finnish Medical Society Duodecim, the Cancer Organizations of Finland, the K. Albin Johansson foundation, the Perklén foundation and Turku University Hospital research funds. Their support is gratefully acknowledged.

Turku, March 2015

Maria Gardberg

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