Exploring Cellular Dynamics
From Vesicle Tethering to Cell Migration

PARHAM ASHRAFZADEH
Cells in the body communicate with each other in order to cooperate efficiently. This communication is in part achieved by regulated secretion of signaling molecules, which when released from a cell may activate receptors present at the plasma membrane of an adjacent cell. Such signals affect both cell fate and behavior. Dysregulated signaling may lead to disease, including cancer. This thesis is focused on how exocytosis and subsequent activation and trafficking of receptors can be regulated, and what the consequences of this regulation may be for cell migration.

Actin filaments are important transport structures for secretory vesicle trafficking. In Paper 1, actin polymerization was shown to induce formation of ordered lipid domains in the plasma membrane. Accordingly, actin filaments may thus create and stabilize specific membrane domains that enable docking of vesicles containing secretory cargo.

The RhoGEF FGD5 regulates Cdc42 which can result in cytoskeletal rearrangements. In Paper II, FGD5 was shown to be selectively expressed in blood vessels and required for normal VEGFR2 signaling. FGD5 protected VEGFR2 from proteasome-mediated degradation and was essential for endothelial cells to efficiently respond to chemotactic gradients of VEGFA.

The exocyst component EXOC7 is essential for tethering secretory vesicles to the plasma membrane prior to SNARE-mediated fusion. In Paper III, EXOC7 was required for trafficking of VEGFR2-containing vesicles to the inner plasma membrane and VEGFR2 presentation at the cell surface.

The ability of tumor cells to escape the primary tumor and establish metastasis is in part dependent on their capacity to migrate. In Paper IV, a method based on time-lapse microscopy and fluorescent dyes was created to analyze single cancer cell migration in mixed cancer cell cultures, and in particular the influence of different types on neighboring cells was assessed.

In conclusion, these studies have enhanced our understanding of the mechanisms behind cellular trafficking, and may be applied in the future to develop more specific therapeutics to treat cancer and other diseases associated with abnormal angiogenesis and cellular migration.

Keywords: angiogenesis, cancer, cell migration, exocyst complex, exocytosis, FGD5, lipid rafts, plasma membrane, receptor trafficking, VEGFR2

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You are today where your thoughts have brought you; you will be tomorrow where your thoughts take you.

-James Allen

To my mother and father
Cover: Trajectories of VEGFR2-mCherry containing vesicles tracked in the TIRF plane for 20 mins in a HEK cell
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Equal contribution

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Related publication by the author not included in this thesis:

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<td>AP</td>
<td>Adaptor protein</td>
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<tr>
<td>ARP</td>
<td>Actin-related proteins</td>
</tr>
<tr>
<td>β-TRCP</td>
<td>Beta-transducin repeat-containing proteins</td>
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<tr>
<td>CAS</td>
<td>CRISPR associated</td>
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<tr>
<td>CATCHR</td>
<td>Complex Associated with Tethering Containing Helical Rods</td>
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<tr>
<td>Cbl</td>
<td>Casitas B-lineage lymphoma</td>
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<tr>
<td>Cdc42</td>
<td>Cell division control protein 42</td>
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<tr>
<td>CLASP</td>
<td>Clathrin-associated sorting proteins</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>CT-B</td>
<td>Cholera toxin B</td>
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<tr>
<td>Dbl</td>
<td>Diffuse B-cell-lymphoma cells</td>
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<tr>
<td>DH</td>
<td>Dbl homology</td>
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<tr>
<td>EB</td>
<td>Embryoid body</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EEA</td>
<td>Early endosome antigen</td>
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<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>Erk</td>
<td>Extracellular signal regulated kinase</td>
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<tr>
<td>ESRP</td>
<td>Epithelial splicing regulatory protein</td>
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<tr>
<td>FGD</td>
<td>FYVE, RhoGEF and PH domain-containing protein</td>
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<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
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<td>GDI</td>
<td>Guanine nucleotide dissociation inhibitors</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GNBP</td>
<td>Guanine nucleotide binding proteins</td>
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<td>GP</td>
<td>Generalized polarization</td>
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<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney cells</td>
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<tr>
<td>HMVEC</td>
<td>Human dermal microvascular endothelial cells</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>Jas</td>
<td>Jasplakinolide</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KDR</td>
<td>Kinase insert domain receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Laurdan</td>
<td>6-Dodecanoyl-2-Dimethylaminonaphthalene</td>
</tr>
<tr>
<td>Lat B</td>
<td>Latrunculin B</td>
</tr>
<tr>
<td>Ld</td>
<td>Lipid/Liquid disordered</td>
</tr>
<tr>
<td>Lo</td>
<td>Lipid/Liquid ordered</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-epithelial transition</td>
</tr>
<tr>
<td>MRCK</td>
<td>Myotonic dystrophy kinase-related Cdc42-binding kinase</td>
</tr>
<tr>
<td>NPF</td>
<td>Nucleation promoting factors</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
</tr>
<tr>
<td>PAO</td>
<td>Phenylarsine oxide</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PI</td>
<td>Phosphoinositides</td>
</tr>
<tr>
<td>PIPs</td>
<td>Phosphatidylinositols</td>
</tr>
<tr>
<td>PtdIns</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI(4,5)P2</td>
<td>Phosphatidylinositol 4, 5-bisphosphate</td>
</tr>
<tr>
<td>PTPs</td>
<td>Protein tyrosine phosphatases</td>
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<tr>
<td>SNAP</td>
<td>Soluble NSF attachment protein</td>
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<tr>
<td>SNARE</td>
<td>Soluble NSF attachment receptor</td>
</tr>
<tr>
<td>TIRF</td>
<td>Total internal reflection fluorescence</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
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</table>
Introduction

In order for cells to coordinate their functions they need to be able to communicate with each other. The mechanism behind this cell-to-cell communication is heavily dependent on the molecules that are secreted from cells, such as growth factors, which can be recognized by receptors at the plasma membrane. Both growth factors and their receptors are delivered to the extracellular space and the plasma membrane via a process called exocytosis. In eukaryotic cells, activated receptors become internalized through the process of endocytosis, whereafter they may be delivered to lysosomes and degraded, or recycled back to the cell surface (Figure 1). Both endocytosis and exocytosis occur through highly organized directional routes on actin cables within the cell [1]. This thesis identifies and explores key regulators of dynamic cellular events and in doing so contributes to our fundamental knowledge of cell signaling, receptor trafficking and cell migration.

Figure 1. General schematic illustration of receptor trafficking in the cell. Filled arrows indicate degradation, open head arrows indicate recycling and double-headed arrows indicate exocytosis.
Lipid components of the plasma membrane

The plasma membrane is of paramount importance for cellular function as it separates the interior of cells from the outside environment. The plasma membrane is comprised of several types of lipids that play a crucial role in its function. In general, lipids are classified into three major classes in eukaryotic cells: glycerophospholipids, sphingolipids and cholesterol (Figure 2). Glycerophospholipids are the most abundant membrane lipids, comprising a hydrophilic head group and two hydrophobic tails that can be saturated or unsaturated. Most sphingolipids are also phospholipids, but instead of containing glycerol they are based on ceramide. Typically, the acyl chain attached to the sphingoid base is saturated and has 16-26 carbons [2]. Cholesterol is one of the most abundant lipids at the plasma membrane, typically comprising 30-40% of plasma membrane lipids. Cholesterol interacts preferentially with sphingolipids rather than with unsaturated phospholipids [3]. The lipid composition of the plasma membrane differs between the exoplasmic (outer) and cytoplasmic (inner) leaflets. For instance, most (if not all) of the sphingolipids are present in the exoplasmic leaflet, while some glycerophospholipids, such as phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine, are limited to the cytoplasmic leaflet of the plasma membrane (Figure 2) [4].

Lipid phases and lipid rafts

In live cells two phases can exist within the plasma membrane: liquid ordered (lo) and liquid disordered (ld) (Figure 2). The lo phase is composed primarily of saturated lipids with low fluidity and fully extended acyl chains. In contrast, the ld phase is comprised of mostly unsaturated lipids with a lower proportion of cholesterol and higher fluidity compared to lo phase [5]. Although it has been reported that the difference in the cholesterol fraction between lo and ld phases is rather small in cells, it is nonetheless an absolute requirement for the formation of lo phase [6, 7]. Lipid rafts, which are also known as lo domains, are plasma membrane nanodomains with an approximate size between 10 to 200 nm that have specific lipid and protein compositions [8]. Approximately twenty years ago, the lipid raft concept was proposed when rafts were demonstrated to form by self-aggregation of
cholesterol and sphingolipids. It has been suggested that lipid rafts are surrounded by a sea of ld domains [9, 10].

**Phosphoinositides**

Phosphoinositides (PIs) are minor lipid components of eukaryotic plasma membranes (*Figure 2*), yet they are still critically important for many cellular functions. There are seven PIs derived from phosphatidylinositol (PtdIns) by PI kinases. PI kinases can phosphorylate PtdIns at one or more of the 3-, 4-, and 5-hydroxyl groups of the inositol head [11]. Microscopy studies suggest PI(4,5)P2 colocalizes with both lipid raft markers and secretory vesicles at the plasma membrane [12, 13]. Also, decreasing the level of PI(4,5)P2 in the plasma membrane results in a dramatic release of the cytoskeleton from the membrane [14].

![Figure 2. The two proposed phases of the plasma membrane in live cells. The lo (liquid ordered) phase is composed of a higher fraction of saturated lipids, cholesterol and PI(4,5)P2 compared to the ld (liquid disordered) phase.](image)

**GTPases**

Small GTPases are molecular switches in the cell that perform essential functions in regulating different biological processes, such as changes in cell shape and motility [15]. They may also be referred to as GTP-binding proteins, G-proteins, and guanine nucleotide binding proteins (GNBPs). GTPases become activated upon GTP binding and inactivated by hydrolytic cleavage of GTP to GDP. Guanine nucleotide exchange factors (GEFs) activate GTPases as they facilitate the release of GDP, thus allowing GTP, which is more abundant in the cytoplasm, to bind. On the other hand, GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of the GTPase itself, resulting in hydrolysis of GTP to GDP, thus returning the GTPase to its inactive state (*Figure 3*). Guanine nucleotide dissociation inhibitors (GDIs) also maintain GTPases in their inactive state by inhibiting dissociation of bound GDP [16, 17].
Figure 3. Activation and inactivation of GTPases. GTPases exist in an active GTP-bound form and an inactive GDP-bound form. GTPases become activated by GEFs, catalyzing the release of GDP and allowing GTP to bind. GAPs can accelerate the return of GTPases to their inactive GDP-bound form.

Rho GTPases and RhoGEFs

The Ras superfamily of small GTPases can be divided into several families according to their function and structure: Ras, Rab, ARF, Sar1, Ran, Rad/Gem and Rho [18]. Among those, the Rho GTPase family has approximately 20 members, including RhoA, Rac1 and Cdc42, which play essential roles in actin cytoskeleton rearrangements [19]. Around 80 RhoGEF proteins that can activate Rho GTPases have been identified. The first RhoGEF was characterized from diffuse B-cell-lymphoma cells and was accordingly designated Dbl [20]. A region of Dbl with approximately 240 residues is homologous to a region in Saccharomyces cerevisiae Cdc42. Dbl together with Cdc42 can facilitate budding and polarity in S. cerevisiae, and Dbl was later found to act as a GEF for human Cdc42 [18, 21]. RhoGEFs contain two types of conserved domains: DH (Dbl homology) and PH (Pleckstrin homology) domains. The DH domain, containing approximately 240 residues, is important for the catalytic function by interacting directly with a switch region of Rho GTPases. The PH domain has about 100 amino acids and facilitates the localization of the RhoGEFs to PI(4,5)P2 at the plasma membrane [22]. Furthermore, small GTPases interact with PI(4,5)P2 to control motility, cell shape and several other processes in the cell [23].

FGD5

FYVE, Rho GEF and PH domain-containing- 5 (FGD5) is a RhoGEF in the FGD family. In addition to the DH and PH domains of RhoGEFs, members of the FGD family have an additional PH domain containing a FYVE zinc-finger domain in between the PH domains. FGD5 is highly expressed in endothelial cells and has been shown to play roles in many cellular processes [24-26]. FGD5 depletion leads to decreased sprouting in endothelial cells [25, 26], and knockdown of FGD5 decreases both VEGFA and FGF2-mediated proliferation [25] and integrin-mediated cell spreading [26]. FGD5 is downstream of the small GTPase Rap1, which mediates actin bundle attachment.
formation and increased cell-cell barrier function. FGD5 activates the myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK) through Cdc42, which in return triggers non-muscle myosin II activation [27]. In contrast, it has been shown that FGD5 plays a critical role in vascular remodeling by promoting apoptosis, resulting in vascular pruning and inhibition of neovascularization [24]. Therefore, whether FGD5 acts to promote or inhibit vascular growth remains a topic of controversy.

Exocytosis

Exocytosis releases growth factors, chemokines and other signaling molecules into the extracellular space. These proteins are used to signal to neighboring cells and stimulate a wide range of cellular behavior depending on the nature of the released cargo. The exocytosis process can be categorized into two types: regulated and constitutive. Regulated exocytosis occurs in response to certain stimuli and happens very quickly after stimulation in most secretory cells. Constitutive exocytosis occurs constantly at the cell membrane to maintain the composition of the plasma membrane, and is required for the delivery and presentation of certain receptors at the cell surface [28]. During constitutive exocytosis no external stimuli is required to trigger cargo release. The mechanisms behind exocytosis and the delivery of secretory vesicles to the plasma membrane are conserved; secretory vesicles are first tethered and then docked before they subsequently fuse with the plasma membrane and in doing so release their cargo (Figure 4).

The exocyst complex

Tethering, which is the initial contact of the vesicles with the plasma membrane, is mediated by the exocyst complex [29, 30]. The exocyst complex is a member of the Complex Associated with Tethering Containing Helical Rods (CATCHR) family [31], and is an octameric protein complex that was first identified in the budding yeast Saccharomyces cerevisiae by biochemical and genetic approaches. The components of the octameric complex in yeast are Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 [32, 33], and in humans they are referred to as EXOC1, EXOC2, EXOC3, EXOC4, EXOC5, EXOC6, EXOC7 and EXOC8, respectively (Table 1). Studies in yeast have shown that Sec3 localizes at exocytotic sites independent of vesicular traffic, while Exo70 is localized both at exocytotic sites and on secretory vesicles. The other six subunits (Sec5, Sec6, Sec8, Sec10, Sec15 and Exo84) tether secretory vesicles to the plasma membrane before vesicle fusion [34]. The tethering of the secretory vesicles at the plasma membrane is through their interactions with several
small GTPases in a GTP dependent manner, including Rho1, Rhp3, Cdc42 in yeast and RalA, TC10 and Rab11 in mammals [35-42]. At the plasma membrane, exocyst complex component EXOC7 binds to PI(4,5)P2 to form a tethering point for secretory vesicles [43].

SNARE proteins present on the intracellular vesicles (referred to as v-SNAREs) and target sites at the plasma membrane (referred to as t-SNAREs) mediate vesicle fusion. The SNARE domains in syntaxin-1 and SNAP25 (at the target membrane) and VAMP2 (on the secretory vesicle) can form a complex, which leads to the formation of fusion pores and exocytosis (Figure 4) [44, 45]. Direct evidence for interactions between the SNAREs and the exocyst subunits have also been described [46].

**Figure 4. Schematic model of secretory vesicle tethering, docking and fusion followed by exocytosis of cargo.** Secretory vesicles containing cargo (eg. VEGFR2) decorated with exocyst components EXOC2-6, 8 and v-SNARE proteins are trafficked to tethering points containing EXOC1 and 7 at the plasma membrane. At the tethering point, all of the exocyst subunits come together to form a bridging complex. Following tethering, interactions of t-SNARES (syntaxin-1 and SNAP25) and v-SNARES (VAMP2) facilitate vesicle docking and ultimately the fusion and exocytosis of cargo.
Table 1. Exocyst complex subunits in yeast and human. The exocyst complex is composed of eight major subunits; notably, the existence of different isoforms and homologs has been described for some of these subunits.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Human</th>
<th>Isoforms and Homologs</th>
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<tbody>
<tr>
<td>Sec3</td>
<td>EXOC1</td>
<td>-</td>
</tr>
<tr>
<td>Sec5</td>
<td>EXOC2</td>
<td>-</td>
</tr>
<tr>
<td>Sec6</td>
<td>EXOC3</td>
<td>EXOC3l1, EXOC3l2</td>
</tr>
<tr>
<td>Sec8</td>
<td>EXOC4</td>
<td>-</td>
</tr>
<tr>
<td>Sec10</td>
<td>EXOC5</td>
<td>-</td>
</tr>
<tr>
<td>Sec15</td>
<td>EXOC6</td>
<td>Sec15b, EXOC6b</td>
</tr>
<tr>
<td>Exo70</td>
<td>EXOC7</td>
<td>EXOC7-1, -2 (M), -4, -5 (E), -6</td>
</tr>
<tr>
<td>Exo84</td>
<td>EXOC8</td>
<td>-</td>
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Cell migration

Cell migration is essential during human development and disease, regulating processes including embryogenesis, wound healing, angiogenesis and cancer metastasis [47-49]. Cells can either move as single entities or together in a process called collective cell migration. The mechanisms of single cell migration have been extensively studied in vitro and informs much of our understanding of how cells move within tissues or secondary growths. Immune cells utilize single cell migration to pass through tissues during immune surveillance. During collective migration, cells stay connected to each other as they move [50]. Apart from random physical cell movements, the location to which cells migrate is often of critical importance. Examples of directed cell migration include chemotaxis, in which cells migrate towards soluble chemoattractants, and haptotaxis, in which cells migrate to gradients of immobilized chemoattractants and/or adhesion sites [51].

Cell migration is also associated with epithelial-mesenchymal transition (EMT). EMT is a process in which adherent epithelial cells switch to a more migratory and thus “mesenchymal-like” state. EMT plays an important role during embryogenesis and organ formation, and has been implicated in cancer metastasis, enabling cancer cells to become more motile and spread to distant organs [52, 53]. It has been shown that epithelial and mesenchymal cells carry certain alternative splicing factors which are important in both EMT and MET (mesenchymal to epithelial transition). Examples of these splicing factors are ESRP1 and ESRP2 (Epithelial Splicing Regulatory Proteins 1 and 2), which can govern the isoform switching events within
genes involved in many cellular processes, such as cell-cell adhesion, cytoskeletal rearrangement and intracellular signaling [54, 55].

**Mechanisms of cell migration**

The mechanisms of cell migration can be described in two alternative processes: membrane flow and actin remodeling [50]. During the membrane flow process, vesicles are continuously transported from the rear of the cell towards the leading edge in the direction of migration, where the plasma membrane is expanded at the front to push the cell forward. In the other process, the actin cytoskeleton needs to be remodeled in order to reorganize the plasma membrane dynamics at the leading edge. The first step in this event of cell migration is the formation of cell surface protrusions, mostly in the form of thin and extensive membrane extensions at the leading edge of the cells termed lamellipodia. It has been shown that lamellipodia have several branched arrays of actin filaments which orient with their plus ends toward the plasma membrane in the most active, motile cells [56].

**FGD5 in cell migration**

To date, there are only a few reports that have addressed the role of FGD5 in different cellular processes. These papers addressed the role of FGD5 in cell migration through regulation of Cdc42 and the subsequent downstream signaling that can lead to actin cytoskeleton rearrangements [24]. In response to cell surface receptor activation, FGD5 can activate Cdc42 from an off-state to an on-state [57], where active Cdc42 facilitates activation of nucleation promoting factors (NPFs) such as WASP family protein members. Arp2/3, which lies downstream of WASP proteins, is then activated and leads to the nucleation and elongation of the cytoskeleton network. Thus, FGD5 mediated activation of Cdc42 followed by a series of signaling cascades is essential for cell adhesion, motility and polarity [58-60]. Deregulation of Cdc42 results in several diseases caused by dysfunctional cytoskeletal formation and membrane trafficking, such as cancer, cardiovascular and neuronal degenerative diseases [17, 18].

Despite the established role of Cdc42 in these processes, there are contradictory reports on how FGD5 affects cell migration [24-27]. Two studies showed that FGD5 depletion leads to a decrease in sprouting and migration of endothelial cells [25, 26]. Knocking down FGD5 either decreased VEGFA or FGF2-mediated proliferation [25] or decreased integrin-mediated cell spreading [26]. However, a separate study proposed that a decrease in the endogenous expression of FGD5 increases sprouting and possibly migration in endothelial cells of the retina [24]. One possible explanation for the discrepancy between these studies could be the model systems that were used; one utilized an *in vivo* system [24], whereas the
other studies were performed *in vitro* or *ex vivo* [25, 26]. In the *in vivo* experiments FGD5 expression was decreased in all cells of the retina, therefore it cannot be excluded that the effects observed on endothelial cells were secondary due to a decrease in FGD5 activity in other cell types, such as perivascular cells.

**The exocyst complex in cell migration**

The exocyst complex plays a crucial role in directed cell migration, where its vesicle tethering properties contribute to the presentation of several proteins at the leading edge of the cell, such as integrins and other adhesion molecules. It has been shown that in the case of prostate cancer cells, disrupting exocyst activity leads to dysfunctional recycling of integrin to the plasma membrane at the leading edge, resulting in decreased tumor cell mobility and subsequent matrix invasiveness [61]. Upon depletion of EXOC7, there is impaired lamellipodia formation as a result of a decrease in the localization of Arp2/3 at the leading edge of the cell. Accordingly, EXOC7 reduction decreases cell migration and directional persistence in wound healing assays [62]. Conversely, overexpression of EXOC7 leads to the formation of spike-like cell surface projections termed filopodia, which can sense the extracellular environment and guide cell movement [63]. Moreover, EXOC7 can deform the membrane in a direct manner to cause filopodia formation [64]. EXOC7 mRNA can be spliced into multiple isoforms by ESRP1 and ESRP2 [55]. Thus far, five EXOC7 isoforms have been identified in human cells: isoform 1, isoform 2 (M), isoform 4, isoform 5 (E) and isoform 6. The E and M terminology originate from their epithelial and mesenchymal expression. mRNA expression of EXOC7-E is high in the luminal epithelial breast carcinoma cell line MCF-7, but low in the claudin-low, invasive breast cancer cell line MDA-MB-231. Interestingly, EXOC7-E overexpression in MDA-MB-231 induced epithelial cell-like phenotypes, while EXOC7-M overexpression promoted tumor cell invasion *in vitro* [65]. Thus, alternative splicing of EXOC7 can regulate EMT, which may be important during tumor cell metastasis.
Angiogenesis

The blood vasculature is a multilayered, complex system comprised of vessels with an innermost layer of endothelial cells (endothelium), surrounded by a basement membrane and mural cells (pericytes and vascular smooth muscle cells) [66]. Vessels form complex branched networks comprised of arteries, capillaries and veins that work together to ensure the delivery of nutrients and oxygen to tissues and removal of waste products. While all vessel types have a number of similar characteristics, they can differ in morphology, gene expression and function between different tissues, resulting in specialized functions to suit their environment.

The formation of the vascular system during embryonic development, or during the physiological response to hypoxia or tissue injury in the adult, occurs through sprouting angiogenesis [67], which is the growth of a newly formed vessel from a pre-existing vessel. The process of angiogenic sprouting occurs when a highly migratory ‘tip cell’ at the vascular front senses extracellular guidance cues through polarized, extended filopodia [68]. These tip cells then migrate out into the environment, where they are followed by neighboring ‘stalk cells’, which contribute to the stable, lumened vessel. In addition to sprouting angiogenesis, the formation of new vessel networks can occur through intussusceptive angiogenesis (splitting of established vessels) and looping angiogenesis [69]. Looping angiogenesis has been proposed as the main mechanism of angiogenesis during wound healing, where contractile myofibroblasts mechanically ‘pull’ adjacent tissue, including existing vessels, into the wound area [70]. Once within the wounded area, these elongated vascular loops remodel to a functional vascular network through sprouting, splitting and regression. Sprouting, intussusception and looping all occur simultaneously, although to what degree each occurs is likely controlled by different physiological cues. For example, it is thought sprouting is the most prominent process during embryonic development, whereas intussusception and looping occur primarily during the initial phases of wound healing.

The process of angiogenesis is primarily controlled by VEGFA binding to VEGF receptor 2 (VEGFR2) expressed on endothelial cells, which triggers signaling pathways controlling a variety of biological outcomes including cell migration, proliferation, survival and identity [71]. During sprouting angiogenesis, VEGFR2 is highly expressed in tip cells, allowing for efficient binding of VEGFA and resulting migration and proliferation [72]. Interestingly, VEGFR2 expression is lower in adjacent stalk cells, rendering them less responsive to VEGFA [73]. VEGFR2 is regulated in a multiple ways including co-receptors, endocytosis, intracellular trafficking and cross signaling with other signaling pathways [74]. Additionally, the expression context of VEGFR2 and its mediators, such as co-receptors, can specify receptor activity. For example, the VEGFR2 co-receptor Neuropilin-
1 promotes VEGFR2 trafficking and signaling when expressed on the same cell, but inhibits internalization when expressed on an adjacent cell [75, 76].

Currently, many therapeutic agents are under development to either suppress or stimulate angiogenesis, depending on the therapeutic need [77]. In settings of ischemia, where tissues are deprived of blood and nutrients due to a lack of vascularization, patient outcome is improved by promoting angiogenesis to the ischemic site. It is advantageous to inhibit vessel growth in settings of cancer. VEGFA is produced at high levels by tumors, promoting the growth of new vessels that in turn promote tumor survival and expansion. In humans, elevated VEGFA correlates with increased metastasis, tumor invasiveness and poor prognosis [78]. While anti-VEGFA treatments have been utilized in the clinic for many years to target tumor-induced angiogenesis, the benefit of this treatment has proved to be modest and patients often do not respond to treatment. This is largely due to compensatory mechanisms upon inhibition of VEGFA, where other pro-angiogenic compounds become highly upregulated [79]. It is clear that given the complexity of the angiogenic response it is essential to improve our understanding of this process in order to develop more effective and efficient treatment strategies.

Vascular endothelial growth factors (VEGFs)

While VEGFA binding to VEGFR2 is the principal angiogenic ligand-receptor complex, the VEGF family consists of four additional members of secreted, dimeric glycoproteins with an approximate size of 40 kDa: VEGF-B, VEGFC, VEGFD and placenta growth factor (PLGF). VEGFA, B and PLGF bind to VEGFR1 and VEGFA binds to VEGFR2 [74]. Family members VEGFC and D primarily bind to VEGFR3 to mediate the growth and development of lymphatic vessels (a process termed lymphangiogenesis) [80], however VEGFC and VEGFD can undergo proteolytic processing and bind to VEGFR2, although with lower affinity compared to VEGFR3 [81]. VEGFs are broadly expressed and are essential for the development of the central nervous system (CNS), and organs including the kidneys, liver and lungs, revealing their global importance during human development and health.

Vascular endothelial growth factor receptors (VEGFRs)

The VEGFRs are receptor tyrosine kinases (RTKs) with an approximately 750 amino acid residue extracellular domain organized into seven Ig-like folds [71]. All VEGFRs share structural similarities, with the extracellular domain accompanied by a transmembrane region, a juxta-membrane domain, two tyrosine kinase domains split by a kinase insert domain and a C terminal tail (Figure 5). In addition to VEGFR2-VEGFR2 homodimers,
VEGFR2 can form heterodimers with VEGFR1 and VEGFR3. While little is known about VEGFR1-VEGFR2 heterodimer formation, processed VEGF-C and D bind to a heterodimerised VEGFR2-VEGFR3 receptor complex with high affinity [82]. In addition to their membrane bound forms, soluble forms of VEGFR1 and VEGFR2 composed of the ligand binding domain, but lacking the seventh Ig domain have been described, sequester ligands and therefore inhibit the angiogenic response [83, 84]. VEGFRs are highly expressed in endothelial cells, but can also be expressed in other non-endothelial cells [71] such as monocytes, smooth muscle cells and neuronal cells (VEGFR1) [85], neuronal cells and tumor cells (VEGFR2) [86], and neuronal progenitors, macrophages and osteoblasts (VEGFR3) [87, 88].

**Figure 5. Structural outline of a VEGFR2 homodimer.** VEGFR2 consists of an extracellular domain, transmembrane domain and cytoplasmic domain (which can be cleaved for proteasomal degradation). VEGFA, presented as a dimer, binds to Ig-like domains 2 and 3 on VEGFR2 (green circles), promoting its dimerization.

**Regulation of VEGFR activity**

Each VEGFR, depending on the local context, displays differential activation leading to a variety of biological outcomes. VEGFR2 dimerization (as a homo- or heterodimer) is a prerequisite for its activation, which is typically induced by ligand binding. Dimerization of the receptor is followed by a conformational change, allowing for activation of receptor-kinase
activity and subsequent autophosphorylation of the receptors within the
dimer at specific tyrosine residues within the intracellular tail [89].
Following phosphorylation, interacting proteins such as adaptors and
additional kinases are recruited by the receptors leading to the activation of a
downstream signaling cascade. In addition to activation of the receptors by
VEGF binding (canonical activation), non-canonical mediators such as
mechanical force (shear stress) or non-VEGF ligands have been shown to
activate VEGF receptors [74, 90].

Negative regulation of VEGFRs is essential for the normal angiogenic
process by limiting the downstream amplitude and duration of transmitted
signals to the target cells. Protein tyrosine phosphatases (PTPs) rapidly
dephosphorylate receptors, and thus can counteract and limit activation of
receptor kinase activity. In the case of VEGFR2, dephosphorylation by PTPs
may occur at cell-cell junctions [91] or within endosomes [92]. Furthermore,
inhibition of signaling can be achieved by rapid receptor degradation
through the proteasome and lysosome [93, 94].

VEGFR2 endocytosis and trafficking
Presence at the plasma membrane is essential for the activation of many
RTKs, including VEGFRs. However, in addition to its activity at the plasma
membrane, VEGFR2 can continue to signal after it has been internalized
within the cell [74, 95, 96]. Therefore, the duration of VEGFR2 presentation
at the membrane and the rate of its endocytosis, trafficking, recycling and
degradation will determine the final signaling output. VEGFR2 is found at
multiple different sub-cellular localizations, including the plasma membrane
(at the abluminal and luminal sides) [97] and various intracellular
compartments, including early and recycling endosomes [98]. Within the
plasma membrane, VEGFR2 may be either freely diffusible or presented at
several different regions/domains, such lipid rafts, focal adhesions or cell-
cell junctions [91, 99]. The specificity of signaling is likely very much
dependent on the receptor localization at various membrane compartments,
yet the mechanisms and importance of differential localization at the plasma
membrane remains to be fully understood.

In order to transmit a large proportion of its signaling, such as VEGFR2-
dependent activation of the ERK1/2 pathway [95, 100], VEGFR2 needs to
be internalized, which occurs via endocytosis primarily after VEGFA
stimulation (Figure 6). It is thought that VEGFR2 endocytosis occurs in a
clathrin-dependent manner when dimerised, VEGFA-bound receptors within
the membrane enter clathrin-coated pits [101, 102]. This process occurs
when clathrin binds indirectly to the receptor via various adaptors, such as
adaptor protein 2 (AP2) or clathrin-associated sorting proteins (CLASPs),
resulting in the formation of clathrin-coated vesicles. These vesicles are
pinched off from the original membrane by dynamin proteins, followed by
rapid shedding of the clathrin coat. The vesicles then fuse with early endosomes whereafter the cargo can proceed through a series of steps for recycling to the membrane through slow or fast pathways [103]. Fast recycling through Rab4 endosomes may also occur in a constitutive, ligand independent manner [103, 104]. Alternatively, VEGFR2-containing vesicles can be transported to lysosome and/or proteasome for degradation.

VEGFR2 degradation

Post-translational modifications such as ubiquitination play a crucial role in VEGFR2 trafficking, as such modifications determine whether the receptor is targeted for degradation or recycling. Activation of VEGFR2 leads to phosphorylation of the ubiquitin ligase Cbl, which in turn ubiquitinates the receptor, targeting it for degradation [105]. The importance of Cbl in the regulation of angiogenesis has been demonstrated in vascular diseases of the eye and during cancer progression [106, 107]. However, Cbl activity is not an absolute requirement for VEGFR2 ubiquitination, and recently, the β-TRCP ligase was found to be essential for ubiquitin-mediated degradation of VEGFR2. β-TRCP was able to decrease cancer cell migration and angiogenesis [108], emphasizing the functional importance of ubiquitin-mediated VEGFR2 degradation in the regulation of angiogenesis.

Ubiquitination occurs in parallel with phosphorylation. Internalized VEGFR2 is either recycled to the plasma membrane after deubiquitination or degraded. Different processes are employed during degradation of VEGFR2. Following VEGFR2 endocytosis the cytoplasmic domain is exposed on the surface of the endosome and susceptible to proteolysis and the fragment subsequently undergoes proteasomal degradation. The extracellular domain is contained within the endosome, which may be trafficked first to multivesicular bodies (MVBs) or directly to the lysosome for degradation [94] (Figure 6). MVBs are specialized endosomes that contain membrane-bound intraluminal vesicles [109].

How a balance is maintained between synthesis, exocytosis, endocytosis, recycling, and degradation of VEGFR2, and how this mediates VEGFR2 activity and subsequent cellular responses, is a subject of intense investigation.
Figure 6. Overview of VEGFR2 degradation. Upon dimer formation stimulated by VEGFA, VEGFR2 becomes phosphorylated and targeted for endocytosis (A). If VEGFR2 is targeted for degradation by ubiquitination, it goes to late endosomes, where it is targeted for lysosomal degradation (B) through multivesicular bodies (MVBs) or directly to the lysosome (B1). Additionally, the proteolytic fragment of VEGFR2 can go through proteasomal degradation (B2). Alternatively, the receptor may be deubiquitinated and recycled back to the plasma membrane (C).
Present investigations

Paper I

Actin filaments attachment at the plasma membrane in live cells cause the formation of ordered lipid domains

The aim of this study was to investigate how altered actin filament dynamics can affect the formation of ordered lipid domains in the plasma membrane. Actin filaments have important biological roles in cells and are required for both intracellular vesicle trafficking as well as for the maintenance and modulation of cell shape required for example in association with cell migration. The actin cytoskeleton is linked to the inner leaflet of the plasma membrane via PI(4,5)P2. In order to study the changes in the fraction of ordered and disordered domains in the plasma membrane upon different treatments, laurdan and di-4-ANEPPDHQ probes were used and generalized polarization (GP) measured as an index to show the relative proportion of lo to ld.

Inhibiting actin polymerization resulted in a significant decrease in the GP values at the plasma membrane, indicating a lower fraction of ordered domains, whereas stabilizing actin filaments led to an increase in GP values at the plasma membrane (Figure 1 and 2). Indeed, these alterations in GP values as a result of different treatments strongly suggested that ordered domains exist in live cells and that their abundance is related to actin dynamics [110]. Accordingly, lowering the association between actin filaments and the plasma membrane resulted in a decrease in laurdan GP values (Figure 3). The existence of these domains have previously been questioned by some investigators, who claim that the size of these domains are beyond the resolution of microscopy (10-200nm) and that single domains therefore cannot be resolved [111, 112]. However, there are different methods that can be used to study the changes in the abundance of lipid domains [113].

Blebs are a section of the plasma membrane that balloon outwards and can form as a result of disconnection of actin filaments from the plasma membrane. Accordingly, blebs are devoid of actin filaments. Blebs had lower GP values compared to the rest of the plasma membrane, indicating a lower fraction of ordered domains as a result of decreased actin filament association (Figure 4 and 5). Aggregation of membrane components by
crosslinking of lipid raft molecules (Cholera toxin B and CD59), a non-lipid raft molecule (CD45) and a molecule that goes to both raft/non-rafts (Concanavalin A) led to a significant increase in the proportion of ordered domains at the plasma membrane compared to control cells (Figure 6 and 7). The change in GP values upon crosslinking showed a strong correlation between increased ordered domains and cell peripheral filamentous actin (Figure 8).

In conclusion, ordered domains can be formed upon actin filament attachments at the plasma membrane, revealing the importance of cytoskeleton-lipid interactions (in addition to lipid-lipid interactions) in the formation and/or stability of ordered domains.

**Paper II**

**FGD5 sustains vascular endothelial growth factor A (VEGFA) signaling through inhibition of proteasome-mediated VEGF-receptor 2 degradation**

The aim of this study was to investigate the role of FGD5 in VEGFR2 signaling. Activation of VEGFR2 signaling via VEGFA and the consequent receptor dimerization and phosphorylation is crucial for biological processes including endothelial cell migration. FGD5 regulates Cdc42 which can result in cytoskeletal rearrangements and actin filament formation [21].

FGD5 was shown to be highly expressed in the endothelium of vascular structures in human tissues, including the rectum, skin, cervix and ovary (Figure 1). FGD5 was not only at the membrane and cell-cell junctions but was also found to be associated with intracellular vesicles in the blood vessels of human brain sections (Figure 2).

The possible colocalization of FGD5 and VEGFR2 in endosomes was investigated. VEGFA-stimulation of HMVEC cells resulted in the formation of numerous endosomes that were shown to be triple-positive for EEA1, VEGFR2 and FGD5. Proximity ligation assay (PLA) confirmed the presence of VEGFR2 and FGD5 in EEA1-positive endosomes (Figure 3). The presence of FGD5/VEGFR2-positive endosomes suggested a link between FGD5 and VEGFR2 trafficking. To show if FGD5 could act as an effector of VEGFR2, loss-of-function experiments were performed in which endogenous FGD5 was depleted in microvascular cells using siRNA. In FGD5 depleted cells, chemotaxis of microvascular cells was reduced towards VEGFA gradients (Figure 4).

Next, experiments were performed to examine whether depleting FGD5 affects the downstream signaling of VEGFR2. Indeed, the amount of phosphorylated Erk1/2 was significantly reduced in FGD5-depleted cells.
upon stimulation with VEGFA. Furthermore, VEGFA-stimulated VEGFR2 phosphorylation was decreased in FGD5 depleted cells (Figure 5). Notably, before VEGFA stimulation, the levels of VEGFR2 protein were lower in cells depleted for FGD5 as compared to control cells. This suggested that FGD5 could play a protective role by reducing the degree of VEGFR2 degradation. Experiments with the proteasome inhibitor lactacystin indeed demonstrated that FGD5 protects VEGFR2 from proteasome-mediated degradation (Figure 6).

Taken together, the present study provides evidence that FGD5 sustains VEGFR2 signaling via inhibition of proteasome-dependent degradation of VEGFR2.

Paper III

Exocyst complex component 7 (EXOC7) regulates vascular endothelial growth factor receptor 2 (VEGFR2) trafficking to the plasma membrane

The aim of this study was to investigate if trafficking of VEGFR2 to the plasma membrane is dependent on EXOC7. EXOC3l2, a homolog of EXOC3, has previously been demonstrated to be required for efficient chemotaxis of endothelial cells toward gradients of VEGFA [114]. At the plasma membrane, EXOC7 binds to PI(4,5)P2 to provide a tethering point for secretory vesicles [34, 43]. EXOC7 expression was efficiently suppressed using treatments with specific siRNAs (Figure 1). Effects of both EXOC7 depletion and EXOC7 overexpression on the localization of VEGFR2-mCherry were investigated in HEK cells. Interestingly, the number of VEGFR2-mCherry containing vesicles that reached the plasma membrane was significantly reduced in EXOC7-depleted cells, as determined by TIRF microscopy (Figure 2). Furthermore, VEGFR2-mCherry vesicles spent less time at the plasma membrane when EXOC7 was depleted (Figure 3). However, the total number of VEGFR2-positive vesicles in the cells was unchanged as determined by confocal microscopy (Figure 2) suggesting that the VEGFR2-positive vesicles were not able to tether properly to the plasma membrane in the absence of EXOC7.

High levels of EGFP-EXOC7 expression affected the distribution and localization of VEGFR2 increasing the amount of VEGFR2-mCherry at the cell membrane, and this phenotype could be reverted by co-transfection with siEXOC7 (Figure 4). Finally, the degree of VEGFR2-mCherry exocytosis with different levels of EXOC7 expression was determined using flow cytometry. Indeed, increased expression of EGFP-EXOC7 correlated with elevated levels of VEGFR2 at the cell surface (Figure 5).
Taken together, in this study we provide evidence that EXOC7 is required for efficient delivery of VEGFR2 to the plasma membrane and subsequent exocytosis. This reinforces the concept that the exocyst complex is of importance in the regulation of VEGFR2 trafficking and could prove to be an important regulator of angiogenesis.

Paper IV

A tracking strategy for analyzing single cancer cell migration behavior in mixed cancer cell cultures

The aim of this study was to develop a new strategy to analyze cell movements in confluent cell layers and determine how the movements of individual cells are affected by their immediate cell neighbors. It has been shown that cells displaying differential EXOC7 isoform expression can have different phenotypic characteristics. Interestingly, in the invasive breast cancer cell line MDA-MB-231, high levels of the EXOC7-M isoform has been linked to a motile and mesenchymal phenotype, whereas in the more epithelial-like breast cancer cell line MCF-7 there is a comparably higher expression of the EXOC7-E isoform [65].

Three cell lines were used in this study: HEK293 cells, MDA-MB-231 cells and MCF-7 cells. Primers were designed to detect and quantify the amounts of EXOC7-E and EXOC7-M using PCR and qPCR. EXOC7-M was much more highly expressed at the mRNA level (approximately 10 fold more) in HEK and MDA-MB-231 cells than EXOC7-E, while EXOC7-E was more highly expressed in MCF-7 cells (Figure 2). EXOC7 isoform expression at the protein level was next examined, and it was shown that only EXOC7-M was expressed in HEK293 and MDA-MB-231, whereas both EXOC7-M and EXOC7-E were expressed in MCF-7 cells. On the grounds that HEK cells have an epithelial-like phenotype, the high expression of the M isoform in these cells was quite surprising.

In Paper III, overexpression of EGFP-EXOC7 isoform 4 led to the exocytosis of VEGFR2 to the cell surface. Accordingly, it was investigated whether overexpression of EXOC7 (isoform 4) could result in increased cell migration in HEK cells. A schematic overview of the strategy that was used for cell tracking together with neighbor analysis can be found in Figure 1. The generated movies were initially analyzed using the CellTracker program, whereafter each resultant dataset was imported into MATLAB and processed using custom made scripts to obtain information about the distances migrated for each cell as well as complete information about their cell neighbors. HEK cells were transfected with an EGFP-EXOC7 construct and studied using time-lapse imaging for 4 hours, 24 hours post transfection.
The transfection efficiency was approximately 40%, which made it possible to look at the number of EGFP-EXOC7 positive as well as negative cells, having different numbers as well as types of cell neighbors over time. Overall, there was no significant difference in distances migrated between EXOC7-positive or EXOC7-negative HEK cells in the chimeric cultures (Figure 3 and 4).

In another approach, MDA-MB-231, and MCF-7 cells were stained with green and red fluorescent CellTracker dyes respectively, whereafter cells were mixed and co-cultured at different ratios followed by time-lapse imaging 48 hours post staining. This allowed us to determine if cells with different EXOC7 isoform expression could influence each others movement when in contact with each other in confluent cell layers. Surprisingly, there was no difference in the total distance or the Euclidian distance migrated by MDA-MB-231 alone or co-cultured with MCF-7 cells at different ratios. Interestingly however, the Euclidian distance travelled by MCF cells was significantly lower when they were surrounded mainly by MDA cells (Figure 5).

In summary, we have developed a new method for automated analysis of cell movements in mixed cell cultures. This method can be used to determine how the migration of single cells in confluent cell layers are affected by the neighbors.
Concluding remarks and future perspectives

The existence and formation of ordered lipid domains in the plasma membrane is important for multiple functions, including cell surface receptor signaling, endocytosis and exocytosis. In Paper I, disrupting actin polymerization resulted in a lower fraction of ordered domains, while stabilizing actin filaments led to an increase of ordered domains at the plasma membrane. Since actin filaments are attached to the inner leaflet of the plasma membrane and the domain reorganization that we quantified was an average derived from both leaflets, it would be interesting to see what the domain organization is exclusively in the inner leaflet of the plasma membrane. In Paper II and III, VEGFR2 trafficking, which plays a crucial role in blood vessel sprouting and stability, was the focus of the studies. It has been shown that VEGFR2 is present in ordered domains and that VEGFR2 signaling could be dependent on these domains [115]. Moreover, ordered domains serve as clustering points in which activated receptors can reside.

Upon internalization, VEGFR2 can be either recycled to the plasma membrane or undergo degradation upon ubiquitination through lysosomes and proteasomes. In Paper II, we showed that FGD5 could protect VEGFR2 from degradation. From the EEA1, VEGFR2, FGD5 immunostaining we formed the hypothesis that FGD5 may be present on VEGFR2-containing endosomes, and through its GEF activity FGD5 may influence whether a VEGFR2-containing endosome is likely to follow the degradation or recycling pathways. In Paper III, we showed that silencing endogenous EXOC7 expression in cells led to a decrease in the number of the VEGFR2-mCherry containing vesicles that were able to reach the plasma membrane. In this study we used HEK cells, which express low levels of endogenous VEGFR2, therefore it would be interesting to see how EXOC7 depletion can affect the VEGFR2 trafficking also in primary endothelial cells and in in vivo models. Sprouting angiogenesis facilitates the vascularization of tumors, connecting them to the circulatory system which accelerates their growth and metastasis. The migratory capacity of endothelial tip cells is central to the success of sprouting angiogenesis and is governed by VEGFR2 expression. Further work to assess the tip and stalk cell profile of EXOC7 expression in a sprouting vessel would help to determine if EXOC7 could be experimentally targeted for the purpose of disrupting pathogenic sprouting angiogenesis. The embryoid body (EB) model of sprouting angiogenesis has
revealed important mechanistic insights into the molecular cues that regulate tip and stalk cell behavior [72]. Further, a CRISPR/Cas system could be applied to the EB model to engineer endogenous EXOC7 and VEGFR2 with fluorescent conjugates (e.g. EGFP and mCherry). The relative and potentially differential expression of EXOC7 and VEGFR2 in the tip and stalk cell positions could then be imaged in real time by fluorescent microscopy. Efforts to generate knockout models of individual exocyst complex components have largely resulted in embryonically lethal phenotypes [116], but conditional knockout systems could be used to elucidate the functional importance of EXOC7 for angiogenesis during the vascularization of the retina, an extensively studied developmental process.

In Paper IV it was investigated if highly migratory cells could promote migration in a less migratory cell type, or vice versa. In the tumor environment, cells with a high migratory capacity can metastasize by migrating to neighboring tissues. However, the non-migratory cells stay in the tumor. One of the factors that has been proposed to alter their migratory behavior in cells is the expression level of certain isoforms of EXOC7. By overexpressing EXOC7-M in epithelial-like cells with less motility, cells can transit to a more motile phenotype in a process called EMT [65]. We have developed a largely automated method that makes it possible to track cell migration in confluent mixed cultures, to correlate migratory behavior with the number and identities of local cell neighbors. For future experiments, other methods may be used to increase or decrease the migratory capacity of cells and their effects on their neighbors, such as overexpressing junction proteins (e.g. E-cadherin). Furthermore, we can alter the environment such that it better represents the tumor milieu, for example to perform analysis in a hypoxic chamber.

In summary, in this thesis we propose a model in which the connection of the actin cables with the plasma membrane promotes the formation of ordered domains where PI(4,5)P2 is enriched [117-119] (Figure 7A). EXOC7 interacts with PI(4,5)P2 to mark the tethering sites at the inner leaflet of the plasma membrane, where secretory vesicles, containing VEGFR2, are trafficked on actin cables to reach these tether sites prior to exocytosis (Figure 7B). Specific isoforms of EXOC7 may promote actin filament polymerization [65]; therefore, a tether site is potentially capable of promoting/stabilizing the actin network in its vicinity (and thus stabilize the plasma membrane domains). VEGFR2 will thus be presented at the surface in an area enriched with PI(4,5)P2, suggesting PI(4,5)P2 is an important component of downstream signaling pathways triggered by VEGFA binding/activation of VEGFR2. VEGFR2 internalization is followed by recycling of the receptor back to the membrane or degradation through lysosomal and proteasomal pathways. This is in part likely regulated by FGD5, which protects VEGFR2 from degradation and may therefore
promote its return to the membrane (*Figure 7C*). FGD5 also activates Cdc42, which is also localized to the membrane through PI(4,5)P2 interactions and promote actin polymerization and recruitment. The activity of EXOC7 and Cdc42 at sites enriched in PI(4,5)P2, and therefore containing VEGFR2, promotes the formation of membrane protrusions, which give rise to filopodia and ultimately cell migration (*Figure 7D*).
Figure 7. **Summary of present investigations.** Association of actin filaments with the inner leaflet of the plasma membrane via PI(4,5)P2 can form ordered domains at the plasma membrane (A). EXOC7, which binds to PI(4,5)P2, is the tethering site for the secretory vesicles containing VEGFR2 reaching the plasma membrane (B). FGD5 impedes VEGFR2 degradation, and may affect the balance between recycling and degradation (C). Specific isoforms of EXOC7 could promote actin filament polymerization in EXOC7/PI(4,5)P2 domains to form membrane protrusions required for cell migration (D).
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