Image-based phenotypic screening for breast cancer metastasis drug target discovery

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Chapter 1

General introduction and scope of this thesis

Cell migration is a complex process consisting of a number of spatially and temporally regulated mechanisms. This complexity arises from the integration of intrinsic signaling, such as regulatory genes and signal transduction pathways, and extrinsic signaling, which include the microenvironment, chemokines and growth factors. Aberrant cell migration plays an important role in dissemination and metastatic spreading of cancer cells. This thesis focusses on the understanding of the signaling programs that determine breast cancer tumor cell migration. Improved understanding of tumor cell migration in breast cancer progression and metastasis formation will ultimately lead to more effective cancer therapies. In the following paragraphs different aspects of breast cancer progression and tumor cell migration will be discussed.

1. Breast cancer

Cancer is one of the leading causes of death in the Western world. In the Netherlands, approximately 105.000 cases of different types of cancer are diagnosed each year (KWF Kankerbestrijding, July 2016). Cancer incidence is almost equal between men and women (54.000 vs 50.000, respectively), however in men the gastro-intestinal tract and prostate are the main sites of cancer, whereas in women, breast cancer is the most prevalent type of malignancy[1]. Approximately 14500 cases of breast cancer are reported each year in the Netherlands.[1] Surgery and radiation therapy are generally effective at early detection, when the tumor is still restricted to its primary site. Even though research in the past decade has improved both early stage cancer detection methods and treatments, almost 3100 patients die each year of breast cancer. The majority of breast cancer mortality can be attributed to cancer cells spreading throughout the body, known as metastasis.

Although chemotherapy and hormone-directed therapies are available, the response to treatment and patient prognosis is variable due to the high heterogeneity of the disease[2,3]. The main subtypes of breast cancer are luminal A, luminal B, ERBB2 over-expressing, the basal A (or basal-like) and basal B subtypes[4,5]. In contrast to the breast cancer with luminal origin, the basal-type are typically referred to as triple negative breast cancers (TNBC) as they are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (ERBB2 or Her2) negative[6]. The work in this thesis is mainly focused on TNBC, as this subtype of breast cancer lacks hormone-directed therapies and shows the worst prognosis in clinic[7].

1.1 Hallmarks of cancer

To understand the remarkable diversity of cancer, a logical framework of different characteristics of cancer pathogenesis and disease progression was described, termed the hallmarks of cancer[8]. Progress in research revealed emerging roles of the tumor microenvironment and immune system, as well as new characteristics of cancer cells. These eight hallmarks comprise distinctive

and complementary capabilities on both the cellular as well as systems level, and enable tumor growth and metastatic dissemination (Fig. 1)[9].

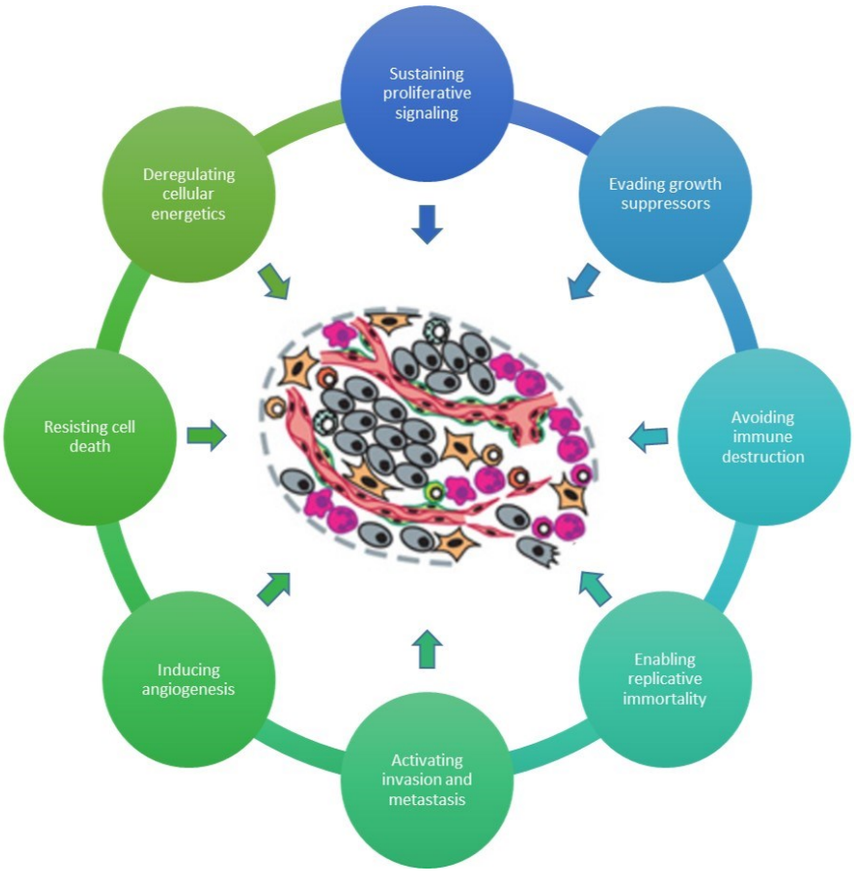

Figure 1. The hallmarks of cancer. The eight hallmarks of cancer comprise biological capabilities acquired during the development of tumors. These hallmarks provide a solid foundation for understanding the biology of cancer and include sustained proliferative signaling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, activating invasion and metastasis, inducing angiogenesis, resisting cell death and deregulating cellular energetics. Acquisition of these hallmarks is required for normal cells to transform into tumor cells (adapted from Hanahan and Weinberg, 2010).

Each hallmark will be discussed briefly to obtain a better understanding of the biology of cancer. For normal cells to develop into highly aggressive tumor cells, they acquire the ability to sustain proliferative signaling. Cancer cells may produce growth factors themselves or stimulate the stromal cells to do so, thereby inducing paracrine signaling. Additionally, mutations in signaling pathways result in constitutive activation and enable unlimited replication. The second hallmark is the evasion of growth suppressors, which are supposed to negatively regulate cell proliferation, and is most likely caused by mutations and/or inactivation of tumor suppressor genes. In addition to altered proliferative capacity, tumor cells acquire the ability to resist cell death (third hallmark). Cell death or apoptosis is a type of programmed cell death that removes unhealthy cells, however tumor cells are able to circumvent this cellular suicide. The fourth hallmark of cancer is replicative immortality, which is normally limited due to shortening of the telomeres.

Telomeres shorten with each cell division, thereby limiting the number of cell divisions in healthy cells. Tumor cells express telomerase, which lengthens the telomeres and thereby counters progressive telomere erosion in highly proliferative tumor cells. These four hallmarks are highly dependent on genome instability and mutations, which is therefore often referred to as an enabling characteristic of tumor cells. The induction of angiogenesis is the fifth hallmark and is required to provide the rapidly expanding tumor with all the nutrients and oxygen it needs. The sixth hallmark is a relatively new one: reprogramming energy metabolism. As cancer cells grow, nutrients and oxygen become scarce, leading to adjustments in energy metabolism to fuel cell growth. Another recent hallmark is the evasion of immune destruction. The innate and adaptive immune system are able to target tumor cells, yet weakly immunogenic tumor cells escape their destruction. The eighth and final hallmark is characterized by tumor cell invasion and metastasis, a multistep process in which cells invade the local tissue and form secondary tumors at distant sites. Dissemination of tumor cells generally has a poor prognostic outcome, as the resulting metastases target and disrupt other organs and are insensitive to therapies.

1.2 Metastasis

Dissemination of tumor cells and the formation of metastases is the underlying cause of death for the majority of breast cancer patients. Metastasis formation is a highly complex phenomenon, influenced by the tumor cells as well as the tumor microenvironment and the other cell types that reside within that environment[9,10]. Nevertheless, the metastatic cascade consists of several distinct steps, which are independent of one another (Fig. 2)[11,12]. After a primary tumor is formed, the tumor cells need to acquire invasive characteristics to be able to metastasize. These changes are caused by genetic alterations as a consequence of genome instability and mutations, which activate oncogenes and inhibit tumor suppressor genes. Furthermore, signals from the microenvironment stimulate an epithelial-mesenchymal transition (EMT) leading to an invasive phenotype[13–16]. This invasive phenotype is generally associated with increased migratory capacity of the tumor cells. Simultaneously, the tumor induces angiogenesis, providing itself with nutrients as well as a route to escape. Both blood and lymphatic vessels provide an escape route by which tumor cells can leave the primary site, a process called intravasation. After tumor cells have invaded the circulatory system, they have to survive in circulation and resist anoikis, until the tumor cells adhere to the vascular wall and extravasate. Once tumor cells have extravasated, they can invade into the distant tissue or organ and undergo a mesenchymal-epithelial transition, which allows the tumor cells to switch back to their epithelial and proliferative state. Once micrometastases are formed, sustained growth and angiogenesis allow these to grow out into secondary tumors. Additionally, tumor cells can also remain dormant for several years and then suddenly re-initiate proliferation, growing into metastatic lesions.

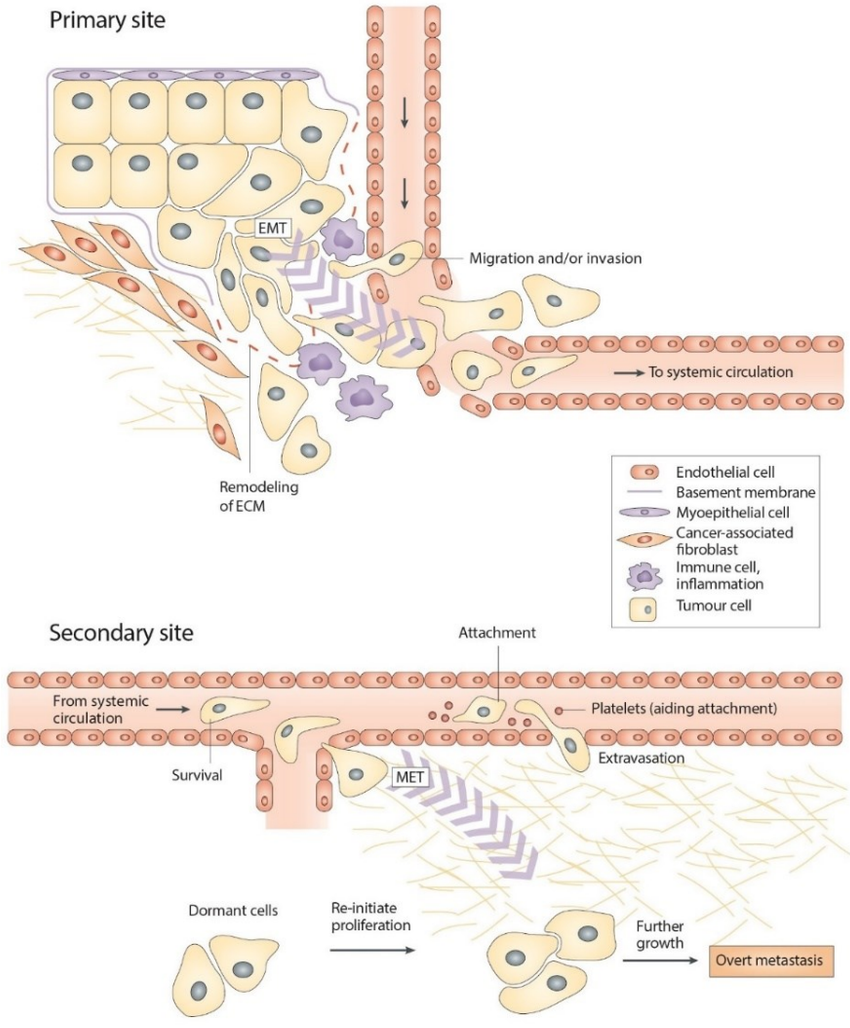

Figure 2. Metastasis formation is a multistep process. Cancer cells in the primary tumor acquire metastatic properties, leading to breakdown of the basement membrane and remodeling of the ECM, through molecular mechanisms such as epithelial-to-mesenchymal transition (EMT). This allows tumor cells to invade the tissue surrounding the tumor and migrate into the circulatory system. At the secondary site, circulating tumor cells extravasate from the vessel into the distant and foreign tissue. Tumor cells undergo a mesenchymal-to-epithelial transition (MET) and can remain dormant for long periods. Nevertheless, once they re-initiate proliferation, these tumor cells can grow into overt metastases (adapted from Eckhardt et al, 2012).

2. Cell migration

Cell migration is a fundamental process that plays an important role in many physiological processes, such as embryonic development, skin renewal and the immune response[17]. Aberrant regulation of cell migration is also the essential component of cancer cell invasion and dissemination, one of the hallmarks of cancer[9,17,18]. Cell migration is involved in several steps of the metastatic cascade, including local invasion, intravasation, extravasation, and dissemination in distant tissue[19]. How tumor cell migration is regulated is therefore of crucial importance to understand metastatic disease.

2.1 Cell migration modes

Tumor cell migration is a complex and profoundly heterogeneous biological process. Tumor cells display different modes of migration, which is dependent on the type of tumor and its genetic and molecular characteristics. As main categories, cells move either collectively (as cohesive strands or a multicellular stream), or individually (single cell migration)(Fig. 3a)[20–22]. Single cell

migration is divided into amoeboid and mesenchymal (also called lamellipodial) migration, and both modes of single cell migration show highly plastic and adaptive subtypes. Amoeboid migration is the movement of rounded cells with little to no adhesion, and depends on bleb formation or pseudopods for movement. Mesenchymal migration is characterized by cellular adhesion, cytoskeletal contractility, and movement in a fibroblast-like manner. These different migration modalities can be observed in 2D (experimental) environments as well as 3D tissue environments[22,23]. Tumor cells are not confined to their migration mode and display switch-like conversion between these distinct modes[24–28]. This adaptive switching is called plasticity, and is a compensatory response of tumor cells to changes in the tumor microenvironment, genetic alterations or molecular targeting. In **chapter 2**, we describe an imaging-based assay for studying single cell migration[29]. In **chapters 3**, **4** and **5**, we describe methodologies for live imaging of cell migration in different types of tumor cells, together with novel image and data analysis tools to interrogate migratory behavior at the single cell level. Ultimately, the capacity of tumor cells to change their migration strategy aggravates the metastatic process, and impairs therapeutic targeting.

2.2 Cell migration cycle

Cell migration is a highly integrated process, consisting of several distinct steps, often referred to as the cell migration cycle[30–32]. This sequence of steps was first observed in fibroblasts and described by Abercrombie in the early 1970s[32]. More specifically, the cell migration cycle describes a few general events of typical lamellipodial or mesenchymal migration. In contrast, amoeboid migration requires little to no cell-matrix adhesion and follows a different set of rules[33]. Unlike amoeboid migration, mesenchymal migration depends on adhesion to the extracellular matrix (ECM) for force application[20]. The first step of the cell migration cycle is extension of the cell membrane at the front of the cell (Fig. 3b). Cell protrusions are driven by actin polymerization and stabilized by attachment of the leading edge to the ECM through cell-matrix adhesions[34]. After maturation of the cell-matrix adhesions, the cell body is translocated forward via actomyosin contractility[35]. The final step of the cell migration cycle, is the retraction of the trailing edge (rear of the cell), which depends on localized disassembly of cell-matrix adhesions[36,37]. After retraction of the rear, the cycle starts again with protrusion of the leading edge.

Cell migration comprises the correct integration of a number of spatiotemporally coordinated molecular events, including cell-matrix adhesion dynamics, actomyosin contractility, Rho GTPase signaling, and microtubule (MT) organization[30,37–40]. Yet, understanding the spatial control of molecular signaling remains a major research challenge throughout cell biology[38,41]. This is because the spatial distributions of signals are often regulated simultaneously by many differentially distributed partners. Rho signaling in cell migration is an extreme case, where

distinctly localized activating and inhibitory proteins act in parallel to regulate RhoA and Rac1 activity distributions, determining the balance between mesenchymal and amoeboid migratory behavior[23,41–43]. Cell-matrix adhesions and adhesion dynamics are equally flexible and dynamic, providing an additional layer of complexity at the molecular and sub-cellular scale[44]. RNA-interference screens provide a way to understand how complex biological processes are functionally regulated. Different screens have been performed to address the role of the kinome and adhesome in context of single and collective cell migration[45,46]. However, a systematic analysis on the signaling landscape in breast cancer cell migration is lacking. In **chapter 4**, we describe a RNA-interference screen in two highly motile breast cancer cell lines in which we identified two sets of genes as novel regulators of cell migration, and subsequently investigated the complexity of tumor cell migration using network analysis approaches.

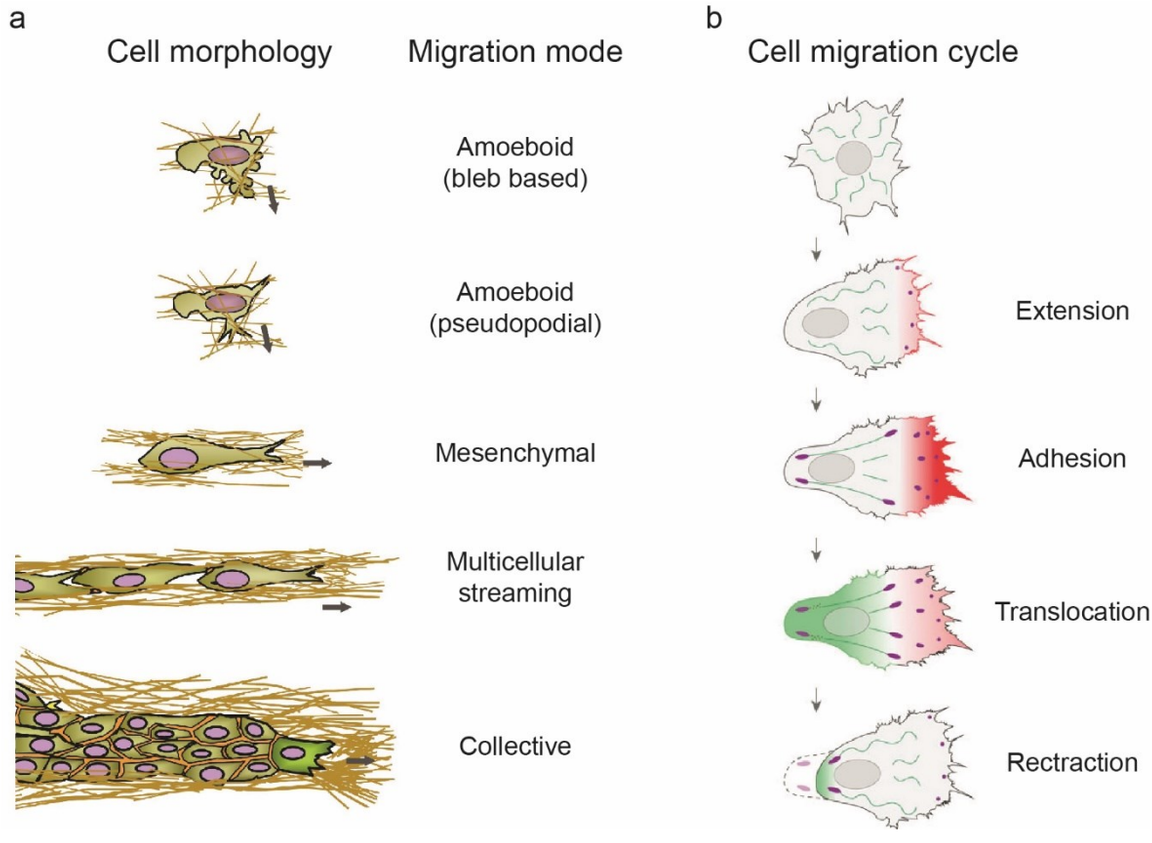

Figure 3: Cell migration modes and the cell migration cycle. (**a**) Tumor cells display different modes of cell migration, which are named after their morphology and/or behavior. Each migration mode is regulated by a set of molecular mechanism (see Friedl et al, 2010, for a thorough review). (**b**) The cell migration cycle as proposed by Abercrombie[32] consists of several steps: extension of the leading edge and initial adhesion to the substrate, maturation of the adhesions, translocation of the cell body via force generation, and retraction of the trailing edge. Actin polymerization-dependent processes are shown in red, cell-matrix adhesions in purple, and myosin II-dependent contractility in green. Figure a was adapted from Friedl et al, 2010; figure b from Reig et al, 2014.

3. Cell adhesion and migration

Cell-matrix adhesions are large protein complexes that mediate cellular adhesion to the ECM. Cell-matrix adhesions, also known as focal adhesions, contain heterodimeric transmembrane receptors called integrins, which provide a physical linkage to the ECM (Fig. 4). On the cytoplasmic side, over 180 proteins make up the cell-matrix adhesions and connect to the actin cytoskeleton[47,48]. Besides forming a physical link, integrin-based adhesions function as signaling hubs for both outside-in and inside-out signaling[49]. As such, cell-matrix adhesions form the core of the migratory machinery in mesenchymal-based tumor cell migration.

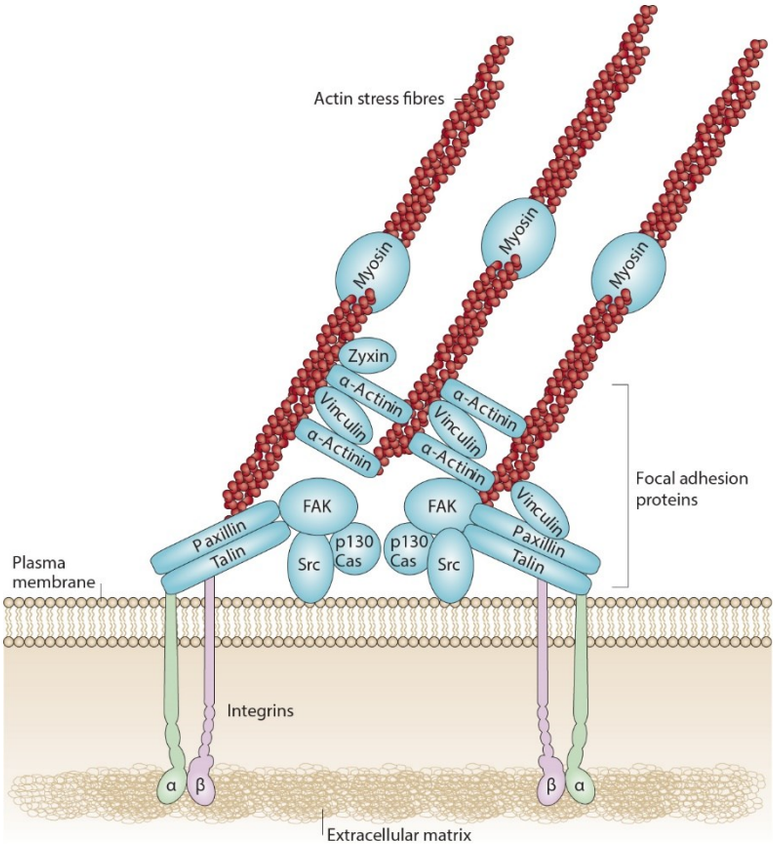

Figure 4: Molecular architecture of cell-matrix adhesions. Cell-matrix adhesions form a physical link between the extracellular matrix and the intra-cellular environment. Many structural and regulatory proteins can interact on the cytoplasmic side of the integrins, resulting in a constantly varying composition of the adhesion. By phosphorylating proteins such as FAK, paxillin, and Src, signals can be relayed across the plasma membrane (outside-in and inside-out signaling). Such signaling events are critical in many biological processes, like cell migration (adapted from Mitra et al. 2005).

Cell-matrix adhesions are not only intricate due to the large number of proteins that make these multi-molecular complexes, they are also highly dynamic and flexible structures[44]. During cell migration, multiple adhesion related processes are simultaneously taking place. At the leading edge of the cell, new adhesions (focal contacts) are being formed, while existing focal contacts undergo maturation into bona fide focal adhesions. At the rear of the cell, adhesions are disassembled allowing retraction of the trailing edge[36,37]. All of these processes (adhesion assembly, maturation, and disassembly) are highly dynamic and tight regulation is required to facilitate cell migration[38,50]. The major components that make up cell-matrix adhesions and their role have been extensively reviewed elsewhere (see [44,48,49,51] for reviews). Advances in the past decade have increased our knowledge on adhesion complexity. An imaging-based RNA-

interference screen in HeLa cells identified novel proteins involved in adhesion formation and cell migration[52]. In another study, a large and integrated proteomic analysis of integrin adhesion complexes revealed a core of 60 adhesion proteins, as well as a meta-adhesome of 2412 proteins involved in integrin-based adhesion[53]. These proteomics studies implicate that a large number of proteins are involved in adhesion organization, yet do not (or partially) show what their role is on a functional level. In **chapter 5**, we describe a high-resolution imaging-based RNA-interference screen in which we investigated the functional implication of knockdown of adhesome components on adhesion organization as well as adhesion assembly and disassembly.

4. Aim and outline of this thesis

With the studies described in this thesis, I aimed to further unravel the signaling- and regulatory networks that drive tumor cell migration. Improved understanding of tumor cell migration will ultimately lead to more effective therapies for breast cancer progression and metastasis formation. The main focus of this thesis is therefore the identification of novel candidate metastasis genes that regulate tumor cell migration and could be used as drug targets. For that reason, we established an imaging-based PhagoKinetic Track (PKT) assay for single cell migration that is amenable for high-throughput screening. The complete protocol is described in **chapter 2**, and includes step-by-step procedures, image acquisition set up, and guidelines for image and data analysis. In **chapter 3**, we describe the role of adhesion G protein-coupled receptor G2 (ADGRG2 or GPR64) in cell adhesion and cell migration. It is shown that ADGRG2 is a functional receptor that constitutively activates two pathways and mediates cell migration via non-canonical NFκB signaling. **Chapter 4** focuses on an imaging-based RNA-interference screen of ~4,200 targets to identify novel regulators of tumor cell migration. We used the PKT assay to classify different migratory phenotypes in the primary screen and performed live cell microscopy to confirm our findings. A novel suite of analysis tools was developed to analyze migratory behavior from live imaging at the single cell level. This led to the identification of two gene sets that are part of a regulatory network responsible for driving tumor cell migration and metastatic dissemination. In **Chapter 5**, we investigated the dynamic organization of cell-matrix adhesions in context of cancer cell migration and identified novel regulators of adhesion dynamics. Downregulation of these genes is shown to regulate cellular traction forces, thereby leading to the formation of large adhesions and reducing motility. Finally, I provide a brief summary and discussion of the findings described in this thesis and describe the future perspectives in **Chapter 6**.

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1. Source: Nederlandse Kankerregistratie, beheerd door IKNL © July 2016.
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