The role of Dock180 in ErbB2-mediated breast tumorigenesis and metastasis

by

Jennifer Huber
Division of Experimental Medicine, Faculty of Medicine
McGill University

Montreal • Canada

A THESIS SUBMITTED TO MCGILL UNIVERSITY IN PARTIAL FULFILLMENT OF THE DEGREE OF MASTER IN EXPERIMENTAL MEDICINE

Master Thesis submitted in August 2010

ABSTRACT

The ErbB2 receptor is found to be overexpressed and/or amplified in 20-30% of all breast cancer cases, which tend to have aggressive behaviors and often metastasize to distant organs making them difficult to treat in the clinic. It has been previously shown that the Dock180 and ELMO1 proteins work together to promote cell motility and invasion in a Rac-dependant manner under physiological and pathological conditions. Rac is a small GTPase well known to act on proliferation, migration and invasion and has been linked to development and metastasis of several types of human cancers. This prompted us to study in more detail the implication of Dock180 in breast cancer progression and metastasis. Here, we use a transgenic MMTV-NIC mouse model, in which we introduced a conditional deletion of Dock180 to investigate its importance in ErbB2-mediated mammary tumorigenesis and metastasis. Our data suggest that the expression of Dock180 is dramatically increased at the periphery and invasive fronts of tumors. We found that deletion of Dock180 did not significantly alter ErbB2-induced tumor onset but does have an impact on tumor growth. Moreover, the loss of Dock180 decreased the number of metastases to lungs by 7-fold. Interestingly, both Dock180 and STAT3, a Rac target, are highly activated at invasive fronts of tumors. Conversely, we found less activation of STAT3 in tumors arising from Dock180 deficient mice, suggesting a role for Dock180 upstream of STAT3 in tumor invasiveness. Here, we identify Dock180 as an important mediator of ErbB2-driven tumor dissemination and suspect that it may be acting by promoting STAT3 activation at the invasive front of breast tumors.

ABRÉGÉ

Le récepteur ErbB2 est surexprimé et/ou amplifié dans 20 à 30% des cas de cancer du sein. Ces cancers du sein ErbB2-surexprimés ont tendance à avoir un phénotype agressif et souvent métastasent vers des organes éloignés, ce qui les rend difficiles à traiter en clinique. Il a été précédemment démontré que les protéines Dock180 et ELMO1 travaillent ensemble pour promouvoir la motilité cellulaire et l'invasion de manière Rac-dépendante dans des conditions physiologiques et pathologiques. Rac est une petite GTPase bien connue pour agir sur la prolifération, la migration et l'invasion et qui a été liée au développement de plusieurs types de cancer et de métastases chez l'homme. Cela nous a incité à étudier plus en détail l'implication de Dock180 dans la progression du cancer du sein et des métastases. Ici, nous utilisons un modèle de souris transgénique, le MMTV-NIC, où nous y avons introduit une délétion conditionnelle de Dock180 afin d'investiguer son importance dans la tumorigénèse mammaire et les métastases médiées par ErbB2. Nos données suggèrent que l'expression de Dock180 est dramatiquement augmentée à la périphérie et aux fronts invasifs des tumeurs. Par ailleurs, l'absence de Dock180 ne modifie pas l'apparition de tumeurs ErbB2-induites mais a un impact sur la croissance tumorale. En outre, la perte de Dock180 diminue de 7 fois le nombre de métastases aux poumons. De plus, Dock180 et STAT3, une cible de Rac, sont fortement activés aux fronts invasifs des tumeurs. Inversement, nous avons trouvé moins d'activation de STAT3 dans les tumeurs provenant de souris déficientes pour Dock180, suggérant un rôle pour cette protéine en amont de STAT3 dans l'envahissement tumoral. Ici, nous avons identifié Dock180 comme un médiateur important de la dissémination tumorale conduite par ErbB2 et nous suspectons qu'il pourrait agir en favorisant l'activation de STAT3 aux fronts invasifs des tumeurs mammaires.

ACKNOWLEDGEMENTS

This thesis would not have been possible without the precious help and support from many people around me for the past two years. First of all, I would like to thank my supervisor, Dr. Jean-François Côté, for giving me the opportunity to work on a subject that touches me profoundly. Throughout my research, he provided me his wise advice, righteous critics and kind encouragements, everytime I needed it. Most importantly, he let me build up my own way of being a scientist, and I am very grateful for that.

Also my dear colleagues, with whom I learned a lot about science and life. Nadine, who guided me since the very beginning and gave me all those scientific tricks. Manishha, with whom I had a very good time, sharing our love for music and cookies, and who I admire for her devotion and genuineness. Melanie, with her perspicacity and her sarcasm, which generated many bursts of laughter. Rosemarie and Ariane, for all the good moments in and outside the lab.

Thanks to my research advisory committee members: Dr. Jean-Philippe Graton, Dr. Marko Horb and Dr. Peter Siegel for their valuable scientific inputs.

Thanks to our collaborators, Dr. William J. Muller for graciously providing the MMTV-NIC mice. Also, thanks to Jill Ranger for her precious advices and for sharing her expertise.

A particular thanks to the Institut de recherches cliniques de Montréal (IRCM) and Invitrogen for the studentship I was awarded for the second year of my Master degree.

A special thanks to all the people from the IRCM who contributed with their expertise to the project: Geneviève Brindle, Nicole Campeau, Claudia Jones, Dominic Fillion, Claudia Toulouse and Annie Vallée. Also, thanks to Manishha and Mélanie for reading the early draft of my thesis.

To all the IRCM students, who helped me out many times and cheered me up. So many nice people I will never forget.

Last but not least, my family, my friends and Julien, who were always there for me in all the good and the bad times. Thank you so much for your support and for believing in me. You all made my life so simple and enjoyable.

CONTRIBUTION OF AUTORS

The Dock180 conditional knock-out mouse (unpublished) used in the present thesis has been designed and generated by Nadine Fradet, research assistant in the laboratory of Dr. Jean-François Côté.

TABLE OF CONTENT

LIST OF FIGURES

Chapter 1 – Review of the literature

Chapter 2 - Results

LIST OF TABLES

Chapter 1

Review of the literature

REVIEW OF THE LITERATURE

1.1 INTRODUCTION TO BREAST CANCER

1.1.1 Introduction

Worldwide, cancer originating in the mammary gland is the most common type of cancer in women. Breast cancer comprises 23% of all cancer incidence among the female population, making it by far the most frequent type of malignancy diagnosed in 2008 and the fifth most common cause of cancer death in women [1]. In Canada, the latest analyses report that approximately 445 women will be diagnosed with breast cancer every week. This means that around 1 out of 9 Canadian women will be diagnosed with breast cancer at some point in her life. Although a notable decline in breast cancer mortality has been observed over the last twenty years, around 20-30% will eventually die of this disease, mainly due to the development of metastases, an incurable condition in most cancer types [2].

In the following paragraphs, factors involved in mammary gland, breast cancer and metastasis development will be discussed. Improved understanding of signaling cascades implicated in these physiological and pathological events will ultimately lead to more effective cancer therapies to combat this life-threatening disease.

1.1.2 Mammary gland development

Much of our knowledge about mammary gland development is based on experiments on mouse models. They can be extrapolated to humans, while accounting for both genetic and physical differences between these species (see chapter 1.2.3).

Mammary gland development is a very dynamic process in which proliferation, apoptosis, differentiation and migration are all essential for the formation of the highly organized branched ductal network of epithelial cells (reviewed in [3-5]). In the mouse, development of the mammary gland begins during foetal development although it undergoes the majority of its maturation after birth. At about embryonic day 11.5 (E11.5) the formation of five placodes begins along each of two milk lines. After one day, these placodes will differentiate into epithelial buds and each bud elongates to form a sprout at E15.5. Invading the underlying fat pad, the sprout will eventually open on the surface of the skin, where epidermal invagination forms the nipple. Until birth, the sprouts will develop ramifications and form small, arborized glands [6]. The major regulatory genes involved in early embryonic mammary gland development are dependent mostly on the canonical Wnt pathway [7, 8] and fibroblast growth factor (FGF) signaling [9].

Newborn mice harbor a rudimentary gland that will grow isometrically with body growth until puberty, starting at approximately 3 weeks of age, which corresponds to the onset of hormone secretion by the ovaries. At this time, the terminal end buds (TEBs, Figure 1.1) appear and start the process of ductal elongation, which continues until 10-12 weeks of age [10]. These highly proliferative structures consist of a single outer layer of undifferentiated cap cells and multiple inner layers of body cells. Those body cells, along with the maturation of the mammary gland, will give rise to the luminal epithelial cells, whereas the trailing edges of the cap cell layer will differentiate into myoepithelial cells [11]. TEBs create the branching architecture of the epithelium and persist until the ducts have reached the outer limits of the fat pad at which point they regress. Lateral branches also form at separate sites along the subtending ducts and can also be referred to as side branches with end buds or alveolar buds. This phase of ductal morphogenesis is dependent on the estrous cycle [12].

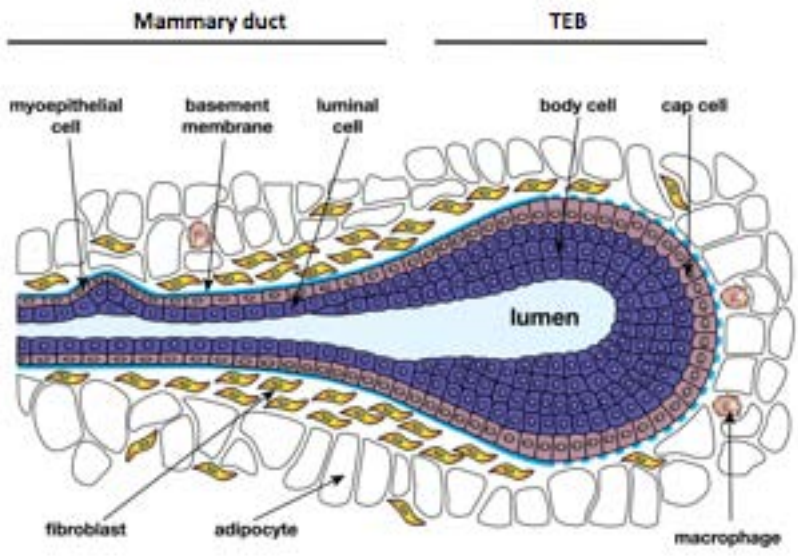

Figure 1.1: *Schematic representation of a terminal end bud and the trailing mammary duct.* The TEBs are located at the tips of the growing ducts, surrounded by a fibroblast- and adipocyte-rich stroma. TEBs consist of multiple layers of epithelium with an outlayer of undifferentiated, pluripotent stem cells called cap cells that contact the basement membrane, and inner layers of body cells. Surrounding these cap cells, the connective stroma is absent (represented by the blue dots). Trailing body cells during ductal elongation will give rise to luminal cells and the cap cells will differentiate into myoepithelial cells. Stromal macrophages are also depicted. Adapted from [13].

The final developmental fate of the mammary gland is accomplished during pregnancy and lactation. Upon stimulation by reproductive hormones, the mammary epithelium expands and differentiates into milk-producing lobular alveoli. At parturition, the alveoli begin copious milk secretion, which continues for about 3 weeks in mice. At weaning, the gland begins a process of important tissue remodeling via the apoptosis of mammary epithelial cells. This process, termed involution, takes approximately 2 weeks, with a peak of apoptosis at 2 days post-weaning. Finally, the gland goes back to a pre-pregnant morphology and is ready to initiate another cycle of pregnancy, lactation and involution (Figure 1.2).

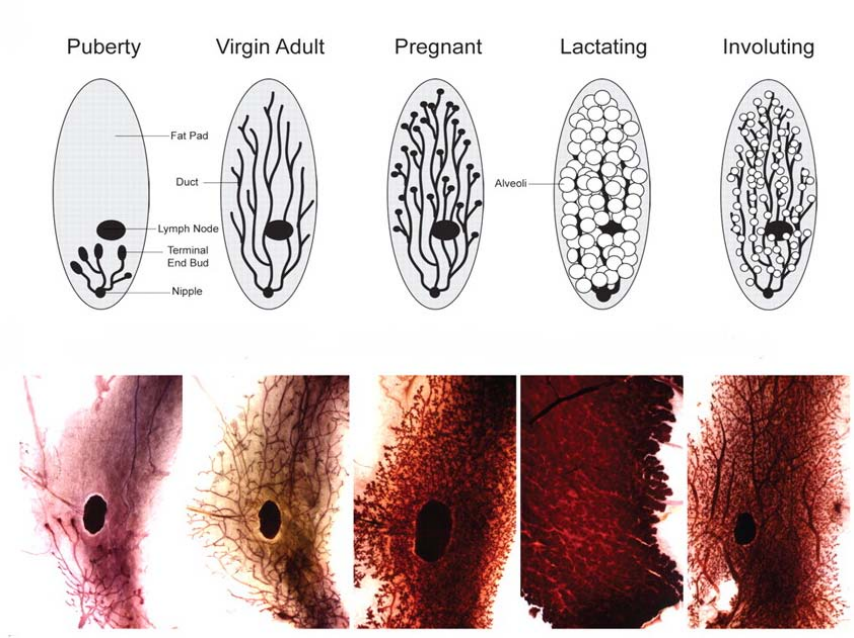

Figure 1.2: *Mouse mammary gland development*. Each stage of the schematic (i.e. puberty, virgin adulthood, pregnancy, lactation and involution corresponds with the whole-mount preparations (below) at the matching time-points. The fat pad, ducts, lymph node, TEB, nipple and alveoli are depicted in the schematic. Adapted from [14].

1.1.3 Breast cancer

1.1.3.1 Multistep mammary tumorigenesis

Breast cancer is a very complex and heterogeneous disease. It is in fact a collection of different types of breast malignancies that have diverse genetic and genomic variations, histopathologies, and clinical outcomes [15]. From a normal mammary epithelium to a transformed - potentially metastatic - tumor, cells have to go through a series of critical steps (reviewed in [16], Figure 1.3). Classic pathology progresses from normal terminal ductal lobular units (TDLUs) to hyperplastic enlarged lobular unit (HELUs), the earliest histologically identifiable potential precursor of breast cancer. HELUs may differentiate to more complex lesions including ductal hyperplasia (ADH) or atypical lobular hyperplasia (ALH), during which time cells exhibit almost

normal histology but display an unusually high cell division rate within the duct or lobule. Selective pressure leads to further damage and/or cell changes, with the epithelium no longer forming a well-ordered cell layer. This abnormally proliferative and shaped region is said to be dysplastic. A much larger and more deviant growth that has dysplastic cells and marked thickening is called ductal carcinoma *in situ* (DCIS), or less frequently lobular carcinoma *in situ* (LCIS). DCIS are not considered invasive as tumor growth is confined within the milk duct. It can be detected by a mammogram and is generally too small to be noticed by physical palpation [17]. These benign tumors may give rise to an invasive ductal, or lobular, carcinoma (IDC or ILC), but it is unclear how to predict which lesions will progress to a more aggressive form of cancer.

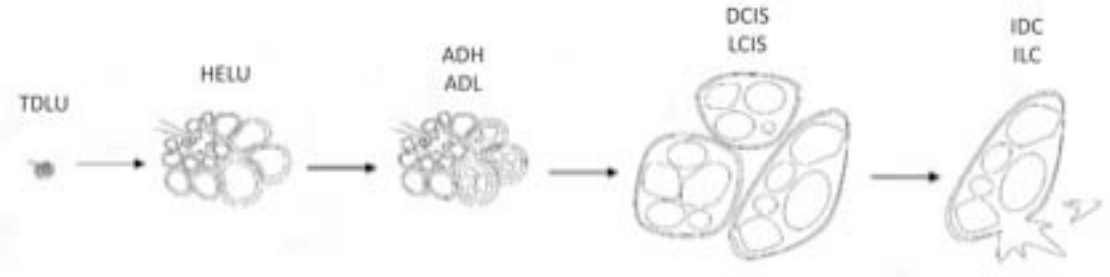

Figure 1.3: *Multistep mammary tumorigenesis*. Breast cancer is a heterogeneous disease that goes through a series of critical steps. From normal TDLUs, hyperplastic breast epithelial cells gradually enlarge to form HELUs. These cells exhibit almost normal histology but a high rate of proliferation. Once a cell or a number of cells acquire irreversible mutations that confer them an uncontrolled growth property, added mutations might arise and a precancerous lesion is formed, named ADH or ALH. With time, this region of atypical hyperplasia may acquire more DNA and phenotypic damages, leading to a ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS). DCIS or LCIS may become invasive and spread to other tissues, termed invasive ductal carcinoma (IDC) or invasive lobular carcinoma (ILC). Modified from [18].

Once cells have broken through the basement membrane and invaded the underlying stromal tissue, the risk of developing metastasis significantly increases. Invasive cells must enter the vasculature (intravasate), survive in the absence of adhesion, exit the vasculature (extravasate) and colonize a foreign environment (see chapter 1.4).

1.1.3.2 Breast cancer classification

The heterogeneity of breast cancer may originate from different target cell populations and/or may be the result of different combinations of oncogene activation and loss of tumor suppressor gene function in a normal breast stem cell or committed progenitor [19]. Over the years, clinicians have been able to classify different aspects of mammary tumors, including the stage [20], histopathology [21], grade [22], receptor status [23], and the presence or absence of genes as determined by DNA testing [24]. As each category hs its own individual prognosis, the field is now moving towards personalized medicine, offering an adapted treatment and increasing overall survival rates.

Breast cancer has been stratified into five major subtypes on the basis of gene expression profiling. These include the luminal A or B, basal-like, HER2/ERBB2-overexpressing, and normal-breast-like subtypes [23, 25, 26]. The differences in tumor subtypes are hypothesized to reflect different mutation profiles, as well as differences in the cell of origin [19, 25]. Most breast cancers, including the common types of invasive ductal and invasive lobular carcinomas, display evidence of luminal cell differentiation. While the luminal A and B subtypes are generally associated with a good prognosis, those tumors that overexpress or show amplification of the HER2 receptor usually display luminal features, but are associated with poor overall survival [27]. The basal-like subtype is very heterogeneous and comprises 15–20% of breast cancers (reviewed in [28]). This group of tumors is among the most clinically aggressive and tends to exhibit a triple-negative phenotype (i.e., lack of expression of ER, PR, and HER2/ErbB2), thus being difficult to treat in the clinic.

1.1.4 Breast cancer metastasis

In 10% of breast cancer diagnoses, tumor cells have migrated invaded out of the primary tumor and are colonizing in distant sites. Among these metastatic breast cancer cases, only 23% of women will survive after five years [29]. Metastatic breast cancer is the most advanced stage of breast cancer, meaning that primary cancer cells have spread past the breast and axillary lymph nodes to other areas of the body where they continue to grow and multiply [20]. The most common targets of human breast cancer metastasis are the bone, followed by the lung, liver and brain [30].

Reorganization of the actin cytoskeleton and the recruitment of the migration machinery at the leading edge of the cancer cell facilitates invasion into the healthy mammary stroma and development of metastasis in distant organs. In order to successfully establish a metastatic focus in a distant location, primary breast cancer cells have to complete a number of successive steps [31, 32]. The sequence of basic steps of the metastatic cascade can be summarized as: (i) local invasion, (ii) intravasation, (iii) survival in the circulation, (iv) extravasation and (v) colonization (Figure 1.4). Although intimately linked to genetic mechanisms, the first steps toward invasion and metastasis are also controlled by the interactions of the cancer cells with their surrounding environment. Cell-intrinsic and external pressure to limit the outgrowth and invasion of tumors at the primary sites drive the selection for traits that enable cancerous cells to bypass such barriers [33]. Acquisition of self-renewing mechanisms, altered cellular adhesions, enhancement in cell motility, resistance to extracellular death signals and disruption of the basement membrane and extracellular matrix are the primary events in the progression from a DCIS to an invasive breast tumor [33, 34].

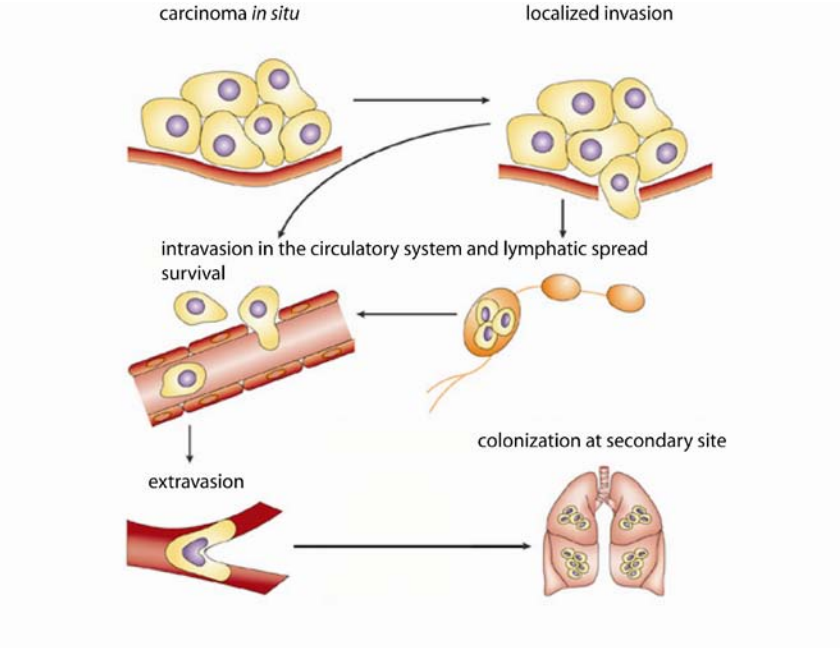

Figure 1.4: *The metastatic cascade*. Beginning with an in situ cancer surrounded by an intact basement membrane, tumor cells invade into the healthy stroma, intravasate via the lymphatic circulation or directly into the systemic circulation, survive and extravasate to colonize secondary sites. Modified from [35].

Of all the processes involved in carcinogenesis, local invasion and the formation of metastases are clinically the most relevant, but remain poorly understood at the molecular level. Revealing their mechanisms is one of the main challenges for exploratory and applied cancer research. Since the subject of the present thesis is focused on the invasion and metastasis of breast cancer, an in depth section on cell motility and the principal molecular mechanisms regulating cell migration and invasion will be presented in chapter 1.4.

1.2 MOUSE MODELS OF BREAST CANCER

Over the past two decades, genetically engineered mice (GEM) have been a precious tool to better understand molecular mechanisms underlying breast cancer

initiation and progression. In this chapter, descriptions of commonly used mouse models of breast cancer, specifically the model used in this thesis, the ErbB2-based MMTV.Neu-NDL2.5-IRES-Cre (MMTV-NIC) mice, and the relevance of mouse models of breast cancer will be discussed.

1.2.1 Commonly used mouse models of breast cancer

Since the development of the first transgenic mouse models of breast cancer in the late 1980s, a panel of GEM encompassing a wide array of targets including growth factors, receptors, oncogenes, tumor suppressor genes and cell cycle regulators have been generated for use in breast cancer research. Being a very heterogeneous disease, no single mouse model can recapitulate all the aspects and stages of human breast cancer. As such, individual models are useful for studying distinct signaling interactions that contribute to breast cancer progression and potentially for testing novel therapies that target these pathways.

The first transgenic models of breast cancer were generated by targeting well-known oncogenes such as c-*myc*, Polyomavirus middle T antigen (PyMT), rat *ErbB2* (also known as *Neu*) and *Hras* under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) and the whey acidic protein (WAP) promoters. Others mammary specific promoters have been used (Table 1.1), but the MMTV and WAP promoters are the most widely used in the field. This section will be limited to the MMTV-PyMT and -ErbB2-based mouse models, whose role is supported by extensive data implicates and which are relevant for the further comprehension of this thesis.

Table 1.1: Mammary-specific promoters

Promoter	Origin	Expression	Activation	References
MMTV-LTR	Mouse mammary tumor virus	Mammary epithelial cells, low expression in several other tissues	Steroid hormones	[36]
WAP	Whey acidic protein	Secretory mammary epithelium	Lactogenic hormones	[37, 38]
C3(l)	Rat prostate steroid-binding protein (PSBP)	Epithelial cells of prostate and mammary gland	Estrogen (ductal and alveolar mammary epithelium)	[39]
B-GL	Bovine ß-lactoglobulin	Mammary gland	Pregnancy and lactation	[40, 41]
MT	Metallothionein	Most mammary cells	Zn^{2+}	[42]

Table 1.1: *List of mammary-specific promoters.* Listed with their corresponding origin, location of expression, means of activation and references.

1.2.2 MMTV-LTR promoter

The most extensively used promoter to target transgene expression into the mammary epithelium is the MMTV-LTR. The mouse mammary tumor virus is a retrovirus associated with the development of mammary carcinomas in infected mice [43]. The long terminal repeat (LTR) of MMTV contains a glucocorticoid hormone responsive element (GRE) and is sensitive to progesterone and dihydrotestosterone but is not induced by estrogen [36]. In fact, the MMTV-LTR drives high levels of transgene expression during all stages of mammary gland development, detectable between 6-21 days post-partum, but has significantly greater activity in the mammary epithelium during puberty, pregnancy and lactation, as a result of hormonal stimulation [44]. Like the vast majority of promoters, the MMTV-LTR is leaky. Depending on its integration site, the MMTV-LTR may not only induce transgene expression in the ductal and alveolar luminal mammary epithelial cells along with myoepithelial cells, but can be also detected, to a lower level, in the skin, salivary gland, ovary, lungs, kidneys, seminar vesicles, testes, prostate, B and T lymphocytes and hematopoietic cells of female mice [45]. Despite its leakiness, tumors induced under the MMTV-LTR promoter only arise in the mammary glands [45]. Since the original description of the MMTV-*c-myc* mice by the Leder Laboratory in 1984, further studies targeted the expression of a wide variety of

genes under the control of the MMTV promoter. Importantly, the majority of these mouse models resemble the human breast adenocarcinomas at the pathological level.

1.2.3 Polyoma virus middle T antigen

One of the most utilized mammary gland tumor mouse models is the MMTV-polyomavirus middle T antigen transgenic mice (MMTV-PyMT). The PyMT was isolated from the murine Polyomavirus and was demonstrated to induce a fully tumorigenic phenotype [46]. This mouse model develops fully penetrant, multifocal mammary tumors as early as 7-8 weeks post-partum (Table 1.2). In addition to its rapid tumor onset, this transgenic mouse exhibits a high rate of metastasis to lungs. Despite its highly aggressive phenotype, tumors progress through four stages, from the induction of hyperplasia, followed by formation of adenomas, early carcinomas and eventually invasive carcinomas [47]. Due to its rapid onset and metastasis development, the MMTV-PyMT model is frequently used to explore the role of potential tumor suppressor proteins, like TGFß1 [48], kinases like Src [49], FAK [50], AKT [51], adaptor proteins like ShcA [52] and integrin-mediated tumorigenesis like ß3 and ß5 integrin [53] and to investigate the progression of metastatic disease like MMPs [54] overexpressing mice.

To exert its highly transforming and metastatic potential, the PyMT has to associate and activate a number of critical signaling molecules including the Src family kinases, the ShcA adapter protein, the p85 subunit of PI3K, PLCγ and members of the 14-3-3 family (reviewed in [55]). The middle T antigen binds the plasma membrane and it essentially functions as a constitutively activated growth factor receptor through its interaction with the aforementioned cellular proteins, despite lacking intrinsic enzymatic activity. After activating several downstream targets, the PyMT can mediate cell survival, proliferation, migration and apoptosis via the Ras/MAPK and PI3K/AKT pathway.

1.2.4 ErbB2-based mouse models

Since ErbB2 is such a prominent human oncogene, several transgenic mouse models using the rat orthologue Neu have been generated to study its molecular mechanisms and downstream targets. ErbB2 was originally identified from a chemically-induced neuroblastoma and demonstrated to be homologous to a retroviral oncogene, the v-ErbB [56]. Since the first generation of MMTV-ErbB2-based mouse models in 1988 by the Leder laboratory (MMTV-Neu-NT mice) [45], advances in genomic technology have provided more systems that recapitulate breast cancer overexpressing the Neu oncogene (reviewed in [57]). The activated form of Neu (Neu-NT) contains a Valine to a Glutamate substitution at the amino acid 664 in its transmembrane domain (Figure 1.5) [58] which permits the aggregation of receptors homodimers at the cell surface [59]. Subsequent studies on the MMTV-Neu-NT clearly showed that the activated Neu is sufficient to induce transformation in mammary epithelial cells, thus developing multiple tumor burden beginning at approximately 12-14 weeks of age (Table 1.2) [60].

Although the MMTV-Neu-NT was able to induce mammary tumors, clinical studies had shown that ErbB2-overexpressing breast cancer patients had no comparable activating mutation. In fact, when overexpressing wt-*Neu* using the MMTV promoter (MMTV-Neu), focal mammary tumors developed only after a long latency (Table 1.2) [61]. Further characterization of the MMTV-Neu mice revealed that close to 100% of the tumors that arose in those strains harboured in-frame deletions or insertions of cysteine residues within a small region of the Neu extracellular domain (Figure 1.5). This imbalance of cysteines promotes Neu homodimerization through the formation of intermolecular disulfide bridges, resulting in a constitutive activation of the receptor [62-64]. Consistent with the transforming ability of these altered Neu proteins, transgenic mice carrying a distinct in-frame deletion of the transgene (MMTV.Neu-NDL1 and -NDL2) were shown to display elevated expression and phosphorylation of Neu and induce rapid tumor onset [65].

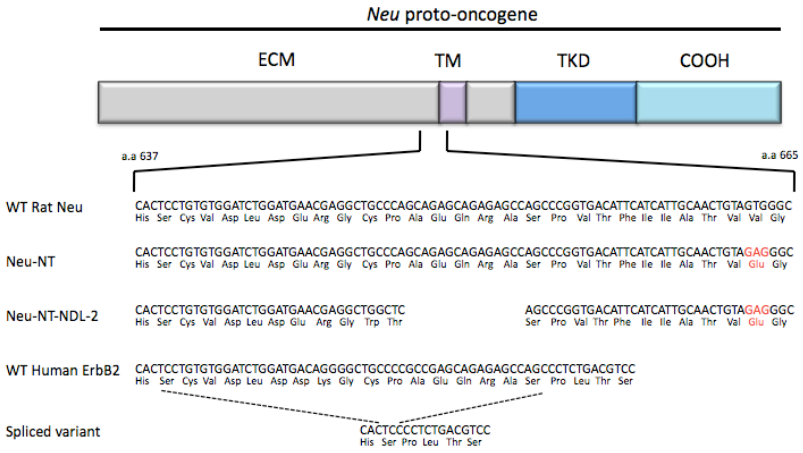

Figure 1.5: *Schematic representation of the Neu proto-oncogene and its mutated variants*. The activated form of Neu (Neu-NT) display a Valine to a Glutamate substitution. The Neu-NDL-2 presents an in-frame deletion, which confers its constitutively active form. A 16 amino-acid deletion seen in the wild-type human ErbB2 of breast cancer patients resembles the Neu-NDL-2 construct.

Moreover, tumors arising in these mice also showed co-expression of Neu and ErbB3 proteins, consistent with frequently high levels of *ErbB2* and *ErbB3* transcripts in human primary breast tumors [65].

More recently, a bigenic mouse that conditionally expressed Neu-NT under the dual control of the MMTV promoter and a tetracycline regulatory element (MMTV-rTA;TetO-Neu-NT mice) has been generated to directly assess whether sustained expression of Neu is required for the maintenance of a transformed phenotype [66]. Doxycycline-induced expression of the Neu-NT transgene resulted in the formation of multifocal and metastatic mammary tumors with kinetics and latency period similar to the MMTV-Neu-NT mice (Table 1.2) [60, 66]. Regression of mammary tumors and metastasis to lungs was observed following doxycycline-free diet. However, animals bearing completely regressed tumors went back on developing Neu-independent mammary tumors,

possibly through the reactivation of dormant tumor cells that remained into the primary tumor.

To circumvent the effect of random insertion and high copies of the Neu transgene in the MMTV-based mice, a transgenic mouse that places the activated Neu under its endogenous mouse ErbB2 promoter has been developed. The MMTV-Cre/FloxNeoNeuNT (herein referred to as ErbB2 knock-in) mice form focal tumors in one mammary gland after an extended period and extremely low metastasis occurrence [67]. Given its non-invasive phenotype, this model does not recapitulate the aggressive phenotype and shortened overall survival that characterize ErbB2-postive human breast carcinomas. Despite these limitations, the ErbB2 knock-in mice may still provide crucial information on the mechanisms that regulate oncogene induction and their downstream signaling pathways. Not only its endogenous promoter better recapitulates ErbB2 expression level as in the endogenous mouse *ErbB2* allele, but most importantly the *ErbB2* amplicon is frequently amplified in this mouse model, such as in human breast cancers.

Table 1.2: Phenotypic characteristics of the ErbB2-based mouse models.

Strain	Average age of mammary tumor onset (days)	Penetrance	Mammary tumor morphology	% metastasis to lungs positive-animals	References
MMTV-NeuWT	288	100	Focal adenocarcinoma	72	[61]
MMTV-Neu-NT	93	100	Multifocal adenocarcinoma	ND	[45]
ErbB2 KI	419	83	Focal, comedo adenocarcinoma	6	[67]
MMTV-PyMT	66	100	Papillary, glandular carcinoma	100	[68]
MMTV-Neu-NDL.2.5	161	81	Multifocal, solid adenocarcinoma	67	[65]
MMTV-NIC	146	100	Multifocal, solid adenocarcinoma	63	[69, 70]

Table 1.2: *Phenotypic characteristics of some ErbB2-based mouse models of breast cancer.* Listed with their corresponding average age of mammary tumor onset, penetrance, tumor morphology and percentage of lung metastasis-positive animals. KI: Knock-in.

Finally, with the first introduction of the Cre-lox system in the eukaryotic genome [71], transgenic mouse models featuring the excisive feature of the Cre recombinase [72, 73]

has been extensively used to efficiently target site-specific recombination of genes implicated in mammary gland development and cancer. The MMTV-Cre transgenic line is able to direct the Cre-mediated recombination in luminal and myoepithelial mammary [74] as early as 6 to 22 days post-partum [75]. However, recombination of conditional alleles following the breeding with the MMTV-Cre mice has been shown to occur incompletely in the mammary epithelium. In fact, animals bearing the GTRosa26 and MMTV-Cre allele revealed expression of the Cre recombinase ranging from 50 to 100% in luminal epithelial cells [76]. Therefore, the Cre-negative cells retain expression of the conditional allele and may be selected for oncogenic transformation [76]. To circumvent the stochastic expression of the Cre recombinase, a novel transgenic mouse harbouring both the Neu-NDL2.5 and the Cre recombinase in the same bicistronic cDNA has been generated. This recent transgenic mouse model of breast cancer is the model used throughout my research and will be discussed in depth in the next section.

1.2.4.1 MMTV.NIC

The latest MMTV-ErbB2-based mouse model to be generated is the MMTV-Neu-NDL2.5-IRES-Cre (MMTV-NIC) transgenic mice [77]. These transgenic mice co-express both activated Neu (NDL2.5) and the Cre recombinase from the same bicistronic transcript, due to the presence of an internal ribosome entry site [78] between the two cDNA sequences. The expression of activated Neu and Cre is coupled within the same mammary epithelial cell, thus avoiding the possibility of obtaining Neu-expressing, Cre-negative “escapee” population. To date, four studies on breast cancer progression and metastasis had used this transgenic line. The direct role of the adaptor protein SchA in Neu-induced mammary tumorigenesis was assessed in the MMTV-NIC mice and was shown to be a critical player during tumor induction [77]. Studies analyzing the importance of PTEN [69], STAT3 [70] and ILK [79] in relation to ErbB2-mediated tumorigenesis using the MMTV-NIC mice gave deeper insights on this novel model. Consistent with the MMTV-Neu-NDL2.5 pathology, the MMTV-NIC mice display multiple

mammary adenocarcinomas, arising at approximately 125 to 198 days of age [69, 70, 77, 79]. All virgin female mice carrying the transgene were tumor-positive and 60 to 67% of them developed lung metastasis (Table 1.2) [69, 70, 79]. These animals also showed high levels of transgene mRNA transcript in the mammary gland, with significantly lower levels in the adrenal gland, lung, ovary, pancreas and salivary gland [77]. In conclusion, the MMTV-NIC mouse model of ErbB2-overexpressing breast cancer is a highly relevant model to investigate the role of a wide array of genes in mammary tumor initiation, progression and metastasis, in bigenic strategies. A thorough description on the ErbB2 receptors and its downstream signaling pathways will be discussed in chapter 1.3.

1.2.5 Relevance of mouse models to study human breast cancer

Some of the most extensively studied mouse models of breast cancer have been described above. Despite the invaluable impact that these experimental models have had on our knowledge of the genetics and biology of breast cancer, some aspects in regard to their mammary morphology and their developed pathology have to be taken into consideration.

The morphological differences between mouse and human mammary tissue are notable. The human breast is characterized by a branching network of ducts that end in clusters of small ductules, which constitute the terminal ductal lobular units (TDLUs). The earliest stages of cancer, commonly termed carcinoma *in situ* or DCIS, begin as small foci of abnormal cells within the TDLUs. In contrast, the mouse mammary epithelial tree does not possess TDLUs but instead consists of alveolar buds that are formed during each estrous cycle. Furthermore, the mouse mammary gland contains less fibrous connective tissue than the human breast, but significantly more adipocytes. However, the precancerous stage is also evident and is called mammary intraepithelial neoplasia (MIN), demonstrating that these pre-transformed cells have their tumorigenic potential pre-encoded at this early stage [57].

A consensus report developed by the US National Institutes of Health [80] Breast Cancer Think Tank and Annapolis Pathology Panel concluded that the histology and the progression of the mouse mammary tumor shows some discrepancies in comparison to human breast cancer [81]. Whereas human breast cancer is strikingly heterogeneous, mouse mammary tumors of GEM are mostly represented by one of the major cancer subtypes. In fact, the hormone receptor status is a component that is lacking in transgenic mice. While roughly 50% of all human breast cancer cases are estrogen receptor (ER) positive and responsive to estrogen, the majority of lesions that arise in GEM are ER negative and hormone-independent [81]. However, the MMTV-Met mice are an exception, where each animal induces mammary tumors with diverse phenotypes [82]. Another major difference between mouse and human mammary cancer is the sites of metastasis. Human breast cancer typically spreads through the local lymph circulation and eventually forms distant metastases mostly to the bone and lung and in a smaller proportion, liver and brain. In contrast, mammary tumors in GEM metastasize exclusively to the lungs via the blood or the lymphatic stream.

Despite the diversity of human breast cancers and species morphological and pathological differences, GEM should be considered as useful tools for investigating precise genes of interest in a given signaling pathway.

1.3 ERBB2 AND ITS IMPLICATION IN BREAST CANCER

The ErbB family of tyrosine kinase receptors (RTKs) has important roles in human cancer. Particularly, the expression and activation of ErbB2 is altered in many epithelial tumors. In fact, 20-30% of all breast cancer cases overexpress and/or amplify this oncogene and correspond to an aggressive phenotype and poor survival rate. Since the establishment of ErbB2 as a potent oncogene in 1987, these receptors have been extensively studied in order to better understand their importance in cancer biology and

as therapeutic targets. In spite of their widely accepted clinical relevance in human breast cancer, the ErbB2 receptors and their downstream signaling pathways still need to be investigated to better understand and treat this disease. In the present chapter, an overview of the ErbB family and the ErbB2 downstream signaling and crosstalks with other pathways will be presented.

1.3.1 ErbB family of receptors

The ErbB family of receptors belong to the type 1 receptor tyrosine kinases and consists of four members who are named for their homology to the v-erbB oncogene: ErbB1 (EGFR, HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). All members have an extracellular region, a single transmembrane region and a cytoplasmic tyrosine-kinase-containing domain. They have molecular weight of 170-185 kDa and share two structural aspects by which they can be distinguished from the other receptor tyrosine kinases: two cysteine rich clusters in the extracellular region and an uninterrupted tyrosine kinase domain in the cytoplasmic region [83, 84]. The highest degree of homology is observed within the kinase domain, with ErbB2 and ErbB4 sharing approximately 80% identity with EGFR [85]. ErbB3 on the other hand, shares only 59% identity and lacks four residues that are conserved in the kinase domain of numerous protein kinases, which confers its status of kinase-inactive receptor [86].

The signaling diversity coming from the four ErbB receptors is generated by the repertoire of ErbB ligands and the combinatorial nature of induced receptor dimers. To date, 10 ErbB-specific ligands have been documented: epithelial growth factor (EGF), amphiregulin (AR) and transforming growth factor-alpha (TFGα), which bind specifically to ErbB1, betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin [87], which exhibit dual specificity for ErbB1 and ErbB4 [88], neuregulin 1 and 2 (NRG-1 and NRG-2), both bind ErbB3 and ErbB4 [89, 90], and NRG-3 [91] and NRG-4 [92] are specific to ErbB4. Despite the large number of ligands described for ErbB1, 3 and 4, no direct ligand

for ErbB2 has been yet discovered.

Ligand binding to the receptor promotes ErbBs homo- or heterodimers and activation of an intrinsic kinase domain that leads to a conformational change resulting in phosphorylation of the tyrosines in the cytoplasmic tail. Unlike other members of the family, heterodimerization is essential in the case of ErbB2 and ErbB3. In fact, ErbB2 ligands have not been identified, whereas ErbB3 is devoid of intrinsic kinase activity. Therefore, numerous studies have established ErbB2 and ErbB3 as obligate partners where their heterodimerization forms the most active signaling partnership of the whole family [93-96]. Overall, ErbB2 is the preferred heterodimerization partner for all other ErbB members and has the strongest catalytic kinase activity [97, 98].

Once they undergo dimerization and transphosphorylation of their intracellular domains, the phosphorylated tyrosine residues of the ErbB receptors can bind Src homology 2 (SH2) or phosphotyrosine binding (PTB) domain-containing-proteins required for the propagation of the signal [99]. Large-scale phosphoproteomic screening illustrated that ErbB receptors potentially bind over 100 proteins [100, 101]. These include adaptor and scaffolding proteins such as Shc, Crk, Grb2, Grb7, Gab1 and Gab2 and kinases such as Src, Chk and PI3K. All ErbB family members couple via Shc and/or Grb2 to the mitogen-activated protein (MAP) kinase pathway [102]. When in complex with the activated receptor, these molecules provide docking sites for the activation and phosphorylation of additional downstream effectors (reviewed in [102, 103]). In this manner, the signaling pathways that ErbB2 activates will lead to a wide array of biological responses including cell division, migration, differentiation and survival [104].

Examination of the diversity and signaling potency emanating from ErbB receptor combinations highlight the synergetic impact on the deregulation of cellular responses. In fact, many human cancers present a hyperactivated ErbB receptor network and their pathogenic potential correlates with a more aggressive disease [105]. In particular, the

ligand-independent activation of overexpressed ErbB2, combined with its preferred role as a partner for the other ErbB members, explain its oncogenic potential. ErbB2 involvement in tumorigenesis will be discussed in the following sections.

1.3.2 ErbB2 structure and function

The *erbB2* gene, located on chromosome 17, encodes for a 185 kDa transmembrane receptor tyrosine kinase, ErbB2, and was originally identified as the transforming component in several rat neuro/glioblastomas [106]. The term ErbB2 is used to refer to the gene across both human and rat species, while HER2 refers to the human gene only and Neu its rodent counterparts. ErbB2 consists of an extracellular domain (with two cysteine-rich regions and two ligand binding regions), a short transmembrane domain, and an intracellular domain with 5 carboxy-terminal tyrosine residues. Interestingly, ErbB2, an orphan receptor, remains in a perpetually "open" or in a ligand-activated-like conformation, with its dimerization arm exposed. In fact, analysis of the crystal structure of a portion of the ErbB2 extracellular domain spanning residues 1 to 509 confirmed an ErbB2 conformation similar to activated ErbB1 [107]. Thus, ErbB2 remains essentially primed for interaction with other ErbB molecules and increases the affinity of ligand binding to all ErbB receptor heterodimers [88]. As might be expected, overexpression of ErbB2 can bias dimer formation and spontaneously generate homodimers in ErbB2-overexpressing cells [108].

1.3.3 ErbB2 in normal development

During normal development, ErbB2 plays physiological roles in mammary gland and heart development, and a minimum threshold level of ErbB2 is necessary for normal development [109, 110]. It has also been shown that ErbB2 is crucial in the development of Schwann cells and proper myelin formation [111]. Total inactivation of the ErbB2 locus is embryonic lethal [112], however targeted ErbB2 knockout restricted

to the mammary gland avoids embryonic lethality and leads to abnormal development of the mammary gland, notably improper duct formation and incomplete ductal branching [113, 114].

1.3.4 ErbB2 overexpression in human breast cancer

The overexpression of the ErbB2 receptor in primary human breast cancers occurs either through gene amplification or through transcriptional deregulation. Clinical reports on ErbB2-overexpressing breast cancers demonstrate that high *erbB2* gene copy number and up to 40- to 100-fold increase in ErbB2 protein expression is seen in these tumors, resulting in up to 2 million receptors expressed at the tumor-cell surface [115]. Previous studies had pointed a unique portrait of ErbB2 amplified breast cancers, correlating with higher tumor grade, the development of metastases, and poor prognostic outcome [27, 116, 117], along with a high proliferation index, negative ER and PR status and p53 mutation [118, 119].

Increased expression of ErbB2 is an essential aspect for its function. Overexpression of ErbB2 in tumors leads to constitutive activation of the receptor, presumably because of increased receptor concentrations at the plasma membrane [105]. Moreover, ErbB2-overexpressing breast cancer and transgenic mice expressing activated Neu display a significant increase of ErbB2-containing heterodimers and ErbB2 homodimers [103]. Indeed, ErbB2 cooperates with ErbB1 and ErbB3, suggesting that this association can contribute to the activation of additional intracellular pathways as well as the maintenance of increased proliferation rates and cell survival [65, 120, 121]. Consistently, the coupling of ErbB2 to ErbB3 receptors, along with the constitutive activation of the ErbB2 homodimers, results in significant prolonged activation of downstream Ras/MAPK and c-Jun, PI3K/AKT, Src kinases, transcriptional activation of the STATs, PAR complex and PKC.

1.3.5 ErbB2 downstream signaling in breast cancer

A number of genes have been implicated in human ErbB2-overexpressing breast cancer development and metastasis. This section focuses on the three main pathways downstream of ErbB2 [122] that are known to be highly activated and to play an important role in ErbB2-overexpressing breast tumor progression and the formation of metastases, along with the p130Cas/Crk pathway which is relevant to the future understanding of the thesis (Figure 1.6).

1.3.5.1 Ras/MAP kinase pathway

The Ras proteins are evolutionarily conserved GTPases implicated in various cellular processes, many of which are growth-regulating signals. Activation of the Ras pathway ultimately leads to the stimulation of the mitogen-activated protein (MAP) kinase, an enzyme with a number of intracellular targets including transcription factors [123, 124]. Activation of Ras by ErbB2 is accomplished, in part, through the recruitement of the Ras exchange factor Sos to the plasma membrane via the binding of Grb2 and Shc within the C-terminal region of activated ErbB2 dimers [125]. Although fewer than 5% of breast cancers have *Ras* mutations, hyperactivation of the Ras signaling pathway in breast cancer has been described [126, 127]. Also, Ras activity being required for efficient ErbB2 and PyMT-mediated signaling, its modulators like Grb2 and Shc also appear to play a critical role in the rapid generation of mammary tumors [77, 128]. Several studies on ErbB2-overexpressing breast cancer argue that the Ras/MAPK pathway is important for tumor cell proliferation, survival, angiogenesis, and regulate the expression of genes associated with invasion and metastasis [52, 122, 129-131].

1.3.5.2 PI3K/AKT pathway

Another critical kinase cascade that plays an essential role in ErbB2-mediated

tumor induction and progression is the PI3K/AKT pathway. This pathway is an important driver of cell proliferation and cell survival by activating a key survival kinase, AKT, and by opposing the tumor suppressor effect of PTEN [132]. PI3K activation is achieved through the binding of the p85 regulatory subunit of PI3K to phosphotyrosine motifs on RTKs, which triggers activation of its p110 catalytic subunit. Apart from being activated following RTKs dimerization, the adaptor protein Grb2 in complex with the scaffolding protein GAB, binds to the p85 subunit of PI3K, leading to the activation of the PI3K/AKT pathway. Moreover, Ras itself can directly activate p110 independently of p85 (reviewed in [132]). PI3K is responsible for the phosphorylation of phosphatidylinositol$_{(4,5)}P_2$ (PIP_2) which generates the second messenger $PI_{(3,4,5)}P_3$ (PIP_3). In turn, PIP_3 is able to recruit to the plasma membrane and interact with many distinct targets that contain PIP_3-binding domains, such as protein kinases, GEFs and phospholipases (reviewed in [133]). For example, the GEF Dock180 gets localized to the plasma membrane upon generation of PIP_3 by PI3K via its DHR-1 lipid-binding domain, thus leading to Rac GTPase-mediated cytoskeletal rearrangement [134, 135]. Tumors from MMTV-Neu mice also have activation of PI3K signaling [136] and apart from inducing proliferation and survival, high PI3K activation correlates with a pro-invasive phenotype [137]. Due to its dramatic importance in breast tumorigenesis, PI3K has been an interesting pharmaceutical target, with many PI3K inhibitors currently in clinical trials (summarized in [138]). In fact, a recent study demonstrated that both trastuzumab, a monoclonal antibody against HER2, and GDC-0941, a novel PI3K inhibitor, treatment on trastuzumab-sensitive and insensitive HER2-amplified breast cancer cells, leads to a drastic synergical antiproliferative effect [139].

Phosphoinositide products of PI3K form high-affinity binding sites for the PH domains of intracellular molecules. Akt is one of the many targets of PI3K products in cells (reviewed in [140]). Following binding of the Akt to PI3K products, Akt gets correctly positioned at the plasma membrane via its PH domain. Akt can then be phosphorylated by its activating kinases, PDK1 and mTORC2. Once activated, Akt activation is channeled

into a plethora of downstream biological responses reaching from angiogenesis, cell survival and proliferation. AKT activation is typically linked to PI3K activity, and has also been associated with overexpression and activation of the Src and FAK kinases [141-144]. For instance, treatment of MCF-7 breast cancer cells with a dominant-negative Src-inhibitor led to decreased proliferation concomitant with decreased AKT phosphorylation, indicating a role for AKT downstream of Src [145]. Additionally, the ability of FAK to interact with integrin subunits may be dependent on the Src-PI3K-AKT pathway [146]. Thus, AKT activation may potentially function at the crux of ErbB2 and integrin signaling via Src and FAK.

1.3.5.3 Src/FAK pathway

Several studies suggest that Src kinases are important second messengers of ErbB2. Src is the prototype of the large family of cytoplasmic tyrosine kinases. Membrane targeting has been shown to be critical for Src's capacity to induce cell transformation, as membrance localization affords Src proximity with both upstream and downstream signal effectors [56,57]. Targeting to the cell membrane may, for instance, enable Src proximity with FAK, an interaction that has been well documented.

Src kinases are potent transformants and oncogenic when constitutively activated and their activation is seen in many human cancers, including mammary cancers with or without ErbB2 overexpression [147-149]. Src proteins associate with ErbB2 in human breast cancer cells [148, 150, 151], an interaction that is required upon the tyrosine phosphorylation of the cytoplasmic and/or kinase domain of ErbB2 and the SH2 domain of Src [152], thus resulting in elevated Src activity [150]. Furthermore, pharmacological inhibition of Src causes a reduction in ErbB2-mediated soft agar colony formation and motility [148, 151]. Additionally, the ability of Src to bind and phosphorylate FAK in breast carcinoma cells requires integrin engagement [153]. FAK is a kinase which functions as both a signaling and scaffolding protein at focal adhesions, where it is

involved in focal adhesion turnover and downstream signaling. Following binding of FAK to the SH2 domain of Src, FAK phosphorylated tyrosine residues can serve as docking sites for further downstream signaling proteins, including PI3K and Grb2 [154]. In addition, FAK can signal via MEK/ERK and JNK to the nucleus thereby regulating transcription of genes that regulate cell growth as well as invasion (reviewed in [155]). FAK has also been shown to be required for ErbB2/3-induced tumorigenesis and metastasis: Cells co-transfected with ErbB2 and ErbB3 and expressing wild-type FAK formed tumors with lung metastasis following transplantation into immunodeficient mice, whereas ErbB2/3-positive but $FAK^{-/-}$ cells failed to form tumors [156]. As well, mammary-specific deletion of FAK in the PyMT model results in a significant block of the transition of premalignant hyperplasias to carcinomas and their subsequent metastases [50].

1.3.4 p130Cas in ErbB2-mediated tumorigenesis and invasion

Interestingly, a recent paper suggested a role for p130Cas in ErbB2-mediated tumorigenesis and invasion, where ErbB2, Src, FAK and p130Cas form a molecular complex which markedly enhance Rac1 and MMP9 signaling [157]. Consistent with these results, the assembly of p130Cas/Crk/Dock180 in a Src-dependant manner drives lamellipodia formation, which ultimately leads to JNK activation and induction of MMPs [158-160]. Therefore, p130Cas seems to be a key player in a novel signaling platform including ErbB2, FAK and Src, which in turn drives migration and invasion. Moreover, double-transgenic mice derived from crossing MMTV-p130Cas with MMTV-Neu-NT mice accelerate tumor onset, mainly via hyperactivation of Src kinase, ERK1/2, MAP kinase (MAPK), and Akt pathways [161].

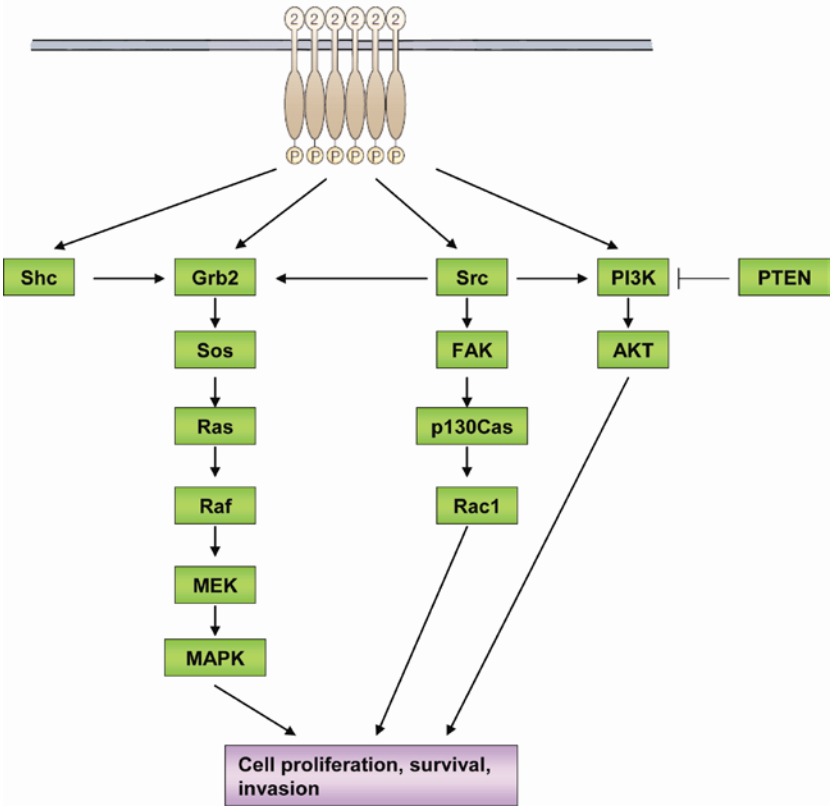

Figure 1.6: *The ErbB2 signaling pathways*. ErbB2-mediated tumorigenesis signals primarily through the Ras/MAPK cascade, PI3K/AKT cascade and the Src/FAK cascade. These pathways ultimately lead to enhanced cell proliferation, survival and invasion.

1.3.5 Crosstalk between ErbB2 and integrins

Integrin engagement has been shown to modulate the activity of various receptor tyrosine kinases (RTKs), including platelet-derived growth factor receptor (PDGFR) [162], vascular endothelial growth factor receptor-2 (VEGFR) [163], Met [164], ErbB1 [165] and ErbB2 [166]. As found by Guo and collegues, α6β4 integrins bind directly with ErbB2 which drive ErbB2-induced mammary tumor cell proliferation and invasion [167]. It is also possible that ErbB2 may cooperate with integrins like α6β4 via intracellular modulators such as Src and FAK, which may serve as both structural and signaling proteins [166]. This study also suggests that the β4 integrin/ErbB2 complex

enhances activation of the transcriptional factor STAT3 and JNK /c-Jun.

1.3.6 Implication of STAT3 in tumor invasion and metastasis

Several studies highlighted a role for the transcriptional factor STAT3 as a key player in tumor cell invasion and metastasis development of ErbB2-mediated breast cancer. In fact, the loss of STAT3 in the MMTV-NIC mouse model impairs lung lesions development due to hindered angiogenesis and cell autonomous defects in colonization [70]. Moreover, recent studies using a constitutively active form of STAT3 in the MMTV-NeuNT transgenic model supports the role of this protein in cell invasion, anchorage-independent growth and metastasis development, but does not affect mammary tumor initiation [168, 169]. As described in the previous section, STAT3 is activated downstream of Src following β4 integrin and ErbB2 stimulation [166]. In this case, STAT3 contributes to the disruption of cellular polarity thus promoting cell invasion, while JNK/c-Jun is required for proliferation. Furthermore, STAT3 is found to be constitutively active in a high percentage of human breast cancers [170, 171]. Due to their important role in invasion and metastasis downstream of ErbB2 signaling, STAT3 is a potential target for molecular therapy of breast cancer.

1.4 MECHANISMS OF TUMOR INVASION AND METASTASIS

Tumor cell motility is the hallmark of invasion and is regarded as the first step in metastatic dissemination [172, 173]. To date, metastasis accounts for over 90% of breast cancer deaths and studying motility mechanisms used by cancer cells would give important information on the key events influencing this dramatic process. This section summarizes how the mechanisms and pathways that regulate cell motility contribute to invasion and metastasis of breast cancer cells, with an emphasis on the implication of

the integrins and the Rho family of small GTPases.

1.4.1 Motility and cytoskeletal rearrangement

Tumor cells move within the surrounding tissues during invasion and metastasis by their own motility machinery. This process of cell migration has been well studied in untransformed cells such as fibroblast and epithelial cells, as well as numerous cancerous cells, and the molecular mechanisms underlying cell motility are common to both normal and cancer cells.

The actin cytoskeleton and the proteins that regulate cytoskeletal rearrangement are essential players in cell migration. Together with the microtubules and the intermediate filaments, the actin filaments compose the cytoskeleton of the cell. In order to move, cancer cells produce protrusive structures at the leading edge of the plasma membrane. These protrusions consist of actin filaments that are organized into different shapes to form lamellipodia, filopodia and invadopodia (Figure 1.7). Lamellipodia are sheet-like membrane protrusions observed at the cell front during cell migration and spreading. Their formation is dependent on actin polymerization by the Arp2/3 complex, which nucleates a new filament from the side of an existing filament. The Apr2/3 complex is regulated by the WAVE complex during assembly of the actin network (reviewed in [174]). This rather large complex interacts with the small GTPase Rac1 and has been proposed to be recruited to the plasma membrane at sites of lamellipodial formation [175]. Conversely, filopodia are thin, finger-like protrusions emerging from the lamellipodial sheet that function as a directional sensor [176]. This structure adheres to the substratum and is thought to be formed from the reorganization of the lamellipodial Arp2/3 complex [177]. Filopods are controlled by various proteins such as fascin, diaphanous and WASP, and are known to be regulated by the small GTPases Rac and Cdc42 [178-180]. Last but not least, invadopodia are intermediate-width extensions present specifically on the ventral side of invasive cells (reviewed in [181]). Each

invadopod present an actin-rich core surrounded by a ring of several actin-associated proteins and signalling proteins. Similar to lamellipodia, formation of invadopodia depends on branched actin assembly by the Apr2/3 complex [182]. Likewise, the Apr2/3 complex is regulated by the N-WASP and WAVE complex but also cortactin, an actin assembly molecule, acting specifically on invadopodia formation [183, 184] and is frequently used as a marker for this structure. These highly dynamic protrusions involve an orchestrated complex of proteins, including integrins, Src, FAK, ILK, Rac1, Cdc42, MMPs as well as uPAR, to migrate over thick extracellular matrices, and mediate matrix degradation to breach physical barriers [185-187].

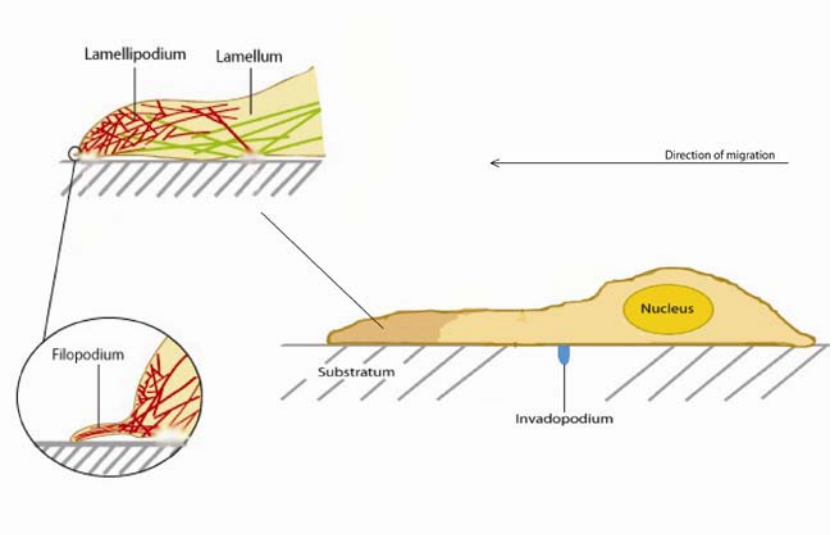

Figure 1.7: *Formation of protrusions during migration*. Actin filament reorganization generates the formation of protrusions at the leading edge of the plasma membrane that drives cell motility in a specific direction. Lamellipods are sheet-like structures at the cell front where filopodia emerge, forming a finger-like protrusion. Invadopods are present on the ventral side and serve for directional movement by invasive tumor cells. Modified from [188].

The currently accepted model of cell motility consists of four successive steps (reviewed in [189, 190]): (1) Protrusion of a broad lamellipodium in the desired direction of movement where *de novo* actin polymerization occurs at the leading edge after activation by extracellular stimuli. Polymerized actin filaments induce the formation of

membrane protrusions such as filopodia and lamellipodia; (2) Adhesion of the leading membrane to the underlying substratum through focal adhesions, integrin-containing adhesive structures at the leading edge; (3) Contraction of the polymerized actin filament (also known as F-actin) stress fiber network which generates sufficient traction to translocate the nucleus and cell body forward; and (4) Disassembly of focal adhesions at the trailing edge of the cell, allowing the cells to retract and be dragged in the direction of migration.

1.4.2 Modes of cell migration in cancer

Different types of cancer cell migration seem to exist, including individual mesenchymal or amoeboid migration and collective cell migration [191]. Structural and molecular determinants of both tissue environment and cell behavior dictate the most relevant mode of migration. In the present section, these modes of migration in cancer are discussed.

Amoeboid migration refers to the movement of rounded or ellipsoid cells that lack mature focal adhesions and stress fibers [192, 193]. Individual cells undergoing this type of migration show a rounded, blebbly shape in 3D substrate and squeeze their body through gaps in the ECM fibers [192, 194, 195]. The enhanced contractility of cells that use amoeboid movement is promoted by the Rho/ROCK signaling pathway [196, 197], and can be conterbalanced by increased Rac activation, which induces cell protrusion and inhibits cell rounding [198]. Amoeboid dissemination allows tumor cells to undergo early detachment and metastatic spread from a small primary tumour, and is most commonly seen in lymphomas and small-cell lung carcinomas [199].

In contrast to amoeboid migration, individual cells with high levels of attachment and cytoskeletal elongation develop mesenchymal migration, which involves cell-matrix interactions and move in a fibroblast-like manner [200, 201]. This type of motility can be

seen in 2D or 3D matrices and is characterized by an elongated, spindle-like and polarized shape [201]. The migration begins with the formation of actin-rich filopodia and lamellipodia at the leading edge, primiraly driven by Rac and Cdc42 [202, 203]. Mesenchymal movement is predominantly found in cells from connective-tissue tumours, such as fibrosarcomas [195], gliomas [204] and in epithelial cancers such as breast cancer [205]. In such cases, the process wherein an epithelial cancer cell switches its mode of migration to a mesenchymal-like migration is termed epithelial-to-mesenchymal transition (EMT).

On the other hand, collective migration is defined by two or more cells that retain their cell-cell junctions and move together across a 2D or a 3D matrix [206, 207]. This type of movement is relevant in many processes such as morphogenesis, tissue repair and cancer invasion [208-210]. Similarly to single-cell migration, collective cell motility results from actomyosin polymerization and contractility coupled to cell polarity [211]. It is possible that most cancer types contain invasive zones, also termed invasive fronts, of intact cell-cell cohesion and collective invasion [212]. These areas display expression of cell-cell adhesion molecules, including claudins 1 and 4, zona occludens 1 (ZO-1) [213], and gap junctions, like connexins CX26 and CX43 [214]. The formation of actin-rich protrusions and focalized proteolysis in collective cell migration ressembles the mesenchymal type of motility. In fact, both of them require lamellipodia and filopodia formation by Rac and Cdc42 at their plasma membrane, as well as the actin-nucleating Arp2/3 complex [215]. “Leader” cells at the tip of the collective front utilize focalized proteolysis by MT1-MMP to generate tracks that enable a group of cells to undergo subsequent collective invasion [216] and show clusters of β1 integrins [217]. These characteristics are histologically detectable in invasive mammary carcinoma [218], colon carcinoma [219] and other forms of invasive cancers.

1.4.3 Integrins in migration

Integrins are a large family of heterodimeric transmembrane glycoproteins that mediate cell-cell or cell-matrix adhesion. These receptors contain two distinct subunits, called the α and β, and 24 combinations of 18 α and 8 β subunits have been characterized in mammals. In addition to their roles in cell survival, proliferation and differentiation, integrins are known to be a critical players in the movement of all motile cell types, such as T cells, macrophages, fibroblast and epithelial cells during wound healing and development [220].

For migration to occur, a protrusion must form and then be stabilized by attaching to its surroundings. Although many receptors are involved in this process, integrins are a major component in cell migration by supporting adhesion to extracellular matrix (ECM) via linking adaptors with actin filaments on the inside of the cell. Activated integrins preferentially localize to the leading edge of the cells [221] and cluster with other integrins or other types of receptors at focal adhesion sites. As previously described, both in normal and transformed cells, integrin-dependent mechanisms have been shown to implicate crosstalk between integrin receptors and other growth promoting molecules such as growth factor receptors [165, 222, 223]. After migration is initiated, integrin clustering at these focal adhesions drive actin polymerization along the leading edge of the cell, which in turn reinforces the formation of integrin-based adhesion complexes [221]. During this process, members of the Rho family of small GTP-binding proteins are activated and contribute to integrin-mediated changes in cell shape and polarity.

The intracellular signaling pathways activated by integrin-ligand interactions give better insight of the function of integrins in cell motility (Figure 1.8). Direct cytoplasmic tail-binding proteins (for example, talin, vinculin and paxillin) act as adaptors and also link integrins to the cytoskeleton by binding F-actin [224-226]. Integrins signal

predominantly through the recruitment and activation of the Src-family kinases (SFKs) and FAK, which causes the phosphorylation of many substrates. First of all, FAK is able to initiate lamellipodia formation. Its activation leads to the phosphorylation of the adaptor protein p130Cas, which in turn recruits Crk. This p130Cas/Crk complex can initiate the activation of the Rac1 GTPase, via the the guanine nucleotide exchange factor Dock180 [227-229] (described in chapter 1.5) thus leading to the formation of lamellipodia at the leading edge of the moving cell. Moreover, FAK can also activate signaling from phosphatidylinositol 3-kinase (PI3K) to AKT/protein kinase B (PKB), through phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P_3 or PIP_3). As well, FAK can activate the protein kinase Src and permit direct interaction with the ß subunit cytoplasmic tail of integrins. These coordinated events activate multiple downstream signaling pathways including MAPK, JNK and the Rho family of GTPases [230-232]. Integrins also associate with other plasma membrane proteins, such as receptor Tyr kinases (RTKs) for coordinated signaling, and with MMPs to connect ECM degradation with cell adhesion and signaling. Urokinase-type plasminogen activator receptor (uPAR) [233] can affect many aspects of integrin function, $\alpha_v\beta_3$ integrin, by regulating activation, signaling, integrin–RTK interactions and pericellular ECM proteolysis.

Being a protypical regulator of inter- and extracellular interactions in a physiological context, the contribution of integrins to tumor progression and metastasis have been studied in great detail. Genetic manipulation of experimental mouse models of breast cancer has demonstrated the requirement of these proteins in cell survival, proliferation and migration in the context of mammary epithelial cells undergoing neoplastic transformation by activated oncogenes such as ErbB2/Neu. For example, targeted ablation of β1 or β4 integrin subunits and their associated intracellular effectors including FAK or Src has been demonstrated to impair tumorigenesis and reduce the invasive phenotype [76], [166].

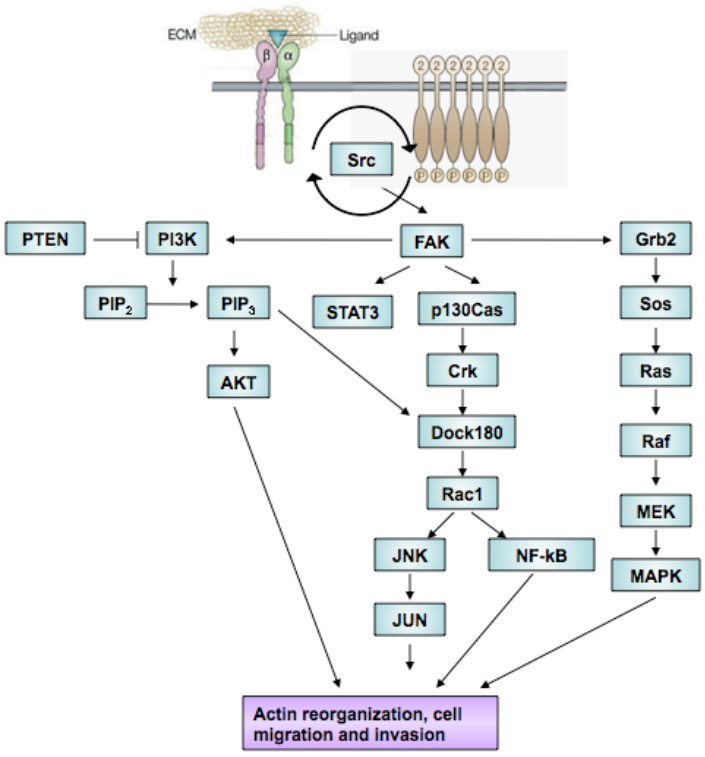

Figure 1.8: *The integrin signaling pathway and crosstalk with ErbB2.* Integrin signaling regulates cytoskeletal reorganization during adhesion and migration and also control cell survival and cell proliferation. Integrins activate FAK and thereby Src, which causes the recruitment and activation of the p130Cas/Crk complex. Subsequently, Dock180 gets recruited to the complex thus activating the Rac GTPase which signals through JNK/JUN. The FAK/Src complex can also signals through PI3K and the Ras/MAPK pathway. Crosstalk between integrins and several RTKs has been shown to enhance RTKs signaling and is responsible for optimal cellular output.

1.4.4 Rho family of small GTPases in breast cancer invasion

The Rho family of small GTPases, a subgroup of the Ras superfamily of GTPases, consists of 22 members in mammals. Rho, Rac and Cdc42, the protypical members of the Rho family, are regulators of the actin cytoskeleton. These GTPases act as molecular switches that are either inactive when bound to GDP or active when bound to GTP. In their active conformation, GTPases can interact and, in turn, activate several effectors [234, 235]. This cycle between the active or inactive form is regulated by the guanine

nucleotide exchange factors (GEFs), the GTPase-activating proteins (GAPs) and the guanine nucleotide-dissociation inhibitors (GDIs) (Figure 1.9) [236]. GEFs promote GTPase activation by catalyzing the exchange of GDP for GTP, GAPs negatively regulate the switch by enhancing the intrinsic GTPase activity of the Rho proteins, returning their equilibrium toward the GDP-bound state, whereas GDIs prevent dissociation of GDP from the GTPases by sequestering the GDP-bound form in the cytosol, keeping them in their inactive form [237]. These proteins are activated, in part, by integrin engagement, contributing to integrin-driven modifications in cell shape and polarity. In 1992, the Hall Laboratory provided evidence of the crucial role of the Rac GTPase in lamellipodia formation and membrane ruffling in fibroblasts [202]. Rac1, a member of the Rac subgroup, is known to directly interact with the WAVE complex, which induces activation of the Arp2/3 complex leading to actin polymerization and lamellipodia formation [238]. Rho induces the formation of stress fibers, whereas Cdc42 induces actin polymerization leading to filopodia formation.

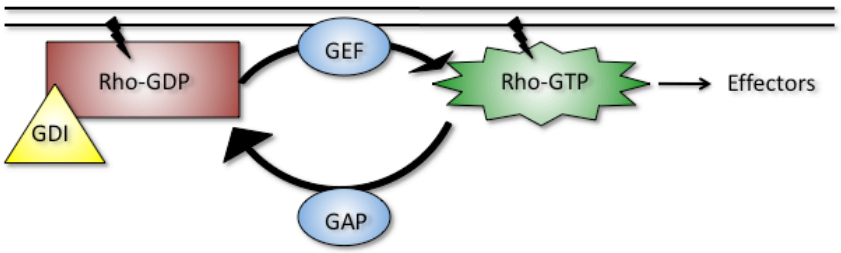

Figure 1.9: *The Rho GTPase cycle*. Actin filament reorganization generates the formation of protrusions at the leading edge of the plasma membrane that drives cell motility in a specific direction.

Several members of the Rho family including RhoA, RhoC, Rac1 and Rac3 have been extensively characterized in breast cancer motility. Although no mutations in the Rho family have been found in breast tumors, these GTPases are often either overexpressed or hyperactive in mammary carcinomas [239, 240]. An increasing body of evidence highlights that Rac is a positive regulator of breast cancer migration. First of all, in a

number of non-invasive (MCF-7 [241, 242], T47D [242]) or highly invasive (MDA-MB-435 [242-244], MDA-MB-231 [241, 242], C3L5 [242], BT549 [245]) breast cancer cell lines, increased activation of Rac1 directly correlates with invasive potential. Conversely, the expression of a dominant negative form of active Rac1 results in a less invasive and motile phenotype [243, 244] and inhibition of Rac1 has powerful inhibitory effect on the invasion of human breast carcinoma cells [241, 244]. Moreover, several reports show that Rac is overexpressed in human tumors. In breast and colon cancers, for example, Rac1 expression is significantly higher in tumor tissues than in corresponding healthy tissue [240, 246]. Interestingly, patients with recurrent breast cancer showed increased membrane localization of Rac1 [246]. All these lines of evidence suggest that the level of activated Rac1 increases along with breast cancer invasion and metastasis.

1.5 DOCK180 PROTEIN

The Dock180 protein is a highly specific activator of the small GTPase Rac during several biological functions such as cell migration, phagocytosis of apoptotic cells and myoblast fusion (reviewed in [247]). Matsuda and colleagues originally discovered it as a binding partner for the SH3 domain of the proto-oncogene CrkII through its C-terminal Proline-rich (PxxP) region [248]. This was further confirmed by independent experiments, all revealing the existence of a CrkII/p130Cas/Dock180 complex responsible for the activation of JNK via Rac in an integrin-dependant fashion [158, 249]. Further study suggested a role for Dock180 as a Rac1 activator, upon integrin signaling through the CrkII/p130Cas complex [229, 250]. In 2002, Côté *et al*. showed that the Dock180 proteins possess a DHR-2 domain which specifically binds to nucleotide-free Rac and activates Rac *in vitro* and *in vivo* [251]. Studies in *C. elegans* and *Drosophila* uncovered several biological processes for the orthologs of Dock180 in these organisms,

Ced-5 and Myoblast City (MBC) respectively, such as phagocytosis, cell migration and myoblast fusion [252, 253].

The Dock family of proteins contains 11 members: Dock1 (also referred as Dock180) to Dock11. The family can be subdivided in 4 groups, depending on their sequence homology, in turn named DockA, B, C and D. The most studied and well characterized of the Dock family member is undoubtedly the Dock180 protein. However, its role in various mammalian biological and pathological events such as breast cancer, is just starting to be uncovered.

1.5.1 Dock180 structure and function

Two distinct families of GEFs for the Rho GTPases have been reported to date: the classical Dbl homology-pleckstrin homology domain (DH-PH)-containing family [254] and the Dock180-related family. Common to all Dock family members are the two evolutionary conserved domains: the Dock homology region (DHR)-1 and -2 domain [255] (Figure 1.10). The DHR-2 of Dock180 is the catalytic domain able to interact with the nuclotide-free form of Rac and promotes its activation, whereas the DHR-1 has a lipid binding domain which permits the translocation of Dock180 to the plasma membrane in a PIP_3-dependant manner [134, 135, 229, 251]. In fact, accumulation of PIP3 at the plasma membrane following PI3K activation, recruits the CrkII/ELMO1/Dock180 complex, which activates Rac and promote directional cell migration [135]. This 180kDa protein also displays an N-terminal SH3 domain, which is the binding site for the ELMO-family of proteins [256] and a C-terminal PxxP region which bind CrkII protein. The ELMO protein, orthologs of Ced-12 in *C. elegans* [257], physically binds Dock180 and is dispensable for its GEF activity towards Rac, impairing Rac signaling [258].

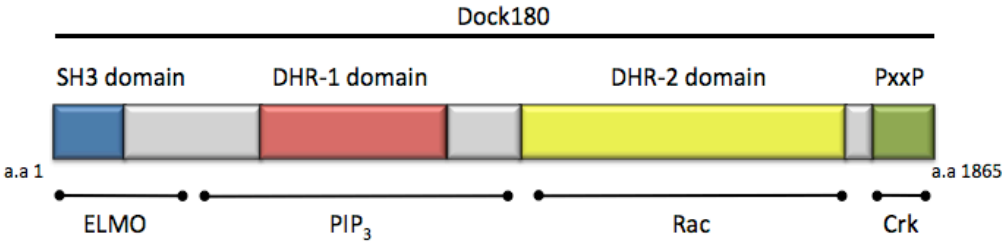

Figure 1.10: *Structure of the Dock180 protein*. The schematic illustration of the Dock180 architecture: the SH3 domain, DHR-1 domain, DHR-2 domain and the Proline-rich domain (PxxP) along with the binding regions of the ELMO protein, the Rac GTPase and the adaptor protein Crk.

As mentioned previously, our knowledge concerning the functions of Dock180 has been mainly uncovered by studies in *Drosophila* and *C. elegans*, but further supported by studies in mammalian cells and mouse models. In fact, a genetic screen in the *Drosophila* aiming to identify novel genes implicated in myoblast fusion identified Myoblast City (MBC), which was subsequently revealed to be the ortholog of Dock180 [259]. Consistent with the role of Dock180 as a GEF for Rac, MBC mutant flies had the same myoblast fusion defects as the Rac (dRac) mutant flies and demonstrated that MBC acts upstream of dRac in a signaling cascade [260, 261]. Conversely, a screen for the discovery of novel genes regulating the phagocytosis of apoptotic cells in *C. elegans* resulted in the identification of Ced-5, where this process requires Ced-2 and Ced-10, later cloned and revealed as orthologues of human CrkII and Rac1, respectively [262]. Beyond phagocytosis defects, mutations in Ced-5/Dock180 impair axon outgrowth and migration of P cells during brain development [263]. Most prominently, mutations in either Ced-2/CrkII, Ced-5/Dock180 or Ced-10/Rac1 compromise distal tip cell migration of developing gonads, by abolishing the polarized extension of cell surfaces [253, 264]. Still in *C. elegans*, Ced-5/Dock180 has been demonstrated to cooperate with Ced-2/CrkII and Ced-12/ELMO1 to promote phagocytosis and collective cell migration [256]. The role of the Dock180/ELMO1 complex in lamellipodia formation, cell polarization and cell migration has also been shown and studied in various mammalian cell lines [135, 265,

266]. Several studies in mammalian cells indicate a role for the Crk/p130Cas/Dock180 complex signaling through Rac1 to induce cell spreading and cell migration [158, 159, 249, 267]. For example, the binding of integrins to fibronectin leads to the activation of Src and FAK and subsequent phosphorylation of p130Cas [268]. After its activation, p130Cas binds to the Crk SH3 domain and recruits Dock180 through its PxxP region and localizes Dock180 to focal adhesions [248].

1.5.2 *In vivo* functions of Dock180

Similar to the myoblast fusion defect in the *Drosophila*, Dock180-deficient mice die at birth, probably due to asphyxia, and present severe reduction in all skeletal muscle tissues, ascribable to a block in myoblast fusion during embryogenesis [269]. More recently, Dock180 has been shown to mediate basement-dependent epiblast survival during embryonic epithelial embryogenesis. This event requires the recruitment of Dock180 by Crk, which in turn activates Rac1 and leads to the activation of the PI3K/AKT pathway [270].

1.5.3 Dock180 and cancer

Many aspects of cellular motility and invasion, including cellular polarity, cytoskeletal reorganization, and transduction of signals from the outside environment, are controlled through an interplay between the Rho-GTPases and their regulatory members. Because of their ability to catalyze the exchange of GDP for GTP, the GEFs of the Rho family of GTPases are good candidates for aberrant GTPase activation in human cancer [271]. Dock180 has been linked to the induction of a pro-invasive phenotype in various malignant human breast cancer cell lines. However, no direct evidence of a role for Dock180 during cell invasion and metastasis in an *in vivo* model of breast cancer has been previously demonstrated. In the present section, studies on Dock180 and its role in tumor invasiveness will be described.

The most direct implication of Dock180 in cancer motility has come from *in vitro* studies with the application of Matrigel assays which mimic basement membrane invasion and migration in the ECM. Firstly, in a metastatic carcinoma cell line, the coupling of p130Cas and Crk signaling through the Dock180-Rac pathway has been shown to be necessary for cell invasion and survival [166, 267, 272]. Moreover, in v-Src transformed fibroblasts, cell invasion was linked to transient FAK accumulation at lamellipodia, formation of a FAK-Src-p130Cas complex which recruits Dock180, thus leading to an elevated Rac and c-Jun activation and increased MMPs expression and activity [159]. Finally, Dock180 is required for cell invasion in the MDA-MB-231 breast cancer cell line, by activating Rac following the formation of the p130Cas/CrkII complex downstream of uPAR, in cooperation with β3 integrin [272]. Additionally, studies in brain cancer demonstrated a role for Dock180 in induction of tumor invasion. Interestingly, in human glioma brain tissue sections, high expression of Dock180, together with ELMO1, is seen at invasive fronts of brain tumors while single tumor cells detaching and migrating out in the surrounding stroma also contain high Dock180 protein levels [273]. As mentioned previously, key proteins in Dock180 activation such as p130Cas and FAK have been correlated with increased cell invasion and metastasis following ErbB2 overexpression in both *in vitro* and *in vivo* models.

1.6 RATIONAL AND OBJECTIVES

RATIONALE

Both cell migration and invasion are highly regulated biological processes where spatial and temporal remodeling of the actin cytoskeleton is essential. Tumor cells that gain the ability to invade the surrounding stroma possess the potential to undergo metastasis. Identification of the molecular players that activate or contribute to the migration machinery is critical in understanding tumor cell dissemination to secondary sites.

Key regulators of actin reorganization are overexpressed in a wide array of human cancers. Proteins such as integrins, FAK, p130Cas, Crk, and PI3K among others are commonly known to promote cancer cell invasion [76, 137, 156, 157, 166]. One of the major players in cell motility, the small GTPase Rac1, is widely known to be activated in several human cancers, including the ErbB2-overexpressing breast cancer [240, 246]. Dock180 is known to mediate Rac activation, in turn leading to lamellipodia formation and cell migration. Moreover, Dock180 has been shown to induce cell invasion in normal and cancerous cell lines. In fact, in complex with p130Cas and CrkII, Dock180 promotes invasion in Matrigel downstream of FAK and Src kinases, by activating the Rac GTPase [159, 274]. Additionally, Dock180 is required for uPAR-driven Rac activation and invasion through the recruitment of p130Cas and Crk via ß3-integrin stimulation in breast MDA-MB-231 cancer cells [272]. Interestingly, one *in vivo* study reports that high levels of ELMO1 and Dock180 are detected in invasive glioma cells in human brain [273]. Experimentally, downregulation of either ELMO1 or DOCK180 dramatically impaired glioma invasion *in vitro*. *While these multiple lines of evidence suggest that DOCK180 contributes to tumor invasion, and potentially metastasis, its role in cancer has never been tested in an experimental* in vivo *model.* Therefore, the straightforward **central**

hypothesis of this thesis is that DOCK180 is a key regulator of tumor cell invasion and metastasis. Preliminary data in the laboratory suggested that DOCK180 is required for ErbB2 transformed cell invasion *in vitro*. To address this hypothesis, we decided to use a mouse model of metastatic breast cancer where an activated version of the oncogene ErbB2 is expressed in the mammary epithelium. With this model in hand, we are in a unique position to address whether DOCK180 is required for breast tumorigenesis and metastasis by crossing a novel conditional allele of Dock180 with this ErbB2 breast cancer model.

OBJECTIVES

AIM 1

Characterization of mammary gland development in the Dock180-conditional knockout mouse. Before investigating the role of Dock180 in ErbB2-overexpressing breast cancer in vivo, it is important to define if Dock180 contributes to mammary gland development, as this could have major implications for further interpretation of tumorigenesis studies. To answer this question, we will use the conditional knockout mice of Dock180 that we developed in our laboratory. This mouse line will be interbred with the MMTV.CRE mice allowing the deletion of the floxed *Dock180* alleles only within the mammary epithelial cells due to its specific promoter in a FVB genetic background. In this in vivo model, we will study the gross development of the mammary gland from post-puberty to verify if the loss of Dock180 impairs its function.

AIM 2

Define the consequences of disrupting Dock180 within the mammary gland in an ErbB2-overexpressing breast cancer model. To address the biological impact of Dock180 in ErbB2-induced mammary tumorigenesis and metastasis, our CKO Dock180 mice will be crossed with the novel ErbB2-based model of breast cancer, the MMTV.NDL2-5-IRES-CRE (MMTV-NIC) animals. These transgenic mice co-express both activated ErbB2 (NDL2-5) and the Cre recombinase via an internal ribosome entry site between the two cDNA sequences. The resulting CKO Dock180:NIC will be studied to evaluate the kinetics of tumor burden appearance by ultra-sound monitoring and physical palpation, tumor multiplicity, tumor invasiveness in the surrounding stroma, activation of downstream effectors of Dock180 and metastasis to lungs.

Chapter 2

Results

RESULTS

Generation of a conditional mutant allele of *Dock180*.

We previously characterized a total knockout mouse of *Dock180* and reported an essential role for this gene in myoblast fusion [269]. To study the role role of Dock180 in cancer, we generated a conditional knockout mouse of this gene (CKO Dock180, described in Materials and Methods) to circumvent embryonic lethality. Briefly, we constructed a targeting vector where the exon1 of the *Dock180* gene was flanked by two LoxP sites (Dock180$^{flx/flx}$), followed by a PGK-neomycin resistance cassette between two FRT sites, and inserted this gene via homologous recombination in embryonic stem (ES) cells (Figure 2.1a). Successfully recombined ES cells were identified by Southern blot and the PKG-neomycin cassette was removed by transient expression of FLP enzyme (Figure 2.1b). Chimeric animals were obtained and crossed with 129/SV mice and offspring with the desired mutation were confirmed by Southern blot analysis (Figure 2.1c). The Dock180$^{flx/flx}$ mice were viable, fertile, and did not show gross abnormalities.

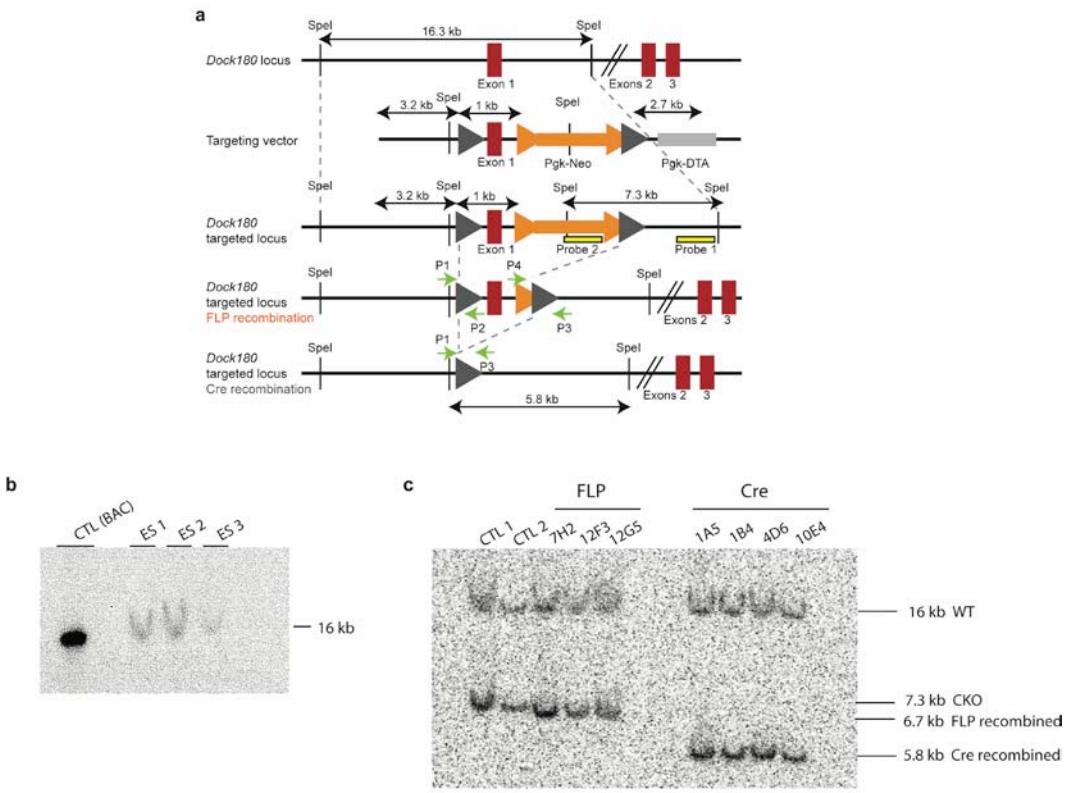

Figure 2.1: *Generation of the CKO Dock180 mice*. a) Partial representation of the *Dock180* locus, structure of the targeting vector, the organization of the rearranged *Dock180* targeted allele after homologous recombination, the targeted *Dock180* locus after FLP recombination and the resulting targeted *Dock180* locus following Cre recombination. b) Screening of targeted ES cells by Southern blot analysis (Probe 1, *Spe*I digestion). First lane represents the control BAC library. Second, third and fourth lanes represent ES cell clones with a targeted *Dock180* locus. c) Screening of targeted ES cells after FLP and Cre recombination. First two lanes represent clones-containing Targeted vector without FLP recombination, lane 3, 4, 5 represent clones after FLP recombination and lane 6 to 9 represent clones after Cre recombination.

Dock180 is not required for mammary gland development but plays a role during the involution phase.

Prior to the investigation of the role of Dock180 in Neu-driven tumorigenesis and metastasis, we verified whether the loss of this gene impaired mammary gland development. This is an important control as such a phenotype would complicate the interpretation of the data obtained in tumorigenesis experiments. To address this question, we interbred our CKO Dock180 mice with transgenic mice carrying the Cre recombinase under the MMTV promoter, and studied the gross morphology of the mammary gland throughout post-pubertal development. We found that whole-mount mammary glands of virgin females from each genotype ($Dock180^{wt/wt}$:CRE; $Dock180^{wt/flx}$:CRE; $Dock180^{flx/flx}$:CRE) at 9, 12, 15 weeks of age presented no gross difference in their morphology (Figure 2.2a, data not shown for $Dock180^{wt/flx}$:CRE). Interestingly, the targeted deletion of *Dock180* in the mammary gland affected the involution phase, as seen in whole-mounts and H&E preparation of inguinal mammary glands of mothers that fed pups for 21 days and were sacrificed 4 days after weaning (Figure 2.2b). At the time of involution, the single layer of epithelium of the alveolar structure in wild-type animals started to collapse and reabsorb residual milk whereas in the Dock180-null mammary gland, the alveoli were still extended and filled with milk. However, the $Dock180^{flx/flx}$:CRE mice pursued normal pregnancy, lactation and the pups had no developmental defects. These observations suggest that Dock180 is not required for the proper development of virgin mammary gland throughout adulthood but hinders the normal involution process.

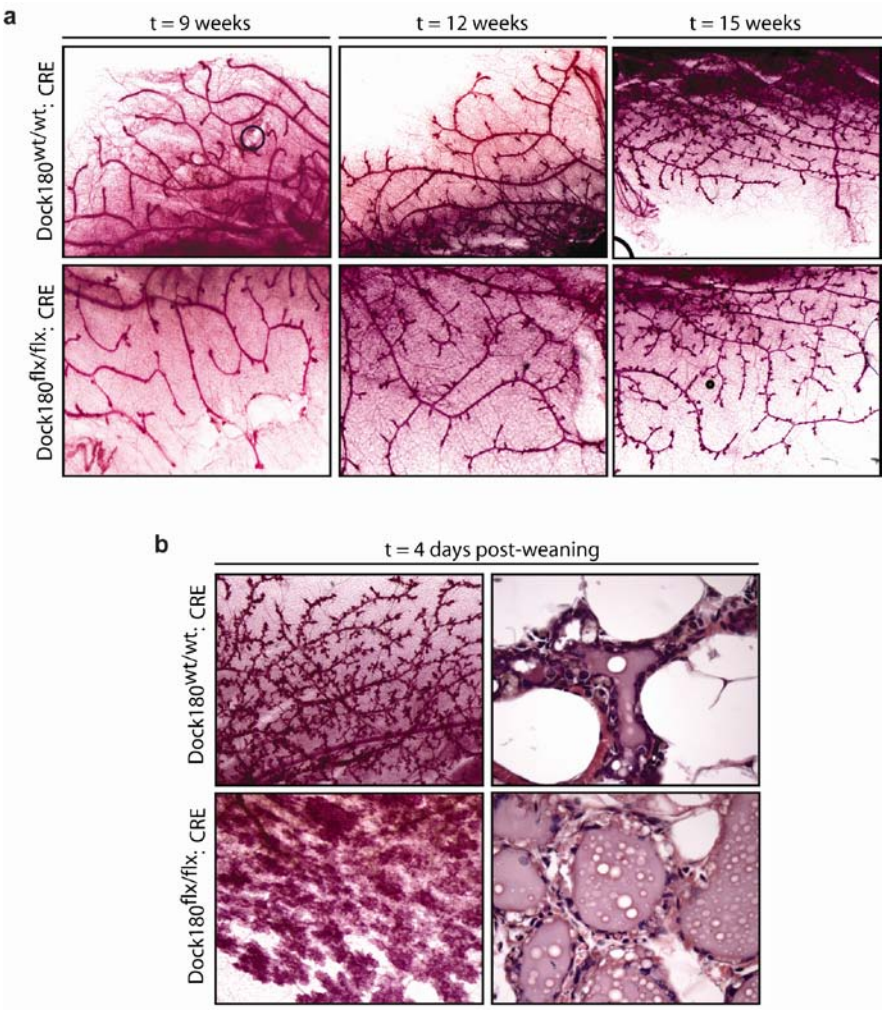

Figure 2.2: *Targeted deletion of* Dock180 *in the mammary gland impairs the involution phase*. a) Whole-mount analyses of inguinal mammary glands of Dock180$^{wt/wt}$:CRE and Dock180$^{flx/flx}$:CRE mice at 9, 12, 15 weeks of age (n=9 for each genotype). b) Whole-mount analyses (*left panel*) and H&E sections (*right panel*) of Dock180$^{wt/wt}$:CRE and Dock180$^{flx/flx}$:CRE inguinal mammary glands at 4 days post-weaning. Dock180$^{flx/flx}$:CRE animals show dilated alveoli filled with milk, whereas alveoli of wild-type mice had undergone normal regression and milk absorption (n=2 for each genotype).

Characterization of breast tumors arising in Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC and Dock180 $^{flx/flx}$:NIC mice.

In this section, we will directly test the contribution of Dock180 in ErbB2-mediated pathogenesis. In order to characterize the ErbB2-overexpressing tumors arising from either the loss of one or two *Dock180* alleles, we interbred the MMTV-NIC mice with our CKO Dock180. The transgenic MMTV-NIC mice can bypass the stochastic expression of the Cre recombinase seen in other ErbB2 based mouse models [77] and developed metastatic mammary tumors starting at approximately 140 days of age [69, 70, 77, 79].

Cohorts of virgin females from each genotype (Dock180$^{wt/wt}$:NIC; Dock180$^{wt/flx}$:NIC; Dock180$^{flx/flx}$:NIC) were monitored weekly by physical palpation. Mice from each genotype were euthanized 5 weeks after the first palpable tumor. Tumors and lungs were harvested as described in Materials and Methods. Complete loss of the floxed *Dock180* allele by the expression of the Cre recombinase in tumors from MMTV-NIC mice was confirmed by PCR (Figure 2.3a) and the expected elimination of Dock180 expression was confirmed by western blot and immunohistological analyses (Figure 2.3b and c). Together, these data illustrate that floxed *Dock180* alleles are properly excised following Cre recombinase expression, resulting in the complete ablation of Dock180 expression in mammary tumors arising from Dock180$^{flx/flx:}$NIC mice.

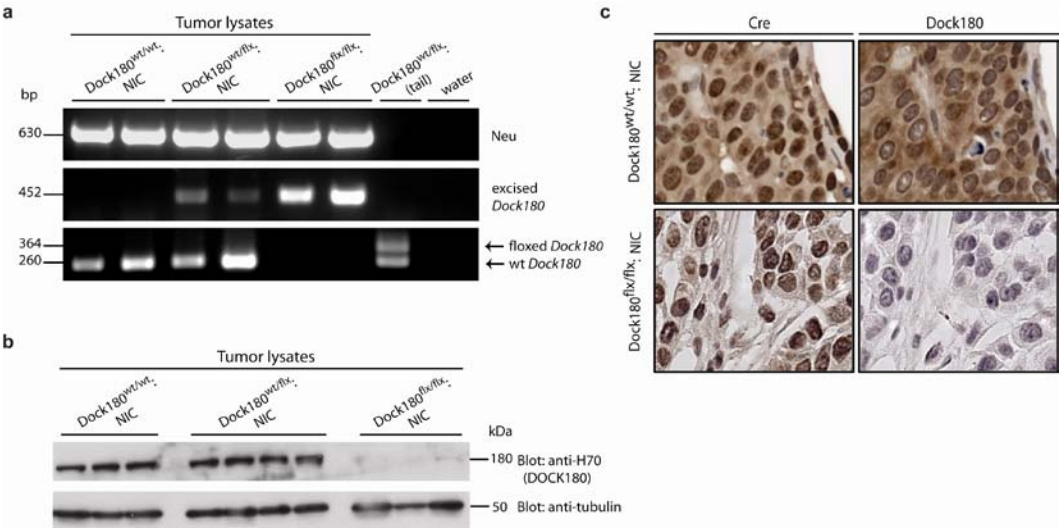

Figure 2.3: *Efficient Cre-mediated excision of the floxed* Dock180 *allele in NIC tumors*. a) PCR analysis of Dock180$^{wt/wt}$, Dock180$^{wt/flx}$ and Dock180$^{flx/flx}$:NIC tumor lysates all showing presence of the Neu transgene (630 bp band). The floxed *Dock180* allele from Dock180$^{wt/flx}$ and Dock180$^{flx/flx}$:NIC tumors was efficiently recombined by the Cre recombinase (452 bp band). Loss of the floxed *Dock180* allele in NIC tumors is illustrated by the absence of a 364 bp band in tumor lysates from Dock180$^{wt/flx}$ and Dock180$^{flx/flx}$:NIC mice. c) Western blot illustrating complete loss of Dock180 expression in Dock180$^{flx/flx}$:NIC mice. d) DAB staining of Dock180$^{wt/wt}$ and Dock180$^{flx/flx}$:NIC tumor sections demonstrating the complete loss of Dock180 expression within the epithelial tumor cells, using anti-Cre antibody (*left panel*) and anti-Dock180 antibody (*right panel*).

Wild-type, heterozygous and homozygous Dock180:NIC mice all developed multifocal mammary tumors at 124, 125 and 132 days of age respectively (Figure 2.4a), and all display characteristics of solid adenocarcinomas (Figure 2.4b), in agreement with previous studies using the MMTV-NIC mice [69, 70, 77, 79]. Finally, these data suggest that the loss of one or both alleles of *Dock180* has no impact on tumor onset and histological properties of the NIC tumors.

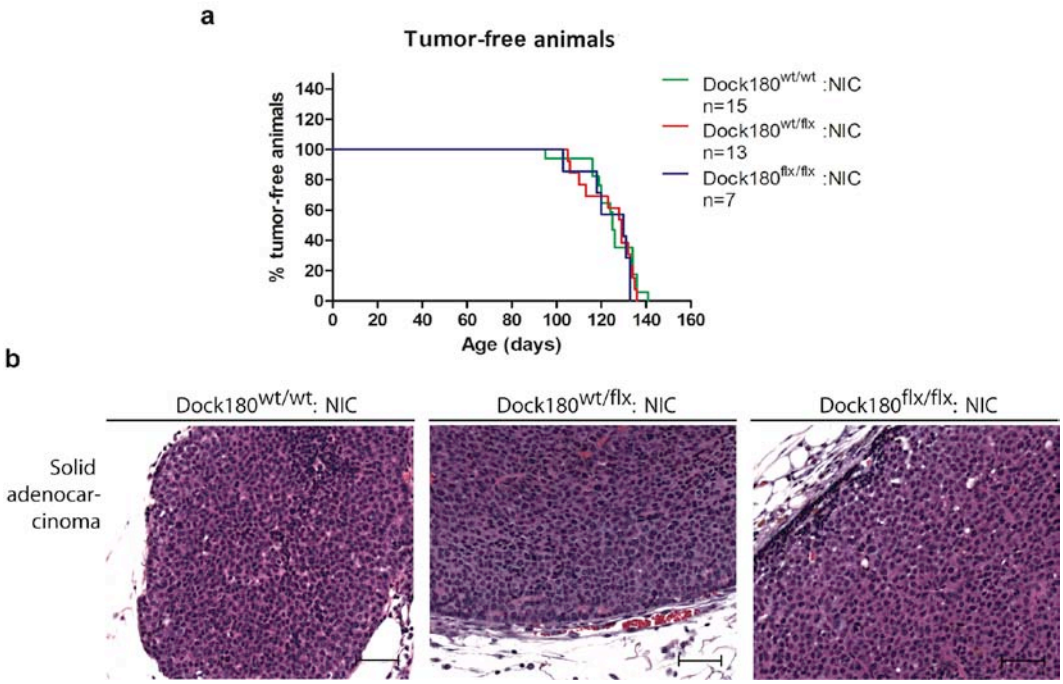

Figure 2.4: *Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC mice display the same tumor onset and histological characteristics as MMTV-NIC mice*. a) Kaplan Meier analysis of tumor onset of Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC cohorts (t= 124, 125, 132 days, respectively). b) H&E staining of mammary tumor sections of each genotype, all displaying characteristics of solid adenocarcinomas. Scale bar represent 10 µm.

Dock180 is not required for tumor initiation and multiplicity but contributes to the outgrowth of Neu-induced tumors.

Although the role of Dock180 in breast carcinoma cell invasion has been previously documented [272], its contribution to tumorigenesis in an *in vivo* setting has not been studied. To precisely determine tumor onset in the mice, ultrasound was used for a group from each genotype (n=6, 9, 7 for Dock180$^{wt/wt}$:NIC; Dock180$^{wt/flx}$:NIC; Dock180$^{flx/flx}$:NIC mice, respectively) as described in Materials and Methods (Figure 2.5a). Contrary to the standard physical palpation of tumors, ultrasound facilitates the visualisation of mammary intraepithelial neoplasia (MIN) lesions as small as 0.02 mm^2 of diameter, 1 to 2 months before first tumor palpation [275]. No significant difference in

tumor onset using this technique is seen between the genotypes, although a small delay is noted for Dock180$^{flx/flx}$:NIC mice. When analysing tumor onset by physical palpation, Dock180$^{flx/flx}$:NIC mouse tumorigenesis also slightly delayed, although this difference was not significant using the student t-test (Figure 2.5b). The number of palpable tumors was not significantly different between the three genotypes, although Dock180$^{flx/flx}$:NIC mice display a tendency to have less tumors than wild-type and heterozygous animals (Figure 2.5c). However, the number of MIN lesions in the Dock180-null mammary tumors is about 2-fold less than the Dock180 wild-type or heterozygous mice, analyzed by whole-mount and quantified by calculating the average MIN lesions of both inguinal mammary glands at necropsy (Figure 2.5d and e). Since the role of Dock180 in tumor growth has not been studied either *in vitro* or *in vivo*, total tumor burden in Dock180$^{wt/wt}$:NIC; Dock180$^{wt/flx}$:NIC; Dock180$^{flx/flx}$:NIC mice was quantified by both mass and tumor volume at necropsy. Dock180$^{wt/wt}$:NIC animals exhibited an average total tumor weight of 1.74 g, whereas the average total tumor weight measured in Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC was 1.89 g and 0.86 g respectively (Figure 2.5f). This decrease in Dock180-deficient tumor mass is significant. Moreover, the total tumor volume is significantly lower in the Dock180-null NIC mice in comparison to the wild-type and heterozygous animals. Dock180$^{wt/wt}$:NIC and Dock180$^{wt/flx}$:NIC mice have a total tumor volume of 1.9 and 2.3 cm^3 respectively, whereas the Dock180$^{flx/flx}$:NIC has a total tumor volume of 1.12 cm^3 (Figure 2.5g). Collectively, these observations suggest that Dock180 is largely dispensable for tumor initiation and multiplicity but contributes to tumor growth.

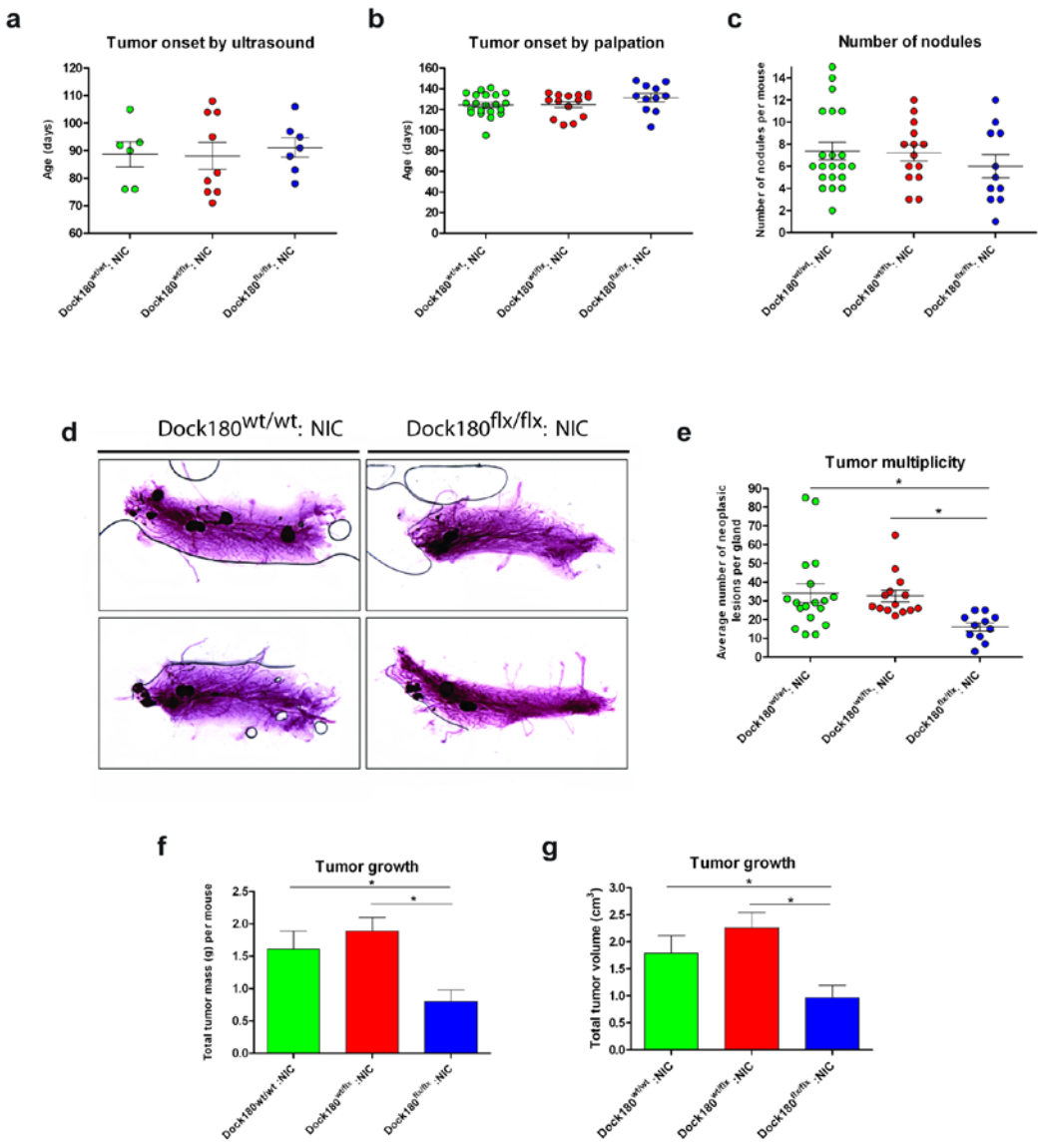

Figure 2.5: *Dock180 is not required for tumor initiation and multiplicity but contributes to tumor growth*. a) Tumor onset for Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC cohorts measured by ultrasound technology (t= 90, 89, 94 days, respectively). b) Tumor onset in Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC mice measured by physical palpation (t= 124, 125, 132 days, respectively). c) Average number of palpable tumors at necropsy for each genotype. d) Whole-mounts of inguinal mammary glands of Dock180$^{wt/wt}$ and Dock180$^{flx/flx}$:NIC mice. e) Average number of MIN lesions per mouse for each genotype. f) Tumor growth measured by total tumor weight per mouse per genotype (n=19, 13, 9, respectively). g) Tumor growth measured by average total tumor volume per mouse per genotype (n=19, 13, 9, respectively). All p-values were calculated using one-way ANOVA (Bonferroni's multiple comparison test). * p-value < 0,05.

Reduced proliferation and increased apoptosis in Dock180-deficient NIC tumors.

To dissect the mechanism by which the loss of Dock180 signaling suppresses tumor growth, average sized tumors (between 0.5 and 1 cm^2 diameter) from Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC (data not shown) and Dock180$^{flx/flx}$:NIC mice were stained with anti-Ki67, a marker of proliferation, and anti-cleaved-Caspase 3, a marker of apoptosis, and quantified as described in Materials and Methods (Figure 2.6a). The percentage of Ki67-positive cells of mammary tumors is significantly lower in Dock180$^{flx/flx}$:NIC mice than Dock180$^{wt/wt}$:NIC and Dock180$^{wt/flx}$:NIC animals (60, 62, 38% for wild-type, heterozygous and homozygous Dock180:NIC mice, respectively; Figure 2.6b). Moreover, the Dock180$^{flx/flx}$:NIC mice had significantly more cells undergoing apoptosis, about 2.3%, compare to wild-type, 0.5%, and heterozygous, 0.3% (Figure 2.6c). Taken together, the loss of Dock180 has a negative effect on proliferation and facilitates apoptosis, which is reflected by smaller tumors at necropsy.

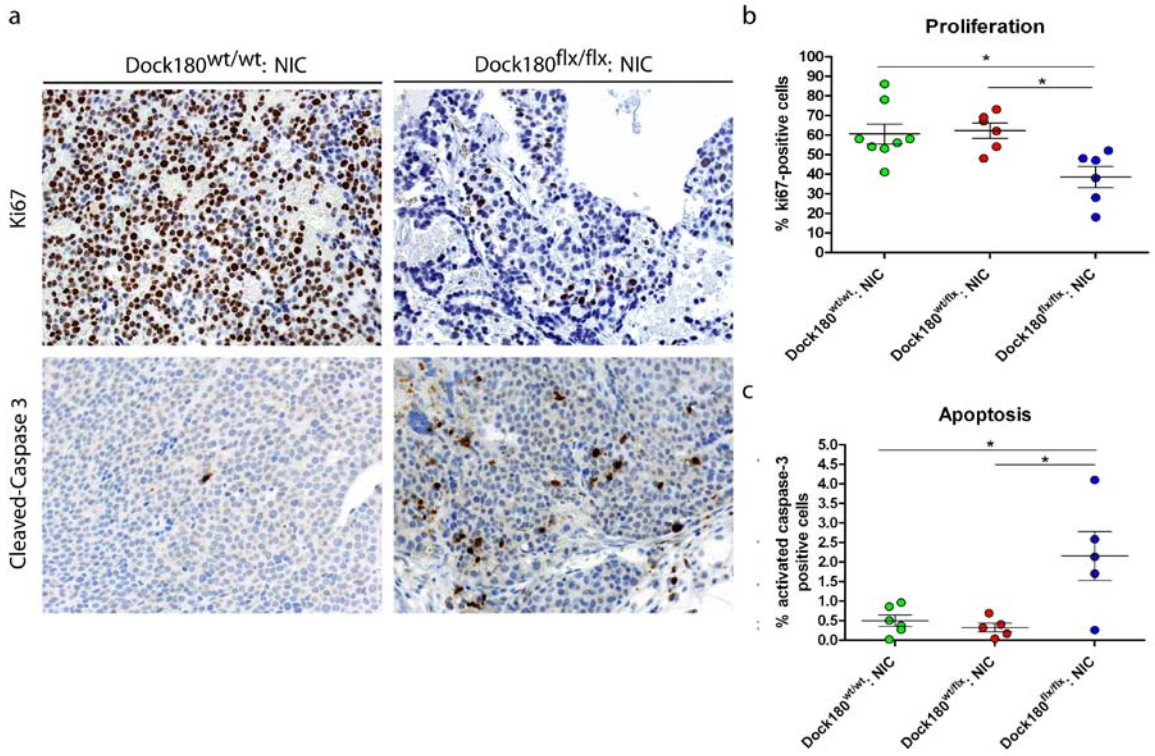

Figure 2.6: *Dock180-deficient NIC tumors present reduced proliferation and increased apoptosis.* a) DAB staining of mammary tumor sections from Dock180$^{wt/wt}$:NIC and Dock180$^{flx/flx}$:NIC mice, using antibody against Ki67 (*top panel*) and cleaved-Caspase 3 (*bottom panel*). b) Percentage of Ki67-positive cells for each genotype. c) Percentage of cleaved-Caspase 3-positive cells for each genotype. All p-values were calculated using one-way ANOVA (Bonferroni's multiple comparison test). * p-value < 0,05.

Dock180 is critical to establish metastases to lungs in MMTV-NIC mice.

Next, we assessed the role of Dock180 in metastases development in the MMTV-NIC mouse model. As previously described, MMTV-NIC mice display metastatic lesions to lungs, with an approximately 60-67% penetrance [69, 70, 77, 79]. In our mice, no difference is seen in the penetrance of the metastatic lesions to lungs between the three genotypes (53%, 54% and 56% for Dock180$^{wt/wt}$:NIC; Dock180$^{wt/flx}$:NIC; Dock180$^{flx/flx}$:NIC mice, respectively; Figure 2.7a). Strikingly, Dock180$^{flx/flx}$:NIC mice only developed an average of 3 metastatic lung lesions per animals, whereas the

Dock180$^{wt/wt}$:NIC and Dock180$^{wt/flx}$:NIC mice exhibit metastases to lungs with an average of 22 and 18 lesions per mouse, respectively (Figure 2.7b). This 7-fold reduction in lung metastases is highly significant ($p<0.02$) and strongly suggests that Dock180 is a key player in metastases development downstream of ErbB2.

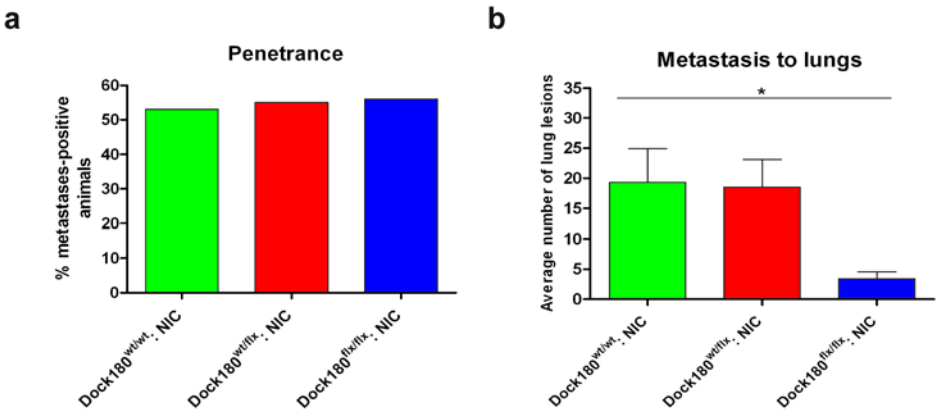

Figure 2.7: *The loss of Dock180 in NIC tumors dramatically decreases the number of metastases to lungs*. a) Percentage of Dock180$^{wt/wt}$:NIC, Dock180$^{wt/flx}$:NIC and Dock180$^{flx/flx}$:NIC mice harboring one or more metastases to lungs (n= 17, 11, 9, respectively). b) Average number of metastatic lung lesions per metastases-positive animals for each genotype (n= 9, 6, 5, respectively). p-value was calculated using one-way ANOVA (Bonferroni's multiple comparison test). * p-value < 0,05.

Dock180 is highly expressed at tumor periphery and invasive fronts of NIC tumors.

Since Dock180 is a specific GEF for the Rac GTPase, we next wanted to investigate if the loss of Dock180 in mammary tumors would reduce the invasive property of ErbB2-overexpressing breast cancer. To address this question, we stained mammary tumors sections from mice of each genotype (Dock180$^{wt/wt}$:NIC; Dock180$^{wt/flx}$:NIC (data not shown); Dock180$^{flx/flx}$:NIC) with an antibody against Dock180

and investigated its overall expression level in tumors (Figure 2.8a). Interestingly, we observed a high level of Dock180 expression at the tumor periphery and at invasive fronts, whereas the center of tumors had low Dock180 expression. Moreover, single (data not shown) and collective cells invading the surrounding stroma typically highly expressed Dock180 (Figure 2.8b). These cells were confirmed to be tumor cells by co-staining of Dock180 and EpCAM, an epithelial tumor cell marker (Figure 2.8b).

The localized Dock180 expression level at invasive areas correlates with disruption of basement membrane and increase of invasive cells.

After examining Dock180 expression at the tumor periphery and invasive fronts, we next evaluated whether this increase in local cell invasion could be explained by differential ability to digest the basement membrane of tumors. First, when we quantified the average number of invading cells within a specific stroma area (described in Materials and Methods) of Dock180$^{wt/wt}$:NIC and Dock180$^{flx/flx}$:NIC mice, a 5-fold reduction in the number of invading cells of the Dock180-null mammary tumors is seen (Figure 2.8b and c). Consistent with these results, the quantification of the loss of basement membrane (described in Materials and Methods) in Dock180$^{wt/wt}$:NIC and Dock180$^{flx/flx}$:NIC mice, uncovered by pan-laminin antibody staining, demonstrated that the loss of Dock180 in mammary tumors diminishes the disruption of the basement membrane (Figure 2.8d and e). All together, these data suggest that Dock180 expression correlates with the pro-invasive characteristic of ErbB2-driven tumors facilitating basement membrane disruption and tumor cells invasion into the surrounding stroma.

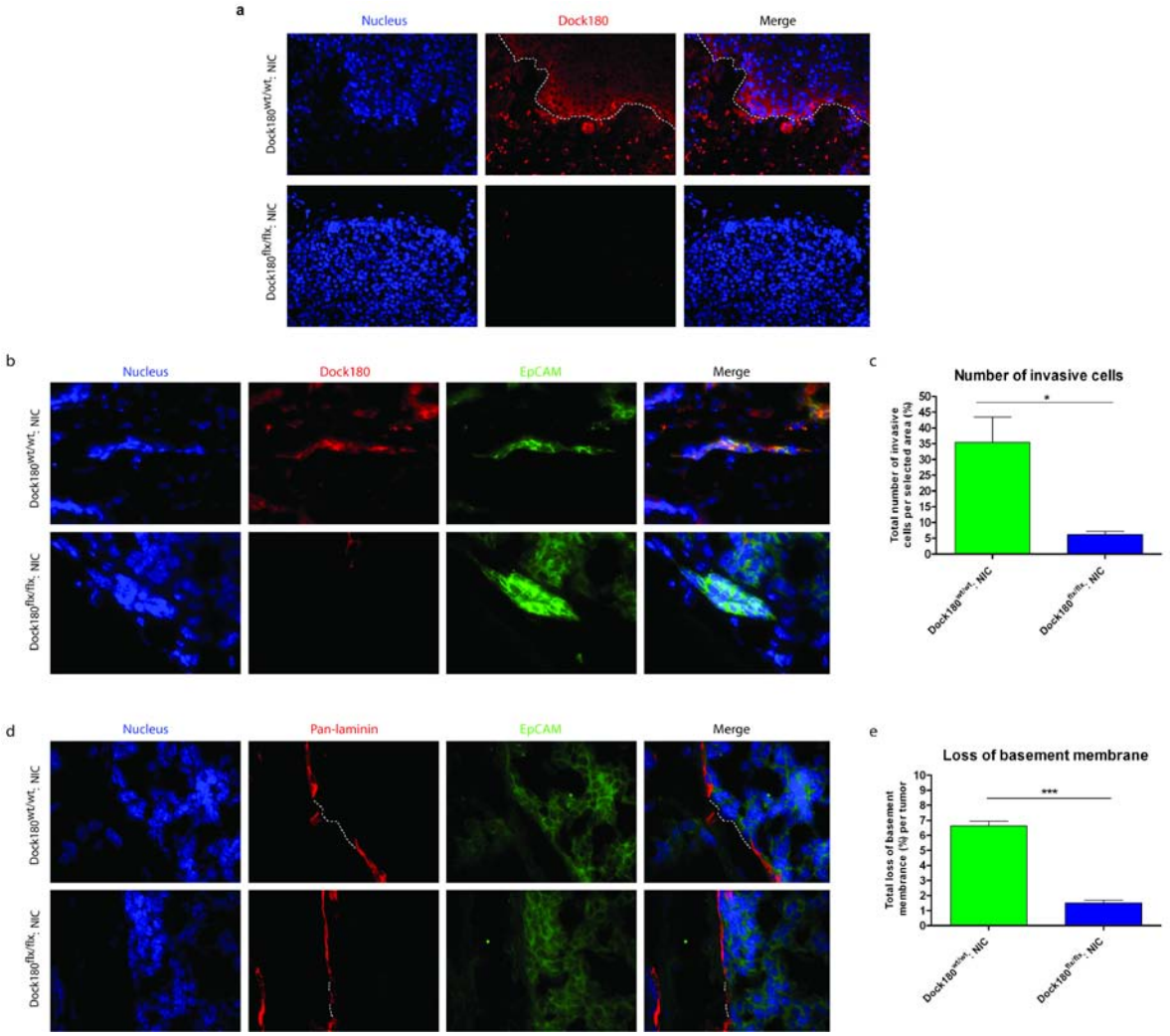

Figure 2.8: *Dock180 is important for the invasive phenotype of ErbB2-driven tumors*. a) Dock180 is highly expressed at tumor periphery and invasive fronts (depicted by surrounded area in white line, *top middle panel*) shown by immunofluoresence staining against Dock180. b) Immunofluoresence staining of mammary tumor sections representing collective invasive tumor cells of Dock180$^{wt/wt}$:NIC mice (*top panel*) expressing both Dock180 and EpCAM, whereas no invasive cells is seen in Dock180$^{flx/flx}$:NIC (*bottom panel*). c) Quantification of the number of invasive tumor cells for each genotype (n=4 for each genotype). d) Immunofluorescence staining of mammary tumor sections representing the surrounding basement membrane of the tumor stained with pan-laminin antibody and tumor cells with an antibody against EpCAM. Dock180$^{wt/wt}$:NIC mice shows disruption of basement membrane (depicted by the dashed white line, *top panel*), whereas Dock180$^{flx/flx}$:NIC basement membrane remains undisrupted. e) Quantification of the loss of basement membrane for each genotype (n= 4 for each genotype). All pictures were taken at 100x magnification, except a) in 40x magnification. Nuclei are stained with Hoescht. All p-values were calculated using Student t-test (unpaired, Welch's correction, two-tailed). * p-value < 0,05. *** p-value < 0,0001.

pSTAT3 might play a role in increasing tumor cell invasiveness downstream of Dock180 in NIC-positive tumors.

Because STAT3 is regulated in part by Rac and is emerging as a key regulator of ErbB2-induced metastasis, we investigated whether the activation level of STAT3 would be altered in Dock180-null/ErbB2-overexpressing mammary tumors. Interestingly, as demonstrated by immonofluorescence staining of frozen tumor sections using an antibody against Dock180 and pSTAT3, activated STAT3 is seen within cells located at the tip of invasive fronts, whereas in the Dock180$^{flx/flx}$:NIC tumors, no expression of pSTAT3 is seen either at the center or the periphery of the tumors (Figure 2.9). This suggest that STAT3 might be activated downstream of Dock180 to promote tumor cell invasion.

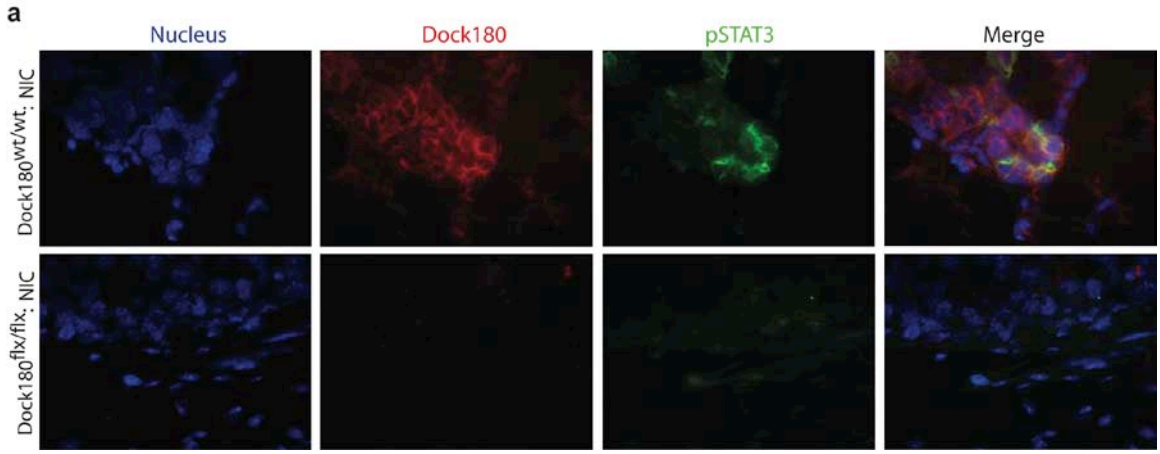

Figure 2.9: *Dock180 and pSTAT3 are co-expressed at invasive areas of NIC mammary tumors*. Immunofluoresence staining of tumor sections from Dock180$^{wt/wt}$:NIC (*top panel*) and Dock180$^{flx/flx}$:NIC (*bottom panel*) mice, using both antibody against Dock180 and pSTAT3. Dock180 is highly expressed at the invasive front (*top panel*), along with elevated levels of pSTAT3 at the tip of the invasive front. Conversely, no pSTAT3 is seen in Dock180$^{flx/flx}$:NIC tumor sections (n=3 for each genotype). Nuclei are stained with Hoescht.

MATERIALS AND METHODS

Generation of CKO Dock180 mouse

The three arms of homology to construct the Dock180 CKO strategy were generated by PCR from C57Bl/6 BAC clones containing the Dock180 locus. All arms were fully sequenced to ensure their integrity before being sequentially inserted into PGKNeoF2L2DTA vector using different restriction enzymes. The construct was linearized by a *Not*I site and electroporated in C57Bl/6 embryonic stem cells (ES). After screening G418-resistant colonies by Southern blot analysis, G418-resistant clones were electroporated with an expression vector of FLP to remove the PGK-Neo cassette, and subsequently screened by Southern blot for positive-FLP recombined clones. The final ES cells were injected into 129/SV blastocysts. Both electroporation and blastocyst injection steps were done with the support of Aurora Burd and the "Cell Migration Consortium". The chimeras were identified on the basis of coat color and were interbred with 129/SV mice. Finally, the mice were backcrossed 6 times in a FVB/NJ background. The genotype of the mice was determined by Southern blot and PCR analysis on tail DNA.

Transgenic mice, genotyping and tumorigenesis studies

Generation of MMTV-CRE and MMTV-NIC transgenic mice has been previously characterized in [67, 74, 77]. Since the MMTV promoter is affected by strains, all mice strain were backcrossed for 6 rounds to FVB/N background. Genotyping of conditional Dock180 and transgenic MMTV-CRE and –NIC mice was determined by PCR as follow: Cre-forward: 5′-GCTTCTGTCCGTTTGCCG-3'; reverse: 5′-ACTGTGTCCAGACCAGGC-3'; Neu-forward: 5′-TTCCGGAACCCACATCAGGCC-3'; reverse: 5′-GTTTCCTGCAGCAGCCTACGC-3'. Virgin females carrying the ErbB2 oncogene were monitored for tumor onset by weekly palpation and ultrasound technology using the Vevo770 (VisualSonics). Tumor

dimensions were measured using a ruler. Tumor volume was calculated as length x width^2 x 0.52 [276]. Mice were euthanized 5 weeks after first palpation. All animal studies were approved by and housed in the animal facility of the Institut de recherches cliniques de Montréal (IRCM) and all experiments were done in accordance with the animal care guidelines of the IRCM.

Antibodies

Antibodies used for DAB staining include goat anti-Dock180 C-19 (Sc-6167; Santa Cruz), rabbit anti-Cre (PRB106C; Covance), rabbit anti-Ki67 (275R-15), rabbit anti-cleaved-caspase3 (9661S; Cell Signaling), biotinylated anti-rabbit (BA1000; Vector Labs), biotinylated anti-goat (BA9200; Vector Labs) and Streptavidin-HRP (554066; BD Pharmigen). Antibodies used for biochemical analysis include rabbit anti-Dock180 H70 (Sc-5625; Santa Cruz), mouse anti-Tubulin (T5168; Sigma) and rabbit anti-pSTAT3 Tyr705 (9145; Cell Signaling). Antibodies used for immunofluorence staining include anti-Dock180 C-19 (Sc-6167; Santa Cruz), rat anti-EpCAM (552370; BD Pharmigen), rabbit anti-pSTAT3 Tyr705 (9145; Cell Signaling), anti-pan-laminin DyLight649 (NB300-144C; Novus), chicken anti-rabbit Alexa488 (A21441; Invitrogen), donkey anti-goat Alexa568 (A11057; Invitrogen), chicken anti-rat488 (A21470; Invitrogen) and Hoescht (33342; Invitrogen).

Immunohistochemical and immunofluorescence analysis

Tumors with adjacent mammary tissue from necropsied mice were either fixed in 4% PFA and embedded in paraffin or frozen in OTC. Embedded tumors were sectioned at 5 µm for histological purposes. Lungs were fixed in 4% PFA, paraffin-embedded, sectioned at 5 µm every 50 µm and stained by H&E to examine metastasis lesions on microscope. For DAB staining, paraffin sections were deparaffinized in 2 changes of xylene and dehydrated. Sections were heated in 10 mM sodium citrate (pH 6) for 30 min at 98^{o}C,

followed by incubation in 3% H_2O_2 for 10 min. Samples were permeabilized with IHC buffer (0.5% Triton-X100/0.02% Tween-20 in PBS) for 10 min at RT, blocked with IHC buffer/1% BSA for 1 hour at RT, incubated with primary antibody in IHC buffer/1% BSA O/N at 4°C, washed with IHC buffer, incubated with biotinylated antibody in IHC buffer/1% BSA for 1 hour at RT, washed with IHC buffer, incubated with Streptavidin-HRP-coupled antibody in IHC buffer/1% BSA for 1 hour at RT, washed with PBS, visualized with DAB Vector kit and counterstained with hematoxylin Mayers for 20 sec. For immunofluoresecnce staining, OCT-sections were fixed in 4% PFA for 10 min, washed with PBS, permeabilized with IHC buffer for 10 min at RT, blocked with IHC buffer/1% BSA for 1 hour at RT, incubated with primary antibody in IHC buffer/1% BSA O/N at 4°C, washed with IHC buffer, incubated with Alexa-conjugated secondary antibody in IHC buffer/1% BSA for 1 hour at RT, washed with IHC buffer, nuclei stained with Hoescht for 1 min at RT, washed with PBS and mounted with mowiol.

Immunoblotting

Mammary tumors were excised at necropsy, either used freshly or kept in liquid nitrogen. Small pieces of tumors were minced with a razor blade in a petri dish before lysis in 1 ml of cold NP-40 buffer (150 mM NaCl, pH 7.5 and Tris 50mM 1% NP-40) and sonicated (twice, 20 sec). Cellular debris were separated by centrifugation (14000 rpm, 20 min, 4°C). The supernatant obtained is the total tumor lysate. The protein concentration in tumor lysates was quantified using the Bradford assay (500-0206; Bio-Rad). 50 µg of total tumor lysate was run on SDS-PAGE gels and transferred onto PVDF membranes.

Whole-mount analysis

Mammary gland no. 4 and 9 were fixed in 4% PFA, delipidated in two changes of acetone and stained with carmine red O/N at 4°C. Tissues were rehydrated, cleared in

xylene and mounted in permount (Fisher). Average of both mammary glands was calculated for the number of neoplastic lesions.

Quantification of invading cells and loss of basement membrane

The quantification of invading cells was calculated by the ratio of the number of tumor cells (labeled by a EpCAM antibody) migrating into the healthy stroma in a given area. In order to better distinguish the tumor cells that are escaping the primary tumor site, frozen tumor with adjacent mammary gland sections were co-labeled with a pan-laminin antibody. The stromal area was delimited and calculated using Northern Eclipse. The same stained sections were used to measure the loss of basement membrane. Each area with loss of basement membrane surrounding the tumor was measured using Northern Eclipse, and subsequently divided by its circumference.

Ultrasound imaging

Sixty day-old mice were anesthetized by inhalation of isoflurane and fur was removed using a mild depilatory cream. A 55 MHz transducer was used for mammary gland imaging. A warm water based ultrasonic gel (Parker Laboratories) was applied between the imaging probe transducer and the mouse skin. All 10 mammary glands were subjected to ultrasound imaging using the Vevo 770 High-Resolution Imaging System for small animal ultrasound (VisualSonics). Orientation of the mammary glands on ultrasound was accomplished by visualizing the lymph node, which is less echogenic and appears as a black hole surrounded by echogenic mammary gland tissue. Mammary preneoplastic lesions were measured with the Vevo770 software. The age at first preneoplastic lesion detection (with a diameter of less than $0.04mm^2$) was assigned as the time of tumor onset.

Microscopy

Whole-mounts were visualized using Leica MZ9.5. Immunofluorescence staining was visualized using Leica Leitz DMRB. DAB and H&E staining were visualized using Zeiss Axiophot.

Statistical analysis

Kaplan-Meier curves were analyzed using Prism 4 (Graph-Pad Software). All other graphs were done with Prism 4 and *p*-value calculated using one-way ANOVA (Bonferroni's multiple comparison test), except Figure 2.8 which used Student t-test (unpaired, Welchs' correction, two-tailed). Proliferation and apoptosis were quantified by calculating the average percentage of positive nuclei relative to total nuclei (4 random fields of 4 independent tumors with adjacent mammary gland samples per genotype; 20x magnification) and analyzed using MATLAB. A *p*-value of less than 0.05 was considered significant. All error bars on graphics represent the SEM.

Chapter 3

Discussion and summary

DISCUSSION

In our study, we uncovered a novel role for Dock180 in ErbB2-induced tumorigenesis and metastasis *in vivo*. We found that Dock180 is not essential for tumor initiation and multiplicity. However, we noted that *Dock180* is required for tumor growth and this phenotype can be explained by decreased proliferation and increased cell death in tumors lacking Dock180. Strikingly, the loss of Dock180 in ErbB2-overexpressing breast tumors dramatically decreases the number of metastatic lung lesions. Importantly, this inefficiency in metastasis correlates with a weak invasive phenotype at primary tumor sites. In fact, high levels of Dock180 expression are seen at the tumor periphery and invasive fronts. Moreover, Dock180-deficient tumors present less invasive cells in the surrounding stroma and less damage to their basement membranes. Our data also suggest that this cellular invasion may be in part mediated by STAT3 downstream of Dock180 since pSTAT3 is localized at invasive fronts of WT mammary tumors but totally absent in Dock180-null tumors. This would represent a novel Dock180-STAT3 pro-invasive signaling axis downstream ErbB2.

Dock180 is not required for ErbB2-induced tumor initiation and multiplicity but is important for tumor growth.

When analyzing tumor progression in a bigenic *in vivo* model, many aspects should be considered. The kinetics of tumor appearance (either shortened or delayed), the multiplicity (having one or more tumors), tumor growth (reflected by tumor mass and size) and the differentiation state of the tumors are variables that define the aggressiveness of the tumors. Importantly, these tumor characteristics are believed to be controlled by specific signaling pathways. This will in turn, lead to a wide array of

cellular responses like proliferation, survival, apoptosis, migration, invasion, etc. In the activated Neu-driven tumorigenesis model, we find that Dock180 is not implicated in tumor initiation and multiplicity but instead appears to affect tumor growth.

To our knowledge, measuring mouse mammary tumor onset with the use of the ultrasound imaging has not been tested in a ErbB2-based model. One publication pointed out that ultrasound technology could be a powerful tool to precisely identify the appearance of mammary intraepithelial neoplasia (MIN) lesions and monitoring their progression into potentially malignant tumors [275]. Also, this imaging technology would permit the monitoring of tumor regression, either at the onset of cancer progression or following anti-cancer treatment. This latter event has been reported in human breast cancer but no experiments allow the real-time following of a mouse mammary tumor in its physiological context. Here, the rationale of using ultrasound live-imaging was to determine the exact time of tumor onset. By monitoring our three groups of mice (starting at 60 days of age), no significant difference has been observed, although Dock180$^{flx/flx}$:NIC mice display a mild delay in MIN lesion onset. Importantly, tumor onset can vary by up to 40 days within the same cohort, suggesting that the technique itself may be at the origin of this variability. In fact, since the MIN lesions are very small, their detection could be missed during this form of monitoring. However, ultrasound live-imaging can be a useful tool to measure tumor initiation, progression and regression, applicable in models other than breast tumorigenesis. All soft tissue tumors such as prostate, pancreatic and hepatocellular carcinoma can be visualized whether they are subcutaneous or orthotopic. As a non-invasive system, ultrasound imaging not only allows for the detection of pre-palpable tumors, but also permits longitudinal studies that monitor the progression and regression of disease in the same animal over time. A good hands-on experience of this technique will open a new area of tumorigenesis studies.

Second, initiation of mammary tumors is commonly monitored by physical palpation. In our study, the loss of Dock180 has not impaired tumor onset, although a slight delay in the appearance of the first solid tumors is observed. This indicates that Dock180 is largely dispensable for mammary tumor initiation. Moreover, we found that Dock180 does not contribute to the induction of multiple mammary tumors and we base this conclusion on the observation that there is no significant decrease in number of solid nodules at necropsy, although the number of MIN lesions in the Dock180-null mammary epithelium is significantly decreased. What is really striking is that the growth of NIC tumors is impaired in the absence of Dock180, as seen in total tumor mass and volume. In fact, we noted that Dock180 plays a role in cell proliferation and apoptosis, depicted by a lower number of Ki67-positive tumor cells and an increased number of cleaved-caspase 3-positive tumor cells in averaged size Dock180-null tumors. This reveals a new role for Dock180 in tumor-induced cell proliferation and apoptosis downstream of ErbB2. In fact, its implication in apoptosis had been revealed previously in our laboratory, where Dock-null embryo display a significant increase in the number of apoptotic cells within the intercostal muscles [269]. Additionally, Dock180 was found to play a crucial role in clearance of apoptotic cells in *C. elegans* [256]. This could be another explanation for the increase of apoptotic cells seen in our Dock180-null tumors, which could be the result of an accumulation of uncleared apoptotic cells. To confirm these *in vivo* data, it would be interesting to look at cell proliferation and apoptosis in an *ex vivo* context. As previously demonstrated, β4-integrin signalling is required for ErbB2-induced epithelial hyperproliferation in a 3D Matrigel of *ex vivo* explants [166]. Such an assay could be exploited to determine the implication of Dock180 downstream of ErbB2 in cell proliferation and apoptosis.

Overall, Dock180 seems to have a minor role in tumor initiation and multiplicity but contributes to tumor growth by maintaining proliferation and preventing apoptosis. Although we did not anticipate any effect resulting from the loss of Dock180 in ErbB2-induced tumor onset, we were surprised to observe a phenotype in proliferation and

apoptosis of Dock180$^{flx/flx}$:NIC mice. This observation might underlie the implication of JNK, which has been previously known to act on proliferation of tumors cells downstream ErbB2. Moreover, Dock180 can activate JNK via the Rac GTPase *in vitro* [274], suggesting that this Dock180-JNK signaling axis may be implicated in tumor proliferation in a ErbB2-dependent manner.

On the other hand, the minor phenotype seen in tumorigenesis studies may suggest another player that is known to compensate for the loss of Dock180 *in vivo*. Silencing of its closely related GEF member, Dock5, has been shown to robustly impair myoblast fusion in a Dock180-heterozygous background [269]. Additionally, interfering with *Dock1* and *Dock5* in zebrafish abrogates myoblast fusion [277]. The two Dock180-related family members display a high degree of conserved regions. In order to analyze whether Dock5 could compensate for the loss of Dock180 in the mammary epithelium, it would be interesting to intercross our CKO Dock180 mice with Dock5 gene trap mice, generated previously in our laboratory [269], and re-evaluate tumorigenesis progression as done in my thesis. However, tumor initiation and multiplicity might be regulated totally independently of Dock180. Deeper investigation of these processes would be required to rule out the implication of Dock5.

To identify molecular mechanisms regulated by Dock180 in ErbB2-induced tumorigenesis, a valuable approach would be to culture primary tumor cells from both Dock180$^{wt/wt}$:NIC and Dock180$^{flx/flx}$:NIC mice and perform biochemical assays. As mentioned previously, β4-integrin amplifies ErbB2-driven tumorigenesis and invasion via the Src kinase that link both receptors [166]. Following Src activation, c-Jun/c-JNK are targeted to promote proliferation whereas pSTAT3 induce cell invasion by disrupting epithelial adhesions. Because activation of STAT3 by the Rac GTPase has been reported in several studies [278-280], it would be interesting to determine if Dock180 is at the center of this pro-invasive signaling cascade.

In our tumorigenesis studies, heterozygous Dock180:NIC mice demonstrated no intermediate phenotype, suggesting that Dock180 signaling is haplosufficient for mammary tumor onset, multiplicity and growth.

Dock180 is a major player in ErbB2-mediated development of metastases to lungs.

Dock180 is a major player in cell migration and has been linked to invasion in an aggressive breast cancer cell line in the context of β1-integrin engagement [272]. Crosstalk between integrins and ErbB2 receptors is known to impact mammary tumorigenesis and metastasis in *in vitro* and *in vivo* models. A recent publication on this topic demonstrated that the loss of β1-integrin in the MMTV-NIC mice impairs tumor growth and metastasis, and these tumors exhibit a dramatic reduction in activation of Src, FAK, p130Cas and paxillin [281].

In our study, we identified a novel role for Dock180 in ErbB2-driven metastasis to lungs. Moreover, the metastatic lung lesions in the Dock180$^{flx/flx}$:NIC mice seem to be smaller than those from the Dock180$^{wt/wt}$:NIC mice, although this has not yet been quantified. While this inefficient metastasis in Dock180-deleted tumor cells is in agreement with the initial hypothesis of this thesis, this phenotype can be the endpoint of several cell behaviour defects such as: 1- penetration into the vasculature surrounding the primary tumor 2- survival in the blood stream and 3- cell adhesion in the lungs.

To decipher the cause of this phenotype, three separate experiments should be conducted. To identify whether the reduction of metastatic lung lesions in Dock180$^{flx/flx}$:NIC mice is due to a defect in entering the blood vasculature at primary tumor sites, intravital studies could be used to monitor this event. In fact, injected labeled-metastatic mammary tumor cells can be observed while they orient themselves towards blood vessels, thus revealing different cell invasive behavior (reviewed in

[282]). This experiment would allow us to confirm whether the loss of Dock180 in mammary tumor cells affects the intrinsic ability of intravasating.

Second, one novel and potentially exciting method to evaluate the survival potential of Dock180 wild-type versus Dock180-deleted explanted mammary tumor cells within the blood circulation is by using *in vivo* flow cytometry [283]. This technique allows for the real-time detection and quantification of circulating fluorescently labeled cells in live animals. A signal from a cell population of interest is recorded as the cells pass through narrow blood vessels and enable tumor cells to be studied in transit in the blood. This *in vivo* flow cytometry assay could reveal the capability of Dock180-null tumor cells to survive in the blood circulation before colonizing the lungs.

Finally, another experiment would be to inject explanted primary tumor cells from both wild-type and Dock180-deficient NIC animals into the tail vein of immunodeficient mice. This would first confirm the phenotype we see in our mouse model, and also directly assess the intrinsic capability of tumor cells to colonize the lungs (extravasation), without having a selective pressure from the tumor microenvironment. In fact, tumor environment may influence the metastatic capability of malignant cells due to external factors, such as the presence of cytokines or a highly vascularised stroma. In parallel, ectopic introduction of Dock180 in Dock180-null explanted tumor cells would confirm that the reduction in metastases to lungs is Dock180-dependent by rescuing the phenotype. We could also use a GFP-labeling system to easily monitor tumor cell adhesion, extravasation and colonization by visualising GFP-positive metastatic tumor cells under a fluorescence microscope shortly after tail-vein injection. This assay could illustrate that the reduction in the number of metastases in Dock180$^{flx/flx}$:NIC mice is due to a problem of extravasation. Since the Dock180-deficient mice show reduced primary tumor size, it would be important to clarify whether decreased number of metastases in this genotype is the result of smaller primary tumors. Tail-vein injection would give an indication on the inerrant behavior of Dock180-deficient tumor cells to colonize the

lungs, discarding other factors such as primary tumor growth. All together, tail vein injection of GFP-positive tumor cells, *in vivo* flow cytometry and intravital studies could identify in which step(s) of metastasis development Dock180 is implicated in.

In the case that the intravasion, extravasion and colonization being normal, a likely possibility to the reduced number of metastases to lungs in Dock180-deficient mice is the result of outgrowth impairment at the site of metastasis. This would explain why the penetrance of this phenotype is equivalent between each cohort. A straightforward experience would be to measure proliferation and apoptosis rate by IHC using Ki67 and activated Caspase-3 antibodies on lung sections of each genotype. Because Dock180-deleted primary tumors show reduced proliferation and increased apoptosis, this observation might be reflected in their metastases as well.

To confirm the importance of STAT3 downstream of Dock180 in metastasis, tail vein and metastatic studies could be performed by overexpressing a constitutively active (CA) form of STAT3 [284] in Dock180-deficient primary tumor cells, we could test whether the number of metastatic lesions to lungs reverts to the wild-type level, thus rescuing the phenotype seen in $Dock180^{flx/flx}$:NIC mice. If this is the case, it would indicate that Dock180 mediates STAT3 activation in order to properly establish metastases to lungs. This interpretation would be in accordance with the role of STAT3 in lung lesions development using this CA form of STAT3. In fact, the CA form of STAT3 has been recently reported to promote invasion and metastasis in the MMTV-NeuNT mouse model [168]. This experiment would consolidate the hypothesis that the activation of STAT3 is Dock180-dependent, thus enhancing metastasis in ErbB2-overexpressing breast cancer cells.

The invasiveness of ErbB2-overexpressing tumors depends on the presence of Dock180.

Here, we showed that Dock180 is a pro-metastatic factor in ErbB2-driven tumor progression. One explanation would be that Dock180 plays a role in tumor invasiveness at primary sites. Remarkably, all Dock180-positive tumors display the same pattern of expression, where levels of Dock180 expression are more important at the tumor periphery and invasive fronts than at the center of the tumor. Moreover, single and collective cells migrating out of the tumors expressed Dock180 at high levels. This pattern of expression has previously been reported in a study on glioblastoma, where Dock180 and ELMO were both highly expressed at invasive areas of human brain tumors and in invading cells [273]. This was also confirmed biochemically by immunobloting against Dock180 and ELMO in either isolated tumor center or invasive border. Interestingly, collective cell migration during *Drosophila* oogenesis mimics Dock180 and ELMO behavior in oncogenic invasion. Border-cell cluster, which consists of about six outer migratory border cells, requires localized signaling from the Dock180 and ELMO following RTKs stimulation for efficient invasive and directional migration of cell groups [285]. Further studies are needed to determine if collective versus individual cell migration is affected in Dock180-null ErbB2-induced tumors.

Although this pattern of expression at invasive fronts is commonly associated with pro-invasive and metastatic cells, no studies to our knowledge have quantified the presence of invasive cells in the surrounding stroma nor the loss of basement membrane of tumors, two characteristics of invasive malignancies. Here, we developed a new quantification model for invasion in an *in vivo* model by calculating the ratio of number of invading cells and the loss of basement membrane. These methods have now been tested on 4 mice from Dock180$^{wt/wt}$:NIC and Dock180$^{flx/flx}$:NIC. Increasing the number of animals in this study would better confirm our results since this is a novel assay. However, a significant 3-fold decrease in the number of invading cells within the stroma

of Dock180-null mammary tumor has been observed. Also, the disruption of basement membrane is less obvious in the Dock180$^{flx/flx}$:NIC mice. The ratio of absence vs total basement membrane is about 6-times less and is highly significant (*p*-value = 0.0003). These observations raise an interesting point: while less cells are migrating out of the tumor in the absence of Dock180 and the basement membrane remains almost intact, it is not obvious how the ratio of metastases-positive animals remains the same between all genotypes. Our explanation at this stage is that less tumor cells reach the circulation and that this correlates with a decreased metastasis to the lungs. As stated above, we will also need to determine if Dock-null cells also have defects in survival within the blood stream or impaired extravasation to lungs.

The invasive phenotype that we see *in vivo* could also be confirmed using *ex vivo* explants of primary tumors cells and invasive assays in 3D Matrigel. We can also look at the level of GTP-loaded Rac, which is commonly known to promote cell migration and invasion downstream of Dock180. Although we tested Rac activation by PAK-pulldown, we failed to detect a reduction of GTP-loaded Rac level when Dock180 is absent. This experiment should be performed again and it would be interesting to see, in parallel, if the *ex vivo* explants of Dock180-deficient tumor cells display a reduction of activated Rac level. To confirm that the loss of Dock180 correlates with a decrease in invasion and activated Rac GTPase level, we could try to rescue the phenotype by overexpressing Dock180 and verify whether GTP-loaded Rac reverts to the wild-type level. However, it is possible that the decrease in total Rac activation is subtle and, most importantly, spatio-temporally regulated, as there are many other Rac GEFs such as Vav family members that can mediate Rac activation. As such, while it is intuitive to expect a massive decrease in Rac GTP loading in the Dock180-deficient NIC tumor cells, in reality, it may not be the case. While performing PAK Pulldown assays in tumor lysate of Dock180-null mammary tumors, a pool of Rac specifically activated at the invasive front is probably missing in the whole tumor lysate, containing tumor cells from its center and

periphery. Therefore, it remains an important challenge of this project to determine the role of the Dock180/Rac pathway in Neu-induced tumor invasion and metastasis.

Dock180 and pSTAT3 are co-expressed at invasive fronts of ErbB2-overexpressing tumors and may promote invasion.

Recent studies identified STAT3 as a regulator of invasion and metastasis in ErbB2-induced breast cancer [70, 166, 168, 169]. In fact, STAT3-deficient NIC tumors showed less metastatic lesions than the wild-type mice, due to a defect in angiogenesis and cell autonomous defects in colonization [70]. Since a body of evidence demonstrated that active Rac1 can directly bind and regulate STAT3 activity [278], we hypothesize that Dock180 might regulate STAT3 by activating the Rac GTPase. In our study, Dock180 and pSTAT3 are co-localized at the invasive fronts of tumors, where conversely, no pSTAT3 staining is seen in Dock180-deficient NIC tumors, suggesting a role for Dock180 upstream of STAT3. However, we can only speculate about the molecular mechanisms regulating STAT3 via Rac, and more experiments on cultured primary tumor cells would definitely be necessary to confirm this Dock180-Rac-STAT3 signaling axis. In parallel, the use of a novel antibody (NewEast Biosciences), that specifically recognizes the GTP-loaded form of Rac, on mammary tumor sections would be an interesting approach to verify if active-Rac is also located with high levels of Dock180 and pSTAT3 at invasion fronts.

Dock180 and the normal development of the mouse mammary gland: A key player during involution?

To date, the role of Dock180 in the normal development of the mammary gland has yet to be studied. Before going further in the investigation of the role of Dock180 in ErbB2-mediated tumorigenesis and metastasis, we had to address whether the loss of Dock180 protein within the mammary epithelial cells would impair its proper development. In a

scenario where Dock180 would be required for normal development, the interpretation of the data in ErbB2-driven tumor formation and progression would be difficult. As demonstrated by our work, Dock180-deficient mammary epithelium did not have any obvious defects throughout its adulthood (from 9 to 15 weeks of age). However, dramatic involution defects were noted in Dock180-null mammary glands from mothers that were euthanized 4 days post-weaning. During the early phase of involution, major tissue remodeling and cell death occur. The day 4 of involution corresponds to a peak of epithelial cells undergoing apoptosis, which are engulfed by neighbouring epithelial cells or by invading macrophages [3]. Interestingly, Dock180 has been shown to mediate engulfment of apoptotic in cells in *C. elegans* by forming a complex with ELMO1 and CrkII, which in turn activates Rac1 and involves cytoskeletal rearrangement required for engulfment [256]. Moreover, conditional deletion of *Stat3* in the mammary epithelium also results in a dramatic delay in mammary gland involution [286]. We might hypothesize that the same molecular complex of CrkII/Dock180/ELMO could also regulate the clearance of apoptotic cells in the involuting mammary gland and that this pathway implicates signaling through STAT3. Investigating the expression level of pSTAT3 during the involution phase by IHC on mammary sections would give us an important insight on the link between Dock180 and STAT3 at this particular stage. As described in the previous section, this potential Dock180/Rac/STAT3 pathway could also be reflected in the invasive phenotype and the appareance of metastases to lungs, seen in our MMTV-NIC mouse model. Since the main focus of our study is on ErbB2-driven tumorigenesis of nulliparous females only, no further experiments were carried out, although deeper investigations would be valuable.

SUMMARY

ErbB2 amplification and overexpression is elevated in 20-30% of breast cancer patients, correlating with an aggressive behavior and poor clinical outcome. Previous studies on human breast cancer cell lines have demonstrated that key players in cytoskeletal rearrangment, such as Rac1, p130Cas, FAK and integrins, are implicated in tumor progression and invasion. Dock180, a highly specific Rac GTPase activator, is known to mediate cell migration and invasion via the p130Cas/CrkII complex following integrin stimulation. However, the role of Dock180 in an *in vivo* model of Neu-mediated breast cancer has not yet been investigated. Using the MMTV-NIC transgenic mouse model, we have demonstrated that loss of Dock180 in an activated ErbB2-induced tumorigenesis did not affect the initiation and multiplicity of mammary tumors but does hinder tumor growth. Strikingly, Dock180-deficient mammary tumor cells are about 7-times less metastatic than their wild-type counterparts and show a weaker invasive phenotype. Dock180 deletion also results in less STAT3 activity at invasive areas of mammary tumors, suggesting that STAT3 might be a downstream effector of Dock180 promoting invasion in breast cancer cells. Taken together, these observations identify a novel role for Dock180 in the control of tumor cell growth, invasion and metastasis downstream of ErbB2.

REFERENCES

1. Ferlay J, S.H., Bray F, Forman D, Mathers C and Parkin DM. *GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet].* 2010; Available from: http://globocan.iarc.fr
2. Canada, C.C.S.N.C.I.o. *Canadian Cancer Statistics 2010*. June 2010; Available from: http://bcsc.ca/.
3. Richert, M.M., et al., *An atlas of mouse mammary gland development.* J Mammary Gland Biol Neoplasia, 2000. **5**(2): p. 227-41.
4. Hennighausen, L. and G.W. Robinson, *Information networks in the mammary gland.* Nat Rev Mol Cell Biol, 2005. **6**(9): p. 715-25.
5. Sternlicht, M.D., *Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis.* Breast Cancer Res, 2006. **8**(1): p. 201.
6. Veltmaat, J.M., et al., *Mouse embryonic mammogenesis as a model for the molecular regulation of pattern formation.* Differentiation, 2003. **71**(1): p. 1-17.
7. van Genderen, C., et al., *Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice.* Genes Dev, 1994. **8**(22): p. 2691-703.
8. Andl, T., et al., *WNT signals are required for the initiation of hair follicle development.* Dev Cell, 2002. **2**(5): p. 643-53.
9. Mailleux, A.A., et al., *Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo.* Development, 2002. **129**(1): p. 53-60.
10. Wiseman, B.S. and Z. Werb, *Stromal effects on mammary gland development and breast cancer.* Science, 2002. **296**(5570): p. 1046-9.
11. Williams, J.M. and C.W. Daniel, *Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis.* Dev Biol, 1983. **97**(2): p. 274-90.
12. Brisken, C., *Hormonal control of alveolar development and its implications for breast carcinogenesis.* J Mammary Gland Biol Neoplasia, 2002. **7**(1): p. 39-48.
13. Visvader, J.E., *Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis.* Genes Dev, 2009. **23**(22): p. 2563-77.
14. Andrechek, E.R., et al., *Patterns of cell signaling pathway activation that characterize mammary development.* Development, 2008. **135**(14): p. 2403-13.
15. Vargo-Gogola, T. and J.M. Rosen, *Modelling breast cancer: one size does not fit all.* Nat Rev Cancer, 2007. **7**(9): p. 659-72.
16. Weinberg, R.A., *The biology of cancer*. 2007: Garland Science, Taylor & francis Group, LLC. 796.
17. Ernie Bodai, H.L.B., Bruce F. Schroeder. *Ductal carcinoma in situ (DCIS)*. 2007; Available from: http://www.imaginis.com/breast-health/ductal-carcinoma-in-situ-dcis-3.

18. Lee, S., et al., *Hormones, receptors, and growth in hyperplastic enlarged lobular units: early potential precursors of breast cancer.* Breast Cancer Res, 2006. **8**(1): p. R6.
19. Gusterson, B.A., et al., *Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer.* Breast Cancer Res, 2005. **7**(4): p. 143-8.
20. Edge, S.B. and C.C. Compton, *The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM.* Ann Surg Oncol, 2010. **17**(6): p. 1471-4.
21. Simpson, J.F., et al., *Prognostic value of histologic grade and proliferative activity in axillary node-positive breast cancer: results from the Eastern Cooperative Oncology Group Companion Study, EST 4189.* J Clin Oncol, 2000. **18**(10): p. 2059-69.
22. Genestie, C., et al., *Comparison of the prognostic value of Scarff-Bloom-Richardson and Nottingham histological grades in a series of 825 cases of breast cancer: major importance of the mitotic count as a component of both grading systems.* Anticancer Res, 1998. **18**(1B): p. 571-6.
23. Sorlie, T., et al., *Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.* Proc Natl Acad Sci U S A, 2001. **98**(19): p. 10869-74.
24. Sotiriou, C. and L. Pusztai, *Gene-expression signatures in breast cancer.* N Engl J Med, 2009. **360**(8): p. 790-800.
25. Perou, C.M., et al., *Molecular portraits of human breast tumours.* Nature, 2000. **406**(6797): p. 747-52.
26. Sorlie, T., et al., *Repeated observation of breast tumor subtypes in independent gene expression data sets.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8418-23.
27. Slamon, D.J., et al., *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene.* Science, 1987. **235**(4785): p. 177-82.
28. Gusterson, B., *Do 'basal-like' breast cancers really exist?* Nat Rev Cancer, 2009. **9**(2): p. 128-34.
29. *Cancer Facts & Figures - 2010*, A.C. Society, Editor. 2010.
30. Paget, S., *The distribution of secondary growths in cancer of the breast. 1889.* Cancer Metastasis Rev, 1989. **8**(2): p. 98-101.
31. Chambers, A.F., A.C. Groom, and I.C. MacDonald, *Dissemination and growth of cancer cells in metastatic sites.* Nat Rev Cancer, 2002. **2**(8): p. 563-72.
32. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nat Rev Cancer, 2003. **3**(6): p. 453-8.
33. Nguyen, D.X., P.D. Bos, and J. Massague, *Metastasis: from dissemination to organ-specific colonization.* Nat Rev Cancer, 2009. **9**(4): p. 274-84.
34. Gupta, G.P. and J. Massague, *Cancer metastasis: building a framework.* Cell, 2006. **127**(4): p. 679-95.
35. Steeg, P.S., *Metastasis suppressors alter the signal transduction of cancer cells.* Nat Rev Cancer, 2003. **3**(1): p. 55-63.

36. Otten, A.D., M.M. Sanders, and G.S. McKnight, *The MMTV LTR promoter is induced by progesterone and dihydrotestosterone but not by estrogen.* Mol Endocrinol, 1988. **2**(2): p. 143-7.
37. Hennighausen, L., *The mammary gland as a bioreactor: production of foreign proteins in milk.* Protein Expr Purif, 1990. **1**(1): p. 3-8.
38. Lipnik, K., et al., *A 470 bp WAP-promoter fragment confers lactation independent, progesterone regulated mammary-specific gene expression in transgenic mice.* Transgenic Res, 2005. **14**(2): p. 145-58.
39. Green, J.E., et al., *The C3(1)/SV40 T-antigen transgenic mouse model of mammary cancer: ductal epithelial cell targeting with multistage progression to carcinoma.* Oncogene, 2000. **19**(8): p. 1020-7.
40. Ali, S. and A.J. Clark, *Characterization of the gene encoding ovine beta-lactoglobulin. Similarity to the genes for retinol binding protein and other secretory proteins.* J Mol Biol, 1988. **199**(3): p. 415-26.
41. Bortner, D.M. and M.P. Rosenberg, *Induction of mammary gland hyperplasia and carcinomas in transgenic mice expressing human cyclin E.* Mol Cell Biol, 1997. **17**(1): p. 453-9.
42. Palmiter, R.D., et al., *Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice.* Mol Cell Biol, 1993. **13**(9): p. 5266-75.
43. Bar-Sinai, A., et al., *Mouse mammary tumor virus Env-derived peptide associates with nucleolar targets in lymphoma, mammary carcinoma, and human breast cancer.* Cancer Res, 2005. **65**(16): p. 7223-30.
44. Cardiff, R.D. and W.J. Muller, *Transgenic mouse models of mammary tumorigenesis.* Cancer Surv, 1993. **16**: p. 97-113.
45. Muller, W.J., et al., *Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene.* Cell, 1988. **54**(1): p. 105-15.
46. Treisman, R., et al., *Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein.* Nature, 1981. **292**(5824): p. 595-600.
47. Lin, E.Y., et al., *Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases.* Am J Pathol, 2003. **163**(5): p. 2113-26.
48. Muraoka-Cook, R.S., et al., *Conditional overexpression of active transforming growth factor beta1 in vivo accelerates metastases of transgenic mammary tumors.* Cancer Res, 2004. **64**(24): p. 9002-11.
49. Guy, C.T., et al., *Activation of the c-Src tyrosine kinase is required for the induction of mammary tumors in transgenic mice.* Genes Dev, 1994. **8**(1): p. 23-32.
50. Lahlou, H., et al., *Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression.* Proc Natl Acad Sci U S A, 2007. **104**(51): p. 20302-7.

51. Hutchinson, J., et al., *Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression.* Mol Cell Biol, 2001. **21**(6): p. 2203-12.
52. Webster, M.A., et al., *Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis.* Mol Cell Biol, 1998. **18**(4): p. 2344-59.
53. Taverna, D., et al., *A direct test of potential roles for beta3 and beta5 integrins in growth and metastasis of murine mammary carcinomas.* Cancer Res, 2005. **65**(22): p. 10324-9.
54. Szabova, L., et al., *MT1-MMP is required for efficient tumor dissemination in experimental metastatic disease.* Oncogene, 2008. **27**(23): p. 3274-81.
55. Marcotte, R. and W.J. Muller, *Signal transduction in transgenic mouse models of human breast cancer--implications for human breast cancer.* J Mammary Gland Biol Neoplasia, 2008. **13**(3): p. 323-35.
56. Shih, C., et al., *Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts.* Nature, 1981. **290**(5803): p. 261-4.
57. Ursini-Siegel, J., et al., *Insights from transgenic mouse models of ERBB2-induced breast cancer.* Nat Rev Cancer, 2007. **7**(5): p. 389-97.
58. Bargmann, C.I., M.C. Hung, and R.A. Weinberg, *Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185.* Cell, 1986. **45**(5): p. 649-57.
59. Weiner, D.B., et al., *A point mutation in the neu oncogene mimics ligand induction of receptor aggregation.* Nature, 1989. **339**(6221): p. 230-1.
60. Guy, C.T., R.D. Cardiff, and W.J. Muller, *Activated neu induces rapid tumor progression.* J Biol Chem, 1996. **271**(13): p. 7673-8.
61. Guy, C.T., et al., *Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease.* Proc Natl Acad Sci U S A, 1992. **89**(22): p. 10578-82.
62. Chan, R., W.J. Muller, and P.M. Siegel, *Oncogenic activating mutations in the neu/erbB-2 oncogene are involved in the induction of mammary tumors.* Ann N Y Acad Sci, 1999. **889**: p. 45-51.
63. Siegel, P.M., et al., *Transforming growth factor beta signaling impairs Neu-induced mammary tumorigenesis while promoting pulmonary metastasis.* Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8430-5.
64. Siegel, P.M., et al., *Novel activating mutations in the neu proto-oncogene involved in induction of mammary tumors.* Mol Cell Biol, 1994. **14**(11): p. 7068-77.
65. Siegel, P.M., et al., *Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer.* EMBO J, 1999. **18**(8): p. 2149-64.
66. Moody, S.E., et al., *Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis.* Cancer Cell, 2002. **2**(6): p. 451-61.

67. Andrechek, E.R., et al., *Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3444-9.
68. Guy, C.T., R.D. Cardiff, and W.J. Muller, *Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease.* Mol Cell Biol, 1992. **12**(3): p. 954-61.
69. Schade, B., et al., *PTEN deficiency in a luminal ErbB-2 mouse model results in dramatic acceleration of mammary tumorigenesis and metastasis.* J Biol Chem, 2009. **284**(28): p. 19018-26.
70. Ranger, J.J., et al., *Identification of a Stat3-dependent transcription regulatory network involved in metastatic progression.* Cancer Res, 2009. **69**(17): p. 6823-30.
71. Sauer, B., *Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(6): p. 2087-96.
72. Orban, P.C., D. Chui, and J.D. Marth, *Tissue- and site-specific DNA recombination in transgenic mice.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 6861-5.
73. Lakso, M., et al., *Targeted oncogene activation by site-specific recombination in transgenic mice.* Proc Natl Acad Sci U S A, 1992. **89**(14): p. 6232-6.
74. Wagner, K.U., et al., *Cre-mediated gene deletion in the mammary gland.* Nucleic Acids Res, 1997. **25**(21): p. 4323-30.
75. Wagner, K.U., et al., *Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice.* Transgenic Res, 2001. **10**(6): p. 545-53.
76. White, D.E., et al., *Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction.* Cancer Cell, 2004. **6**(2): p. 159-70.
77. Ursini-Siegel, J., et al., *ShcA signalling is essential for tumour progression in mouse models of human breast cancer.* EMBO J, 2008. **27**(6): p. 910-20.
78. Osiecki, K., et al., *Identification of granulocyte-macrophage colony-stimulating factor and lipopolysaccharide-induced signal transduction pathways that synergize to stimulate HIV type 1 production by monocytes from HIV type 1 transgenic mice.* AIDS Res Hum Retroviruses, 2005. **21**(2): p. 125-39.
79. Pontier, S.M., et al., *Integrin-linked kinase has a critical role in ErbB2 mammary tumor progression: implications for human breast cancer.* Oncogene, 2010. **29**(23): p. 3374-85.
80. Nihei, M., et al., *[Clinical study of CA15-3 in human breast cancer].* Nippon Gan Chiryo Gakkai Shi, 1987. **22**(5): p. 987-95.
81. Cardiff, R.D., et al., *The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting.* Oncogene, 2000. **19**(8): p. 968-88.
82. Ponzo, M.G., et al., *Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer.* Proc Natl Acad Sci U S A, 2009. **106**(31): p. 12903-8.

83. Carpenter, G. and S. Cohen, *Epidermal growth factor.* J Biol Chem, 1990. **265**(14): p. 7709-12.
84. Ullrich, A. and J. Schlessinger, *Signal transduction by receptors with tyrosine kinase activity.* Cell, 1990. **61**(2): p. 203-12.
85. Earp, H.S., et al., *Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research.* Breast Cancer Res Treat, 1995. **35**(1): p. 115-32.
86. Guy, P.M., et al., *Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity.* Proc Natl Acad Sci U S A, 1994. **91**(17): p. 8132-6.
87. Leprince, C., et al., *Characterization of molecular components associated with surface immunoglobulin M in human B lymphocytes: presence of tyrosine and serine/threonine protein kinases.* Eur J Immunol, 1992. **22**(8): p. 2093-9.
88. Jones, J.T., R.W. Akita, and M.X. Sliwkowski, *Binding specificities and affinities of egf domains for ErbB receptors.* FEBS Lett, 1999. **447**(2-3): p. 227-31.
89. Riese, D.J., 2nd, et al., *The cellular response to neuregulins is governed by complex interactions of the erbB receptor family.* Mol Cell Biol, 1995. **15**(10): p. 5770-6.
90. Carraway, K.L., 3rd, et al., *Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases.* Nature, 1997. **387**(6632): p. 512-6.
91. Zhang, D., et al., *Neuregulin-3 (NRG3): a novel neural tissue-enriched protein that binds and activates ErbB4.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9562-7.
92. Harari, D., et al., *Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase.* Oncogene, 1999. **18**(17): p. 2681-9.
93. Wallasch, C., et al., *Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3.* EMBO J, 1995. **14**(17): p. 4267-75.
94. Sliwkowski, M.X., et al., *Coexpression of erbB2 and erbB3 proteins reconstitutes a high affinity receptor for heregulin.* J Biol Chem, 1994. **269**(20): p. 14661-5.
95. Horan, T., et al., *Binding of Neu differentiation factor with the extracellular domain of Her2 and Her3.* J Biol Chem, 1995. **270**(41): p. 24604-8.
96. Keely, S.J. and K.E. Barrett, *ErbB2 and ErbB3 receptors mediate inhibition of calcium-dependent chloride secretion in colonic epithelial cells.* J Biol Chem, 1999. **274**(47): p. 33449-54.
97. Tzahar, E., et al., *A hierarchical network of interreceptor interactions determines signal transduction by Neu differentiation factor/neuregulin and epidermal growth factor.* Mol Cell Biol, 1996. **16**(10): p. 5276-87.
98. Graus-Porta, D., et al., *ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling.* EMBO J, 1997. **16**(7): p. 1647-55.
99. Lamorte, L. and M. Park, *The receptor tyrosine kinases: role in cancer progression.* Surg Oncol Clin N Am, 2001. **10**(2): p. 271-88, viii.
100. Schulze, W.X., L. Deng, and M. Mann, *Phosphotyrosine interactome of the ErbB-receptor kinase family.* Mol Syst Biol, 2005. **1**: p. 2005 0008.
101. Olsen, J.V., et al., *Global, in vivo, and site-specific phosphorylation dynamics in signaling networks.* Cell, 2006. **127**(3): p. 635-48.

102. Yarden, Y. and M.X. Sliwkowski, *Untangling the ErbB signalling network.* Nat Rev Mol Cell Biol, 2001. **2**(2): p. 127-37.
103. Olayioye, M.A., et al., *The ErbB signaling network: receptor heterodimerization in development and cancer.* EMBO J, 2000. **19**(13): p. 3159-67.
104. Pawson, T., *Protein modules and signalling networks.* Nature, 1995. **373**(6515): p. 573-80.
105. Hynes, N.E. and H.A. Lane, *ERBB receptors and cancer: the complexity of targeted inhibitors.* Nat Rev Cancer, 2005. **5**(5): p. 341-54.
106. Schechter, A.L., et al., *The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen.* Nature, 1984. **312**(5994): p. 513-6.
107. Garrett, T.P., et al., *The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors.* Mol Cell, 2003. **11**(2): p. 495-505.
108. Hendriks, B.S., et al., *Quantitative analysis of HER2-mediated effects on HER2 and epidermal growth factor receptor endocytosis: distribution of homo- and heterodimers depends on relative HER2 levels.* J Biol Chem, 2003. **278**(26): p. 23343-51.
109. Chan, R., et al., *Modulation of Erbb2 signaling during development: a threshold level of Erbb2 signaling is required for development.* Development, 2004. **131**(22): p. 5551-60.
110. Negro, A., B.K. Brar, and K.F. Lee, *Essential roles of Her2/erbB2 in cardiac development and function.* Recent Prog Horm Res, 2004. **59**: p. 1-12.
111. Morris, J.K., et al., *A disorganized innervation of the inner ear persists in the absence of ErbB2.* Brain Res, 2006. **1091**(1): p. 186-99.
112. Lee, K.F., et al., *Requirement for neuregulin receptor erbB2 in neural and cardiac development.* Nature, 1995. **378**(6555): p. 394-8.
113. Jackson-Fisher, A.J., et al., *ErbB2 is required for ductal morphogenesis of the mammary gland.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17138-43.
114. Andrechek, E.R., D. White, and W.J. Muller, *Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth.* Oncogene, 2005. **24**(5): p. 932-7.
115. Moasser, M.M., *The oncogene HER2: its signaling and transforming functions and its role in human cancer pathogenesis.* Oncogene, 2007. **26**(45): p. 6469-87.
116. Moreno, A., et al., *Ductal carcinoma in situ of the breast: correlation between histologic classifications and biologic markers.* Mod Pathol, 1997. **10**(11): p. 1088-92.
117. Mack, L., et al., *Relationship of a new histological categorization of ductal carcinoma in situ of the breast with size and the immunohistochemical expression of p53, c-erb B2, bcl-2, and ki-67.* Hum Pathol, 1997. **28**(8): p. 974-9.
118. Masood, S. and M.M. Bui, *Prognostic and predictive value of HER2/neu oncogene in breast cancer.* Microsc Res Tech, 2002. **59**(2): p. 102-8.
119. Hayes, D.F. and A.D. Thor, *c-erbB-2 in breast cancer: development of a clinically useful marker.* Semin Oncol, 2002. **29**(3): p. 231-45.

120. Salomon, D.S., et al., *Epidermal growth factor-related peptides and their receptors in human malignancies.* Crit Rev Oncol Hematol, 1995. **19**(3): p. 183-232.
121. Lemoine, N.R., et al., *Expression of the ERBB3 gene product in breast cancer.* Br J Cancer, 1992. **66**(6): p. 1116-21.
122. Niu, G. and W.B. Carter, *Human epidermal growth factor receptor 2 regulates angiopoietin-2 expression in breast cancer via AKT and mitogen-activated protein kinase pathways.* Cancer Res, 2007. **67**(4): p. 1487-93.
123. Johnson, G.L. and R.R. Vaillancourt, *Sequential protein kinase reactions controlling cell growth and differentiation.* Curr Opin Cell Biol, 1994. **6**(2): p. 230-8.
124. Ben-Levy, R., et al., *A single autophosphorylation site confers oncogenicity to the Neu/ErbB-2 receptor and enables coupling to the MAP kinase pathway.* EMBO J, 1994. **13**(14): p. 3302-11.
125. Dankort, D.L. and W.J. Muller, *Signal transduction in mammary tumorigenesis: a transgenic perspective.* Oncogene, 2000. **19**(8): p. 1038-44.
126. Clark, G.J. and C.J. Der, *Aberrant function of the Ras signal transduction pathway in human breast cancer.* Breast Cancer Res Treat, 1995. **35**(1): p. 133-44.
127. O'Regan, R.M. and F.R. Khuri, *Farnesyl transferase inhibitors: the next targeted therapies for breast cancer?* Endocr Relat Cancer, 2004. **11**(2): p. 191-205.
128. Rauh, M.J., et al., *Accelerated mammary tumor development in mutant polyomavirus middle T transgenic mice expressing elevated levels of either the Shc or Grb2 adapter protein.* Mol Cell Biol, 1999. **19**(12): p. 8169-79.
129. Cheng, A.M., et al., *Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation.* Cell, 1998. **95**(6): p. 793-803.
130. Mansour, S.J., et al., *Transformation of mammalian cells by constitutively active MAP kinase kinase.* Science, 1994. **265**(5174): p. 966-70.
131. Westermarck, J. and V.M. Kahari, *Regulation of matrix metalloproteinase expression in tumor invasion.* FASEB J, 1999. **13**(8): p. 781-92.
132. Cully, M., et al., *Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis.* Nat Rev Cancer, 2006. **6**(3): p. 184-92.
133. Hinchliffe, K.A., *Cellular signalling: stressing the importance of PIP3.* Curr Biol, 2001. **11**(9): p. R371-2.
134. Kobayashi, S., et al., *Membrane recruitment of DOCK180 by binding to PtdIns(3,4,5)P3.* Biochem J, 2001. **354**(Pt 1): p. 73-8.
135. Cote, J.F., et al., *A novel and evolutionarily conserved PtdIns(3,4,5)P3-binding domain is necessary for DOCK180 signalling.* Nat Cell Biol, 2005. **7**(8): p. 797-807.
136. Amundadottir, L.T. and P. Leder, *Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes.* Oncogene, 1998. **16**(6): p. 737-46.
137. Ignatoski, K.M., et al., *ERBB-2 overexpression confers PI 3' kinase-dependent invasion capacity on human mammary epithelial cells.* Br J Cancer, 2000. **82**(3): p. 666-74.

138. Wu, P., T. Liu, and Y. Hu, *PI3K inhibitors for cancer therapy: what has been achieved so far?* Curr Med Chem, 2009. **16**(8): p. 916-30.
139. Junttila, T.T., et al., *Ligand-independent HER2/HER3/PI3K complex is disrupted by trastuzumab and is effectively inhibited by the PI3K inhibitor GDC-0941.* Cancer Cell, 2009. **15**(5): p. 429-40.
140. Franke, T.F., *PI3K/Akt: getting it right matters.* Oncogene, 2008. **27**(50): p. 6473-88.
141. Bouchard, V., et al., *Fak/Src signaling in human intestinal epithelial cell survival and anoikis: differentiation state-specific uncoupling with the PI3-K/Akt-1 and MEK/Erk pathways.* J Cell Physiol, 2007. **212**(3): p. 717-28.
142. Lin, H.J., et al., *Elevated phosphorylation and activation of PDK-1/AKT pathway in human breast cancer.* Br J Cancer, 2005. **93**(12): p. 1372-81.
143. Schmitz, K.J., et al., *High expression of focal adhesion kinase (p125FAK) in node-negative breast cancer is related to overexpression of HER-2/neu and activated Akt kinase but does not predict outcome.* Breast Cancer Res, 2005. **7**(2): p. R194-203.
144. Diaz-Montero, C.M., J.N. Wygant, and B.W. McIntyre, *PI3-K/Akt-mediated anoikis resistance of human osteosarcoma cells requires Src activation.* Eur J Cancer, 2006. **42**(10): p. 1491-500.
145. Gonzalez, L., et al., *Role of c-Src in human MCF7 breast cancer cell tumorigenesis.* J Biol Chem, 2006. **281**(30): p. 20851-64.
146. Thamilselvan, V., D.H. Craig, and M.D. Basson, *FAK association with multiple signal proteins mediates pressure-induced colon cancer cell adhesion via a Src-dependent PI3K/Akt pathway.* FASEB J, 2007. **21**(8): p. 1730-41.
147. Ottenhoff-Kalff, A.E., et al., *Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product.* Cancer Res, 1992. **52**(17): p. 4773-8.
148. Belsches-Jablonski, A.P., et al., *Src family kinases and HER2 interactions in human breast cancer cell growth and survival.* Oncogene, 2001. **20**(12): p. 1465-75.
149. Reissig, D., et al., *Elevated activity and expression of Src-family kinases in human breast carcinoma tissue versus matched non-tumor tissue.* J Cancer Res Clin Oncol, 2001. **127**(4): p. 226-30.
150. Muthuswamy, S.K., et al., *Mammary tumors expressing the neu proto-oncogene possess elevated c-Src tyrosine kinase activity.* Mol Cell Biol, 1994. **14**(1): p. 735-43.
151. Sheffield, L.G., *C-Src activation by ErbB2 leads to attachment-independent growth of human breast epithelial cells.* Biochem Biophys Res Commun, 1998. **250**(1): p. 27-31.
152. Kim, H., et al., *The c-Src tyrosine kinase associates with the catalytic domain of ErbB-2: implications for ErbB-2 mediated signaling and transformation.* Oncogene, 2005. **24**(51): p. 7599-607.
153. Lin, E.H., et al., *Disruption of Ca2+-dependent cell-matrix adhesion enhances c-Src kinase activity, but causes dissociation of the c-Src/FAK complex and*

dephosphorylation of tyrosine-577 of FAK in carcinoma cells. Exp Cell Res, 2004. **293**(1): p. 1-13.
154. Parsons, J.T., *Focal adhesion kinase: the first ten years.* J Cell Sci, 2003. **116**(Pt 8): p. 1409-16.
155. McLean, G.W., et al., *The role of focal-adhesion kinase in cancer - a new therapeutic opportunity.* Nat Rev Cancer, 2005. **5**(7): p. 505-15.
156. Benlimame, N., et al., *FAK signaling is critical for ErbB-2/ErbB-3 receptor cooperation for oncogenic transformation and invasion.* J Cell Biol, 2005. **171**(3): p. 505-16.
157. Cabodi, S., et al., *p130Cas is an essential transducer element in ErbB2 transformation.* FASEB J, 2010.
158. Dolfi, F., et al., *The adaptor protein Crk connects multiple cellular stimuli to the JNK signaling pathway.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15394-9.
159. Hsia, D.A., et al., *Differential regulation of cell motility and invasion by FAK.* J Cell Biol, 2003. **160**(5): p. 753-67.
160. Nakamoto, T., et al., *CIZ, a zinc finger protein that interacts with p130(cas) and activates the expression of matrix metalloproteinases.* Mol Cell Biol, 2000. **20**(5): p. 1649-58.
161. Cabodi, S., et al., *p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis.* Cancer Res, 2006. **66**(9): p. 4672-80.
162. Sundberg, C. and K. Rubin, *Stimulation of beta1 integrins on fibroblasts induces PDGF independent tyrosine phosphorylation of PDGF beta-receptors.* J Cell Biol, 1996. **132**(4): p. 741-52.
163. Borges, E., Y. Jan, and E. Ruoslahti, *Platelet-derived growth factor receptor beta and vascular endothelial growth factor receptor 2 bind to the beta 3 integrin through its extracellular domain.* J Biol Chem, 2000. **275**(51): p. 39867-73.
164. Bertotti, A., P.M. Comoglio, and L. Trusolino, *Beta4 integrin is a transforming molecule that unleashes Met tyrosine kinase tumorigenesis.* Cancer Res, 2005. **65**(23): p. 10674-9.
165. Wang, F., et al., *Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14821-6.
166. Guo, W., et al., *Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis.* Cell, 2006. **126**(3): p. 489-502.
167. Falcioni, R., et al., *Alpha 6 beta 4 and alpha 6 beta 1 integrins associate with ErbB-2 in human carcinoma cell lines.* Exp Cell Res, 1997. **236**(1): p. 76-85.
168. Barbieri, I., et al., *Constitutively active Stat3 enhances neu-mediated migration and metastasis in mammary tumors via upregulation of Cten.* Cancer Res, 2010. **70**(6): p. 2558-67.
169. Barbieri, I., et al., *Stat3 is required for anchorage-independent growth and metastasis but not for mammary tumor development downstream of the ErbB-2 oncogene.* Mol Carcinog, 2010. **49**(2): p. 114-20.

170. Hsieh, F.C., G. Cheng, and J. Lin, *Evaluation of potential Stat3-regulated genes in human breast cancer.* Biochem Biophys Res Commun, 2005. **335**(2): p. 292-9.
171. Yeh, Y.T., et al., *STAT3 ser727 phosphorylation and its association with negative estrogen receptor status in breast infiltrating ductal carcinoma.* Int J Cancer, 2006. **118**(12): p. 2943-7.
172. Fidler, I.J., *Critical determinants of cancer metastasis: rationale for therapy.* Cancer Chemother Pharmacol, 1999. **43 Suppl**: p. S3-10.
173. Woodhouse, E.C., R.F. Chuaqui, and L.A. Liotta, *General mechanisms of metastasis.* Cancer, 1997. **80**(8 Suppl): p. 1529-37.
174. Ibarra, N., A. Pollitt, and R.H. Insall, *Regulation of actin assembly by SCAR/WAVE proteins.* Biochem Soc Trans, 2005. **33**(Pt 6): p. 1243-6.
175. Kunda, P., et al., *Abi, Sra1, and Kette control the stability and localization of SCAR/WAVE to regulate the formation of actin-based protrusions.* Curr Biol, 2003. **13**(21): p. 1867-75.
176. Pertz, O., et al., *Spatiotemporal dynamics of RhoA activity in migrating cells.* Nature, 2006. **440**(7087): p. 1069-72.
177. Svitkina, T.M., et al., *Mechanism of filopodia initiation by reorganization of a dendritic network.* J Cell Biol, 2003. **160**(3): p. 409-21.
178. Adams, J.C. and M.A. Schwartz, *Stimulation of fascin spikes by thrombospondin-1 is mediated by the GTPases Rac and Cdc42.* J Cell Biol, 2000. **150**(4): p. 807-22.
179. Hashimoto, Y., M. Parsons, and J.C. Adams, *Dual actin-bundling and protein kinase C-binding activities of fascin regulate carcinoma cell migration downstream of Rac and contribute to metastasis.* Mol Biol Cell, 2007. **18**(11): p. 4591-602.
180. Yamakita, Y., et al., *Phosphorylation of human fascin inhibits its actin binding and bundling activities.* J Biol Chem, 1996. **271**(21): p. 12632-8.
181. Weaver, A.M., *Invadopodia: specialized cell structures for cancer invasion.* Clin Exp Metastasis, 2006. **23**(2): p. 97-105.
182. Yamaguchi, H., et al., *Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin.* J Cell Biol, 2005. **168**(3): p. 441-52.
183. Weaver, A.M., et al., *Cortactin promotes and stabilizes Arp2/3-induced actin filament network formation.* Curr Biol, 2001. **11**(5): p. 370-4.
184. Uruno, T., et al., *Activation of Arp2/3 complex-mediated actin polymerization by cortactin.* Nat Cell Biol, 2001. **3**(3): p. 259-66.
185. Mueller, S.C., et al., *A novel protease-docking function of integrin at invadopodia.* J Biol Chem, 1999. **274**(35): p. 24947-52.
186. Nakahara, H., et al., *Involvement of Cdc42 and Rac small G proteins in invadopodia formation of RPMI7951 cells.* Genes Cells, 2003. **8**(12): p. 1019-27.
187. Hauck, C.R., et al., *v-Src SH3-enhanced interaction with focal adhesion kinase at beta 1 integrin-containing invadopodia promotes cell invasion.* J Biol Chem, 2002. **277**(15): p. 12487-90.

188. Murphy, B., and O'Boyle D. *Lamellipodia in Manual of Cellular and Molecular Function*. 2008; Available from: http://handbook.blueprint.org/Home/lamellipodia.
189. Jiang, P., A. Enomoto, and M. Takahashi, *Cell biology of the movement of breast cancer cells: intracellular signalling and the actin cytoskeleton.* Cancer Lett, 2009. **284**(2): p. 122-30.
190. Yamazaki, D., S. Kurisu, and T. Takenawa, *Regulation of cancer cell motility through actin reorganization.* Cancer Sci, 2005. **96**(7): p. 379-86.
191. Friedl, P., *Prespecification and plasticity: shifting mechanisms of cell migration.* Curr Opin Cell Biol, 2004. **16**(1): p. 14-23.
192. Friedl, P., S. Borgmann, and E.B. Brocker, *Amoeboid leukocyte crawling through extracellular matrix: lessons from the Dictyostelium paradigm of cell movement.* J Leukoc Biol, 2001. **70**(4): p. 491-509.
193. Lammermann, T. and M. Sixt, *Mechanical modes of 'amoeboid' cell migration.* Curr Opin Cell Biol, 2009. **21**(5): p. 636-44.
194. Mandeville, J.T., M.A. Lawson, and F.R. Maxfield, *Dynamic imaging of neutrophil migration in three dimensions: mechanical interactions between cells and matrix.* J Leukoc Biol, 1997. **61**(2): p. 188-200.
195. Wolf, K., et al., *Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis.* J Cell Biol, 2003. **160**(2): p. 267-77.
196. Sahai, E. and C.J. Marshall, *Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis.* Nat Cell Biol, 2003. **5**(8): p. 711-9.
197. Wyckoff, J.B., et al., *ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo.* Curr Biol, 2006. **16**(15): p. 1515-23.
198. Sanz-Moreno, V., et al., *Rac activation and inactivation control plasticity of tumor cell movement.* Cell, 2008. **135**(3): p. 510-23.
199. Rintoul, R.C. and T. Sethi, *The role of extracellular matrix in small-cell lung cancer.* Lancet Oncol, 2001. **2**(7): p. 437-42.
200. Kaye, G.I., L.F. Siegel, and R.R. Pascal, *Cell replication of mesenchymal elements in adult tissues. I. The replication and migration of mesenchymal cells in the adult rabbit dermis.* Anat Rec, 1971. **169**(3): p. 593-611.
201. Grinnell, F., *Fibroblast mechanics in three-dimensional collagen matrices.* J Bodyw Mov Ther, 2008. **12**(3): p. 191-3.
202. Ridley, A.J., et al., *The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.* Cell, 1992. **70**(3): p. 401-10.
203. Nobes, C.D. and A. Hall, *Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia.* Cell, 1995. **81**(1): p. 53-62.
204. Paulus, W., et al., *Diffuse brain invasion of glioma cells requires beta 1 integrins.* Lab Invest, 1996. **75**(6): p. 819-26.

205. Morishige, M., et al., *GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion.* Nat Cell Biol, 2008. **10**(1): p. 85-92.
206. Friedl, P., Y. Hegerfeldt, and M. Tusch, *Collective cell migration in morphogenesis and cancer.* Int J Dev Biol, 2004. **48**(5-6): p. 441-9.
207. Friedl, P. and D. Gilmour, *Collective cell migration in morphogenesis, regeneration and cancer.* Nat Rev Mol Cell Biol, 2009. **10**(7): p. 445-57.
208. Vaughan, R.B. and J.P. Trinkaus, *Movements of epithelial cell sheets in vitro.* J Cell Sci, 1966. **1**(4): p. 407-13.
209. Friedl, P., et al., *Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants in vitro.* Cancer Res, 1995. **55**(20): p. 4557-60.
210. Weijer, C.J., *Collective cell migration in development.* J Cell Sci, 2009. **122**(Pt 18): p. 3215-23.
211. Ilina, O. and P. Friedl, *Mechanisms of collective cell migration at a glance.* J Cell Sci, 2009. **122**(Pt 18): p. 3203-8.
212. Christiansen, J.J. and A.K. Rajasekaran, *Reassessing epithelial to mesenchymal transition as a prerequisite for carcinoma invasion and metastasis.* Cancer Res, 2006. **66**(17): p. 8319-26.
213. Langbein, L., et al., *Tight junction-related structures in the absence of a lumen: occludin, claudins and tight junction plaque proteins in densely packed cell formations of stratified epithelia and squamous cell carcinomas.* Eur J Cell Biol, 2003. **82**(8): p. 385-400.
214. Ito, A., et al., *Increased expression of connexin 26 in the invasive component of lung squamous cell carcinoma: significant correlation with poor prognosis.* Cancer Lett, 2006. **234**(2): p. 239-48.
215. Yamada, S. and W.J. Nelson, *Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell-cell adhesion.* J Cell Biol, 2007. **178**(3): p. 517-27.
216. Wolf, K., et al., *Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion.* Nat Cell Biol, 2007. **9**(8): p. 893-904.
217. Hegerfeldt, Y., et al., *Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies.* Cancer Res, 2002. **62**(7): p. 2125-30.
218. Bell, C.D. and E. Waizbard, *Variability of cell size in primary and metastatic human breast carcinoma.* Invasion Metastasis, 1986. **6**(1): p. 11-20.
219. Nabeshima, K., et al., *Cohort migration of carcinoma cells: differentiated colorectal carcinoma cells move as coherent cell clusters or sheets.* Histol Histopathol, 1999. **14**(4): p. 1183-97.
220. White, D.E. and W.J. Muller, *Multifaceted roles of integrins in breast cancer metastasis.* J Mammary Gland Biol Neoplasia, 2007. **12**(2-3): p. 135-42.
221. Kiosses, W.B., et al., *Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration.* Nat Cell Biol, 2001. **3**(3): p. 316-20.

222. Moro, L., et al., *Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival.* EMBO J, 1998. **17**(22): p. 6622-32.
223. Miyamoto, S., et al., *Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors.* J Cell Biol, 1996. **135**(6 Pt 1): p. 1633-42.
224. Ezzell, R.M., et al., *Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton.* Exp Cell Res, 1997. **231**(1): p. 14-26.
225. Panetti, T.S., *Tyrosine phosphorylation of paxillin, FAK, and p130CAS: effects on cell spreading and migration.* Front Biosci, 2002. **7**: p. d143-50.
226. Calderwood, D.A., et al., *The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation.* J Biol Chem, 1999. **274**(40): p. 28071-4.
227. Sakai, R., et al., *A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner.* EMBO J, 1994. **13**(16): p. 3748-56.
228. Tachibana, K., et al., *Tyrosine phosphorylation of Crk-associated substrates by focal adhesion kinase. A putative mechanism for the integrin-mediated tyrosine phosphorylation of Crk-associated substrates.* J Biol Chem, 1997. **272**(46): p. 29083-90.
229. Brugnera, E., et al., *Unconventional Rac-GEF activity is mediated through the Dock180-ELMO complex.* Nat Cell Biol, 2002. **4**(8): p. 574-82.
230. Almeida, E.A., et al., *Matrix survival signaling: from fibronectin via focal adhesion kinase to c-Jun NH(2)-terminal kinase.* J Cell Biol, 2000. **149**(3): p. 741-54.
231. Arias-Salgado, E.G., et al., *Src kinase activation by direct interaction with the integrin beta cytoplasmic domain.* Proc Natl Acad Sci U S A, 2003. **100**(23): p. 13298-302.
232. Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer, *Focal adhesion kinase: in command and control of cell motility.* Nat Rev Mol Cell Biol, 2005. **6**(1): p. 56-68.
233. Li, H., et al., *Endocytosis of urokinase-plasminogen activator inhibitor type 1 complexes bound to a chimeric transmembrane urokinase receptor.* J Biol Chem, 1994. **269**(11): p. 8153-8.
234. Bourne, H.R., D.A. Sanders, and F. McCormick, *The GTPase superfamily: conserved structure and molecular mechanism.* Nature, 1991. **349**(6305): p. 117-27.
235. Neal, S.E., et al., *Kinetic analysis of the hydrolysis of GTP by p21N-ras. The basal GTPase mechanism.* J Biol Chem, 1988. **263**(36): p. 19718-22.
236. Heasman, S.J. and A.J. Ridley, *Mammalian Rho GTPases: new insights into their functions from in vivo studies.* Nat Rev Mol Cell Biol, 2008. **9**(9): p. 690-701.
237. Jaffe, A.B. and A. Hall, *Rho GTPases: biochemistry and biology.* Annu Rev Cell Dev Biol, 2005. **21**: p. 247-69.
238. Takenawa, T. and S. Suetsugu, *The WASP-WAVE protein network: connecting the membrane to the cytoskeleton.* Nat Rev Mol Cell Biol, 2007. **8**(1): p. 37-48.

239. Fritz, G., I. Just, and B. Kaina, *Rho GTPases are over-expressed in human tumors.* Int J Cancer, 1999. **81**(5): p. 682-7.
240. Fritz, G., et al., *Rho GTPases in human breast tumours: expression and mutation analyses and correlation with clinical parameters.* Br J Cancer, 2002. **87**(6): p. 635-44.
241. Han, G., et al., *Positive regulation of migration and invasion by vasodilator-stimulated phosphoprotein via Rac1 pathway in human breast cancer cells.* Oncol Rep, 2008. **20**(4): p. 929-39.
242. Zuo, Y., S.K. Shields, and C. Chakraborty, *Enhanced intrinsic migration of aggressive breast cancer cells by inhibition of Rac1 GTPase.* Biochem Biophys Res Commun, 2006. **351**(2): p. 361-7.
243. O'Connor, K.L. and A.M. Mercurio, *Protein kinase A regulates Rac and is required for the growth factor-stimulated migration of carcinoma cells.* J Biol Chem, 2001. **276**(51): p. 47895-900.
244. Baugher, P.J., et al., *Rac1 and Rac3 isoform activation is involved in the invasive and metastatic phenotype of human breast cancer cells.* Breast Cancer Res, 2005. **7**(6): p. R965-74.
245. Chan, A.Y., et al., *Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion.* Oncogene, 2005. **24**(53): p. 7821-9.
246. Schnelzer, A., et al., *Rac1 in human breast cancer: overexpression, mutation analysis, and characterization of a new isoform, Rac1b.* Oncogene, 2000. **19**(26): p. 3013-20.
247. Cote, J.F. and K. Vuori, *GEF what? Dock180 and related proteins help Rac to polarize cells in new ways.* Trends Cell Biol, 2007. **17**(8): p. 383-93.
248. Hasegawa, H., et al., *DOCK180, a major CRK-binding protein, alters cell morphology upon translocation to the cell membrane.* Mol Cell Biol, 1996. **16**(4): p. 1770-6.
249. Cheresh, D.A., J. Leng, and R.L. Klemke, *Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells.* J Cell Biol, 1999. **146**(5): p. 1107-16.
250. Meller, N., et al., *Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins.* Nat Cell Biol, 2002. **4**(9): p. 639-47.
251. Cote, J.F. and K. Vuori, *Identification of an evolutionarily conserved superfamily of DOCK180-related proteins with guanine nucleotide exchange activity.* J Cell Sci, 2002. **115**(Pt 24): p. 4901-13.
252. Rushton, E., et al., *Mutations in a novel gene, myoblast city, provide evidence in support of the founder cell hypothesis for Drosophila muscle development.* Development, 1995. **121**(7): p. 1979-88.
253. Wu, Y.C. and H.R. Horvitz, *C. elegans phagocytosis and cell-migration protein CED-5 is similar to human DOCK180.* Nature, 1998. **392**(6675): p. 501-4.
254. Rossman, K.L., C.J. Der, and J. Sondek, *GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors.* Nat Rev Mol Cell Biol, 2005. **6**(2): p. 167-80.

255. Meller, N., S. Merlot, and C. Guda, *CZH proteins: a new family of Rho-GEFs.* J Cell Sci, 2005. **118**(Pt 21): p. 4937-46.
256. Gumienny, T.L., et al., *CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration.* Cell, 2001. **107**(1): p. 27-41.
257. Chung, S., et al., *A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in C. elegans.* Nat Cell Biol, 2000. **2**(12): p. 931-7.
258. Komander, D., et al., *An alpha-helical extension of the ELMO1 pleckstrin homology domain mediates direct interaction to DOCK180 and is critical in Rac signaling.* Mol Biol Cell, 2008. **19**(11): p. 4837-51.
259. Erickson, M.R., B.J. Galletta, and S.M. Abmayr, *Drosophila myoblast city encodes a conserved protein that is essential for myoblast fusion, dorsal closure, and cytoskeletal organization.* J Cell Biol, 1997. **138**(3): p. 589-603.
260. Hakeda-Suzuki, S., et al., *Rac function and regulation during Drosophila development.* Nature, 2002. **416**(6879): p. 438-42.
261. Nolan, K.M., et al., *Myoblast city, the Drosophila homolog of DOCK180/CED-5, is required in a Rac signaling pathway utilized for multiple developmental processes.* Genes Dev, 1998. **12**(21): p. 3337-42.
262. Ellis, R.E., D.M. Jacobson, and H.R. Horvitz, *Genes required for the engulfment of cell corpses during programmed cell death in Caenorhabditis elegans.* Genetics, 1991. **129**(1): p. 79-94.
263. Wu, Y.C., et al., *Distinct rac activation pathways control Caenorhabditis elegans cell migration and axon outgrowth.* Dev Biol, 2002. **250**(1): p. 145-55.
264. Reddien, P.W. and H.R. Horvitz, *CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans.* Nat Cell Biol, 2000. **2**(3): p. 131-6.
265. Sanders, M.A., D. Ampasala, and M.D. Basson, *DOCK5 and DOCK1 regulate Caco-2 intestinal epithelial cell spreading and migration on collagen IV.* J Biol Chem, 2009. **284**(1): p. 27-35.
266. Grimsley, C.M., et al., *Dock180 and ELMO1 proteins cooperate to promote evolutionarily conserved Rac-dependent cell migration.* J Biol Chem, 2004. **279**(7): p. 6087-97.
267. Cho, S.Y. and R.L. Klemke, *Extracellular-regulated kinase activation and CAS/Crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix.* J Cell Biol, 2000. **149**(1): p. 223-36.
268. Vuori, K. and E. Ruoslahti, *Tyrosine phosphorylation of p130Cas and cortactin accompanies integrin-mediated cell adhesion to extracellular matrix.* J Biol Chem, 1995. **270**(38): p. 22259-62.
269. Laurin, M., et al., *The atypical Rac activator Dock180 (Dock1) regulates myoblast fusion in vivo.* Proc Natl Acad Sci U S A, 2008. **105**(40): p. 15446-51.
270. He, X., et al., *Rac1 is essential for basement membrane-dependent epiblast survival.* Mol Cell Biol, 2010. **30**(14): p. 3569-81.

271. Lin, M. and K.L. van Golen, *Rho-regulatory proteins in breast cancer cell motility and invasion.* Breast Cancer Res Treat, 2004. **84**(1): p. 49-60.
272. Smith, H.W., P. Marra, and C.J. Marshall, *uPAR promotes formation of the p130Cas-Crk complex to activate Rac through DOCK180.* J Cell Biol, 2008. **182**(4): p. 777-90.
273. Jarzynka, M.J., et al., *ELMO1 and Dock180, a bipartite Rac1 guanine nucleotide exchange factor, promote human glioma cell invasion.* Cancer Res, 2007. **67**(15): p. 7203-11.
274. Kiyokawa, E., et al., *Activation of Rac1 by a Crk SH3-binding protein, DOCK180.* Genes Dev, 1998. **12**(21): p. 3331-6.
275. Tilli, M.T., et al., *Comparison of mouse mammary gland imaging techniques and applications: reflectance confocal microscopy, GFP imaging, and ultrasound.* BMC Cancer, 2008. **8**: p. 21.
276. Brantley-Sieders, D.M., et al., *The receptor tyrosine kinase EphA2 promotes mammary adenocarcinoma tumorigenesis and metastatic progression in mice by amplifying ErbB2 signaling.* J Clin Invest, 2008. **118**(1): p. 64-78.
277. Moore, C.A., et al., *A role for the Myoblast city homologues Dock1 and Dock5 and the adaptor proteins Crk and Crk-like in zebrafish myoblast fusion.* Development, 2007. **134**(17): p. 3145-53.
278. Simon, A.R., et al., *Regulation of STAT3 by direct binding to the Rac1 GTPase.* Science, 2000. **290**(5489): p. 144-7.
279. Faruqi, T.R., et al., *Rac1 mediates STAT3 activation by autocrine IL-6.* Proc Natl Acad Sci U S A, 2001. **98**(16): p. 9014-9.
280. Debidda, M., et al., *A role of STAT3 in Rho GTPase-regulated cell migration and proliferation.* J Biol Chem, 2005. **280**(17): p. 17275-85.
281. Huck, L., et al., *{beta}1-integrin is dispensable for the induction of ErbB2 mammary tumors but plays a critical role in the metastatic phase of tumor progression.* Proc Natl Acad Sci U S A, 2010.
282. Sahai, E., *Illuminating the metastatic process.* Nat Rev Cancer, 2007. **7**(10): p. 737-49.
283. Novak, J., et al., *In vivo flow cytometer for real-time detection and quantification of circulating cells.* Opt Lett, 2004. **29**(1): p. 77-9.
284. Bromberg, J.F., et al., *Stat3 as an oncogene.* Cell, 1999. **98**(3): p. 295-303.
285. Bianco, A., et al., *Two distinct modes of guidance signalling during collective migration of border cells.* Nature, 2007. **448**(7151): p. 362-5.
286. Chapman, R.S., et al., *Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3.* Genes Dev, 1999. **13**(19): p. 2604-16.