Regulation of actomyosin contraction as a driving force of invasive lobular breast cancer

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General Introduction

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Breast cancer heterogeneity

Cancer is global health problem with more than 24 million cases and close to 10 million deaths world wide[1]. The chance of developing cancer during a lifetime is 1 in 3 for men and 1 in 4 four women[1]. In women, the second most common type of cancer is breast cancer[1]. Despite substantial improvement of available interventions, breast cancer remains responsible for the most cancer related deaths in women[1]. Breast cancer is a very heterogeneous disease that comprises multiple tumor types which can be classified based on different characteristics[2].

The most common method for breast cancer classification is via histopathology. Classification by histopathology relies on morphology, size, grade and biomarker expression of the tumors[3,4]. The first distinction made in breast cancers is whether the cancer cells are retained within the borders of the mammary duct. Carcinomas that are contained within the lumen of the mammary ducts are referred to as *in situ* carcinomas. Tumors that have breached the mammary ducts are termed invasive carcinomas. The second distinction based on morphology is the presence of a specific growth pattern. The majority of breast cancers (70-75%) are classified as carcinomas of no special type (formerly known as ductal carcinomas). Most of the special subtypes of breast cancer are relatively rare and often associated with specific genetic alterations[2]. Besides morphological classification, breast tumors are also divided by the expression of the Estrogen (ER) and progesterone (PR) receptors and more recently by expression of ERBB2 (also referred to as HER2)[5]. Tumors that express none of these receptors are referred to as triple-negative tumors. The classification by expression of these receptors is important because expression of ER and PR makes patients eligible for hormonal therapies while expression of HER2 enables treatment with HER2-blocking antibodies.

At the turn of the 21st century, molecular profiling based on gene expression enabled classification of breast tumors into two different subtypes driven by the presence or absence of active ER signaling[6]. Shortly thereafter, the molecular subtypes were further refined into 5 intrinsic subtypes namely: Luminal A and B, basal-like, HER2 and normal-like[7,8]. Confirmation that molecular classification can be a useful tool for treatment came when a gene signature assay (PAM50) was developed based on these intrinsic subtypes enabling risk of relapse prediction for ER positive breast cancer patients[9]. In addition, a 70-gene signature was generated that was capable of predicting which breast cancer patients had a good or poor

survival outcome[10,11]. Next to gene expression analysis genome wide copy number analysis has also been used to classify patients. Analysis based on copy number alterations (CNAs) found in human breast cancer patients yielded subgroups with differential patient survival rates[12,13]. The subgroups identified using CNA data were different from the subtypes identified using gene expression indicative that better classification was possible. Integration of both copy number analysis and gene expression data yielded 10 subtypes with differential clinical outcomes[14,15]. Finally DNA sequencing data are an additional source of information which can be used to classify tumors[16–18]. Mutual exclusivity and co-occurrence of mutations enable identification of mutations with similar or synergistic underlying mechanisms and allow stratification of tumors based on the involvement of specific signaling pathways.

While all of the above described techniques have greatly contributed to our knowledge of the mutational spectrum of breast cancers, many of the mechanisms underlying the various subtypes remain unclear. To establish causal relationships between mutations clinical outcomes we require representative models that recapitulate the complexity of patient tumors but also enable assessment the contribution of specific mutations. To this end, genetically engineered mouse models (GEMMs) were created that harbor the same mutations found in the tumors of human cancer patients. GEMMs can be used to study most aspects of tumor biology including initiation of tumor development, tumor progression and drug response[19]. *Ex vivo* analysis of GEMM derived material is an additional method that allows the assessment of specific conditions in a controlled setting while maintaining a relevant background.

Invasive lobular Carcinoma

After breast cancer of no special type, invasive lobular carcinoma (ILC) is the second most common subtype of breast cancer accounting for 8-14% of all cases[20–22]. Several morphological subtypes of ILC have been identified[23]. The most frequent morphological subtype of ILC is classic ILC characterized by non-cohesive cells invading the surrounding stroma in single files also referred to as 'Indian files'. The invasive nature of ILC complicates diagnosis since ILCs typically do not form a solid mass and have no calcifications making it difficult to detect these lesions by palpation or mammography[24]. ILCs also frequently develop multifocally and are more often bilateral at presentation than other breast cancers[25–27]. ILCs typically have low grade

histology, low proliferation rates and are ER positive[28]. Classic ILCs usually fall in the luminal A subgroup which have a good prognosis. Less common ILC subtypes like pleomorphic ILC are part of the luminal B group which have a poorer prognosis compared to classical ILCs[2]. Because most ILCs are ER- and PR-positive the standard of care is endocrine based therapy[29]. ILCs generally respond well to endocrine-based therapies and have a favorable 5 year survival rate compared to breast cancers of no special type but have lower long-term survival rates[30,31]. Furthermore, If hormone receptor expression is lost ILCs are less responsive to chemotherapy[32–34]. ILCs have a tendency to disseminate to different organs than other types of breast cancer. Metastasis to the bone, peritoneum and ovaries are particularly more frequent in ILC[21,35].

ILCs are not only distinct on a morphological level but also have different genetic alterations than other breast cancers. Loss of the cellular adhesion protein E-cadherin (encoded by *CDH1*) is the most common molecular characteristic of ILC (discussed in more detail in the next paragraph)[36]. Recurrent gains of chromosomes 1q, 16p and loss of chromosome 16q (where *CDH1* is located*)* are also more common in ILC patients[37,38]. Next generation sequencing of human ILCs have yielded a wealth of information[39–41]. After *CDH1,* the most common mutations are found in Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*) which is an important member of the PI3K pathway. Also, other members of the PI3K pathway *PTEN* and *AKT1* are frequently mutated in ILC patients indicating that the Pi3K pathway is an important pathway for this type of breast cancer. In addition, mutations in *TP53*, *FOXA1*,*TBX3* and *RUNX1* are also found in ILC patients.

E-cadherin as a Tumor suppressor

Intercellular adhesion is essential for the integrity of epithelial tissues. Adherens junctions ensure that the barrier function of the epithelium is maintained. The most prominent component of adherens junctions are the cadherens[42]. Cadherins are transmembrane proteins that interact in the presence of calcium with other cadherins on neighboring cells via their extracellular domains. The intracellular domains of cadherins bind to P120- and β-catenin. β-catenin in turn binds to α-catenin which facilitates the interaction with the actin cytoskeleton. However, adherens junctions are more than just structural proteins, they also mediate multiple signaling pathways[43–45]. The most studied cadherin is found in epithelial tissues,

and therefore named E-cadherin. The importance of adherens junctions is underscored by the fact that inactivation of the E-cadherin gene results in embryonic lethality[46]. In the mammary gland, the luminal epithelial cells express E-cadherin while the myoepithelial cells express P-cadherin[47].

In the early 1990's, it became apparent that E-cadherin acts as a tumor suppressor. Patient analysis revealed that E-cadherin was frequently inactivated in multiple solid tumor types[48–50]. There are several mechanisms through which E-cadherin can be inactivated in tumors. Promotor hyper methylation can reduce the expression levels resulting in loss or reduction of E-cadherin function[51]. The chromosomal region 16q is also frequently lost in multiple types of cancer either homo- or heterozygously[52–54]. Finally, mutations can also result in E-cadherin inactivation by generation of a truncated protein or exon skipping[55–57]. Often it is a combination of these events that results in complete loss of E-cadherin. The two tumor types most clearly associated with loss of E-cadherin are diffuse gastric cancer and ILC. In these cancer types, mutations are observed frequently and are known to result in complete loss of E-cadherin expression[39–41,58]. Furthermore, hereditary diffuse gastric cancer syndrome is characterized by heterozygous germline mutations in E-cadherin[59]. Women with germline E-cadherin mutations not only have a very high risk of developing gastric cancer but also have a greater than 40% chance of developing lobular breast cancer during their lifetime[60,61].

A functional role for E-cadherin loss in tumorigenesis became clear when comparison of tumor cells revealed that more invasive tumor cells often express no E-cadherin and became less invasive when E-cadherin was expressed[62,63]. E-cadherin loss is also a well-known characteristic of epithelial to mesenchymal transition (EMT). EMT is a process where epithelial cells lose their epithelial properties and gain mesenchymal properties. EMT was discovered as a vital process involved in multiple phases of embryonic development[64]. This process can also be hijacked by tumor cells to promote their dissemination. While loss of E-cadherin is an essential step in EMT it is a common misconception that loss of E-cadherin by itself results in EMT. It has been shown that knockout of E-cadherin in epithelial cells is insufficient to induce EMT[65–67]. Furthermore, ILCs and diffuse gastric cancers typically do not display a mesenchymal phenotype[23,68]. This indicates that loss of E-cadherin is a consequence of EMT rather than a driving event. Nonetheless, it is clear E-cadherin mediated cell-cell interactions play an important role in the regulation of

motility in epithelial cells.

Besides increased invasion, loss of E-cadherin has also been shown to have an impact on cell proliferation. In tissue homeostasis, an epithelial cell needs to 'know' when to proliferate or not. Adherence junctions provide critical information of the direct spatial cellular surrounding. In wild-type epithelial cells, the growth is halted when cells become confluent, a phenomenon known as contact inhibition of proliferation. E-cadherin has been proposed to play an important role in this phenomenon as loss of E-cadherin was required for the growth of epithelial cancer cells when they are confluent[69,70]. However, there is also evidence that loss of E-cadherin alone is insufficient to induce contact inhibition in untransformed epithelial cells, indicating other alterations are required[71]. Contact inhibition has been linked to multiple signaling pathways. One pathway may be related to growth factor signaling, as E-cadherin engagement has been shown to inhibit ligand-based activation of EGFR and other receptor tyrosine kinase receptors[72,73]. Furthermore, E-cadherin also activates the hippo signaling pathway via α-catenin, which upon activation inhibits the transcription factors YAP and TAZ that stimulate cell growth[74]. However, there is also evidence indicating that E-cadherin interactions between cells are involved in cell cycle progression[75]. Benham-pyle et al. showed that homophilic trans interactions between E-cadherin molecules is required for the transition from G1/0 to S phase that is induced by mechanical strain in confluent epithelial cells[75]. Altogether, E-cadherin may inhibit or induce cell proliferation depending on the physiological context.

Finally, E-cadherin also plays an important role in the induction of cell-extrinsic apoptosis[76]. Cell-extrinsic apoptosis is initiated by specific members of the tumor necrosis factor ligand family, including Apo2L/TRAIL. These ligands bind to the death receptors DR4 and DR5, leading to the formation of a death-inducing signaling complex (DISC) which includes several protein including caspase 8. To induce apoptosis, DISC has to be coupled to the actin cytoskeleton. E-cadherin containing adherens junctions were shown to be required for coupling of the DISC complex to the cytoskeleton. This interaction was dependent on the presence of β-catenin. Knockdown of E-cadherin significantly reduced the sensitivity of cancer cells to TRAIL-induced apoptosis. It should be noted that cells that have lost E-cadherin are still going into apoptosis via cell-intrinsic apoptosis pathways.

Overall E-cadherin has been shown to be an important tumor suppression

that acts via different mechanisms. The ample evidence for *CDH1* mutations in ILC made E-cadherin a prime candidate for the generation of ILC mouse models.

Actomyosin contractility

The actin cytoskeleton is a primary component of every cell, providing cell structure and controlling cell signaling by mechanotransduction. The actin cytoskeleton controls cell shape and movement but also cell survival and expansion. The actin cytoskeleton is composed of actin monomers which are polymerized into actin filaments[77,78]. Two actin filaments wind around each other to form a helical structure. The actin filaments can be used as anchoring points for myosins. Myosins are the motor proteins that can cause contraction of different actin filaments enabling motility and shape changes. Myosins were originally identified in muscle cells but later found to be present in all eukaryotic cells[79]. Epithelial cells express non-muscle myosin II (NM II) which is a complex of two myosin heavy chains, two regulatory light chains and two essential light chains[80]. The heavy chains form a homodimer with their c-terminal tails and bind one essential and regulatory light chain each. The essential light chains stabilize the heavy chains while the regulatory light chains (RLCs) determine the activity of the complex. In its active state, NM II can self-associate with other NM II via the c-terminal domain while the N-terminal heads tether to actin filaments. ATP hydrolysis in the heads of the heavy chains causes a conformational change resulting in the anti-parallel movement of the actin filaments.

The activity of myosins has to be tightly controlled in order to result in directed motility. The primary determinant of NM II activity is the phosphorylation of Serine 19 (Ser19) of RLCs which greatly increases the ATPase activity by controlling the conformation of the myosin heads[81,82]. The phosphorylation Ser19 of the RLCs can be performed by a number of kinases, including myosin light chain kinase (MLCK), Rho-associated coiled coil-containing kinase (ROCK), citron kinase, leucine zipper interacting kinase and myotonic dystrophy kinase related CDC42-binding kinase[81–84]. These kinases are activated by different signals. MLCK is activated by calcium binding protein calmodulin while ROCK and citron kinase are activated by Ras homolog gene family member A (RhoA), which is a small GTP-binding protein[85–87]. While MLCK can only phosphorylate RLCs, both ROCK and citron kinase have multiple substrates. ROCK not only activates NM II by RLC phosphorylation but also by phosphorylation of the myosin light chain

phosphatase (MLCP) complex. Myosin phosphatase is a protein complex containing protein phosphatase 1 (PP1), myosin phosphatase targeting subunit 1 (MYPT1 or PPP1R12A) and a small subunit M20[88]. As the name suggests, MLCP dephosphorylates RLC thereby inhibiting NM II. ROCK can phosphorylate MYPT1 at Threonine 697 and 855 inhibiting MLCP activity[89]. Further regulation of NM II activity is controlled by phosphorylation of C-terminal residues of the heavy chains, which prevents myosin filament formation or induces their dissociation[90–92].

Adherens junctions are not just tethered to the actin cytoskeleton but are also involved in actin filament assembly and actin contraction[93]. The effects of cadherens on actomyosin contractility seem to type dependent. The formation of adherens junctions by E-cadherin engagement has been shown to reduce RhoA signaling while upregulating the Rho GTPase Rac1[94]. Inhibition of RhoA is partly mediated by sequestering of the GTPase activating protein (GAP) p190RhoGAP to the cell junction by E-cadherin[95]. In addition, loss of E-cadherin has been shown to increase cytoplasmic and nuclear localization of p120 catenin[96]. In E-cadherin-deficient mammary tumor cells, cytoplasmic localization of p120 can result in the activation of RhoA by binding and inhibiting the Rho antagonist myosin phosphatase Rho-interacting Protein (MRIP)[97]. While E-cadherin seems to decrease RhoA activity, engagement of N-cadherin and VE-cadherin results in increased RhoA activation[98,99]. It appears therefore that E-cadherin reduces actomyosin contractility while N-cadherin and VE-cadherin activate actomyosin contractility. This is in line with the observation that luminal epithelial cells have low MLC phosphorylation compared to mesenchymal and endothelial cells[100].

Next to intercellular adhesion the adhesion of cells to their environment also plays an important role in actomyosin contractility[101]. Stiffer matrices have been shown to induce actomyosin contractility in fibroblasts and lead to higher migration rates of mesenchymal stem cells[102,103]. The stiffness of the extracellular matrix (ECM) is in part dependent on the components making up the matrix. For example fibrillar collagen (collagen 1) is much stiffer than basement membrane components laminin and collagen IV[104]. ECM components themselves also have differential effects on actomyosin contractility. For example, adhesion on fibrillar collagen results in increased actomyosin contraction while laminin 332 a component of the basement membrane of the mammary gland reduces actomyosin contractility[105]. This is caused by differential integrin activation. Integrin α3β1 adhesion

to laminin 332 leads to activation of Rac1, which in turn inhibits RhoA signaling[106–109]. In contrast, adhesion of integrin α2β1 to collagen 1 induces RhoA activation[105].

The role of actomyosin contractility in cancer

Actomyosin contractility has been studied intensively in the context of tumorigenesis[110]. High actomyosin contractility has been associated with multiple steps of the metastatic cascade in multiple types of cancer. Increased activation of ROCK via RhoA has been shown to increase invasion of cancer cells by increasing migration and matrix deformation[111–114]. Actomyosin contractility is also required for cells to enter and exit blood vessels and for their survival in circulation[113,115–117]. Finally the colonization of distant organs is also favored by cells with higher actomyosin contractility[116,118–120]. While there is extensive evidence that actomyosin contractility is important during metastasis, there is also evidence for a role in tumor growth. Inhibition of ROCK by shRNA-mediated knockdown or pharmaceutical inhibition not only reduced metastasis but also reduced tumor outgrowth[97,121]. ROCK activity has also been shown to increase tissue stiffness and activation of β-catenin, which increased the growth of melanoma[122]. Actomyosin contractility is also an important factor for the maintenance of nuclear integrity. Nuclear deformation and genomic instability are features of cancer and correlate with increased malignancy. Inhibition of MLCP by knockdown of either MYPT1 or PP1 has been shown to increase nuclear deformation and genomic instability[123]. Overall, actomyosin contractility has been primarily associated with migration and invasion whereas it clearly also impacts other aspects of tumorigenesis like cell adhesion.

Mouse models in breast cancer research

The first transgenic mouse models of breast cancers were generated by mammary specific overexpression of strong oncogenes, such as Myc, Ras and Neu (now known as ERRB2)[124–126]. To achieve this, a part of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) was cloned in front of the *Myc*, *Ras* and *Neu* sequences. The development of gene knockout technology enabled analysis of tumor suppressor genes (TSGs) such as p53[127]. The limitations of these models were that the TSG knockouts were not tissue specific and the transgenic mice expressed the transgenes in the majority of mammary epithelial cells. The latter is a problem because

cancer is viewed to arise in single cells that progressively gain multiple genetic mutations. Another issue frequently observed with the deletion of tumor suppressor genes is that loss of these genes often causes embryonic lethality[128–131].

To circumvent the limitations of conventional GEMMs, new systems were developed that allowed induction oncogenes or knockout of TSGs in specific tissues and at the time of interest. The most commonly used system combines a bacterial enzyme called Cre recombinase with recombinase recognition sites (LoxP sites), which are integrated into the genome[132]. These loxP sites do not affect normal gene function but in the presence Cre recombinase a recombination event occurs that excises the region in between the loxP sites. If the loxP sites flank a gene or part of it then its expression will be perturbed. To ensure tissue specificity, Cre recombinase was coupled to promoter sequences only active in the tissue of interest. For the mammary gland, the most common promoter sequences are mouse mammary tumor virus (MMTV), Whey acid protein (WAP) and beta-lactoglobulin (BLG)[133–135]. Alternatively, Cre recombinase can also be expressed by transducing mammary epithelial cells with lenti- or adeno-viruses encoding Cre recombinase administered via intraductal injection[136,137]. To induce overexpression of (onco)genes with the Cre/LoxP system, it is possible to use a so called lox-stop-lox (LSL) system, which utilizes a transcription termination sequence flanked by loxP sites[138–140]. In the absence of Cre expression, the transgene downstream of the LSL system is not expressed. Besides the LSL system, it is also possible to use the mutant loxP sites Lox66 and lox71[141,142]. When Cre recombinase interacts with Lox66 and lox71 sites it results in an irreversible inversion. An inverted promotor sequence flanked by Lox66 and lox71 sites upstream of an oncogene will therefore only result in expression if Cre recombinase is active[143]. Overall, the Cre/loxP system has been used successfully to generate multiple models of breast Cancer[144–148].

While germline mouse models have given us valuable information they are also expensive and time-consuming. This holds true especially if multiple mutations are combined. The development of CRISPR/Cas technology has greatly expanded our options for mouse model development. The system was originally discovered as a prokaryotic defense mechanism against foreign genetic elements[149–152]. During recent years, this system has been successfully utilized to edit the genome of mammalian cells[153]. In this system, short RNA sequences called single guide RNAs are used to target

a nuclease called Cas9 to a specific site in genome. Once the nuclease is at this specific site it makes a double-strand break in the DNA, which can be repaired by the DNA repair pathways present in the cell. The true power of this technique lies in the fact that If repair of the break is done correctly it can be targeted again by the CRISPR system until mistakes are made during DNA repair. The repair with the non-homologous end joining pathway is relatively error-prone and can lead to small deletions or insertions that can cause a frame shift resulting in inactivation of the targeted gene. The CRISPR/Cas system has been utilized for modeling of multiple cancer types including breast cancer[154–157].

Modeling invasive lobular carcinoma in mice

Loss of E-cadherin is not only a hallmark of ILC, it is also considered to be an early event in ILC tumorigenesis[158]. Therefore, modeling of ILC in mice started with the generation of E-cadherin floxed alleles that enabled somatic loss of E-cadherin in the mammary gland[145,159]. It quickly became apparent that despite being the main driver event in ILCs, loss of E-cadherin alone is insufficient to induce mammary tumors in mice. The first ILC mouse model therefore combined epithelium-specific loss of E-cadherin with the loss of tumor suppressor p53 which proved sufficient to induce ILC[145]. A limitation of this model is the Cre recombinase expression was under control of the K14 promoter the activity of which is not exclusive to the mammary gland. Most mice therefore not only developed ILCs but also skin tumors. To address this issue, a new model was generated in which deletion of E-cadherin and p53 was induced by Cre recombinase expressed from the Whey acid protein (WAP) promotor, which is exclusive to mammary epithelial cells[146]. While the tumors generated by combined loss of E-cadherin and p53 were ILCs, there remained several limitations. The tumors were typically had a pleomorphic morphology which is a relatively rare subtype observed in the clinic. Furthermore, tumors driven by combined loss of p53 and E-cadherin grow very rapidly while ILCs are typically marked by low proliferation rates. Finally most human ILCs are characterized by expression of the estrogen receptor (ER) but the tumors generated by combined loss of E-cadherin and p53 were mostly ER negative. Analysis of human tumors has shown that the most common mutations in human ILCs (after E-cadherin) are found in members of PI3K pathway. Combinations of these PI3K pathway mutations with loss of E-cadherin yielded additional models of ILC[147,148,156]. Importantly, tumors derived from these models did show a morphology closely resembling human classic ILC the most frequently observed

subtype. Additionally, ER expression was observed in most of these tumors although these models were never tested for their dependency on ER signaling. Overall, there have been made substantial advances in modeling ILC in mice over the past fifteen years, yielding multiple models that resemble different subtypes of ILC.

Insertional mutagenesis as a tool for cancer driver discovery

The extensive analysis of patient tumors has yielded a wealth of information on the mutational landscape of cancer. For some genes the sheer number of specific mutations makes establishing causality relatively easy. However not all mutations have such clear causal links. Copy-number alterations are particularly difficult since these mutations often involve large number of genes. An alternative method to identify genes involved in cancer development or progression is insertional mutagenesis in mice[160]. This method relies on the random insertion of genetic elements called transposons in the genome. These transposons can interfere with gene transcription leading to either inactivation or activation of the gene. Integration of transposons near/into oncogenes and tumor suppressor genes can result in tumor development. To establish which genes are affected by transposon insertions, the location of insertions needs to be determined. The location of insertions can be determined using DNA or RNA derived from the generated tumors[161,162]. Analysis of multiple tumors allows one to determine common insertion sites which are likely to contain candidate driver genes.

Several insertional mutagenesis systems have been developed over the years. The initial insertional mutagenesis screen were conducted using slow transforming retroviruses[163,164]. While successful, these systems mainly identified oncogenes and were limited to tissues that could be readily infected[165]. These limitations were circumvented when DNA transposons were engineered to work in mammalian cells[166,167]. The most frequently used systems in mice are the *Sleeping Beauty* (SB) and *piggyBac* (PB) systems[168–171]. They are two-part systems that rely on a concatemer of transposons and an enzyme called transposase. The transposase facilitates excision of transposons and the integration into another part of the genome. The transposons have been engineered to contain splice acceptor sites followed by splice donor sites in both orientations, allowing for premature termination of transcription resulting in loss of gene expression or the expression of a truncated version of the protein. The transposon also

harbor a unidirectional promoter sequence upstream of a splice donor, enabling gene expression only if the transposon is inserted in the sense orientation. In this case, the orientation of the inserted transposon can provide a hint whether the gene is an oncogene or a tumor suppressor gene. One limitation of insertional mutagenesis is that it is not possible to generate point mutations. Overall, insertional mutagenesis screens in mice have proven to be a powerful tool for the discovery of genes involved in tumor development[171–174].

Chapters of this thesis

In this thesis, we made use of genetically engineered mouse models and in vivo insertional mutagenesis to identify drivers of ILC. Using lineage tracing and intravital imaging, we discovered the consequences of E-cadherin loss in luminal mammary epithelial cells and the requirement for rebalancing of actomyosin contractility to drive ILC development. We used in vivo insertion mutagenesis to discover new mechanisms of resistance against FGFR inhibition.

In **Chapter 2**, we identified novel drivers of ILC by combining insertional mutagenesis with germline mouse modeling. We introduced the Sleeping Beauty system in mice that have mammary gland specific loss of E-cadherin. We showed that insertional mutagenesis in these mice results in the formation of ILCs that resemble human ILC. Analysis of the common insertion sites in these tumors revealed mutually exclusive insertions in a group of four genes, indicating a shared mechanism of action. Transposon insertions in *Ppp1r12a/b* (MYPT1/2) and *Trp53bp2* (ASPP2) resulted in the expression of truncation variants while insertion in *Myh9* resulted in reduced protein expression. We validated that alterations in these genes are indeed sufficient to induce ILC when combined with E-cadherin loss. Three of these four genes are involved in actomyosin contractility implicating this pathway as an important component of ILC development.

In **Chapter 3,** we delve deeper into the consequences of E-cadherin loss in the mammary epithelium and the requirements for ILC development. By combining our conditional E-cadherin knockout mouse models with a GFP reporter, we were able to identify the fate of luminal murine mammary epithelial cells (MMECs) that lose E-cadherin. We discovered that E-cadherin loss not only results in luminal extrusion followed by apoptosis as reported earlier, but also basal extrusion allowing survival of E-cadherin deficient

MMECs in the fibrous stroma. The basally extruded cells formed clusters of cells but did not progress into tumors over time. We discovered that increased actomyosin contractility inhibits adhesion and survival on fibrillar collagen and invasion into the mammary stroma. Expression of truncated MYPT1 partially reduced actomyosin contractility of E-cadherin deficient MMECs, resulting in rapid ILC formation.

In **Chapter 4,** we investigated the molecular mechanism underlying ILC formation by truncated ASPP2 (*Trp53bp2*). We show that expression of truncated ASPP2 causes actomyosin relaxation similar to truncated MYPT1, enabling the survival and adhesion of E-cadherin deficient MECs on stiff substrates. Expression of truncated ASPP2 also decreased tumor related survival compared to tumors induced by expression of truncated MYPT1. We found that truncated ASPP2 not only decreases actomyosin relaxation but also induces activation of YAP1, which enhances tumor growth. Activation of YAP1 by truncated ASPP2 enhances tumor growth but is not required for tumor initiation.

In **Chapter 5,** we used insertional mutagenesis to identify mechanisms of resistance to the FGFR inhibitor AZD4547. Tumors with active FGFR signaling were initially sensitive to FGFR inhibition but eventually developed resistance to AZD4547. Analysis of the resistant tumors yielded multiple candidate genes for resistance. We were able to identify 4 mechanisms of resistance, mostly converging on reactivation of the canonical MAPK-ERK pathway. Two of these mechanisms, inactivation of RASA1 and activation of the drugs efflux pump ABCG2, were only found by *de novo* transposon insertions in these genes.

In **Chapter 6** I discuss the findings of this thesis and put them into perspective. I also discuss some of the remaining issues and new questions.

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