Characterizing the disruption of tissue organization during early breast cancer development

Or

"How to be an oncogene"

A thesis by

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Abstract

Breast cancer is a common disease affecting women worldwide, and although overall mortality is decreasing, incidence is increasing. The latter is partially due to better detection methods identifying lesions earlier, at pre-cancerous stages which are then resected. Since not all pre-cancerous lesions will progress to malignancy, I sought to better understand these earlier stages of breast cancer.

Using human breast biopsy samples, as well as inducible the polyoma middle T (MIC) *in vivo* mouse model and organotypic cultures, I report in this thesis diverse mechanisms that contribute to loss of cell and tissue polarity in the development of carcinoma. We identified that a major mechanism observed in generating solid ducts involved progressive loss of cell polarity. This occurs through an initial increase in baseline proliferation rates, followed by altered cell division orientation. Next, the lumen collapses through reduced RhoA/myosin II activity at the apical membrane. Moreover, loss of tissue and cell polarity was reversible upon removal of oncogene stimulation.

Furthermore, we found that depletion of Src tyrosine kinase in the MIC mouse model fails to produce mammary tumours. The mammary glands form enlarged ducts that fail to generate stratified epithelia by re-establishing proper mitotic spindle orientation, with no direct effect on proliferation. To examine if disrupted spindle orientation is necessary for tumour progression, we depleted the spindle orientation regulator LGN from the mammary epithelium. In normal tissue, $LGN^{-/-}$ glands resulted in randomized mitotic spindles, but was not sufficient to induce tumours or even disrupt tissue organization. However, LGN-deficient mammary glands unblocked tumour formation in MIC/$Src^{-/-}$ mice. This supports that spindle mis-orientation is a necessary and early step in breast cancer initiation.

Together, these results demonstrate dynamic event of epithelial remodeling during the development of carcinoma, and reveal a previously unappreciated mechanism involving progressive polarity loss, manipulation of oriented cell division, and luminal collapse as key characteristics.

Résumé

Le cancer du sein est une maladie courante qui touche les femmes du monde entier et bien que la mortalité globale diminue, son incidence augmente. Cette dernière est partiellement due à de meilleures méthodes de détection identifiant les lésions aux stades précancéreux, qui seront ensuite réséqués. Comme toutes les lésions précancéreuses ne dégénèrent pas en tumeur maligne, j'ai cherché à mieux comprendre ces stades antérieurs du cancer du sein.

En utilisant des biopsies de glandes mammaires humaines, ainsi que des cultures organotypiques et le modèle murins polyoma middle T inductible (MIC), je rapporte dans cette thèse de divers mécanismes qui contribuent à la perte de polarité cellulaire et tissulaire dans le développement du carcinome. Nous avons identifié qu'un mécanisme majeur observé dans la génération de canaux solides impliquait une perte progressive de polarité cellulaire. Cela se produit par une augmentation initiale des taux de prolifération de base, suivie d'une orientation modifiée de la division cellulaire. Ensuite, le lumen s'effondre par l'activité réduite de RhoA / myosine II au niveau de la membrane apicale. De plus, la perte de polarité tissulaire et cellulaire était réversible lors du retrait de la stimulation oncogénique.

En outre, nous avons constaté que l'invalidation de la tyrosine kinase Src dans la souris MIC ne produit pas de tumeurs. Les glandes mammaires forment des canaux élargis sans épithélium stratifié en rétablissant une orientation correcte du fuseau mitotique, sans effet direct sur la prolifération. Pour examiner si l'orientation perturbée du fuseau est nécessaire à la progression d'une tumeur, nous avons de plus invalidé le régulateur d'orientation du fuseau mitotique LGN. Dans les tissus normaux, les glandes $LGN^{-/-}$ ont résulté en fuseaux mitotiques randomisés, mais n'étaient pas suffisants pour induire des tumeurs ou même pour perturber l'organisation des tissus. Cependant, les glandes mammaires déficientes en LGN n'ont pas bloqué la formation de tumeurs chez les souris MIC/$Src^{-/-}$. Cela confirme

que la perturbation de l'orientation du fuseau mitotique est une étape nécessaire et précoce de l'initiation du cancer du sein.

Ensemble, ces résultats démontrent un événement dynamique de remodelage épithélial au cours du développement du carcinome et révèlent un mécanisme jusqu'alors méconnu comprenant une perte de polarité progressive, la manipulation de la division cellulaire orientée et un effondrement du lumen comme caractéristiques clés.

Acknowledgements

I dedicate this thesis to my two grandmothers, Sania Dbouk and Atifa Habbal, who passed away during my studies. I miss you!

I have always been curious about science, even as a child, so I would first like to thank my beautiful parents Safi and Roula for constantly encouraging me to seek knowledge, and never ceasing to answer all the weird questions I asked growing up. Thank you also for constantly supporting me throughout this process, along with my brother Jad and sister Nada. And a special thanks goes out to my mother and sister who suffered with me through all the ups and downs that this degree has put me through, and to all my amazing friends, too many to name, who have listened to me rant and cheered me up, each in their own way. I love you all.

I would also like to thank all my teachers who have instilled and driven this passion for science within me, especially my two high school teachers, Mr. Yves Mouffron who helped me discover that a career in health sciences is truly my path in life, and Mr. Abdelkarim Jarjini for teaching me to push myself and never give up.

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This has truly been an amazing experience, through which I have not only learned science, but also grown into the person I have always wanted to be, I would not trade it in for the world!

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Contribution to Original Knowledge

During my studies, I have made five main contributions to original knowledge that I highlight in this thesis.

1) I have identified a frequent, yet previously unappreciated, cellular mechanism of ductal filling during breast cancer, in which ducts stratify and the lumen collapses through a loss of apical tension, rather than shedding of cells into the lumen;

2) I established that epithelial stratification occurs through asymmetric divisions generating non-polarized cells that ultimately form invasive cancers;

3) I determined that disruption of cell division orientation is required for breast tumour initiation;

4) I discovered loss of epithelial organization is reversible, demonstrating remarkable tissue plasticity in early breast cancer lesions.

5) I developed a CRISPR/Cas9 strategy to target genes in the mammary epithelium to study cancer initiation and progression.

Contribution of Authors

All the work presented in this thesis is my original research, conceived, designed, performed, and analyzed by myself, supervised by Dr. Luke McCaffrey. Published work from two articles in the journals Oncogene (Halaoui and McCaffrey 2015) and Genes & Development (Halaoui et al. 2017) have been used throughout the thesis.
Christina Kalos contributed to the imaging and data analysis of figures 3.1, 3.2, and 3.3.
Dr. Carlis Rejon aided in the performance of the orthotopic transplants in figure 2.5C.
Dr. Sudipa Chatterjee performed the TUNEL staining and quantification from figure S2.5H.
Joseph Szymborski performed the analysis in figures 2.4B, E, and G.
Siying Li provided technical assistance with measuring spindle orientation angles in figure 3G.
Dr. William Muller kindly provided us with the MIC and MIC/Src$^{-/-}$ mouse models studied.
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Refereed Journal Publications

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*co-first authors

Halaoui R, Rejon C, Chatterjee SJ, Szymborski J, Meterissian S, Muller WJ, Omeroglu A, McCaffrey L. "Progressive polarity loss and luminal collapse disrupt tissue organization in carcinoma". *Genes and Development,* 2017.

W Lee, N Kalashnikov, S Mok, **R Halaoui**, E Kuzmin, AJ Putnam, S Takayama, M Park, L McCaffrey, R Zhao, RL Leask, C Moraes. "Dispersible hydrogel force sensors reveal patterns of solid mechanical stress in multicellular spheroid cultures". *Nature Communications,* 2019

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Halaoui R*, Kalos C*, Muller WJ, McCaffrey LM. "Spindle misorientation is an essential step in cancer initiation". *Manuscript in preparation, 2019.*

Refereed Conference Abstracts

R Halaoui, S Li, WJ. Muller, LM McCaffrey. "Characterization of polarity protein changes in mammary tumour progression". The Terry Fox Research Institute Annual Scientific Meeting (TFRI ASM) *(2013, Ottawa, ON)*

R Halaoui, S Li, WJ Muller, LM McCaffrey. "Characterizing Epithelial Cell Polarity Changes During Breast Cancer Progression". The Terry Fox Research Institute Annual Scientific Meeting (TFRI ASM) *(2014, Montreal, QC)*

R Halaoui, J Szymborski, S Li, N Bertos, M Park, S Meterissian, WJ Muller, A Omeroglu, LM McCaffrey. "Mechanisms of Breast Cancer Initiation". The 3rd Canadian Cancer Research Conference (CCRC) *(2015, Montreal, QC)*

R Halaoui, C Rejon, J Szymborski, S Meterissian, WJ Muller, A Omeroglu, LM McCaffrey. "Mechanisms of Breast Cancer Initiation". The Canadian Student Health Research Forum (CSHRF) *(2017, Winnipeg, MB)*

- Nominated as top 10% of PhD students from the Department of Medicine at McGill University
- Won Gold Award

R Halaoui, C Rejon, J Szymborski, S Meterissian, WJ Muller, A Omeroglu, LM McCaffrey. "Epithelial Polarity Remodeling and Luminal Collapse Generate Solid Ducts in Early Mammary Tumourigenesis. The 4th Canadian Cancer Research Conference (CCRC) *(2017, Vancouver, BC)*

- Selected to give s 15min oral presentation given during the first concurrent session of the conference entitled "cellular Mechanisms of tumour Cell Migration/Invasion"

Chapter 1: Introduction and Literature Review

Epithelial cells form organized structures including tubes, alveoli and stratified sheets. Robust cell-cell and cell-matrix adhesions create permeability barriers and maintain mechanically durable tissues (Carthew 2005; Nelson et al. 2013). Epithelial cells exhibit asymmetric distribution of protein, lipid, and RNA macromolecules, a property called cell polarity, which organizes the plasma membrane into discrete compartments. In cells with apical-basal polarity, the apical membrane faces the central lumen of a duct whereas the basolateral membrane contacts adjacent cells and the basement membrane, and is separated from the apical membrane by tight junctions (Figure 1.1). A key function of cell polarity is to spatially organize signaling pathways within cells, which provides an important mechanism for cells to interpret and integrate cues from their surrounding microenvironment to control proliferation, apoptosis, metabolism, differentiation, and motility (Martin-Belmonte et al. 2008; Jansen et al. 2009; St Johnston and Ahringer 2010; Laprise and Tepass 2011; Martin-Belmonte and Perez-Moreno 2011; McCaffrey and Macara 2011; Nance and Zallen 2011; Tepass 2012).

Epithelial cells line the major organ surfaces and are the origin for 80-90% of all human cancers, called carcinoma. Epithelial cancers display a loss of growth control, tissue organization, and cell polarity (Bostwick and Cheng 2012; Siziopikou 2013). Carcinogenesis initiates in epithelial tissues that exhibit an otherwise normal histology and progress into structures with highly disrupted cell and tissue organization. However, the mechanisms that underlie the early development and progression of carcinoma remain poorly characterized. In my doctoral studies, I have used breast cancer as a model to study early stages of carcinogenesis, focusing on understanding tissue structure changes that occur during cancer initiation, in order to better appreciate the process. Increasing our knowledge and understanding of how cancer initiates may reveal new opportunities for preventative therapies.

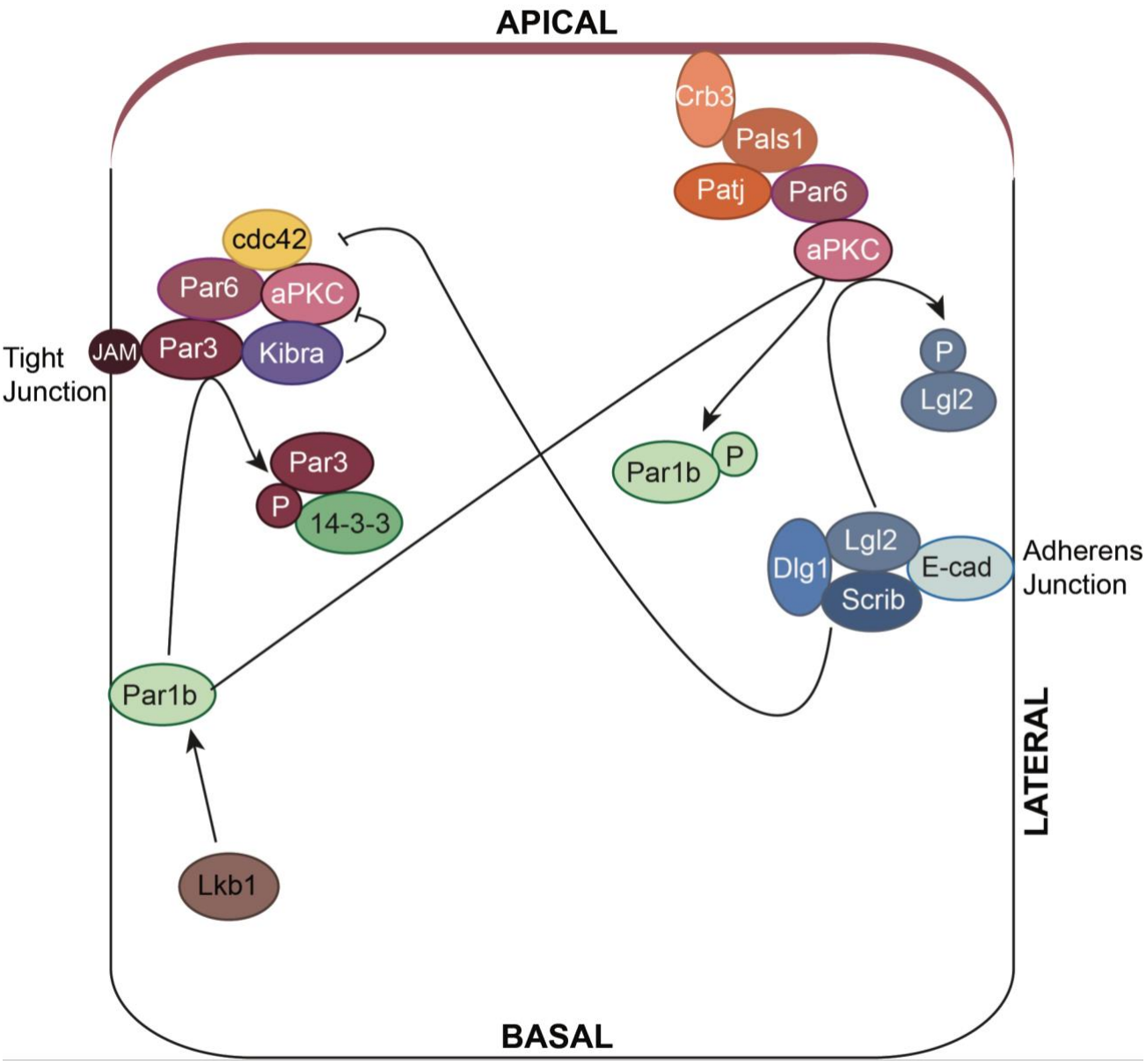

Figure 1.1: Core regulators of cell polarity

Core regulators of cell polarity. Apical–basal polarity complexes dynamically regulate polarization by mutual exclusion. Thus, epithelial homeostasis is maintained by ensuring proper localization and function of polarity protein complexes. Major polarity complexes include the Par complex (red/pink), Crumbs complex (orange), and Scribble complex (blue).

1.1 Breast Cancer Pathophysiology

1.1.1 Mammary gland anatomy and development

The mammary gland is a complex secretory organ made up of a network of branched milk ducts interspersed in stromal adipose tissue which itself is infiltrated with vascular endothelial cells, fibroblasts, and immune cells (Macias and Hinck 2012). Each mammary duct consists of a bilayer of epithelial cells: an inner secretory layer of polarized luminal cells surrounded by contractile myoepithelial cells, which contact the basement membrane (BM) (Macias and Hinck 2012). At birth, there exists a rudimentary ductal structure which resumes development at puberty; structures termed terminal end buds located at the tips of the growing duct commence expansive proliferation, invading and bifurcating into the adipose tissue (Macias and Hinck 2012). Then, secondary branches sprout from the ducts forming a tree-like structure that fills up about 60% of the tissue, leaving space for a third round of expansion during pregnancy (Macias and Hinck 2012). In humans, the terminal part of the duct is called an acinus, which is a ductal-lobular unit embedded in fibroblastic rich stroma, unlike the adipose rich stroma that surrounds rodent ductal branches (Macias and Hinck 2012).

This complex network of ducts undergoes dynamic regulation to uphold its morphology. Tension at the apical membrane works to maintain lumen shape and size by modulating the actin cytoskeleton through RhoA family GTPases, thus supporting tissue integrity and morphogenesis (Martin and Goldstein 2014). Myosin II associates with actin filaments and contracts following phosphorylation of its light-chain by Rho-associated protein kinase (ROCK), which itself is activated by RhoA in its active GTP-bound state (Martin and Goldstein 2014). RhoA activity is regulated at the apical membrane through guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which enable an appropriate balance of RhoA in its inactive GDP- and active GTP-bound states (Martin and Goldstein 2014). Importantly, altered RhoA activity has been associated with both anti- and pro-

tumourigenic functions, indicating it behaves as an oncogene or tumour suppressor in different contexts (Zandvakili et al. 2016).

1.1.2 Early breast cancer progression

Breast cancer is the most common malignancy affecting women worldwide, and although breast cancer-associated mortality has reduced, incidence is increasing (Global Burden of Disease Cancer et al. 2015).

Breast cancer is a state in which the mammary gland becomes disorganized due to the loss of growth control and differentiation of these epithelial cells (Chatterjee and McCaffrey 2014). It presents in two types depending on the location of the cells of origin: The infamous ductal carcinomas account for about 85% of all breast cancer cases, originate from cells located in the mammary duct, and are more aggressive; while the less prevalent lobular carcinomas originate from cells in the lobules, are less aggressive with a high survival rate, but are more difficult to detect by screening (McCart Reed et al. 2015). That is why I chose to focus my research on the more prevalent and more aggressive ductal carcinomas.

Ductal breast cancers progress stepwise, leading to gradual tissue disorganization commonly referred to as luminal filling. It initiates through precursor stages including flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS), which are high risk factors for development of invasive ductal carcinoma (IDC) (Ellis 2010; Lopez-Garcia et al. 2010; Bombonati and Sgroi 2011b). FEA and ADH are precursor lesions identified by the expansion of the ductal size and shape, then multilayering of the luminal cells creating a stratified epithelium. Cells continue to divide and fill up the luminal space, thus progressing to DCIS. Progression to IDC is characterized by the breach of the basement membrane, allowing the cancer cells to invade and metastasize (Figure 1.2)

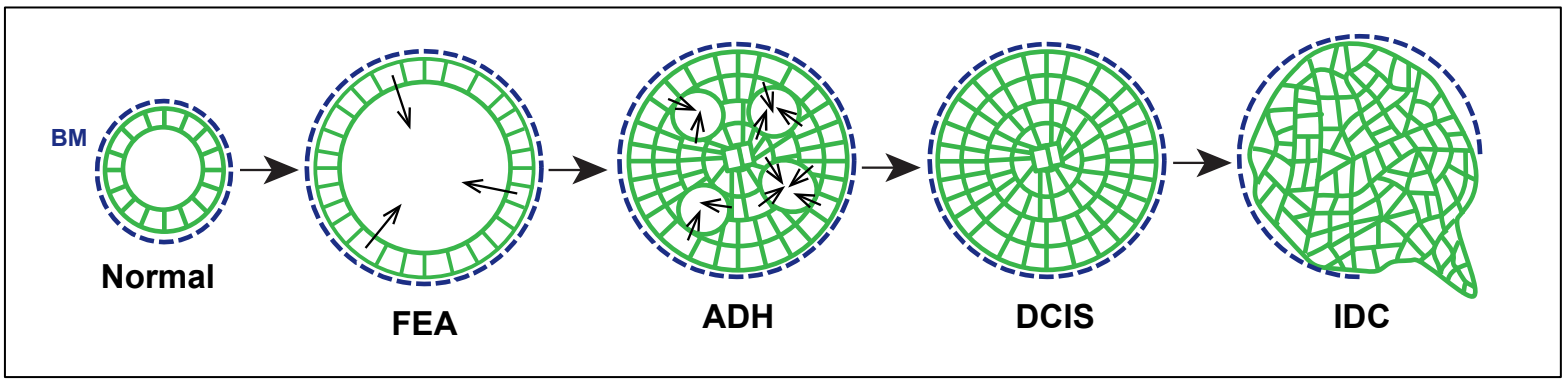

Figure 1.2: Early Breast Cancer Progression

Breast Cancer is a step-wise process, initiating ductal dilation at the stage of flat epithelial atypia (FEA), then progressing to atypical ductal hyperplasia (ADH) where the ducts become stratified, followed by the appearance of solid ducts at ductal carcinoma in situ (DCIS), and ending in the potentially lethal stage of invasive ductal carcinoma (IDC) marked by the breach of the basement membrane.

(Sgroi 2010; Bombonati and Sgroi 2011a). We know very little about the transitions between each of the stages of progression, especially during early preinvasive lesions.

In some cases, structure disorganization may be benign and can be identified by traditional histology. The management of these lesions is controversial since their presence increases the risk of patients developing cancer (Sickles 1995). There have been several large-scale prospective studies that established the validity of managing these lesions, concluding periodic mammography surveillance as a safe and effective approach, instead of immediate resection (Sickles 1995). However, this relies on constant monitoring of the lesions without proper knowledge of whether signs exist on how to distinguish the ones that will progress from ones that will not.

Indeed, cancerous lesions are often detected at DCIS, due to improved biomedical technologies with higher resolution, as well as better public awareness of the importance of screening (Chatterjee and McCaffrey 2014). At this point, epithelial polarity is often considered to be lost when ducts fill, although whether or not individual cells are polarized has yet to be examined. Pure DCIS itself is not considered imminently lethal, as it is a well encapsulated mass that, if removed, has close to a 100% survival rate (Roses et al. 2011). From the few studies when small numbers of DCIS where misdiagnosis led to omission of surgery, 14–53% progressed to IDC (Gorringe and Fox 2017). However, it is currently not possible to predict which lesions will progress to IDC.

1.1.3 Breast cancer subtypes

Breast cancer is a heterogeneous group of diseases; therefore, it is divided into distinct molecular subtypes with prognostic differences in patient outcomes, depending on histological features, molecular expression signatures, and clinical outcomes (Eroles et al. 2012). There are multiple classification types, the most prevalent method is to classify histologically in the clinic based on the

presence or absence of hormone receptors estrogen (ER) and progesterone (PR), and the human epidermal growth factor receptor 2 (HER2), as well as the prevalence of proliferation marker Ki67. This results in the stratification of the disease into the following subtypes (Anderson et al. 2014): (1) Accounting for 40% of the cases, Luminal A expresses ER+ and PR+, is HER-, and has low Ki67. It exhibits the best prognosis of all subtypes as it is slow growing, has a low recurrence rate, a high survival rate, and is responsive to endocrine therapy. (2) Luminal B is also hormone receptor positive, but either HER2+ or HER- with high Ki67. It has a 10-20% prevalence, and with its high proliferation rates comes in at a worse prognosis than Luminal A, but still responds to endocrine therapy. (3) About 10% of cases present as having HER2 overexpressing subtype; as its name clearly states, is HER2+, ER-/PR-, and tend to grow and spread more aggressively. It has a poor short-term survival, but targeted therapies do exist and are improving the outcomes of affected patients. (4) Lastly, this subtype is termed Triple Negative Breast Cancer (TNBC) as it does not express any of the three receptors above-mentioned. It accounts for 10-20% of cases, presents in younger patients as it is fast growing and aggressive, and has a poor prognosis because of its high rates of recurrence after surgery and lack of targeted therapy (Anderson et al. 2014).

Evidence supports that these subtypes are already present at the DCIS stage, indicating that drivers of different subtypes may act earlier during disease progression (Hannemann et al. 2006; Allred et al. 2008; Lopez-Garcia et al. 2010; Tang et al. 2016). The occurrence of different molecular subtypes at these early stages could be explained by the existence of distinct tumour pathways, such as a unique cell of origin for each subtype, and/or subtype-specific genetic and epigenetic events. In addition, DCIS is heterogeneous, and multiple clones harboring different genetic aberrations may exist (Lopez-Garcia et al. 2010). This genetic instability could be one of the predictors of progression, as is the case in oesophageal adenocarcinoma (Maley et al. 2004).

In general, hormone receptor-positive cancers (HR+) carcinoma represent the majority of breast cancers. They generally have a better prognosis than other subtypes in the short-term, survival declines over 10 years, and due to the high prevalence of this subtype, it still accounts for a majority of breast cancer-related deaths (Haque et al. 2012), indicating that it is still important to invest efforts in studying this subtype, especially at early stages. In fact, the cellular and molecular mechanism by which carcinoma develop in the breast and other epithelia remain poorly understood, which impedes progress to prevent cancer development.

1.1.4 Treatment methods

Currently, there is routine screening, and treatment methods cater to most breast cancer subtypes, but a major goal is to develop improved personalized medicine through more specific treatments with fewer side-effects, and to overcome resistance to existing drugs, which is unfortunately a common occurrence. The guidelines put together by the Canadian Task Force on Preventative Health Care for breast cancer screening, updated in 2018, no longer recommend monthly self-exams for women, as this failed to show any impact on breast cancer associated mortality. Screening with mammography is the current method of choice, which is recommended every 2-3 years for women over 50, and every year for women with high-risk familial cancers as of age 30.

As for treatment methods, it is recommended that luminal subtypes are resected, and treated using endocrine therapy if found early, or chemotherapy if the disease is already metastatic (Bonotto et al. 2017). Targeted therapies are also used for HER2+ breast cancers, with the developments of drugs such as lapatinib and trastuzumab that inhibit the Her2 receptor driving this subtype (Bonotto et al. 2017). As for TNBC, classical cytotoxic chemotherapy is the recommended first-line treatment, to which it is initially responsive; however, a high rate of recurrence is observed, most of which is metastatic

(Griffiths and Olin 2012). Currently, no targeted therapies exist, although many possibilities are being investigated (Griffiths and Olin 2012). These possibilities include PARP inhibitors, which take advantage of synthetic lethality (Lord and Ashworth 2017), EGFR inhibitors (Nakai et al. 2016), and anti-angiogenic agents (Guo et al. 2016), to which resistance to therapy or low response rates are observed. More recent avenues include several applications in immunotherapy (Tolba and Omar 2018).

However, diagnosis of early lesions remains problematic: every lesion found in women by mammography is biopsied and assumed to have the potential to progress; it is thus is often removed and treated with drugs that have severe side effects, even if the lesion is still at an early pre-invasive stage and may not be at high risk for progression. When early lesions are not removed, the women are considered at high risk of developing breast cancer and could qualify for preventative treatments such as tamoxifen (Freedman et al. 2003; Ravdin 2010). However, the serious side-effects of these drug have resulted in few women taking advantage of these strategies. Therefore, we are in need of stratification methods for pre-invasive lesions in order to differentiate between ones that are likely to progress, and ones that are dormant, and it would be advantageous if we were able to stop the progression of detected lesions before they become malignant.

1.2 Polarity and Cancer

1.2.1 Regulators of cell polarity

A conserved group of core polarity proteins assemble into complexes to establish and maintain apical-basal polarity (Figure 1.1). The apical Crumbs complex consists of Crumbs3 (Crb3), a trans-membrane protein that associates with the multi-domain scaffolding proteins: protein associated with Lin-7 (Pals1) and Pals1-associated tight junction protein (Patj, also called InaD-like, INADL) (Laprise and Tepass 2011; Pocha and Knust 2013).

The Par complex consists of Par3, Cdc42, Par6, and atypical protein kinase C (aPKC), with multiple isoforms of each (Par3, Par3L, Par6α, Par6β, Par6γ, aPKCι, aPKCζ) (Goldstein and Macara 2007; McCaffrey and Macara 2009b; Nance and Zallen 2011). Par3 is a multi-domain scaffolding protein that localizes to tight junctions through junction adhesion molecule A (JAM-A) and phospholipids (Ebnet et al. 2001; Krahn et al. 2010). Par3 interacts directly with Par6, an adaptor protein that is constitutively bound to aPKC and can regulate the kinase's activity, and in turn aPKC stabilizes Par6 and protects it from degradation (Durgan et al. 2011). The polarity complexes are dynamic and Par6 also binds Crb3 and Pals1, which anchors Par6 to the apical domain (Hurd et al. 2003; Morais-de-Sa et al. 2010; Hayase et al. 2013). Other Par proteins include Liver kinase B1 (Lkb1, also called Par4), which phosphorylates and activates basolateral microtubule affinity-regulating kinase 2 (MARK2, also called Par1b). Activated Par1b phosphorylates Par3 to enable binding of Par3 to 14-3-3ζ (also called Par5), which disrupts the interaction of Par3 with the plasma membrane and therefore excludes the Par complex from the basolateral membrane (Benton and St Johnston 2003; Hurov and Piwnica-Worms 2007; Yang et al. 2012). Conversely, apically restricted aPKC directly phosphorylates Lethal Giant Larvae Homolog 2 (Lgl2), Par1b, and other basolateral proteins to exclude them from the apical domain and maintain apical identity (Plant et al. 2003; Suzuki et al. 2004; Smith et al. 2007). Cdc42 and Kibra associate with the Par complex to positively or negatively regulate aPKC activity, respectively, which controls the size of the apical membrane (Joberty et al. 2000; Yoshihama et al. 2011).

The Scribble complex consists of the multi-domain scaffolds Scribble (Scrib) and Discs large (Dlg; five isoforms), as well as Lgl2 (HUGL in humans; two isoforms), which is implicated in intracellular trafficking (Humbert et al. 2006). Basolateral localization of Scrib is dependent on cell-cell adhesions mediated by E-cadherin and reciprocally, Scrib maintains E-cadherin-mediated adhesions to specify the

basolateral membrane and oppose apical membrane identity (Bilder and Perrimon 2000; Navarro et al. 2005; Qin et al. 2005).

1.2.2 Disrupted cell polarity in cancer

Accumulating evidence indicates that apical-basal polarity is a barrier to carcinogenesis (Halaoui and McCaffrey 2015). Formal evidence that disrupting cell polarity proteins contributes to tumourigenesis initially came from *Drosophila* and revealed an intricate relationship between tumour suppressor polarity proteins, oncogenes, cell survival and proliferation (reviewed in (Vidal and Cagan 2006; Fan and Bergmann 2008; Bergstralh and St Johnston 2012; Elsum et al. 2012; Levayer and Moreno 2013). Recent studies in mammalian cell lines and mouse models have built upon this foundation to further our understanding of how polarity proteins contribute to cancer progression.

Loss of apical-basal polarity is an early event in epithelial cancers and can occur at pre-invasive stages (Huang and Muthuswamy 2010a; Bostwick and Cheng 2012; Siziopikou 2013; Huebner et al. 2014). Indeed, depletion of polarity proteins Par3 or Scrib is sufficient to disrupt cell polarity and epithelial architecture *in vivo* in mice that resembles pre-invasive lesions observed in patients (Aranda et al. 2006b; Godde et al. 2014; Huebner et al. 2014). However, whether loss of cell polarity at the earliest stages is widespread in human carcinoma has not been established.

Several polarity proteins have altered expression due to gene amplification, deletion or epigenetic regulation, which frequently correlates with disease progression (Table 1.1). Transcriptional regulation of polarity genes is also a means of altered expression in tumour cells. For example, aPKCι has Elk-1 and Gli binding sites in its promoter, which regulate its expression downstream of Mek/Erk and Hedgehog signaling in chronic myelogenous leukemia and basal cell carcinoma, respectively (Gustafson et al. 2004; Atwood et al. 2013). Moreover, Zeb1 and Snail directly suppress expression of

CRB3, *LGL2*, and *INADL* during epithelial-to-mesenchymal transitions (Russ et al. 2012; Kashyap et al. 2013).

A key mechanism by which polarity proteins function is by spatially restricting signal transduction components; therefore, mutations that alter the localization or protein-protein interactions of polarity proteins may also affect downstream signaling. For example, Scrib is mislocalized in ductal carcinoma *in situ* (DCIS) and expression of a mutant that does not correctly localize to the plasma membrane disrupts 3D acini formation and fails to block Ras-induced invasion of MCF10 cells (Zhan et al. 2008; Elsum and Humbert 2013). However, mis-localized Scrib retains the ability to suppress Ras-induced anchorage-independent growth, indicating that some, but not all, tumourigenic effects of Scrib may be regulated by localization (Elsum and Humbert 2013). Furthermore, intragenic deletions in *PAR3* that remove the PDZ domains or key phosphorylation sites were identified in squamous carcinoma cells, and re-expression of full-length Par3 restores tight junction formation, reduces proliferation, and inhibits migration (Rothenberg et al. 2010). Moreover, the most frequent mutation in aPKCι occurs at Arg471, and expression of an aPKCι-R471C mutant reduces Lgl2 phosphorylation and induces polarity defects in 3D cultures (Linch et al. 2014). Additionally, a S514F mutation in aPKCζ impairs aPKCζ kinase activity and increases the tumourigenicity of Ras-transformed fibroblasts (Galvez et al. 2009). In sum, polarity protein expression and function are disrupted through multiple events in cancer, which is permissive to cancer development by deregulating diverse signaling pathways.

Table 1.1: Alterations in polarity protein expression in human cancers

Common Name	Gene Name	Alteration	Type of Cancer	Phenotype	References
Par3	*PARD3*	Gene deletion Down-regulation	Esophageal squamous carcinoma	Cell junctions disruption	(Zen et al 2009)
		Deletion	Glioblastoma, esophageal, head and neck lung cancers		(Rothenberg et al 2010)
		Down-regulation	Breast cancer	Promotion of tumorigenesis and metastasis. Cell-cell cohesion compromised, promotion of metastasis	(McCaffrey et al 2012, Xue et al 2012)
		Over-expression	Renal cancer	Poor prognosis	(Dugay et al 2013)
Par6	Par6 (protein)	Phosphorylation	BRCA1 associated tumor tissues	Protein phosphorylation, lumen filling, cell junction disruption, increased metastasis	(Viloria-Petit et al 2009)
	Par6 (protein)	Over-expression	Lung (NSCLC)	Stroma: good prognosis	(Al-Saad et al 2008)
	PARD6B	Amplification Over-expression	Breast cancer	Cell hyper-proliferation. Amplification in luminal subtypes.	(Cunliffe et al 2012, Nolan et al 2008)
	PARD6G	Deletion	Adrenal and lung cancers		(Rothenberg et al 2010)
aPKCζ	*PRKCZ*	Over-expression	Hepatocellular carcinoma		(Tsai et al 2000)
		Over-expression	Urinary bladder cancer	Invasive tumor	(Langzam et al 2001)
		Down-regulation	Superficial bladder cancers	Increased tumor recurrence	(Namdarian et al 2013)
		Down-regulation	Colorectal cancer	Poor prognosis	(Ma et al 2013)
		Over-expression	Squamous cell carcinoma or head and neck tumors	Protein phosphorylation, increased cell proliferation, squamous carcinoma	(Cohen et al 2006)
		Over-expression	Pancreatic cancers		(Evans et al 2003)
		Over-expression	Breast cancer	Increased cell proliferation	(Nolan et al 2008)
		Over-expression	Prostate cancer	Over-expression of a splice variant Predictive marker for survival	(Yao et al 2010, Yao et al 2012)
		Mislocalization	Ovarian cancer		(Grifoni et al 2007)
aPKCι/λ	*PRKCI*	Over-expression	Hepatocellular carcinoma	Protein phosphorylation, metastasis and invasion	(Du et al 2009)
		Amplification Over-expression	Lung (NSCLC)	Cell transformation, poor prognosis	(Regala et al 2005b)
		Over-expression	Pancreatic cancers	Malignant transformation, prognostic marker	(Kato et al 2013)
		Amplification Over-expression Mislocalization	Ovarian cancer	Low survival	(Eder et al 2005)
		Amplification	Esophageal cancer	Associated with lymph-node metastasis.	(Yang et al 2008)
		Over-expression Mislocalization	Breast cancer	Increased tumor size, invasion, and metastasis.	(Kohjima et al 2002)
		Over-expression	Basal cell carcinoma	GLI regulator, activation of HH signaling	(Atwood et al 2013)
Scrib	*SCRIB*	Mislocalization Down-regulation	Colon adenocarcinoma	Neoplastic colon mucosa	(Gardiol et al 2006)
		Mislocalization Down-regulation	Cervical cancers	HPV-positive cervical high grade squamous intraepithelial lesions, invasive cervical cancer	(Nakagawa et al 2004)
		Mislocalization Down-regulation	Endometrial cancer	Association with clinical stage, histopathological differentiation, and lymph node metastasis	(Ouyang et al 2010)
		Mislocalization Down-regulation	Breast cancer		(Zhan et al 2008)
		Over-expression	Breast cancer	Invasive phenotype, correlates with relapse and decreased survival	(Anastas et al 2012)
		Mislocalization Down-regulation	Prostate cancer	Levels and localization correlates with tumor stage, Gleason grade and PSA levels	(Pearson et al 2011)

Dlg	*DLG1*	Mislocalization Down-regulation	Cervical cancer	Protein targeted for degradation by HPV E6	(Gardiol D 1999, Lin Ht 2004, Watson Ra 2002)
		Mislocalization Down-regulation	Colon adenocarcinoma		(Gardiol et al 2006)
		Mislocalization Down-regulation	Cervical, colon and kidney cancers		(Cavatorta et al 2004)
	DLG2	Over-expression	Kidney (Oncocytoma, benign tumor)		(Zubakov et al 2006)
		Deletion	Cervical and lung cancers		(Rothenberg et al 2010)
	DLG3	Down-regulation	Colorectal cancer	Promotor methylation	(Feng et al 2012)
Lgl	*LLGL1*	Down-regulation	Lung, ovarian, prostate, breast and colon cancers		(Grifoni et al 2004)
		Down-regulation	Colorectal cancer		(Schimanski et al 2005)
		Exon Deletion	Hepatocellular carcinoma	Poor differentiation, larger tumor size	(Lu et al 2009)
		Down-regulation	Melanoma	Malignant phenotype	(Kuphal et al 2006)
	LLGL1 *LLGL2*	Down-regulation	Breast cancer	Overgrowth and luminal filling	(Russ et al 2012)
	LLGL2	Mislocalization Down-regulation	Gastric cancer		(Lisovsky et al 2009, Lisovsky M 2010)
		Down-regulation	Endometrial cancer	Loss of lgl2 induces/is caused by EMT. When transfected back, induces MET	(Kashyap et al 2012, Tsuruga et al 2007)
Patj	*INADL*	Down-regulation	Colon and breast cancer	ZEB1 represses expression of PATJ and other polarity proteins, leads to dedifferentiation and EMT	(Aigner et al 2007)
		Down-regulation	HPV	Degradation target of viral protein E6	(Storrs and Silverstein 2007)
Lkb1	*STK11*	Mutation	Gastrointestinal cancer		(Korsse et al 2013a, Park et al 1998)
		Mutation Deletion	Colorectal cancer		(Avizienyte et al 1998, Dong et al 1998)
		Mutation Down-regulation	Esophageal carcinoma	Enhanced Wnt signaling	(Liu et al 2013)
		Down-regulation	Head and neck cancer		(Ekizoglu et al 2013)
		Down-regulation	Hepatocellular carcinoma		(Huang Yh 2013)
		Mutation	Pancreatic cancers	Peutz-Jeghers Syndrome	(Korsse et al 2013b, Sato et al 2001, Su et al 1999)
		Down-regulation	Renal cancer	Growth advantage	(Duivenvoorden et al 2013)
		Mutation (inactivating)	Lung (NSCLC)		(Gonzalez-Sanchez et al 2013, Ji et al 2007, Koivunen et al 2008, Sanchez-Cespedes et al 2002)
		Down-regulation	Ovarian cancer	High grade histotypes, lower differential status	(Tanwar et al 2013, Wang et al 1999)
		Down-regulation	Cervical cancer	Poor prognosis	(Avizienyte et al 1999, Mack and Munger 2013)
		Down-regulation	Breast cancer		(Andrade-Vieira et al 2013, Forster et al 2000, Sobottka et al 2000, Sohn et al 2013, Zhuang et al 2013)
		Mutation Deletion	Testicular cancer		(Avizienyte et al 1998)
		Mutation Deletion	Melanoma		(Guldberg P 1999, Rowan et al 1999)
		Down-regulation	Brain cancer		(Sobottka et al 2000)

1.2.3 Tissue polarity

Epithelial ducts exhibit polarity at both cellular and tissue levels, both of which are disrupted in carcinoma. We have tackled the concept of apical-basal polarity above. Tissue polarity, on the other hand, is characterized by the presence of a central lumen formed by a layer of epithelial cells, and loss of the space during cancer progression is frequently referred to as luminal filling (Taraseviciute et al. 2010; Leung and Brugge 2012b; Venugopalan et al. 2014). One model proposes that luminal filling arises from cells shedding or migrating into the lumen to create solid structures (Taraseviciute et al. 2010; Leung and Brugge 2012b). This model was established in the MCF10A cell line, where cells are not fully polarized and do not have tight junctions, and depends on cells overcoming anoikis and stimulating proliferation in the lumen (Danes et al. 2008; Leung and Brugge 2012a; Pradeep et al. 2012). More recently, it was demonstrated in mouse models of breast cancer that misoriented cell divisions perpendicular to the plane of a mammary duct can contribute to stratification of luminal epithelial cells in the absence of cell shedding or migration into the lumen (i.e. luminal filling), however stratification is not sufficient to generate solid ducts (Godde et al. 2014; Shore et al. 2016). Therefore, there is controversy regarding the mechanisms that contribute to the generation of solid ducts in experimental models, and how this occurs in human patients is not understood.

1.2.4 Cell polarity signaling in cancer

1.2.4.1 Tumour suppressive functions of Lkb1

The first mammalian polarity protein to have a demonstrated role in cancer was Lkb1/Par4. Heterozygous mutations in *LKB1* cause Peutz-Jeghers Syndrome, in which patients develop gastrointestinal polyps and are predisposed to cancer in multiple tissues (Beggs et al. 2010; Herrmann et al. 2011). Heterozygous deletion of *Lkb1* in mice induces gastrointestinal polyps, but rarely develop

mammary tumours, whereas homozygous deletion of *Lkb1* in the mammary gland results in tumour formation in about 30% of mice following a long latency (Bardeesy et al. 2002; McCarthy et al. 2009). Furthermore, loss of *Lkb1* in the pancreas induces pancreatic intraepithelial neoplasia (PIN), which fails to progress to more advanced tumours (Lo et al 2012). Therefore, loss of *Lkb1* is sufficient to promote the early stages of cancer in multiple organs, but likely requires cooperating events for tumour progression. Indeed, in combination with Myc or Neu/ErbB2 oncogenes, deletion of *Lkb1* reduces mammary tumour latency compared to oncogene expression alone (McCarthy et al. 2009; Partanen et al. 2012; Andrade-Vieira et al. 2013).

Loss of Lkb1 deregulates multiple cellular properties that could contribute to tumour progression, including cell polarity, metabolism (see below), extra-cellular matrix deposition (see below), and Hedgehog (Hh) signaling. Hedgehog signaling has an important function during embryonic development and is deregulated in numerous cancer types (Briscoe and Therond 2013). Binding of Hh ligands to the receptor Patched relieves inhibition of the transmembrane protein Smoothened (Smo), which activates intracellular signaling complexes to stimulate gene-expression through Gli transcription factors. In human breast cancers, *LKB1* expression negatively correlates with Hh and depleting Lkb1 increases Hh, Smo and Gli expression (Zhuang et al. 2013) (Figure 1.3). Interestingly, knockdown of Lkb1 in MDA-MB-231 breast cancer cells enhances tumour growth in mammary fat pad xenografts, which is blocked by cyclopamine, a Smo inhibitor (Zhuang et al. 2013).

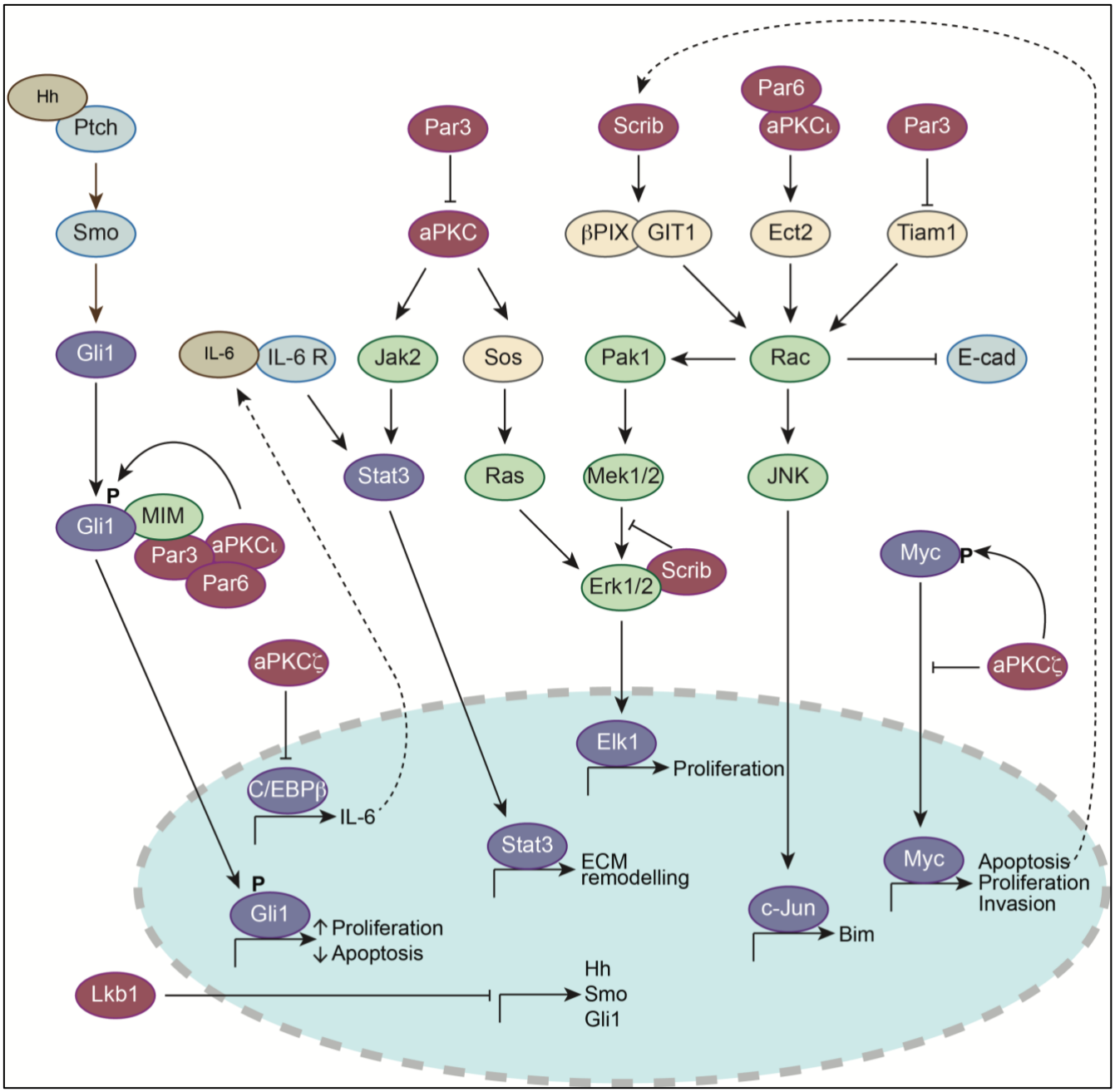

Figure 1.3: Polarity signaling in tumourigenesis

Polarity proteins are implicated in diverse signaling pathways that modulate transcriptional programs regulating proliferation, apoptosis, and survival. Signaling molecules are color-coded: Polarity proteins (red); Transcription factors (purple); Guanine nucleotide exchange factors (yellow); Signal transducers (green); Membrane receptors (blue); Ligands (brown).

1.2.4.2 Tumour promoting and tumour suppressing roles for aPKCs

aPKCι is amplified or over-expressed in multiple cancer types, and contributes to tumour growth through multiple pathways (Murray et al. 2011). In skin basal cell carcinoma (BCC), aPKCι, but not aPKCζ, is up-regulated, and suppressing aPKCι expression or activity reduces Hh signaling and BCC growth (Atwood et al. 2013). Interestingly, aPKCι, Par3 and Par6 all localize to centrosomes and associate with Missing in Mitosis (MIM), a molecular scaffold that potentiates Hh signaling by binding Gli1 (Callahan et al. 2004; Atwood et al. 2013). aPKCι directly phosphorylates Gli1 and increases its DNA-binding and transcriptional activity in BCC (Figure 1.3). Remarkably, aPKCι is up-regulated in Smo-inhibitor-resistant BCC cells, and inhibiting aPKCι reduces both proliferation of resistant cells and tumour growth, indicating that aPKCι up-regulation may be a mode of resistance in BCC, making it an attractive therapeutic target (Atwood et al. 2013). Hh signaling has been linked with polarity in many developmental contexts, indicating that a link between polarity and Hh signaling may have widespread importance in cancer progression (Krauss et al. 1993; Hashizume and Hieda 2006; Park et al. 2008; Kosinski et al. 2010; Atwood et al. 2013; Geisbrecht et al. 2013).

aPKCι also has a role in other cancers, and homozygous deletion of aPKCι suppresses K-Ras-induced lung and $Apc^{Min/+}$-induced intestinal tumourigenesis, whereas mice expressing a constitutively active form of aPKCι are more susceptible to intestinal tumours (Murray et al. 2004; Regala et al. 2005). Moreover, aPKCι is required for expansion and growth of ovarian tumour-initiating cells (TIC), and suppressing aPKCι expression or activity impairs ovarian TIC expansion and growth *in vitro* and tumour growth *in vivo* (Wang et al. 2013). In lung and ovarian tumours, the Par6/aPKCι complex associates with Ect2, a guanine nucleotide exchange factor (GEF) that activates Rac1 and downstream Pak1-Mek1/2-Erk1/2 signaling to regulate tumour growth (Figure 1.3) (Murray et al. 2004; Regala et al. 2005; Justilien

and Fields 2009; Justilien et al. 2011; Wang et al. 2013). These studies provide strong evidence that aPKCι is an oncogene in multiple organs.

Unlike aPKCι, aPKCζ acts as a tumour suppressor in many contexts. In K-Ras-mediated lung tumourigenesis, deleting aPKCζ enhances proliferation and tumourigenesis by repressing histone acetylation of a C/EBPβ element within the IL-6 promoter, which increases IL-6 production and subsequent pro-inflammatory signaling through Stat3 (Figure 1.3) (Galvez et al. 2009). Furthermore, although $PTEN^{+/-}$ mice develop prostate intraepithelial neoplasia (PIN) that does not progress to invasive carcinoma, loss of aPKCζ enables progression to invasive carcinoma (Kim et al. 2013). Interestingly, aPKCζ directly phosphorylates Myc and negatively regulates its transcriptional function. Loss of aPKCζ, expression of kinase-dead aPKCζ, or expression of a non-phosphorylatable Myc mutant promotes Myc activity and subsequent proliferation, invasion, and metastasis, whereas aPKCζ over-expression inhibits prostate invasion and metastasis (Figure 1.3) (Powell et al. 1996; Kim et al. 2013). Importantly, protein expression of aPKCι does not change in aPKCζ-deficient tumour cells, indicating that the tumourigenic effect of loss of aPKCζ does not act through up-regulation of aPKCι (Ma et al. 2013). Therefore, aPKCs can act to promote or suppress tumourigenesis depending on the specific signaling pathways regulated.

1.2.4.3 Scrib and tumour progression

Apoptosis and proliferation are tightly controlled in polarized epithelia and become deregulated in cancer, in part through disrupted polarity signaling. Scrib also modulates tumourigenesis by regulating Myc-dependent signaling. Myc expression induces both proliferation and apoptosis, and loss of Scrib prevents Myc-induced apoptosis and promotes tumourigenesis (Zhan et al 2008). Myc induces apoptosis by up-regulating βPIX and GIT1, which requires Scrib to assemble a βPIX/GIT1 complex that activates Rac1 and subsequently stimulates the JNK/c-Jun pathway to induce expression of pro-

apoptotic Bim (Figure 1.3) (Zhan et al. 2008). Interestingly, mis-localizing Scrib phenocopies Scrib-depletion, highlighting that Scrib spatially regulates the Myc-induced apoptotic pathway and that disrupting the organization of apoptosis signaling promotes tumourigenesis (Zhan et al. 2008).

Depleting Scrib also accelerates K-Ras driven prostate tumour progression to invasive cancer, and loss of Scrib results in activation of Erk1/2 and the downstream transcription factor Elk1 (Pearson et al. 2011). Scrib can bind directly to Erk1 through a pair kinase-interacting motifs (KIM), which prevents Erk1 activation and nuclear translocation (Nagasaka et al. 2010), and treatment of mice bearing Scrib-deficient tumours with a Mek inhibitor suppresses proliferation and prostate tumour burden, indicating that Scrib may also act as a scaffold to inhibit Erk1/2 activation by upstream Mek kinases (Figure 1.3) (Pearson et al. 2011).

These studies indicate a tumour suppressive role for Scrib; however, Scrib may also have pro-tumourigenic activity. High expression of *SCRIB* mRNA is associated with increased risk of breast cancer relapse, and loss of Scrib in aggressive MDA-MB-231 breast cancer cells inhibits mammary tumourigenesis *in vivo* (Anastas et al. 2012). One explanation for opposing roles for Scrib is that its scaffolding function may be context specific, which can have distinct effects on tumourigenesis. As described above, Scrib binds to βPIX/GIT1 to regulate apoptosis, but Scrib also binds to the scaffolding protein NOS1AP and the planar cell polarity protein VANGL2 (Richier et al. 2010; Anastas et al. 2012). However, unlike in other cell types, Scrib binds βPIX/GIT1 and NOS1AP independently in MDA-MB-231 breast cancer cells and forms a Scrib/NOS1AP/VANGL2 complex that localizes to lamellipodia to promote cell invasion (Anastas et al. 2012). Yet an opposite effect is observed in MCF10A cells expressing an oncogenic form of Ras (RasV12), where Scrib suppresses invasion (Dow et al. 2008). These studies highlight the complexity of Scrib-mediated signaling in cancer, and demonstrate that Scrib may have pro- or anti-tumourigenic functions, dependent on the tumourigenic signaling context of the cells.

1.2.4.4 Tumour promoting and suppressing roles for Par3

Par3 also has tumour promoting and tumour suppressive functions. In a DMBA/TPA-induced skin cancer model, loss of Par3 protects mice from papilloma but dramatically increases the formation of keratoacanthomas, a rare form of skin cancer (Iden et al. 2012). The tumour promoting function of Par3 is through its scaffolding function in skin. In keratinocytes, Ras and its GEF, Sos, are enriched at sites of cell-cell contacts, but in Par3-knockout cells they are delocalized and Ras activation of downstream Erk1/2 and Akt signaling is impaired, which reduces proliferation and increases apoptosis (Iden et al. 2012). Interestingly, loss of Par3 delocalizes aPKC, and blocking aPKC activity or the aPKC-Par6 interaction can restore Erk1/2 activation. This is consistent with lung cancer, where aPKC also promotes Erk1/2 activation, and indicates that a key function of Par3 may be restricting aPKC activity (Figure 1.3) (Regala et al. 2005; Iden et al. 2012). In Ras and Notch models of mammary cancer, loss of Par3 also delocalizes and activates aPKC, but in contrast to skin, breast tumourigenesis is potentiated in these models by activating Jak/Stat signaling (McCaffrey et al. 2012). Moreover, loss of Par3 induces multi-layering of mammary ducts and differentiation defects, with a concomitant increase in both proliferation and apoptosis (McCaffrey and Macara 2009a).

1.2.4.5 Cell polarity in invasion and metastasis

A role for cell polarity proteins in invasion and metastasis was first identified in *Drosophila*, which revealed that inactivation of a number of cell polarity genes leads to metastatic behavior (Pagliarini and Xu 2003; Stefanatos and Vidal 2011). Recent studies in mammalian cell lines and mouse models confirm a role for apical-basal proteins in invasion and metastasis, and further reveal diverse signaling pathways that participate as a consequence of disrupted polarity.

1.2.3 Epithelial-Mesenchymal Transition (EMT)

Elegant studies have indicated that loss of epithelial and gain of mesenchymal characteristics can promote intravasation and early steps of metastasis, but that re-epithelialization is necessary for proliferation of disseminated cells at metastatic sites (Ocaña et al. 2012; Tsai et al. 2012). EMT is characterized by a loss of cell polarity and cell-cell adhesion molecules like E-cadherin, with increased expression of mesenchymal markers like vimentin, N-cadherin and fibronectin. EMT can be induced by TGFβ/SMAD signaling or transcription factors, including Zeb1, Twist and Snail (Figure 1.4) (Thiery et al. 2009). However, it has been shown that cells do not necessarily need to undergo EMT for metastasis (Fischer et al. 2015). Clearly cells can invade locally as groups of cells (Friedl and Wolf 2010; Ewald et al. 2012; Nguyen-Ngoc et al. 2012a) and tumour cells isolated from the circulating blood of cancer patients can express epithelial markers (Yu et al. 2013). It is therefore likely that multiple modes of invasion and metastasis exist within a spectrum of epithelial and mesenchymal characteristics (Li and Kang 2016).

Loss of polarity is considered a hallmark of EMT and several polarity proteins are transcriptionally repressed during this process. For example, Zeb1 and Snail can directly repress the expression of Crb3, Lgl2 and Patj (Figure 1.4), as well as other epithelial genes (Aigner et al. 2007; Russ et al. 2012; Kashyap et al. 2013). Strikingly, some polarity proteins are able to suppress EMT; re-expression of Lgl2 blocks Snail-induced EMT and reduces mammary tumourigenesis and metastasis (Kashyap et al. 2013). Furthermore, over-expression of Scrib acts through MAPK to down-regulate Zeb1, which restores Crb3 expression and tight junction formation in MCF10A cells (Elsum et al 2013). However, an important question is whether loss of apical-basal polarity proteins is sufficient to induce EMT. This appears not to be the case since loss of one, or even simultaneous depletion of two polarity proteins does not induce EMT *in vitro* or *in vivo* (Chatterjee et al. 2012; McCaffrey et al. 2012; Xue et al.

2013). Therefore, suppression of apical-basal polarity may be necessary, but is not sufficient, to induce EMT.

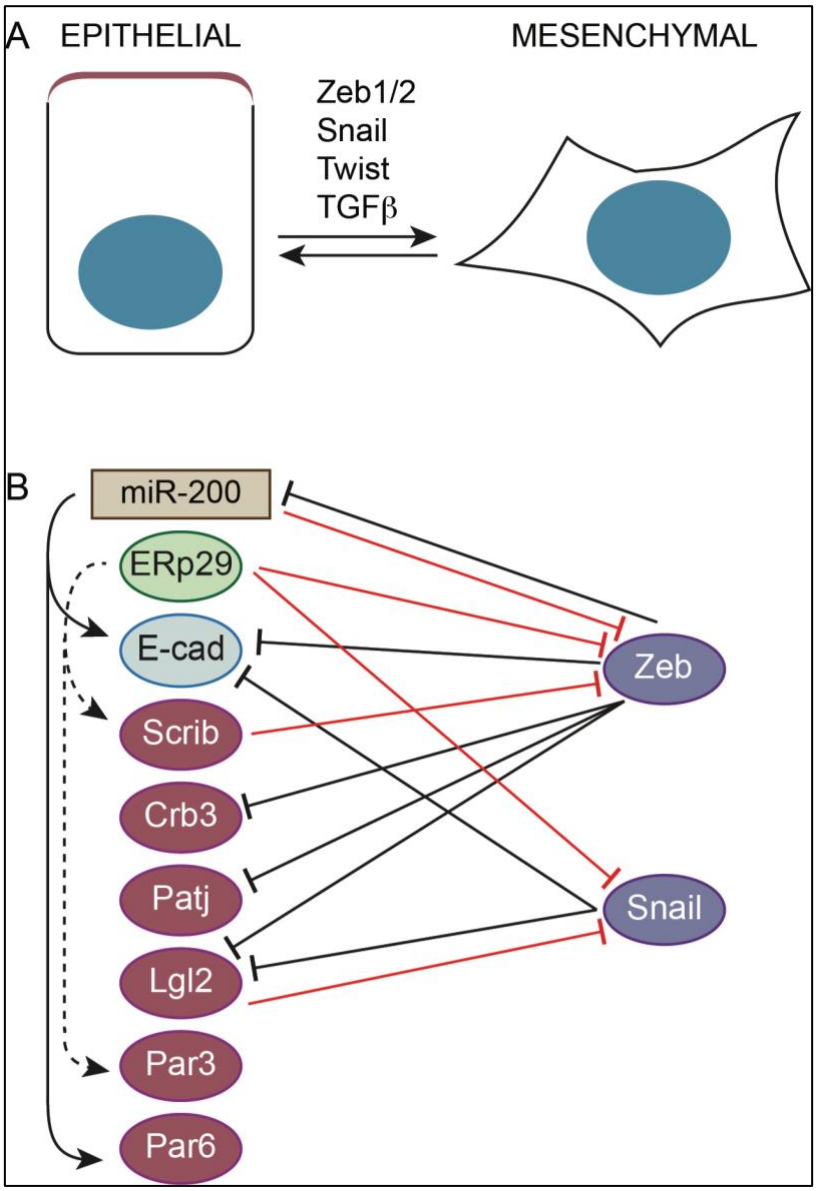

Figure 1.4: Apical-basal polarity and EMT

A) Factors such as Zeb, Snail, Twist and TGFβ reprogram cells by suppressing epithelial and promoting mesenchymal characteristics. B) Bidirectional feedback loops between EMT factors, miRNA, and polarity proteins.

Unlike the Scrib and Crbs complexes, there is little evidence that the Par complex is targeted for destruction by EMT-inducing factors. In fact, the opposite is observed where EMT is inhibited through Par complex-mediated SNAI1 degradation (Jung HY, Nature Cell Biol 2019). Par6 is also required for certain aspects of EMT. For example, Par6 is phosphorylated by TGFRII on Ser345, which recruits Smurf1 to tight junctions and enables RhoA-dependent disassembly of the tight junction complex (Figure 1.5) (Ozdamar et al. 2005; Viloria-Petit and Wrana 2010). This requires an intact Par6/aPKC complex, and aPKC kinase activity increases the Par6 function (Gunaratne et al. 2013). A similar requirement for Par6/aPKC activity is seen in ErbB2-driven breast cancers, where ErbB2 binds Par6/aPKC to uncouple it from Par3 to disrupt apical-basal polarity (Aranda et al. 2006a). These data suggest that apical-basal polarity is a controlled process downstream of growth factor receptors, which may have developmental origins since apical-basal polarity can be transiently lost without inducing EMT during growth factor-driven mammary branching morphogenesis (Ewald et al. 2008).

Additional pathways function to maintain an epithelial state and suppress the mesenchymal program. For example, the miR200 family is part of a double feedback loop in which miR200 inhibits TGFβ signaling and Zeb1 to maintain epithelial differentiation, whereas Zeb1 suppresses miR200 to induces a mesenchymal phenotype (Figure 1.4) (Brabletz and Brabletz 2010). In the mammary epithelium, knockdown of miR-200a down-regulates E-cadherin, claudin-3, and Par6β impaired lumen formation (Nagaoka et al. 2013), indicating that the miR-200 family regulates apical-basal polarity as part of the epithelial program. Furthermore, Endoplasmic Reticulum protein 29 (ERp29) is a chaperone protein that suppresses Snail, Twist, and Zeb2 expression and can regulate tight junction formation and membrane localization of Par3 and Scribble in MDA-MB-231 breast cancer cells (Figure 1.4) (Bambang et al. 2013).

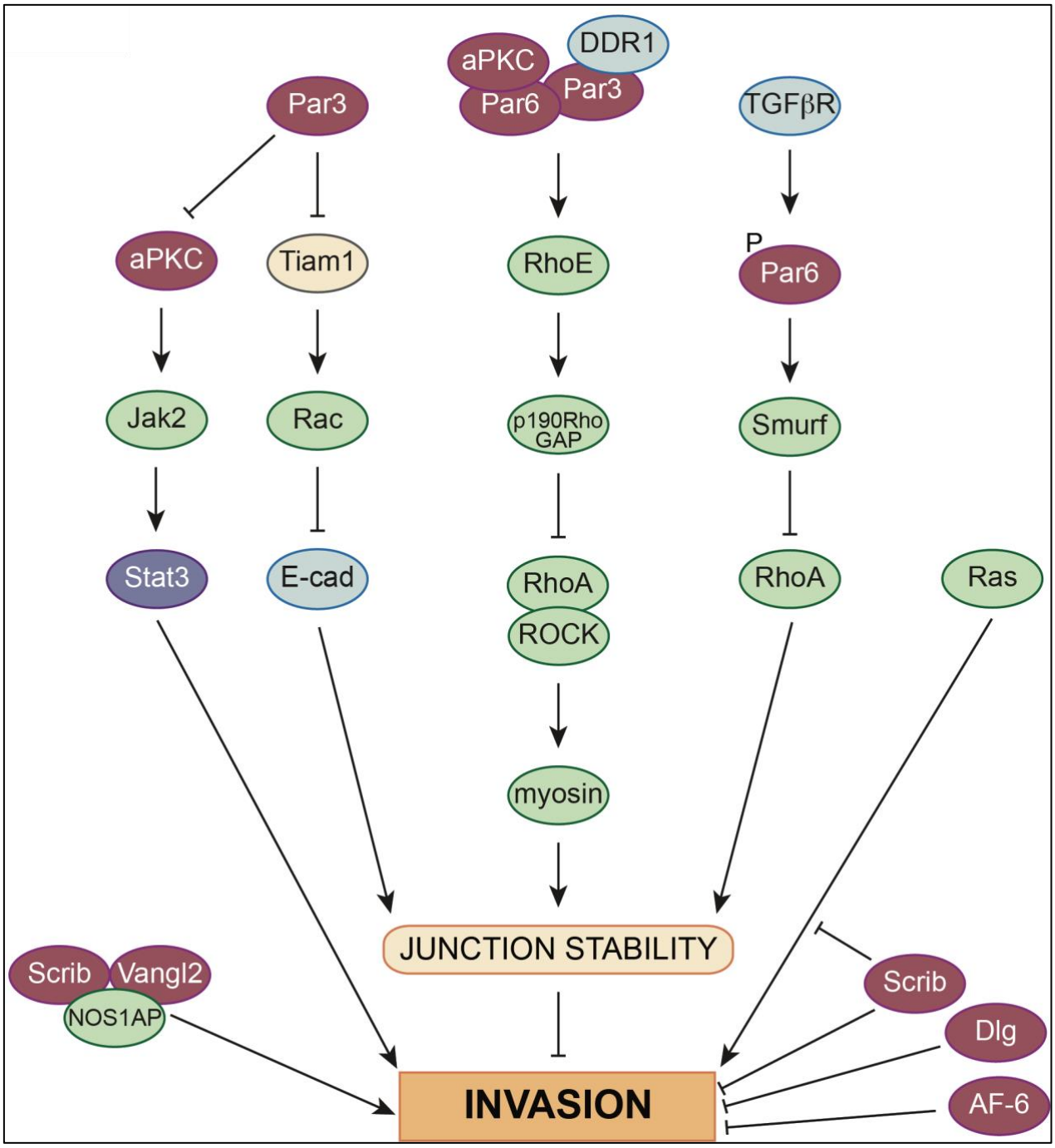

Figure 1.5: Polarity proteins in cancer invasion and metastasis

Integration of pathways regulated by polarity proteins that promote invasion and metastasis. Proteins are color-coded: Polarity proteins (red); Transcription factors (purple); Signal transducers (green); Membrane proteins (blue) and Guanine nucleotide exchange factors (yellow).

1.2.4 The Par complex in invasion and metastasis

In addition to a mesenchymal single-cell mode of invasion, tumour cells invade collectively as groups of cells, and cells can switch between invasive modes (Friedl and Gilmour 2009; Friedl and Wolf 2010; Nguyen-Ngoc et al. 2012a). The Par complex is required to maintain collective invasion in squamous cell carcinoma (SCC) by maintaining reduced actomyosin contraction at cell-cell contacts. The Discoidin Domain Receptor 1 (DDR1) binds Par3 and Par6, which is necessary to recruit RhoE to cell-cell contacts, thereby limiting actomyosin contraction by recruiting p190RhoGAP to inhibit Rho/ROCK activation of myosin (Figure 1.5) (Hidalgo-Carcedo et al. 2011). Loss of Par3, Par6, aPKC or Tiam-1 uncouples this pathway and decreases collective invasion of A431 SCC cells (Hidalgo-Carcedo et al. 2011). In some ways, this mechanism parallels the TGFβ receptor-Par6-Smurf1-RhoA pathway, in which Par proteins recruit Rho-family GTPases to control cell junction dynamics (Figure 1.5).

In breast cancer, the Par complex is also involved in invasion through regulating cell-junction stability. Loss of Par3 in ErbB2 breast cancer cells mis-localizes the Rac1 GEF Tiam1, which affects actin remodeling and E-cadherin stability to increase cell invasion and metastasis *in vivo* (Xue et al. 2013). Furthermore, Par6 is not only required to dissolve tight junctions, but is also for invasion and metastasis, and inactivating mutants of Par6 block metastasis *in vivo* (Viloria-Petit et al. 2009). These data reveal an important role for polarity proteins in regulated cell-cell junction integrity as a mechanism to control invasion and metastasis (Figure 1.5).

The Par complex can also promote invasion and metastasis by altering cell-matrix interactions. Strikingly, whereas mammary tumours formed in a Notch model do not metastasize to lungs, loss of Par3 enables robust lung metastasis (McCaffrey et al. 2012). This occurs, in part, by the ability of Par3-loss to stimulate Jak/Stat3 signaling downstream of aPKC, and knockdown of Stat3 impairs metastasis of Par3-depleted cells (Figure 1.5) (McCaffrey et al. 2012). Activation of Jak/Stat in Par3-knockdown

cells deregulates the expression of a number of extracellular matrix-related genes, including MMPs, which enables Par3-deficient cells to efficiently degrade the extracellular matrix (McCaffrey et al. 2012). Therefore, altering cell-cell and cell-matrix interactions may cooperate to enable invasion and metastasis of Par3-depleted cells.

1.2.5 Microenvironment and cell polarity

The tumour microenvironment and extracellular matrix are critical regulators of cancer progression, and breaching the laminin-rich basement membrane is a feature of invasive carcinomas. Basement membrane protein gels, like Matrigel, act as a barrier to tumour cell invasion, however, cells readily invade in collagen I, which is highly expressed in many tumours (Nguyen-Ngoc et al. 2012a). Remarkably, even normal epithelial cells can become invasive in collagen, indicating that the composition of the ECM is a critical modulator of invasive behavior (Nguyen-Ngoc et al. 2012a). Moreover, simultaneously depleting two polarity proteins (AF6/Scrib, AF6/Scrib) or Patj alone induces invasion in stiff Collagen I-containing gels, but not in soft Matrigel (Chatterjee et al. 2012).

The extracellular matrix can provide a cue for proper orientation of apical-basal polarity. When laminin deposition is disrupted, cells exhibit inverted polarity, with apical markers localizing to the outside of 3D epithelial cysts (Weaver et al. 1995; O'Brien et al. 2001; Bissell et al. 2002). Collagen can also polarize cells, which requires β1-integrin mediated laminin organization in MDCK cells (Yu et al. 2005). However, in mammary epithelial cells with an intact basement membrane, loss of β1-integrin still inverts polarity, indicating that not all collagen/β1-integrin polarizing effects act through organizing laminin (Akhtar and Streuli 2013). Instead, β1-integrin-deficient mammary cells fail to engage integrin-linked kinase (ILK), which is necessary to correctly orient microtubules that establish apical-polarity by directing endocytosis of apical determinants from the basal membrane (Akhtar and Streuli 2013).

Therefore, cell-ECM interactions are important regulators of apical-basal polarity, and defects in these interactions may have important implications for tumourigenesis, invasion and metastasis.

Importantly, cell polarity proteins can reciprocally affect the ECM. Loss of Par3 in Notch and Ras models of breast cancer causes numerous changes in extracellular matrix proteins and regulators, including collagens, laminins, periostin, and MMPs 2, 3, 9 and 12 (McCaffrey et al. 2012). In addition, aPKC can regulate MMP expression through Rac1-dependent signaling (Frederick et al. 2008). Moreover, loss of Lkb1 disrupts apical-basal polarity and causes basement membrane fragmentation by up-regulating the serine protease Hepsin (Partanen et al. 2012). Therefore, there is an intricate relationship between cell polarity and the extracellular matrix that can promote epithelial disorganization and invasion when disrupted.

The tumour microenvironment also contains various cell types including fibroblasts and immune cells that can condition the epithelial tumour cells to promote tumourigenesis, invasion and metastasis. Interestingly, suppression of polarity protein expression in tumour cells enables dendritic cell or macrophage conditioned medium to induce invasion, potentially by up-regulating EGFR and Akt signaling (Chatterjee et al. 2012). This is not observed in normal cells, indicating that apical-basal polarity proteins act as a barrier to invasion induced by cytokine secreted by the tumour stroma (Chatterjee et al. 2012).

Polarity proteins may also have a function in the stromal compartment of some tumours. High Par6 in the stroma correlates significantly with good outcome in non-small-cell lung cancer, whereas Par6 has no prognostic value in the lung tumour epithelium, which is different than breast, where high Par6 in the tumour epithelium correlates with poor outcome (Al-Saad et al. 2008). Furthermore, several components of the Par and Scrib complexes are expressed in fibroblasts, macrophages, and monocytes

and can regulate their migration (Allen et al. 1998; Liu et al. 1998; Wang et al. 2003; Petit et al. 2005; Schmoranzer et al. 2009; Tamehiro et al. 2009; Solinet et al. 2011).

1.3 Spindle Orientation and Early Cancer Progression

1.3.1 Spindle orientation complex

An early event of epithelial cancers is the multi-layering and loss of the central lumen, which correlates with loss of apical-basal polarity. To maintain proper tissue organization, epithelial cells divide within the plane of the epithelium, and disrupting polarity may contribute to early cancer stages by misorienting cell divisions (Figure 1.6) (Knoblich 2010).

Proper spindle orientation during mitosis is thought to be crucial for the proper establishment and maintenance of epithelial tissue morphology. In fact, divergent mechanisms have come into play in order to ensure proper spindle alignment, and many cellular mechanisms exist to protect against aberrant spindle orientation, such as cell delamination or re-integration after abnormal division (Nakajima 2018). Asymmetric divisions are highly controlled and rely on the polarized state of cell fate determinants and as a result, the segregation of core polarity proteins leading to the correct spindle orientation (Albertson and Doe 2003; Januschke and Gonzalez 2008). In contrast, when dividing symmetrically, epithelial cells orient their spindle perpendicularly to the apical-basal axis in order for both daughter cells to remain within the epithelium (Bergstralh et al. 2013). Imbalance in the percentage of symmetric versus asymmetric divisions can lead to tissue overgrowth in Drosophila, implicating spindle orientation defects at the initiation step of human cancers (Albertson and Doe 2003; Januschke and Gonzalez 2008).

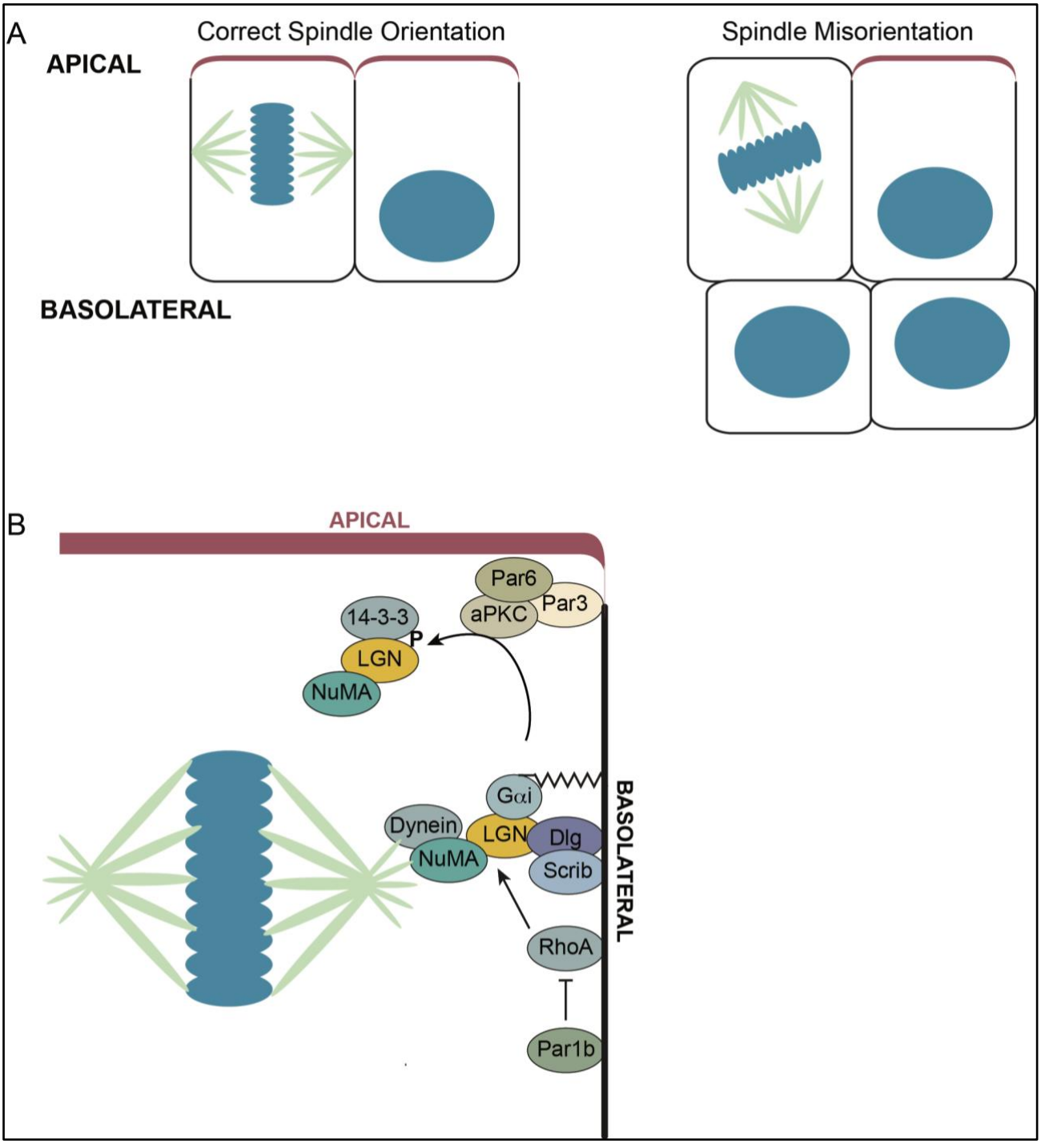

Figure 1.6: Oriented cell division

A) Model for how misoriented mitotic cell divisions may contribute to disorganization of the epithelial tissues. B) Apical and basolateral polarity complexes are implicated in positioning the mitotic spindle poles to the lateral plasma membrane.

In the last 25 years, work has defined a core ternary complex that participates in the orientation of the mitotic spindle, whether symmetric or asymmetric divisions are occurring. Orienting the mitotic spindle requires coupling the polar astral microtubules to the lateral cell cortex, which is achieved by the binding of the adaptor LGN to spindle microtubules through a NuMA/dynein complex at the cortex via myristoylated Gαi (Du et al. 2001; Du and Macara 2004).

LGN (Leucine-Glycine-Asparagine (LGN) repeat-enriched protein) was first identified in Drosophila (named Pins; Partner of Inscuteable) and is associated with the astral microtubules spindle and the cell cortex during cell mitosis (Du et al. 2001; Kaushik et al. 2003; Siller and Doe 2009). It functions as a scaffolding protein for the spindle orientation machinery, and is composed of an N-terminal domain made up of tetratricopeptide repeats (TPRs) that bind NuMA (Nuclear Mitotic Apparatus), and a C-terminal domain containing three GoLoco motifs that interact with GDP-bound Gαi at the plasma membrane (Kaushik et al. 2003; Nipper et al. 2007; Johnston et al. 2009). Myristolated Gα_i is bound to the plasma membrane through the modification, which LGN utilizes to anchor to the plasma membrane. LGN's two motifs are bound together through an unstructured linker domain; in said state, the protein is held in an inactive conformation which is released upon interaction with NuMA and Gα_i (Du et al. 2001; Du and Macara 2004; Nipper et al. 2007).

NuMA, on the other hand, was first identified as the mammalian orthologue of Drosophila protein Mud, initially identified as a key factor in neuroblast spindle orientation (Bowman et al. 2006; Izumi et al. 2006; Siller and Doe 2009). It was also observed at the cortex of dividing cells, where it binds the dynein/dynactin complex in order to modulate spindle orientation and position the mitotic spindle (Bergstralh et al. 2013).

1.3.2 Cell polarity and spindle orientation

In mammalian epithelial cells, loss of Par3, Par6, aPKC, or Cdc42 misorients the mitotic spindle (Jaffe et al. 2008; Hao et al. 2010; Qin et al. 2010; Durgan et al. 2011). Par3 and Par6 are required to localize aPKC to the apical membrane where aPKC phosphorylates LGN, promoting 14-3-3 binding and LGN's dissociation from $G\alpha_i$ and is excluded from the apical membrane (Figure 1.6) (Hao et al. 2010). Par3 also associates with Hippo pathway protein Lats1 and its loss causes mitotic spindle randomization by inhibiting Lats1 phosphorylation which impairs Lats1 interaction with LGN (Zhou et al. 2019). Par1b also determines correct spindle orientation by regulating RhoA activity at the cell cortex, which affects $G\alpha_i$ and LGN/NuMA recruitment (Lazaro-Dieguez et al. 2013). In the *Drosophila* follicular epithelium, the mechanism of spindle orientation is rather different. aPKC disappears from the apical membrane during mitosis and is not required for horizontal spindle orientation (Bergstralh et al. 2013). Instead, LGN interacts with Dlg, which positions it at the lateral membrane. Interestingly, loss of Scrib or Dlg in *Drosophila* imaginal discs results in misoriented cell divisions, causing cells to delaminate from the epithelial sheet and undergo apoptosis (Nakajima et al. 2013). However, if apoptosis is blocked, over-growth occurs from cells that arise from misoriented cell divisions (Nakajima et al. 2013), indicating that loss of spindle orientation control may contribute to tissue disorganization during early stages of cancer development.

1.3.3 Spindle orientation proteins and cancer

Little evidence exists directly implicating proteins of the spindle orientation complex in cancer growth and progression. Based on TCGA data, expression levels of LGN, for example, have different prognoses depending on cancer type, while high expression of NuMA seems to point towards a favourable outcome (cBioPortal).

One study using clinical breast cancer samples suggests that phosphorylation of LGN by PBK/TOPK during mitosis is critical for tumour growth, and that this pathway could be a promising molecular target for breast cancer treatment (Fukukawa et al. 2010). As for NuMA, circumstantial evidence points towards its involvement in cancer development. Its expression has been shown to be upregulated in ovarian cancers, which correlates with increased mitotic defects and aneuploidy (Bruning-Richardson et al. 2012); the mechanism leading to this process, however, has yet to be established. Another study performed in cell lines indicates that NuMA modulates p53-mediated transcription in cancer cells (Endo et al. 2013), but again the mechanism of this has not been elucidated. Further reports find NuMA as a biomarker for colorectal and bladder cancers (Keesee et al. 1996; Briggman et al. 1999).

Therefore, an attractive idea is that altered spindle orientation contributes to the development of cancer. However, little data directly addresses this, and there is a need to further understand the involvement of spindle orientation proteins in cancer and whether or not this complex is a possible therapeutic target.

1.4 Hypothesis and Project Aims

Apical-basal polarity proteins have essential roles in maintaining epithelial integrity and feed into diverse cellular pathways that control cell proliferation, apoptosis, invasion and metastasis. The complexity of how polarity proteins regulate signaling pathways in cancer progression is becoming apparent, which converge on a number of signaling nodes, including Ras, Erk, Rac1, Stat3, Myc, and Hedgehog signaling. Importantly, multiple polarity proteins (i.e. Par3, Scrib, and aPKC isoforms) have both tumour promoting and tumour suppressing functions, depending on the tissue or cell type involved. These apparently opposing roles for polarity proteins in cancer reflect their abilities to

regulate diverse signaling pathways. Many of the studies on polarity proteins to date have focused on breast, lung, prostate and colon cancer models in the context of a narrow range of oncogenes, and future studies should provide a more thorough understanding of the role of polarity signaling in different cancer types and subtypes that are driven by diverse oncogenes and tumour suppressor genes. Furthermore, it is apparent that polarity proteins function beyond simply regulating intracellular signaling, allowing cells to appropriately interact with the surrounding microenvironment by coupling cell organization with metabolism and interactions with the extracellular matrix.

All of the above brings to light a fundamental question: is polarity a barrier to tumour progression? An emerging concept is that loss of polarity is a permissive step in cancer progression that uncouples normal tumour suppressive cellular functions like apoptosis, and contributes to pro-tumourigenic signaling through deregulated proliferation, invasion and metastasis. Therefore, **the overarching hypothesis of this body of work is that polarity disruption is a necessary step enabling tumour development**. I aimed to answer this question in two ways. Firstly, I evaluated the loss of polarity, characterized the cellular mechanisms behind the step-by-step process of early tumourigenesis, and evaluated the reversibility of tumour growth in Chapter 2; and examined whether spindle orientation contributes to early breast cancer progression in Chapter 3.

Chapter 2: Progressive polarity loss and luminal collapse disrupt tissue organization in carcinoma

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Abstract

Epithelial cancers (carcinoma) account for 80-90% of all cancers. The development of carcinoma is associated with disrupted epithelial organization and solid ductal structures. The mechanisms underlying the morphological development of carcinoma are poorly understood, but it is thought that loss of cell polarity is an early event. Here we report the characterization of the development of human breast lesions leading to carcinoma. We identified a unique mechanism that generates solid ducts in carcinoma through progressive loss of polarity and collapse of the luminal architecture. This program initiates with asymmetric divisions of polarized cells that generate a stratified epithelium containing both polarized and depolarized cells. Stratified regions form cords that penetrate into the lumen, subdividing it into polarized secondary lumen. Secondary lumen then collapse with a concomitant decrease in RhoA and myosin II activity at the apical membrane, and ultimately lose apical-basal polarity. By restoring RhoA activity in mice, ducts maintained lumen and cell polarity. Notably, disrupted tissue architecture through luminal collapse was reversible and ducts with a lumen were established after oncogene suppression *in vivo*. This reveals a novel and common mechanism that contributes to carcinoma development by progressively disrupting cell and tissue organization.

2.1 Introduction

Epithelial cells line many organ and tissue surfaces, and are the origin for 80-90% of all human cancers, called carcinoma. Carcinogenesis is the process in which normal epithelial cells progressively evolve to become cancer cells, which is associated with disrupted cell and tissue organization. However, the mechanisms that underlie the early development and progression of carcinoma remain poorly characterized.

Epithelial ducts are highly organized structures at both cellular and tissue levels, which is disrupted in carcinoma. Apical-basal membrane polarity demarcates different cellular membrane compartments, with an apical membrane that faces the lumen and a basolateral membrane that contacts adjacent cells and the extracellular matrix (Rodriguez-Boulan and Macara 2014). Multiple protein complexes cooperate to establish distinct membrane domains, which are essential to organize intracellular signaling pathways for proper growth control, tissue organization, and to suppress invasion and metastasis (Rodriguez-Boulan and Macara 2014; Halaoui and McCaffrey 2015). The Par complex consists of a core scaffold Par3 that recruits the adaptor protein Par6 and its effector kinase aPKC. After being recruited by Par3, Par6 and aPKC dissociate from Par3 and associate with the Crumbs complex (Crb3/Pals1/Patj) at the apical membrane in mature epithelial cells (Rodriguez-Boulan and Macara 2014). The Scrib complex consists of Scribble, Discs large 1 (Dlg1) and Lethal Giant Larvae (Lgl2) and acts to mutually restrict the spatial organization of the apical complexes (Rodriguez-Boulan and Macara 2014). Accumulating evidence indicates that apical-basal polarity is a barrier to carcinogenesis (Halaoui and McCaffrey 2015). For example, depletion or mislocalization of individual polarity proteins in the mammary epithelium is sufficient to induce pre-malignant lesions and can accelerate tumour progression in the presence of oncogenes (Zhan et al. 2008; McCaffrey and Macara 2009a; Xue et al. 2013; Feigin et al. 2014; Godde et al. 2014; Archibald et al. 2015). It has been proposed that loss of

polarity is an early event in carcinogenesis (Huang and Muthuswamy 2010b; Huebner et al. 2014). Indeed, ErbB2 expression or depletion of polarity protein Scrib are capable of disrupting cell polarity prior to hyperplasia in organoids or *in vivo* in mice (Aranda et al. 2006b; Godde et al. 2014; Huebner et al. 2014). However, whether loss of cell polarity at the earliest stages of carcinoma development is widespread in human disease has not been established.

The organization of mammary ducts is characterized by the presence of a central lumen formed by a layer of epithelial cells that is surrounded by a basal myoepithelial cell layer, and loss of the luminal space during cancer progression is frequently referred to as luminal filling (Taraseviciute et al. 2010; Leung and Brugge 2012b; Venugopalan et al. 2014). One model proposes that luminal filling arises from cells shedding or migrating into the lumen to create solid structures (Taraseviciute et al. 2010; Leung and Brugge 2012b). This depends on cells overcoming anoikis and stimulating proliferation inside the lumen (Danes et al. 2008; Leung and Brugge 2012a; Pradeep et al. 2012). More recently, it was demonstrated in mouse models of breast cancer that cell divisions oriented perpendicular to the plane of the duct can contribute to stratification of luminal epithelial cells in the absence of cell shedding or migration into the lumen (i.e. luminal filling), however stratification is not sufficient to generate solid ducts (Godde et al. 2014; Huebner and Ewald 2014; Shore et al. 2016). Therefore, there is controversy regarding the mechanisms that contribute to the generation of solid ducts in experimental models, and how this occurs in human patients is not understood.

Tension at the apical membrane works to maintain lumen shape and size by modulating the actin cytoskeleton through RhoA family GTPases, thus controlling tissue integrity and morphogenesis (Martin and Goldstein 2014). Myosin II associates with actin filaments and contracts following phosphorylation of its light-chain by Rho-associated protein kinase (ROCK), which itself is activated by RhoA in its active GTP-bound state (Martin and Goldstein 2014). RhoA activity is regulated at the apical

membrane through guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which enable an appropriate balance of RhoA in its inactive GDP- and active GTP-bound states. Importantly, altered RhoA activity has been associated with both anti- and pro-tumourigenic functions, indicating it behaves as an oncogene or tumour suppressor in different contexts (Zandvakili et al. 2016).

Breast cancer is a heterogeneous disease and is the most common malignancy affecting women worldwide. Although breast cancer-associated mortality has reduced, incidence is increasing (Global Burden of Disease Cancer et al. 2015). Genetic, epidemiological, and histological studies indicate that ductal breast cancers can develop through pre-invasive stages that include flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS), which are risk factors for development of invasive ductal carcinoma (IDC) (Ellis 2010; Lopez-Garcia et al. 2010; Sgroi 2010; Sinn et al. 2010; Bombonati and Sgroi 2011b). However, not all lesions will lead to invasive disease and it is currently not possible to predict which are most likely to progress (Sgroi 2010), highlighting the need for a better understanding of the mechanisms involved in early breast cancer progression.

Based on the presence or absence of hormone receptors to estrogen and progesterone, and the human epidermal growth factor receptor 2 (HER2), breast cancers are broadly classified into subtypes that exhibit diverse phenotypes and clinical outcomes (Kos and Dabbs 2016). Hormone receptor-positive luminal-type carcinoma represent the majority of breast cancers (60-70%), and evidence supports that different subtypes are already evident at the DCIS stage, indicating that drivers of different subtypes may act prior to development of invasive disease (Hannemann et al. 2006; Allred et al. 2008; Lopez-Garcia et al. 2010; Tang et al. 2016). Although hormone receptor positive cancers (HR+) generally have a better prognosis than other subtypes in the short-term, survival declines over 10 years, and due to the high prevalence of this subtype, it accounts for a majority of breast cancer-related

deaths (Haque et al. 2012). The cellular and molecular mechanism by which carcinoma develop in the breast and other epithelia remain poorly understood, which impedes progress to prevent cancer development.

Using human breast biopsy samples, as well as *in vivo* mouse models and organotypic cultures, we report diverse mechanisms that contribute to loss of cell and tissue polarity in the development of carcinoma. We identified that a major mechanism observed in generating solid ducts involved progressive loss of cell polarity through asymmetric cell divisions, and the collapse of lumen through reduced RhoA/myosin II activity at the apical membrane. In contrast, early loss of apical cell polarity and shedding of cells into the lumen were rarely observed in HR+ breast lesions. Finally, we report that loss of tissue organization and cell polarity were reversible upon removal of oncogene stimulation. Together these results highlight the dynamics of epithelial remodeling during the development of carcinoma, and reveal a previously unappreciated mechanism involving progressive polarity loss and luminal collapse as key characteristics.

2.2 Results

2.2.1 Diverse polarity phenotypes are present in DCIS

To investigate the apical-basal membrane polarity in DCIS we examined 49 hormone receptor-positive (HR+) DCIS lesions from breast biopsies by immunostaining for Par6 and E-cadherin to mark the apical and basolateral membranes, respectively (Figure 2.1, Supplemental Figure S2.1). We characterize apical-basal membrane polarity, herein referred to apical-basal polarity for simplicity, as segregation of apical and basolateral plasma membrane domains based on established apical (Par6, Ezrin) and basolateral (E-cadherin, Dlg1) markers. In most of the DCIS we analyzed (47/49), we observed populations of cells that exhibited apical-basal polarity (range: 0.3-80%, Figure 2.1 A,B). The presence

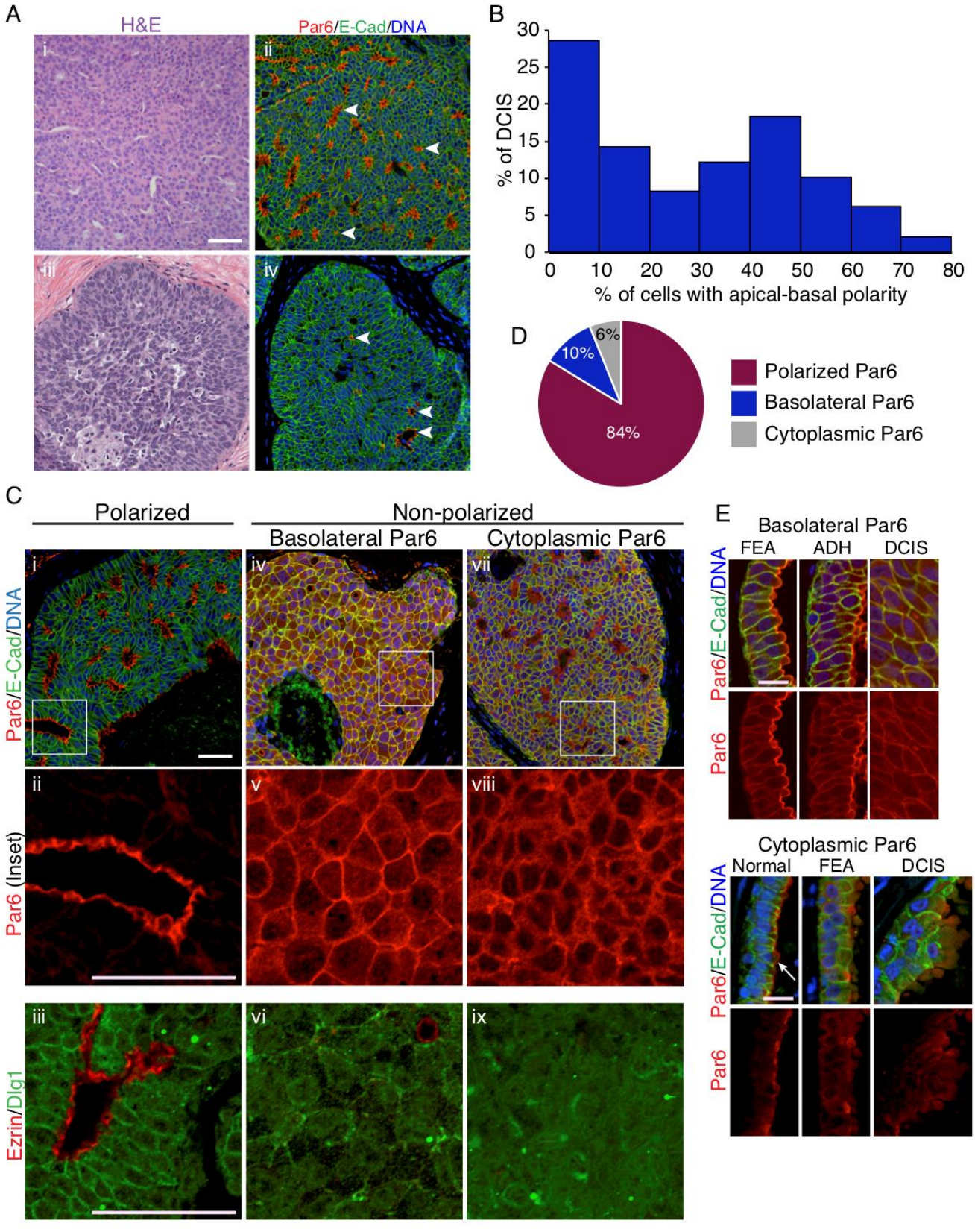
A
H&E
Par6/E-Cad/DNA
i
ii
iii
iv
B
% of DCIS
% of cells with apical-basal polarity
0
5
10
15
20
25
30
0
10
20
30
40
50
60
70
80
D
6%
10%
84%
Polarized Par6
Basolateral Par6
Cytoplasmic Par6
C
Polarized
Non-polarized
Basolateral Par6
Cytoplasmic Par6
Par6/E-Cad/DNA
Par6 (Inset)
Ezrin/Dlg1
i
ii
iii
iv
v
vi
vii
viii
ix
E
Basolateral Par6
FEA
ADH
DCIS
Par6/E-Cad/DNA
Par6
Cytoplasmic Par6
Normal
FEA
DCIS
Par6/E-Cad/DNA
Par6

Figure 2. 1: Diversity of apical-basal polarity in DCIS. (A) Histological sections (i and iii) and immunofluorescence staining (ii and iv) of adjacent sections of DCIS showing examples of apical-basal polarity in DCIS. Arrows show examples of cells with polarized Par6. (B) Histogram of the distribution of DCIS with varying frequencies of cells with apical-basal polarity (n=49). (C) Immunofluorescence staining of DCIS showing polarized apical Par6 (i, ii), basolateral Par6 (iv, v), and cytoplasmic Par6 (vii, viii). Lower panels (iii, vi, ix) show immunofluorescence of serial sections stained for Ezrin and Dlg1. (D) Frequency of Par6 phenotypes observed in DCIS. (E) Immunofluorescence of Par6 and E-cadherin on individual patient samples containing DCIS and earlier stages. Arrow shows polarized Par6 in adjacent normal tissue. Bars: A and C, 50μm; E, 20μm.

of cells with apical-basal polarity in DCIS reveals that complete loss of polarity is not necessarily an early event during development of carcinoma, indicating that previously unappreciated mechanisms exist to disrupt cell and tissue organization to generate solid ducts.

Less frequently, we also observed additional Par6 staining patterns that were uniform in all cells of the lesion, including Par6 colocalizing with the basolateral marker E-cadherin on membranes (3/49) and Par6 highly expressed in the cytoplasm (5/49) (Figure 2.1 C,D). As expected, an inverse correlation existed between lesions with membrane or cytoplasmic Par6 and the proportion of polarized cells (membrane: Pearson's ρ = -0.39, p=0.006; cytoplasmic: Spearman's ρ = -0.45, p=0.001). To determine if these staining patterns were specific to Par6 or were shared by other apical polarity proteins, we immunostained for Ezrin, a sub-apical protein that associates with the actin cytoskeleton. In samples with polarized Par6, Ezrin was also detected in a polarized pattern, indicating that apical-basal membrane polarity is present in these cells. However, in cells with basolateral Par6, Ezrin was not detected in the basolateral membrane, indicating specificity of mislocalized apical proteins (Figure 2.1C). Ezrin was also not detected in cells from DCIS with cytoplasmic Par6 (Figure 2.1C), indicating that these cells have lost general apical membrane identity, not just Par6. Since the apical Par polarity complex is known to cross-regulate basolateral polarity proteins, we examined the localization of Dlg1 in DCIS within lesions with different Par6 phenotypes. In DCIS with polarized Par6, Dlg1 was expressed on the basolateral membrane, as expected. In contrast, Dlg1 showed weak and fragmented localization to the plasma membrane or was cytoplasmic in samples with basolateral or cytoplasmic Par6, indicating that apical-basal membrane polarity is disrupted in these lesions (Figure 2.1 C,D). To understand if Par6 localization is altered in other pre-invasive lesions, we immunostained samples that contained less advanced stages adjacent to DCIS. We observed that in cases with basolateral or cytoplasmic Par6 in DCIS, Par6 mislocalization was also detectable in the FEA stage (Figure 2.1E). Similarly, in DCIS with

polarized Par6, less advanced lesions also retained apical-basal membrane polarity (Figure 2.2 A-C). Therefore, most DCIS examined retained a population of cells with apical-basal membrane polarity, even when tissue organization was disrupted and ducts appeared solid by standard histological analysis.

2.2.2 Apical-basal membrane polarity is progressively lost during breast carcinoma development

Solid ducts are a feature of carcinoma progression, and it has been proposed that depolarized cells can shed into the lumen to generate solids ducts (Danes et al. 2008; Leung and Brugge 2012a; Pradeep et al. 2012). We examined 114 pre-invasive lesions and observed only two that showed epithelial cells populating the luminal cavity (Figure S2.2A). Interestingly, in both examples, the ducts also had disrupted apical membrane identity, indicating that early loss of apical-basal polarity is a rare event, but may be associated with cells entering the lumen. Our finding that the majority of DCIS examined were heterogeneous for cells with and without apical-basal polarity prompted us to examine the apical-basal polarity status in a spectrum of pre-invasive human breast lesions including FEA, ADH, DCIS, and IDC. FEA are enlarged hyperplastic ducts with one to several layers of abnormal cells, but lack architectural alterations like cribiform patterns or solid ducts that are present in ADH and DCIS (Figure 2.2).We identified samples that contained DCIS with adjacent FEA or ADH, but with no invasive carcinoma, as well as DCIS adjacent to invasive carcinoma, and invasive ductal carcinoma, and scored the proportion of cells exhibiting apical-basal membrane polarity in each type of lesion (Figure 2.2 B,C). Whereas we observed ~80% of epithelial cells exhibiting apical-basal polarity in FEA, this was reduced to ~60% in ADH. In FEA and ADH, non-polarized cells are positioned basally to polarized cells as part of stratified regions surrounding round luminal structures (Figure 2.2 B,C, Supplemental Figure S2.2B). DCIS lesions had regions of polarized cells that were much smaller than in ADH. In ADH, cells with apical-

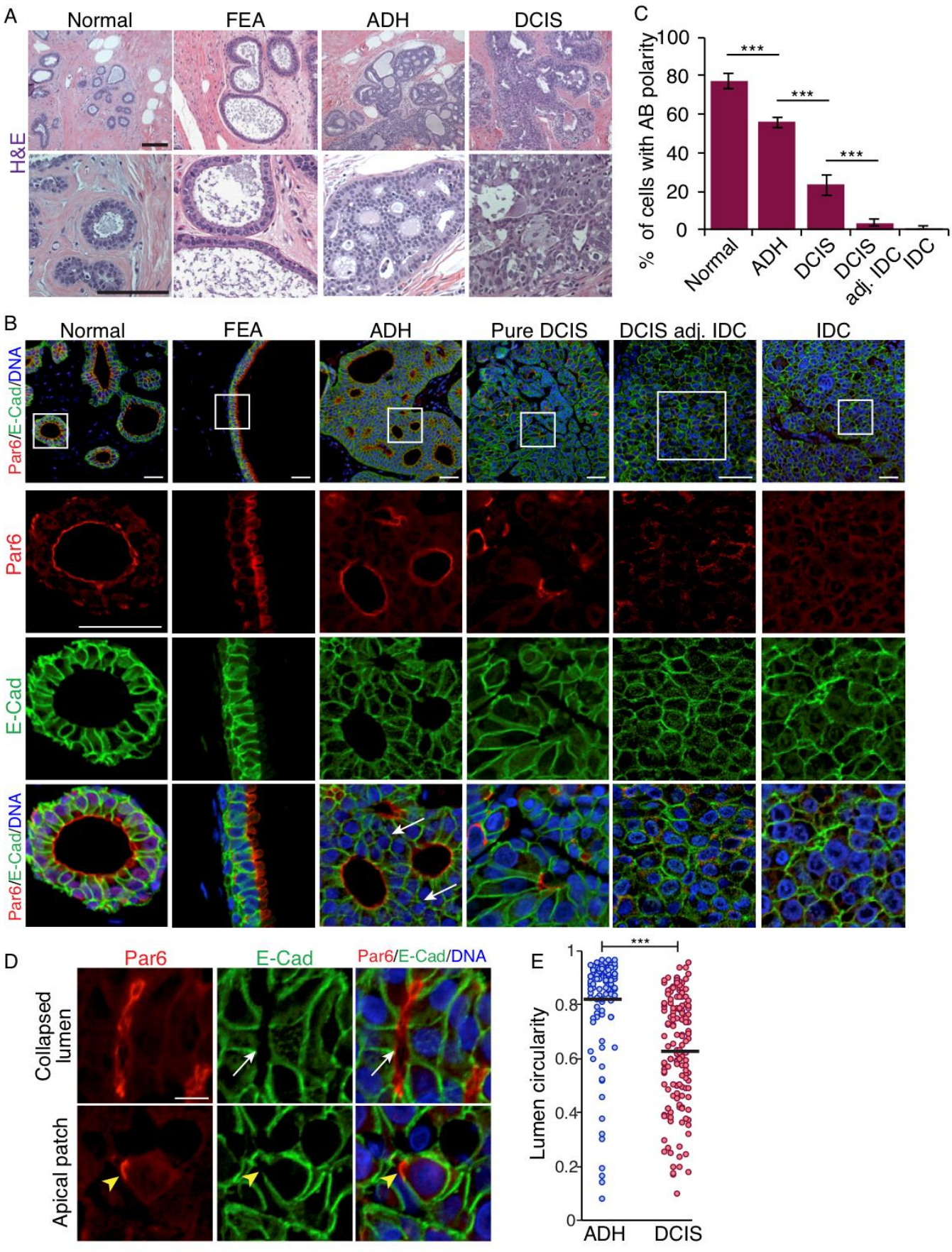
A
Normal
FEA
ADH
DCIS
H&E
C
% of cells with AB polarity
100
80
60
40
20
0
***
***
***
Normal
ADH
DCIS
DCIS adj. IDC
IDC
B
Normal
FEA
ADH
Pure DCIS
DCIS adj. IDC
IDC
Par6/E-Cad/DNA
Par6
E-Cad
Par6/E-Cad/DNA
D
Par6
E-Cad
Par6/E-Cad/DNA
Collapsed lumen
Apical patch
E
***
Lumen circularity
1
0.8
0.6
0.4
0.2
0
ADH
DCIS

Figure 2.2: Apical-basal polarity is progressively lost in pre-invasive breast lesions. (A) Histological sections from biopsy material containing normal, flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS). Asterisks indicate lumen. (B) Fluorescence images of tissue sections immunostained for Par6 (red) and E-Cadherin (green) at indicated stages, including DCIS adjacent to invasive carcinoma (DCIS adj. IDC), and invasive ductal carcinoma (IDC). Arrows show examples of cells lacking apical polarity. (C) Quantification of the percentage of cells that exhibit apical polarity at indicated stages of progression. At least 6 image fields from 5 subjects were analyzed for each. Error bars: sem. D, representative fluorescence images from DCIS showing collapsed lumen (white arrow) and apical patch (yellow arrowhead). (E) Quantification of lumen shape (circularity) between ADH and DCIS. Four subjects with both ADH and DCIS were used to measure 114 lumen (ADH) and 153 lumen (DCIS). Black bars represent the mean. ***, $p<0.001$. Bars: A, 100μm; B, 50μm; D, 20μm.

basal polarity surrounded well-defined round lumen, however in DCIS, the lumen were often collapsed, showing thin luminal space or were present as an apical patch, with no intercellular space detected (Figure 2.2 D,E). Although elements of apical-basal polarity persisted in DCIS without invasive disease, we detected very few cells with apical-basal polarity in invasive carcinoma or DCIS adjacent to invasive carcinoma (Figure 2.2 B,C). Therefore, we propose that loss of apical-basal membrane polarity occurs progressively during breast carcinoma development. Furthermore, solid ducts rarely result from cells shed into the lumen, but rather the lumen collapses following stratification of the epithelium.

2.2.3 Polarized cells collectively penetrate the lumen to generate secondary lumen

Since cell shedding into the lumen is an uncommon event in luminal breast lesions, we next explored the process by which a duct with a single large lumen (FEA) may generate stratified ducts with numerous smaller lumen (ADH). FEA are considered precursors to ADH (Sgroi 2010) and we therefore examined 3 patient samples where FEA was adjacent to ADH. Of the 16 FEA lesions adjacent to ADH, we observed stratified FEA with finger-like cords of cells extending into the lumen in 4 cases (25%), and bridges that generate secondary lumen in all ADH (Supplemental Figure S2.3 A,B). In all cases the cords contained cells with apical-basal polarity contacting the lumen (Supplemental Figure S2.3B). We did not observe any finger-like projections in FEA that were not adjacent to ADH. A distinguishing feature of FEA and ADH is the absence complex architecture in FEA (Sgroi 2010); therefore we consider the presence of finger-like projects as an intermediate between FEA and ADH.

We next examined whether the growth properties of simple or stratified epithelia in FEA were altered. Staining for Ki67 revealed that stratified epithelial were more proliferative in FEA (Supplemental Figure S2.3 C,D), suggesting that the stratified epithelium may represent an active state that is amenable to morphogenesis in pre-invasive lesions.

2.2.4 Polarity is progressively lost in a mouse model of breast cancer progression

To further understand the spatiotemporal events leading to loss of apical basal polarity, we examined tumours from mice expressing the Polyoma virus middle-T antigen (PyMT) under the mouse mammary tumour virus (MMTV) promoter/enhancer (Lin et al. 2003), or as a doxycycline-inducible system with a cross of two mouse strains: the PyMT-IRES-CRE (MIC) and the MMTV-reverse tetracycline transactivator (rtTA) (MTB) (Rao et al. 2014). These mice form multi-focal, hormone receptor-positive, luminal-type tumours with high penetrance (>75%) that progress through stages including hyperplasia, mammary intraductal neoplasia (MIN), and adenocarcinoma that are morphologically similar to human pre-invasive breast lesions (Guy et al. 1992; Lin et al. 2003; Herschkowitz et al. 2007; Rao et al. 2014). To determine if this model resembles human cancer with regards to progressive loss of apical-basal polarity and tissue organization we immunostained tissue sections from different stages with a panel of apical-basal polarity markers including ZO1, Par6, aPKC, Ezrin, E-cadherin and Dlg1 (Figure 2.3A, Supplemental Figure S2.4 A-C). In hyperplasia, we observed an increase in the number and size of ducts, indicating that branching morphogenesis is increased at the earliest steps. These ducts then became multilayered with the appearance of collapsed structures or apical patches at later stages, with eventual complete loss of apical-basal membrane polarity, similar to human breast progression (Supplemental Figure S2.5A). Like human progression, the proportion of cells exhibiting polarity was gradually lost and progressed through lumen collapse and apical patches in more advanced lesions (Figure 2.3 A,B, Supplemental Figure 2.5A,B). All apical markers showed similar localization patterns and we did not observe any mislocalization of the basolateral markers examined, confirming the presence of apical-basal membrane polarity in a subset of cells.

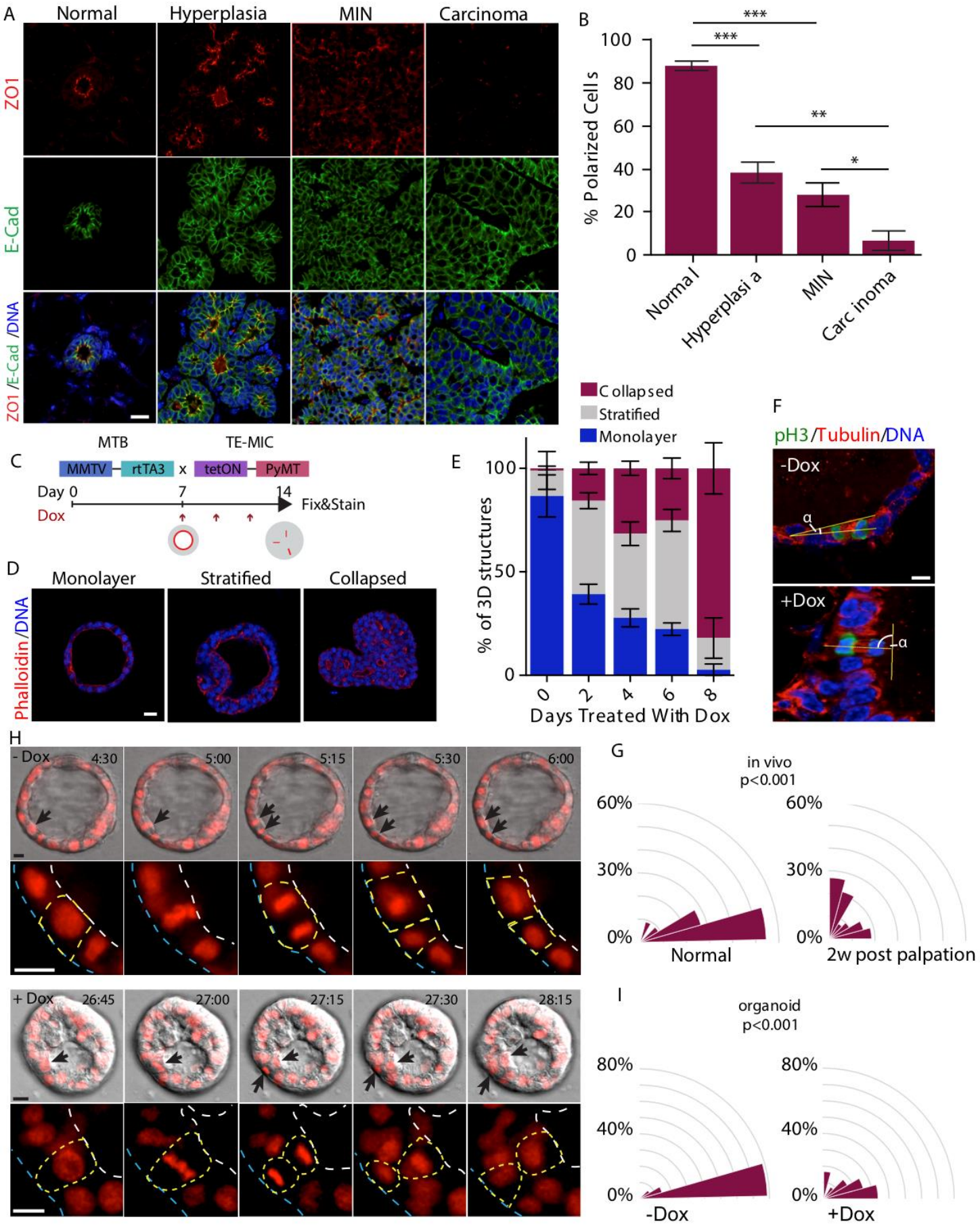
A
Normal
Hyperplasia
MIN
Carcinoma
ZO1
E-Cad
ZO1 /E-Cad /DNA
B
% Polarized Cells
Normal
Hyperplasia
MIN
Carcinoma
C
MTB
TE-MIC
MMTV
rtTA3
x
tetON
PyMT
Day 0
7
14
Fix&Stain
Dox
D
Monolayer
Stratified
Collapsed
Phalloidin/DNA
E
Collapsed
Stratified
Monolayer
% of 3D structures
Days Treated With Dox
F
pH3/Tubulin/DNA
-Dox
+Dox
H
- Dox
4:30
5:00
5:15
5:30
6:00
+ Dox
26:45
27:00
27:15
27:30
28:15
G
in vivo
p<0.001
Normal
2w post palpation
I
organoid
p<0.001
-Dox
+Dox

Figure 2.3: Progressive polarity loss and luminal collapse during mammary tumour progression in mice.

(A) Images of normal and tumour tissue from PyMT mice immunostained for ZO1 (red) and E-Cadherin (green). (B) Quantification of polarized and non-polarized cells at the indicated stages during mammary tumour progression. (C) Schematic diagram of experimental system for 3D organotypic cultures. (D) Confocal images of organotypic cultures stained with phalloidin to mark the apical membrane following induction of PyMT. (E) Quantification of indicated phenotypes at different time points following doxycycline-induced PyMT expression. 50 structures from 3 mice were counted. (F) Images of tissue samples immunostained for α-Tubulin (red), phospho-Histone-H3 (green), and DNA (blue). (G) Quantification of the cell division angle relative to the plane of the lumen; n=11 normal and 98 hyperplastic lesions. (H) Image series from time-lapse microscopy showing cell division in 3D organotypic cultures from MIC-PyMT cultures induced or not with doxycycline. Arrows show dividing cells. White dotted lines mark the apical surface and blue dotted lines mark the basal surface. Yellow dotted lines outline cells during division. (I) Quantification of the cell division angle relative to the plane of the lumen in organotypic cultures from MIC-PyMT mice. Cell divisions (n=24) from 52 organotypic cultures were examined. Bars: A, 20μm; D, F, H, 10μm. Error bars represent sem.

The basement membrane can provide cues to establish or maintain apical-basal polarity and it is altered during breast cancer progression (Lee and Streuli 2014). Therefore, to further understand changes in cell polarity and tissue organization we investigated the basement membrane and myoepithelial cells by immunostaining mouse tumour sections for laminin, a prominent basement membrane marker. Normal ducts and hyperplasia/MIN had robust laminin staining around the outside of ducts (Supplemental Figure S2.5C). Although some cells in stratified epithelia appeared to lack contact with laminin, laminin was detected surrounding collapsed lumen, supporting the idea that collapsed lumen maintain elements of a cell polarity program, and that the myoepithelial cell layer is not responsible for depositing the laminin in these lesions (Supplemental Figure S2.5 C,D).

As shown above, the fraction of cells with apical-basal polarity and lumen size is reduced as breast lesions progress. We predicted that non-polarized cells may have a proliferative advantage, allowing them to outgrow the polarized epithelial population. We immunostained for Ki67 and found that non-polarized cells had a ~4-fold increase in the proportion of proliferating cells compared to their polarized counterparts (Supplemental Figure S2.5 E,F). To determine if polarized cells may be eliminated by apoptosis, we stained for both cleaved-Caspase 3 and TUNEL, and quantified the number of positive cells that are contacting the lumen or not. Polarity markers were lost in most apoptotic cells, therefore the quantification was conducted with respect to their position, whether contacting the lumen or more basally positioned. However, we found that cells contacting the lumen had lower apoptosis compared to more basally positioned cells (Supplemental Figure S2.5 G, H), suggesting that polarized cells are unlikely eliminated through cell death during carcinoma development.

To further examine key steps in the progression, we established 3D organotypic cultures from mammary epithelial cells from MMTV-rtTA-PyMT mice, which drive PyMT oncogene expression through a doxycycline-dependent inducible promoter (Rao et al. 2014). Cells from non-induced mice

were cultured to establish polarized structures with a lumen, and then doxycycline was added to the medium to induce PyMT expression (Figure 2.3C). Cells were fixed every 2 days and the lumen were visualized by staining F-actin with Phalloidin. Before addition of doxycycline, ~90% of organotypic cultures exhibited a single large primary lumen (Figure 2.3 D,E). After 2 days, ~50% of of organotypic cultures were multilayered, but still contained a large primary lumen. Between 6 and 8 days, organotypic cultures dramatically shifted phenotype and ~90% existed as multilayered structures with collapsed lumen. Importantly, the stages from 3D culture were morphologically similar to tumours *in vivo* (i.e. stratification and luminal collapse). Although the lumen appeared as multiple microlumen in confocal image slices, 3D reconstructions from z-stacks confirmed that they are mostly connected and convoluted collapsed lumen (Supplemental Figure S2.5H). Although tumours from PyMT mice do not go through stages that resemble human FEA or ADH, they do form stratified epithelial ducts, progressively lose cell polarity and undergo luminal collapse, key elements observed in the majority of human HR+ breast lesions examined.

2.2.5 Asymmetric divisions contribute to stratification

Our data from human and mouse lesions indicate that establishing stratified ducts may be one of the earliest events in early breast cancer progression. Oriented cell divisions are essential to maintain normal epithelial architecture in many tissues, including the mammary gland, and cell divisions perpendicular to the duct can generate multilayered mammary ducts (Godde et al. 2014; Huebner et al. 2014). We therefore examined whether misoriented divisions occurred *in vivo* in the PyMT model. We immunostained tissue sections for phospho-Ser10-Histone to label mitotic cells, and α-tubulin to label the mitotic spindle in normal and PyMT lesions (Figure 2.3F). In normal ducts, cells predominantly divided within the plane of the duct (0-30°), whereas there were rare oblique (30-60°) or perpendicular

(60-90°) divisions (Figure 2.3 F,G). Interestingly, rare out-of-plane divisions in normal ducts have also been observed previously (Godde et al. 2014). In contrast, cells in PyMT-expressing ducts that were not yet stratified, divided in random orientations (Figure 2.3 F,G), supporting that misoriented cell divisions contribute to the formation of stratified ducts and disrupt epithelial organization.

To further examine whether the products of misoriented divisions were sufficient to establish and maintain multilayered ducts, we labeled cell nuclei of mammary cells with mCherry-tagged Histone-H2B and tracked the fate of cell divisions in 3D organoid cultures (Figure 2.3 H,I, Supplemental Figure S2.6A). In non-induced cultures, cells predominantly divided within 30° of the plane of the lumen, with rare oblique divisions occurring at an angle of 30-60°. However, following oblique divisions the cells resolved to maintain a simple epithelial organization (Supplemental Figure S2.6A). Dox induction of PyMT resulted in cells more frequently dividing in oblique or perpendicular orientations to the apical domain, which resulted in one daughter cell retaining an apical position and one adopting a basal position (Figure 2.3 H, I, Supplemental Figure S2.6B). Importantly, we tracked cell fates for several hours after division and found that basal cells were permanently excluded from an apical position. Moreover, we did not observe any stratification arising from cell rearrangement or migration alone.

Loss of cell polarity can disrupt cell division orientation and previous reports indicate that loss of apical-basal polarity occurs prior to stratification and hyperplasia in mouse models of breast cancer (Godde et al. 2014; Huebner et al. 2014). To determine whether polarity was disrupted prior to misoriented cell divisions in PyMT mammary glands, we examined Par6 staining in dividing cells in hyperplasia. We found that Par6 was present at the apical membrane, indicating that apical-basal polarity is intact during randomly oriented cell divisions in this model (Supplemental Figure S2.6C). Therefore, this reveals that non-polarized cells in the stratified epithelium arise from asymmetric

divisions, in which one cell retains contact with the lumen and apical identity and the other attains a basal position and lacks apical identity.

2.2.6 Reduced RhoA activity is associated with luminal collapse

Since the actomyosin cytoskeleton controls apical integrity and lumen size (Martin and Goldstein 2014), we investigated its putative role in luminal collapse. Myosin II is activated through phosphorylation by Rho kinase (ROCK1/2) to control tight junction and apical integrity (Zhan et al. 2008). To investigate whether Myosin II activity was altered in collapsed lumen we immunostained for phospho-Myosin II in tissue sections from induced or non-induced PyMT mice. Whereas normal ducts exhibit robust phospho-Myosin that localized to the apical membrane, phospho-Myosin was less intense and diffuse in Dox-induced glands (Figure 2.4 A,B). To confirm and extend this observation, we also examined RhoA-GTP by immunostaining tissue sections with an active-state specific antibody (Kuipers et al. 2014). Indeed, we also observed that active RhoA-GTP was reduced in ducts induced with PyMT, and closer examination indicated that RhoA-GTP was low on both open and collapsed lumen in PyMT-expressing glands (Figure 2.4 C-E). In early transformed ducts that were not yet stratified and collapsed, we also observed low RhoA-GTP (Figure 2.4D), indicating that it precedes lumen collapse in this model. In human premalignant lesions, ADH exhibits well-developed secondary lumen, but lumen were frequently collapsed in DCIS. We therefore evaluated samples containing both ADH and DCIS with collapsed lumen to determine if RhoA-GTP was also reduced in human samples (Figure 2.4 F,G). Indeed, we observed a reduction in RhoA activity in collapsed lumen, supporting that loss of apical integrity associated with luminal collapse may result from reduced RhoA/myosin II activity.

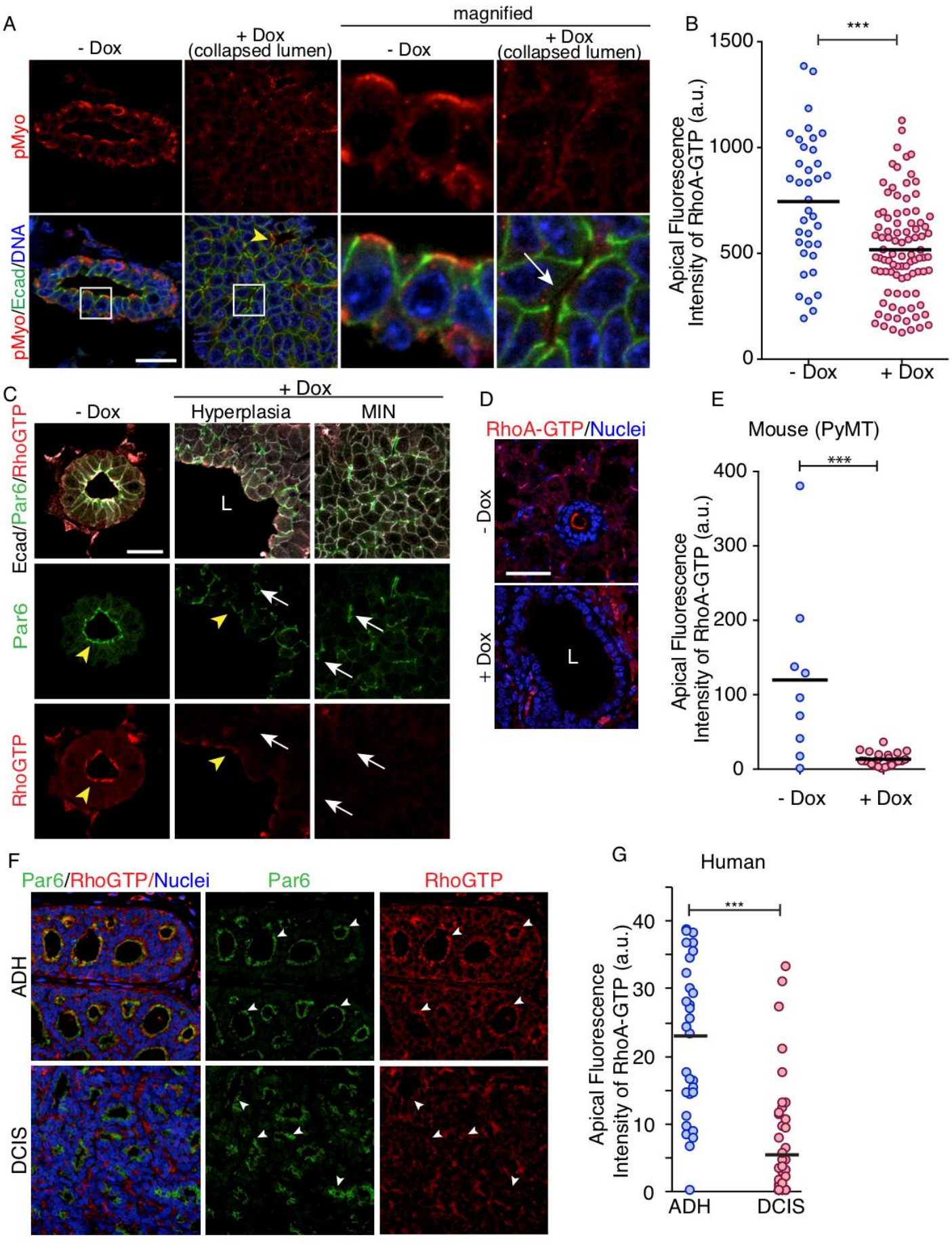
A
magnified
- Dox
+ Dox (collapsed lumen)
- Dox
+ Dox (collapsed lumen)
pMyo
pMyo/Ecad/DNA
B
***
Apical Fluorescence Intensity of RhoA-GTP (a.u.)
1500
1000
500
0
- Dox
+ Dox
C
+ Dox
- Dox
Hyperplasia
MIN
Ecad/Par6/RhoGTP
L
Par6
RhoGTP
D
RhoA-GTP/Nuclei
- Dox
+ Dox
L
E
Mouse (PyMT)
***
Apical Fluorescence Intensity of RhoA-GTP (a.u.)
400
300
200
100
0
- Dox
+ Dox
F
Par6/RhoGTP/Nuclei
Par6
RhoGTP
ADH
DCIS
G
Human
***
Apical Fluorescence Intensity of RhoA-GTP (a.u.)
40
30
20
10
0
ADH
DCIS

Figure 4: Luminal collapse is associated with disrupted apical integrity. (A) Image of mouse mammary glands with or without Dox-induced PyMT expression immunostained for phospho-Myosin II (red) and E-Cadherin (green). Yellow arrowheads show open lumen and white arrows show collapsed lumen. (B) Quantification of phospho-myosin II intensity at the apical membranes. Five tissue sections were analyzed from each of 3 mice. (C) Images of mouse mammary gland with or without Dox-induced PyMT expression immunostained for Par6 (red) and active RhoA (RhoGTP; green). Yellow arrowheads show open lumen and white arrows show collapsed lumen. (D) Image showing RhoA-GTP in open ducts in glands with or without Dox-induced PyMT. The lower panel shows a duct that has not collapsed. (E) Quantification of active RhoA (RhoGTP) at the apical membrane. Three issue sections were analyzed from each of 3 mice. (F) Images of adjacent ADH and DCIS immunostained for Par6 (green) and RhoA-GTP (red). (G) Quantification of apical intensity of RhoA-GTP in ADH (n=29 lumen) and DCIS (n=52 lumen) from 4 human subjects. Bars: A and C, 20μm; E, 50μm.

We next sought to test if restoring RhoA activity could block luminal collapse. Reduction of RhoA/myosin activity at the apical membrane could be due to either reduced RhoGEF or increased RhoGAP activity. We first investigated whether there was a change in a RhoGEF at the apical membrane in collapsed lumen. One candidate GEF is p114RhoGEF, which is expressed on the apical domain in epithelial cells and is required to maintain apical integrity and regulates epithelial morphogenesis (Nakajima and Tanoue 2011; Terry et al. 2011). However, immunostaining of tumour sections from PyMT tumours showed strong expression of p114RhoGEF on both open and collapsed lumen (Supplemental Figure S2.7A). The presence of p114RhoGEF on the apical membrane of collapsed lumen suggests that loss of apical GEF activity is unlikely responsible for reduced RhoA and myosin II activity in collapsed lumen, although we cannot exclude the possibility that other apical GEFs are altered. Instead, we predicted that altered GAP activity may affect RhoA activity during luminal collapse. p190BRhoGAP regulates RhoA during mammary gland morphogenesis and increased p190-B expression was found in poorly differentiated mammary tumours in mice (Chakravarty et al. 2000; Vargo-Gogola et al. 2006). Moreover, p190-B haploinsufficiency in the mammary epithelium was reported to inhibit ErbB2-mediated mammary tumour formation (Heckman-Stoddard et al. 2009). We therefore investigated whether p190-B was altered following doxycycline-induction of PyMT. Following Dox-induced PyMT expression in mammary glands we observed p190-B immunostaining was more intense and diffusely distributed throughout cells (Supplemental Figure S2.7B). Specificity of the antibody was confirmed by knockdown (Supplemental Figure S2.7 C,D). Therefore, we investigated whether reducing p190-B could block luminal collapse in the 3D organoid model. We identified two independent shRNA that efficiently reduced p190-B in mammary epithelial cells and noted that knock-down alone had little effect on cyst growth and structure. (Supplemental Figure S2.7 C,D). Upon PyMT induction by adding

doxycycline to the culture medium, we noted that p190-B knockdown impaired both lumen collapse and the generation of solid organoids (Figure 2.5 A,B).

Finally, we examined whether depleting p190-B could reduce luminal collapse *in vivo*. We depleted p190RhoGAP from mammary epithelial cells and performed transplants into the cleared mammary fat pad. After regeneration of the epithelium for 5 weeks in the absence of doxycycline, we induced PyMT for an additional 2 weeks. We observed the ducts depleted of p190-B retained a more organized structure with significantly more lumen maintained compared to glands expressing PyMT with control shRNA (Figure 2.5 C,D). Importantly, we confirmed that RhoA-GTP was retained in ducts depleted of p190-B (Figure 2.5C). Depletion of p190B in vivo did not significantly affect the proliferation rate or orientation of cell division (Figure S2.7 E, F), indicating that reduced apical RhoA activity is not required for stratification. Despite the retention of lumen, in some ducts we observed cells invading basally from ducts in p190-B-depleted glands, which was not observed with control shRNA or in parental PyMT tumours, nor in 3D organoid cultures *in vitro* (Figure 2.5A and not shown). We attribute this to sustained RhoA activity, caused by GAP depletion, having multiple effects *in vivo*, which highlights the tight spatio-temporal control of RhoA during mammary gland development and cancer progression. Nonetheless, our *in vitro* and *in vivo* data support that luminal collapse and disrupted tissue organization are at least partially due to reduced RhoA activity.

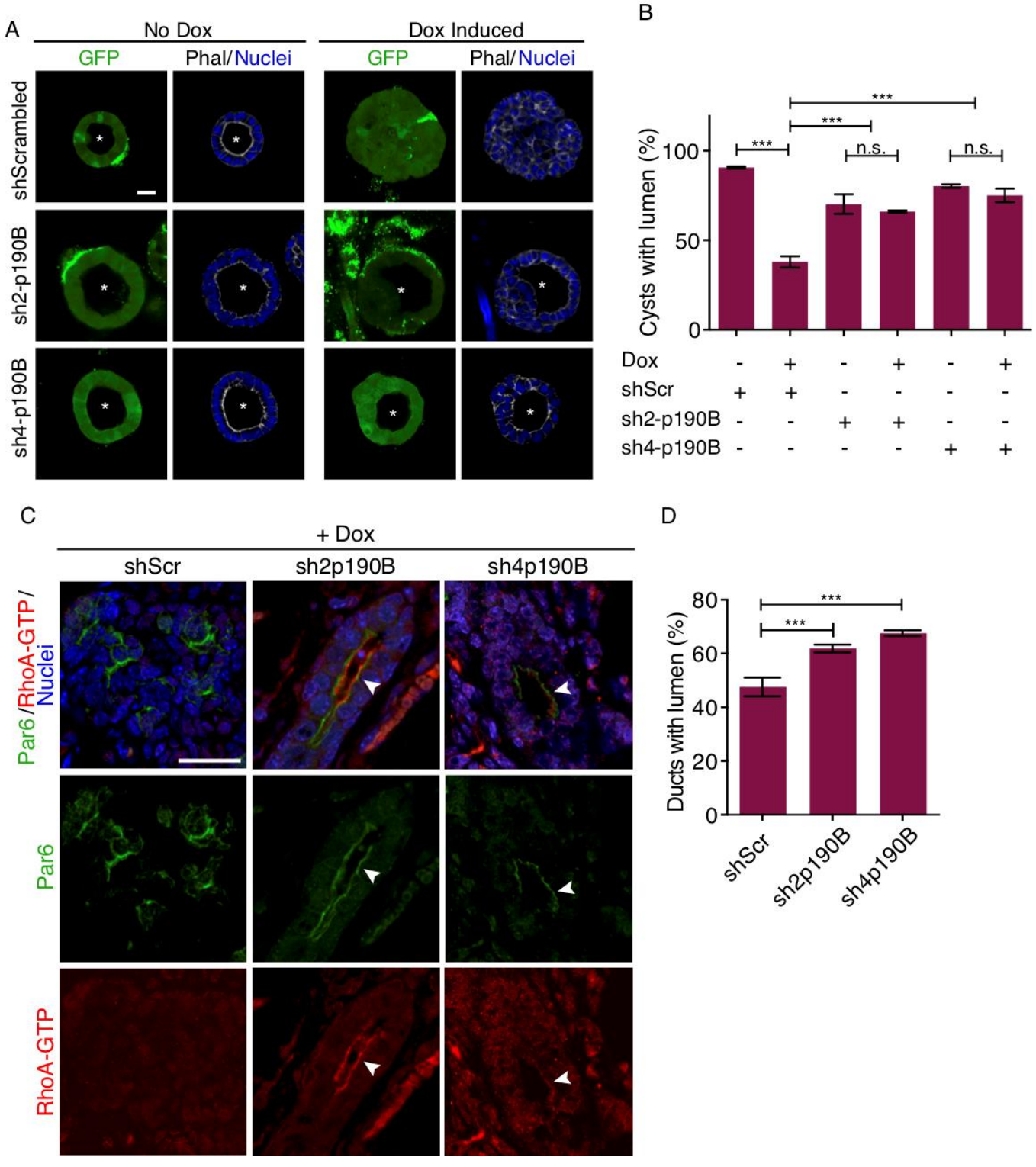
A
No Dox
Dox Induced
GFP
Phal/Nuclei
GFP
Phal/Nuclei
shScrambled
sh2-p190B
sh4-p190B
B
Cysts with lumen (%)
100
50
0
***
***
***
n.s.
n.s.
Dox - + - + - +
shScr + + - - - -
sh2-p190B - - + + - -
sh4-p190B - - - - + +
C
+ Dox
shScr
sh2p190B
sh4p190B
Par6 /RhoA-GTP / Nuclei
Par6
RhoA-GTP
D
Ducts with lumen (%)
80
60
40
20
0
***
***
shScr
sh2p190B
sh4p190B

Figure 2.5: Regulation of RhoA activity regulates lumen collapse. (A) Images of 3D organotypic cultures with or without Doxycycline-induced PyMT expression in the presence of scrambled (shScr) or two different p190B-RhoGAP shRNA and immunostained for Par6. GFP marks cells expressing shRNA. Asterisks show open lumen. (B) Quantification of lumen phenotypes in cysts. (C) Images of sections of mouse mammary gland with or without induction of PyMT and expression of p190B-RhoGAP shRNA immunostained for Par6 (green) and RhoA-GTP (red). (D) Quantification of ducts with open lumen. 2 tissue sections were analyzed from each of 4 mice. ***, $p<0.001$. Bars: 25μm.

2.2.7 Loss of apical-basal polarity is reversible

We observed a series of morphological steps that occur during progression of breast cancers that contribute to loss of apical-basal polarity and generating solid ducts. We next asked whether this process was reversible if the stimulating oncogene was removed. To test this, we induced PyMT mice for 2 weeks to generate early stage mammary tumours with multilayered, collapsed epithelial ducts, then de-induced for 3-days, 1-week, or 4-weeks by removing doxycycline from the drinking water (Figure 2.6A). In all mice examined, we found that that de-induction rapidly reverted to ducts with apical-basal cell polarity and a hollow lumen (Figure 2.6 B-D). Within 3-days of doxycycline withdrawal, a ring of cells formed that contained a heterogeneous population of cells with and without apical identity (Figure 2.6 B-C). However, by 1-week after deinduction, the proportion of cells exhibiting apical-basal membrane polarity was not significantly different that control normal ducts (Figure 2.6 B,C). Deinduction of oncogenes can result in apoptosis and lumen clearing in culture (Jechlinger et al. 2009), and we therefore examined whether apoptosis was also involved in the more complex in vivo environment (Figure 2.6 E,F). At 3-days deinduction, we observed that the cores of the ducts were filled with apoptotic cells. At 1-week post induction, the cores were mostly free from apoptotic cells, but retained debris, which was cleared by 4-weeks deinduction. Interestingly, during the spike in apoptosis at 3-days deinduction, a relatively high proportion of cells in the reforming duct were also apoptotic (Figure 2.6E, arrows). Finally, we examined whether RhoA-GTP was restored to the apical membrane of reforming ducts. Although there was a significant increase in the number of cells with apical-basal polarity at 3-days deinduction, RhoA was not restored at this timepoint (Figure S2.8 A,B). At 4-weeks deinduction, RhoA-GTP was significantly higher than in the induced glands, however, it had not returned to levels observed in normal ducts by this time point (Figure S2.8 A,B). We next asked whether more advanced tumours that had lost apical basal polarity were also able to restore polarity epithelial

organization. We induced PyMT for 8-weeks, then de-induced for an additional 9-weeks. Surprisingly, the de-induced glands were able to restore the number of polarized cells back to 70-80%, which was slightly lower than in normal ducts, and the number of ducts containing lumen back to normal levels (Figure 2.6 B-D). This demonstrates that epithelial remodeling in mammary tumour progression is dynamic and disruption of cell and tissue polarity is reversible, at least in this model.

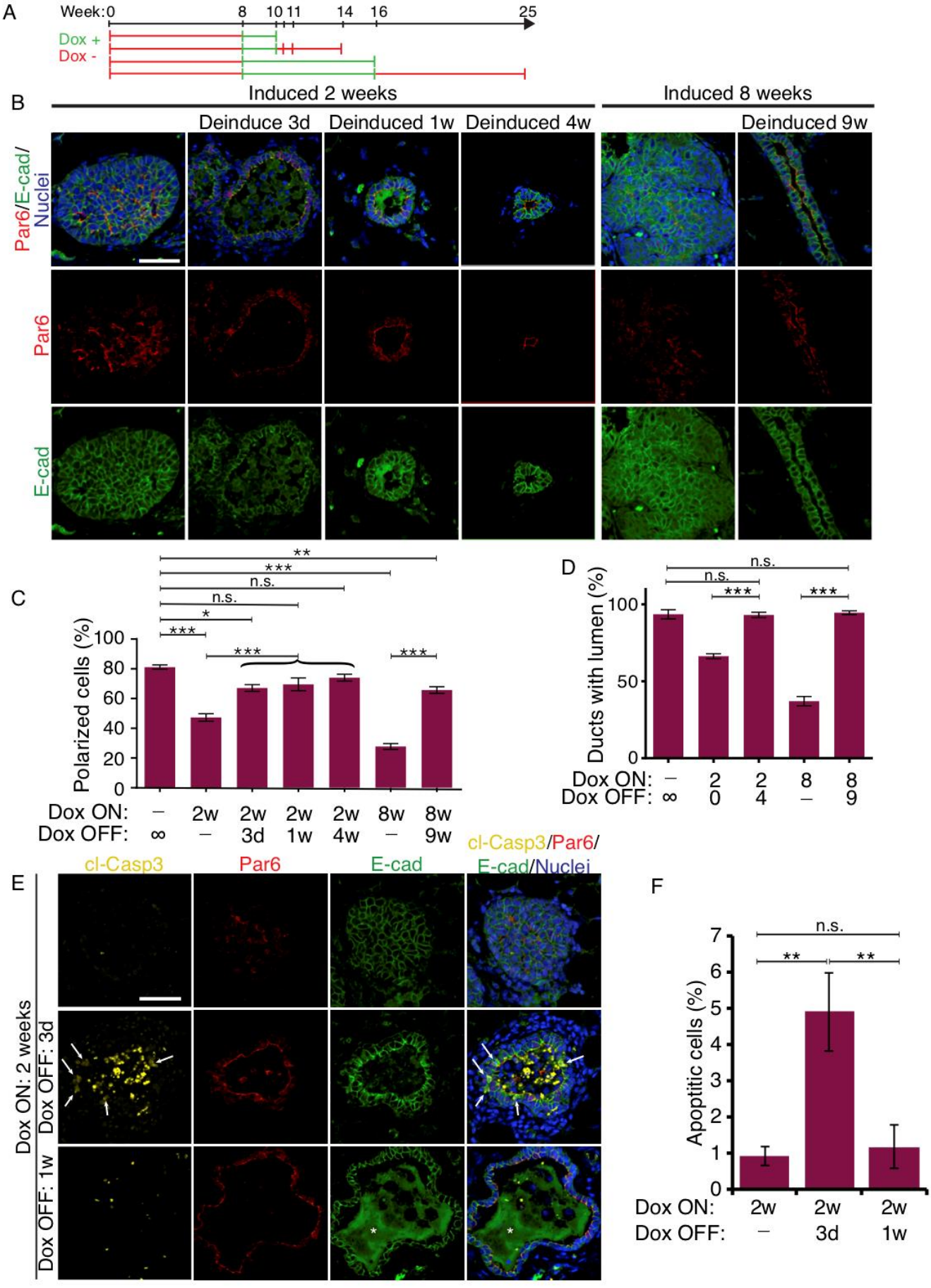
A
Week: 0
8
10 11
14
16
25
Dox +
Dox -
B
Induced 2 weeks
Induced 8 weeks
Deinduce 3d
Deinduced 1w
Deinduced 4w
Deinduced 9w
Par6/E-cad/ Nuclei
Par6
E-cad
C
Polarized cells (%)
Dox ON: – 2w 2w 2w 2w 8w 8w
Dox OFF: ∞ – 3d 1w 4w – 9w
D
Ducts with lumen (%)
Dox ON: – 2 2 8 8
Dox OFF: ∞ 0 4 – 9
E
cl-Casp3
Par6
E-cad
cl-Casp3/Par6/ E-cad/Nuclei
Dox ON: 2 weeks
Dox OFF: 3d
Dox OFF: 1w
F
Apoptitic cells (%)
Dox ON: 2w 2w 2w
Dox OFF: – 3d 1w

Figure 2.6: Disruption of tissue organization and cell polarity is reversible. (A) Scheme showing doxycycline treatment schedule for PyMT induction and de-induction. (B) Images of mammary tissue immunostained for Par6 and E-cadherin after Dox-mediated induction/de-induction for specified times. (C) Quantification of cells with apical-basal membrane polarity from induction/de-induction cycles. (D) Quantification of ducts with open lumen from induction/de-induction cycles. (E) Images of mammary tissue immunostained for Par6, E-cadherin, and cleaved Caspase 3 after Dox-mediated induction/de-induction for indicated times. The white star indicates fluorescence from debris in the lumen. (F) Quantification of the number of cleaved Caspase-3 positive cells in E-cadherin positive ductal cells. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Bars: 50μm.

2.3 Discussion

2.3.1 Loss of polarity is a progressive event in luminal breast carcinoma development

The development breast carcinoma is associated with loss of growth control and disrupted tissue organization in the form of solid ducts that lack a central lumen. To understand loss of polarity in early progression of breast cancers, we examined apical-basal polarity markers in HR+ DCIS and made the unexpected discovery that many DCIS contained populations of cells with elements of apical-basal membrane polarity. A closer analysis revealed that polarized cells were organized around multiple small collapsed lumen or apical patches distributed throughout the lesions. This reveals that although tissue organization is largely disrupted in DCIS, individual cells can retain elements of cell polarity. However, these cells unlikely are normal, and they may have other aspects of polarity disrupted.

Invasive breast cancers are heterogeneous, which has a significant impact on therapeutic response and relapse (Zhang et al. 2017), but heterogeneity in pre-invasive lesions is less well characterized. We report that within pre-invasive lesions cellular heterogeneity exists with respect to apical-basal polarity. Since apical-basal membrane polarity is an important regulator of cell behavior, including control of signaling, proliferation, survival, and invasion (Halaoui and McCaffrey 2015) heterogeneity in polarity status may enable altered signaling in polarized and non-polarized cells which may promote their progression to invasive carcinoma. In support of this, we observed that non-polarized cells were more proliferative than their polarized counterparts. Moreover, invasive breast cancers examined by us here and previously (McCaffrey et al. 2012) were found to be comprised of cells that lack apical-basal polarity. Therefore, disrupted polarity cues may contribute to heterogeneous cell behaviors in the progression of pre-invasive breast lesions that may promote development of invasive carcinoma. Moreover, although pre-invasive lesions in which some cells retain apical-basal polarity was most common in our dataset, we also observed other phenotypes, including basolateral or cytoplasmic

Par6, and rare ducts in which apical-basal polarity was lost and cells shed into the lumen. Whether these different phenotypes are associated with different potential to progress to invasive carcinoma is not known at present, and will need to be evaluated in the future.

Luminal filling caused by cells shedding or migrating into the lumen and evading anoikis has been proposed as a mechanism to generate solid lumen (Leung and Brugge 2012a; Pradeep et al. 2012). In our dataset of pre-invasive luminal breast lesions, we observed intraluminal epithelial cells only rarely (2/114 lesions) indicating that although this process can occur, it is not the primary mechanism that generates solid lesions in DCIS. Instead, we found that luminal collapse of stratified ducts and progressive loss of apical-basal polarity contribute to generate solid ductal structures. The differences in luminal filling versus stratification and luminal collapse may be explained by the presence or absence of tight junctions. Luminal filling has been characterized predominantly in MCF10A cells, which lack tight junctions and apical membrane identity (Fogg et al. 2005; Mailleux et al. 2008). In support of this, the two lesions we identified with luminal filling had disrupted apical polarity. However, disruption of Scrib or expression of ErbB2 also eliminate tight junctions and cell polarity, but do not induce cell shedding into the lumen (Godde et al. 2014; Huebner et al. 2014), suggesting that other factors may also contribute.

The PyMT model is a widely used mouse model of breast cancer that recapitulates signaling features of luminal breast cancer, and progresses through pre-invasive stages that are histologically and molecularly similar to human breast cancer progression (Maglione et al. 2001; Lin et al. 2003; Herschkowitz et al. 2007; Fluck and Schaffhausen 2009; Rao et al. 2014). Human cancers are thought to initiate from alterations in individual cells, and although this initiating event may not be recapitulated in transgenic models that have uniform expression of an oncogene, the PyMT model is a useful tool to study progression because of its high penetrance, short latency and highly reproducible progression to

carcinoma (Guy et al. 1992; Rao et al. 2014). Based our observations from PyMT mice in vivo and in organotypic cultures, we propose that increased proliferation initially expands the number and size of ducts, while maintaining relatively normal tissue organization. The next step involves breaking tissue polarity and creating a stratified epithelium. It was previously reported that mammary stratification is induced by cell divisions oriented perpendicular to the duct plane as an early event in hyperplastic growth in response to oncogene activation or tumour suppressor inactivation (Godde et al. 2014; Huebner et al. 2014). In these models, misorientation of the mitotic spindle was associated with loss of apical-basal polarity, either by directly disrupting the polarity machinery, or through expression of the ErbB2 oncogene, which can bind to and disrupt the Par polarity complex (Aranda et al. 2006b; Godde et al. 2014; Huebner et al. 2014). Our results from PyMT mouse lesions similarly show that perpendicular divisions are the primary driver that generats stratified epithelial structures early during tumour progression. However, one important difference with the PyMT model is that apical-basal membrane polarity is not lost from the luminal cells. Instead, cells undergo an asymmetric cell division to generate a daughter cell that retains an apical position and apical membrane identity, and a basally positioned daughter that does not contact the lumen and lacks apical-basal polarity. The contribution of asymmetric divisions that maintain a population of polarized cells and generate non-polarized cells is consistent with gradual reduction in the proportion of polarized cells we observed during progression in humans and PyMT mice.

We propose that similar processes may take place during pre-invasive breast cancer progression in humans (Figure 2.7). Genetic and histological data support a model by which stepwise progression through FEA, ADH, and DCIS is a non-obligate precursor of invasive carcinoma (Sgroi 2010; Sinn et al. 2010). FEA present as enlarged ducts that are organized as one to several layers. Since cell divisions are rare in human samples, we were unable to identify any cells dividing in FEA to determine if division

orientation is altered in stratified samples. However, perpendicular division can lead to mammary gland stratification in a variety of normal and tumourigenic contexts, as described here and previously (Godde et al. 2014; Huebner and Ewald 2014), and it seems likely that this may also contribute to stratification in humans as well. In our human experimental dataset, we observed that stratified FEA retained apical-basal polarity in cells adjacent to the lumen and cells that lack apical-basal polarity in a more basal position, similar to what we observed in PyMT mice and organotypic cultures, raising the possibility that that asymmetric divisions generate cells that lack apical-basal polarity in human breast lesions as well. The formation of a stratified epithelium may initiate an activated epithelial state that permits dynamic tissue remodeling during progression to later stages. Indeed, the generation of a stratified epithelium is coincident with dynamic cell rearrangements that promote branching morphogenesis and ductal growth during mammary development, and collective migration occurs from stratified epithelial structures in advanced carcinoma (Nguyen-Ngoc et al. 2012b; Cheung et al. 2013; Huebner and Ewald 2014). Our observations indicate that stratified epithelia may be involved in tissue remodeling during pre-invasive breast cancer progression, since we observed finger-like cords extending from stratified regions that form intraluminal bridges and secondary lumen in ADH, a process we call lumen splitting (Figure 2.7). Importantly, the luminal edges of finger-like projections retain apical identity, maintaining polarized secondary lumen surrounded by non-polarized cells.

2.3.2 Luminal collapse generates solid duct structures in HR+ carcinoma progression.

Importantly, we observed a progressive reduction in the proportion of cells exhibiting apical-basal membrane polarity in more advanced human lesions, suggesting that similar to the PyMT model, human pre-invasive cancer develop with progressive loss of apical-basal polarity. Moreover, we also observed collapsed lumen in more advanced lesions in both the PyMT model and human DCIS. Lumen

collapse appear to depend on reduced RhoA/Myosin activity at the apical membrane, since both were reduced at the apical membrane following PyMT induction, and restoring RhoA at the apical membrane, by depleting p190B-RhoGAP, prevented luminal collapse. Intriguingly, loss of RhoA/myosin was observed prior to lumen collapse, indicating that it is permissive to collapse, but not sufficient. RhoA has been shown to have both oncogenic and tumour suppressive functions (Zandvakili et al. 2016). Our data support a mechanism whereby RhoA has tumour suppressive activity in early stages of breast cancer progression to maintain lumen architecture of ducts. Consistent with this, a decrease in the progression of pre-neoplastic lesions in MMTV-Neu mice was also observed with p190-B haploinsufficiency (Heckman-Stoddard et al. 2009). In some p190-B-depleted ducts we observed increased invasion of cells, likely due to increased global RhoA activity. This highlights the significance of spatio-temporal regulation of RhoA that contribute to tumour suppressive and oncogenic functions during cancer development. In our human dataset, we found that collapsed lumen were only observed in DCIS. Interestingly, we observed high apical RhoA at open lumen in ADH, but it was significantly reduced in collapsed lumen of DCIS, indicating that reduced apical RhoA is relevant may be development of DCIS in humans.

Apical constriction is a force generating mechanism that uses actomyosin contractility to reduce lumen sizes (Martin and Goldstein 2014). Our data showing reduced apical RhoA/myosin II argue against apical constriction as a force to generate the collapsed lumen phenotype in pre-invasive breast lesions. The force required to collapse lumen is unknown at present, but we hypothesize it could result from forces within the stratified epithelium or increased proliferation of non-polarized cells, which would cooperate with reduced apical integrity to collapse lumen. In support of this concept, tissue forces in the Drosophila wing pouch were found to arise from differential cell proliferation rates, rather than apical constriction (Mao et al. 2013).

2.3.3 Loss of apical-basal polarity and tissue organization is reversed upon oncogene removal.

Deinduction of oncogene expression in PyMT and other transgenic mouse models results in tumour reversion in vivo, but it is not known if cell polarity and duct organization is restored (Liu et al. 2011; Rao et al. 2014). Remarkably, we found that disruption of tissue and cell polarity was reversible, and that de-inducing PyMT resulted in polarized ducts, even after advanced carcinoma that mostly lacked cell polarity were established. This suggests that polarity was not irreversibly suppressed in these lesions, but that cells were transiently impaired in their ability to maintain cell polarity. Re-establishing duct organization with a lumen was rapid and involved apoptosis of internal cells to clear the luminal space. Similar clearing of a lumen through apoptosis was observed for other oncogenes in 3D culture (Jechlinger et al. 2009), indicating that there may be a common underlying mechanism. At present we do not understand how some cells are protected from apoptosis, but their position at the outside suggests that interactions with the extracellular matrix may provide survival cues. However, we observed that many cells adjacent to the stroma in the emerging ductal epithelium were also apoptotic, suggesting other factors may be required for survival and that additional epithelial remodeling may contribute. Interestingly, RhoA was not present at the lumen of newly emerging ducts, indicating that it is not involved in reforming the lumen, but we cannot exclude that it may have other functions in epithelial remodeling during this phase.

Our finding that pre-invasive stages are reversible raises the possibility that early breast lesions may be manipulated pharmacologically to restore or maintain organized ducts to prevent progression of pre-malignant lesions or recurrence of breast cancer in the future.

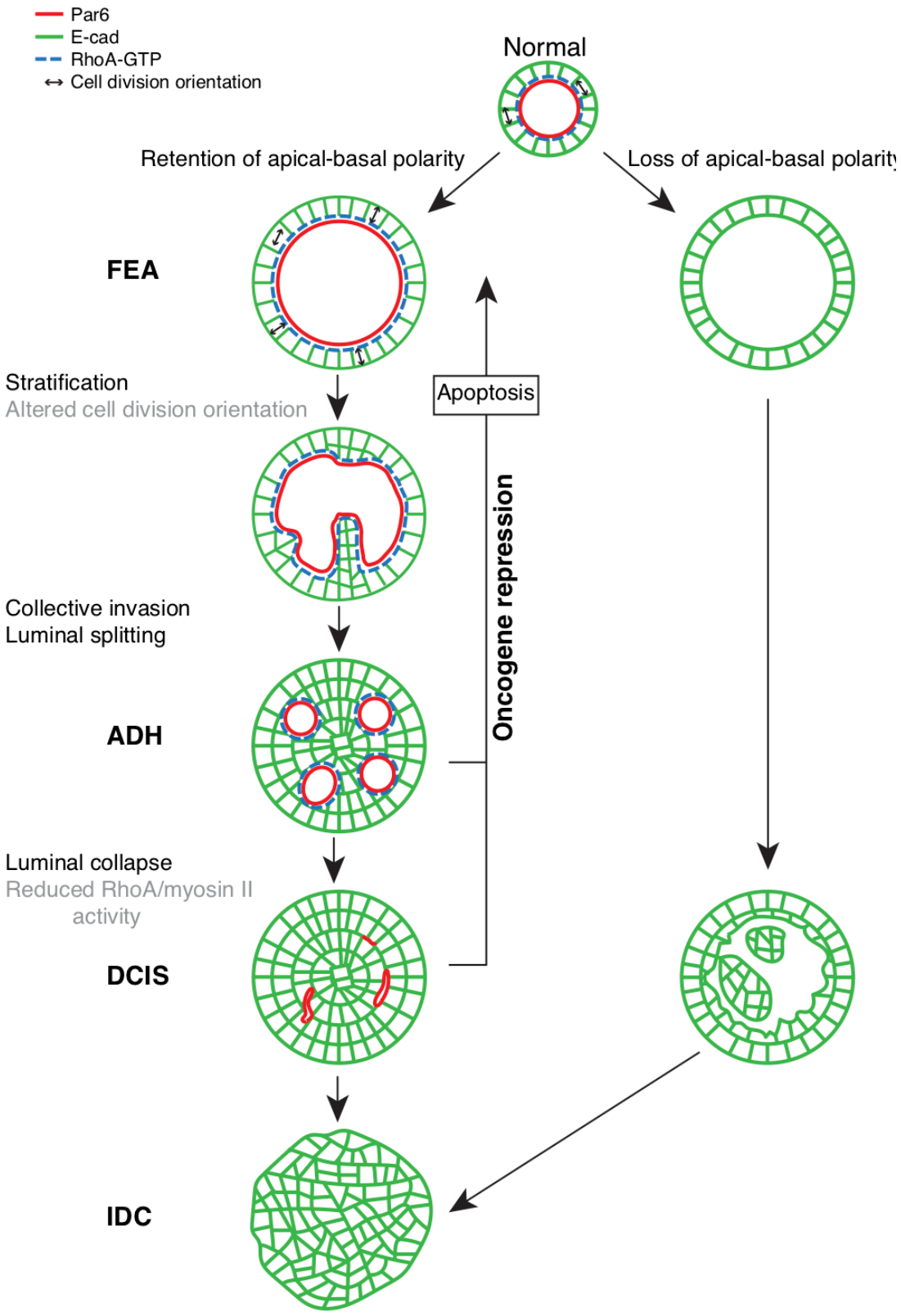

Figure 2.7: Model for loss of apical polarity and lumen filling in ER+ breast cancers. Model showing progressive loss of polarity (left) and early loss of polarity. See discussion for details.

2.4 Materials and Methods

2.4.1 Human breast cancer tissue

Biopsy samples containing normal tissue, FEA, ADH, DCIS, IDC were analyzed by a certified pathologist who is director of breast pathology at the McGill University Health Centre, using standard histological scoring parameters. Tissue sections adjacent to those for histological assessment and were immunostained for polarity markers and other proteins described below. Luminal lesions were confirmed by the presence of ERa and PR, as assessed by immunohistochemistry. All procedures involving human patient tissue were approved by the McGill Institutional Review Board (IRB # A03-M24-15A).

2.4.2 Mouse Models

All mice used were in an FVB background. Tumours from MMTV-PyMT mice were collected at 4- and 8-weeks post-tumour first detection for hyperplasia/MIN and carcinoma, respectively. For mice with inducible expression of PyMT, mT-IRES-Cre (MIC) mice were crossed with MMTV-rtTA (MTB) mice (Rao et al. 2014), and pups heterozygous for both genes were selected. Mice 8-weeks of age and older were given 2mg/ml of doxycycline in their water and were sacrificed after 2- or 8-weeks induction for hyperplasia/MIN and carcinoma, respectively. All procedures involving animals were approved by the McGill University Animal Care Committee.

2.4.3 Immunostaining and imaging

Human breast samples were fixed in formalin and cut at 3□m thickness. Mouse mammary tissue was fixed in either 4% PFA or Carnoy's fixative, as described previously (McCaffrey et al. 2012) and cut at 8□m thickness. Deparafinization, and immunostaining was performed as previously described (McCaffrey et al. 2012). The primary antibodies were used as follows: cleaved-Caspase-3 1/300 (Cell Signaling #9661), Dlg1 1/100 (US Biologicals # S0095-30), E-Cadherin 1/500 (BD Transduction #610181),

Ezrin 1/300 (Cell Signaling #3145), Ki67 1/300 (Abcam #ab15580), Par3 1/250 (Millipore #07-330), Par6B 1/300 (Santa Cruz # sc-67393), Phalloidin 1/100 (Invitrogen #A34055), phospho-Histone-H3 (pH3) 1/250 (Cell Signaling #9701), aPKCι 1/500 (BD Transduction #610175), phospho-Myosin Light Chain II (Cell Signaling #3671), pan Laminin (Abcam #ab14055), RhoA-GTP (NewEast Biosciences #26904), p190-B(BD Transduction #611613), □-Tubulin 1/250 (Millipore #MAB1864), and □-Tubulin 1/500 (Sigma #T9026). Secondary antibodies (Alexa Fluor 488, 555, and 647) were used at a 1/750 dilution at room temperature for one hour. For TUNEL staining, tissue sections were prepared and stained using the TumourTACs kit according to the manufacturer's instructions (Trevigen), except 3,3'-Diaminobenzidine (DAB) chromogen was replace with AlexaFluor-555 Tyramide Signal Amplification (ThermoFisher) for fluorescence detection. Confocal imaging was performed using LSM700 from Zeiss with 20X/0.8NA or 40X/1.4 NA lenses. Image processing of brightness and contrast was performed in ImageJ and was applied uniformly to the whole image.

2.4.4 Tissue section measurements

The number of polarized cells in human and the mouse tissue, sections were taken from normal and tumour tissue, stained with apical markers (i.e. Par6, Ezrin, aPKC), tight junction (ZO1), and basolateral (E-Cadherin, Dlg1) markers. For each cell in a mammary duct or lesion, we traced the plasma membrane encircling the nucleus and scored it as polarized or not-polarized. To be considered polarized in our study, cells met all of the following criteria: 1) Both apical and basolateral membrane markers were detected in the cell; 2) the apical marker was enriched on a part of the membrane and excluded from another part of the membrane; 3) the basolateral marker was enriched on a part of the membrane and was excluded from another part of the membrane; 4) enrichment of apical and basolateral proteins on regions of the plasma membrane were mutually exclusive. A protein was considered enriched if the intensity was higher on the membrane than in the cytoplasm of that cell. A protein was considered

excluded from the membrane if intensity at the membrane was equal to or less than the cytoplasmic intensity in that cell. Non-polarized cells were considered to have basolateral Par6 if cells expressed overlapping Par6 and E-cadherin uniformly on the plasma membrane. Non-polarized cells were considered to have high cytoplasmic Par6, if the cytoplasmic intensity was greater than two-fold higher than the average cytoplasmic intensity of Par6 in normal duct cells. Measurements were performed manually using Image J on tissue sections stained in parallel and imaged sequentially using identical microscope settings. For the human samples, the percentage of cells with apical-basal membrane polarity was determined from 9 normal, 6 ADH, 6 Pure DCIS, 17 DCIS adjacent to IDC, and 5 IDC all characterized by a pathologist. For the mouse samples, 5 normal, 3 hyperplasia/MIN, 3 early carcinomas, and 2 late carcinoma sections were examined from 2-3 different mice each.

To measure the number of polarized and non-polarized cells that were proliferating, PyMT mouse tumour tissue were stained with the Ki67 proliferation marker and with PKC to mark the apical membrane. Using ImageJ, counts were performed on 4 mice with 6 different fields of view per section at the hyperplasia/MIN stage.

To measure the lumen size and number of cells per lumen, the lumen circumference determined by the apical Par6 signal was measured using the polygon selection tool in ImageJ. We examined 31 images of human sections, and >11 images from at least 3 mice, at different stages of progression.

To measure the spindle orientation of dividing cells, sections from FVB (control) and PyMT mice 4 weeks post-palpation were stained for pH3 to mark diving cells and α-tubulin to visualize the spindle pole. The lumen was used as the planar reference, and only dividing cells in regions that were not stratified were measured. Using ImageJ, a line was drawn tangent to the lumen, and another through the spindle poles; the resulting angle was measured. We measured the angles of 46 dividing cells from 7 PyMT mice and 11 dividing cells from 6 glands from 3 FVB mice.

The phospho-Myosin II and RhoA-GTP intensity was measured from tissue sections from 3 mice or 5 patients per condition, with 3-15 images each. For mice, non-induced mammary glands were compared to tissue following 2-weeks doxycycline treatment to induce PyMT expression. Human patient breast lesions were examined for cases containing ADH and DCIS in the same sample. Phospho-myosin II or RhoA-GTP were co-stained Par6 to identify the apical membrane. The average pixel intensity of the phospho-myosin II or RhoA-GTP channel along the apical membrane was measured using the polyline measurement tool in ImageJ.

Circularity was calculated using the Shape Descriptors feature in ImageJ measurements and given by the formula Circularity = $3A/4\pi r^2$, where A is area and r is radius.

2.4.5 Mammary epithelial cell isolation

Mammary glands from 8-12 week old mice were chopped and added to digestion medium (10ml Phenol Free DMEM/F12, 1X Penicillin/Streptomycin, 50ug/ml Gentamycin, 2mg/ml collagenase A, 600U/ml Nystatin) for 1-1.5 hours. The epithelial cells were then purified into pure epithelial organoids by sequential washes in PBS+ 5% FBS and centrifugations (15s at 1500rpm). To obtain single cells, the organoids were resuspended in 0.25% Trypsin for 20min at 37^0C. The trypsin was then quenched with FBS, and the cells were resuspended in media. Finally, single epithelial cells were passed through a 40μm mesh to eliminate cell clusters.

2.4.6 Ex-vivo 3D organotypic culture

Following primary mammary epithelial cell extraction, cells were plated in an 8-well chambered coverglass (LabTek II, ThermoFisher Scientific #155409) or 8 well µ-slide (Ibidi #80826) at a density of 5000 cells per well on top of a layer of 100% GelTrex (ThermoFisher Scientific # A1413202) in media supplemented with 2% GelTrex. The cyst medium consists of Epicult-B mouse medium (StemCell Technologies #05610), Knockout Serum Replacement (Gibco # 10828010), Pen/Strep, 10 ng/ml EGF, 25

µg/ml Insulin, and 1 µg/ml Hydrocorticone. Cysts formed after 5-7 days in culture. The PyMT oncogene was induced with 2µg/ml doxycycline for an additional 5-10 days. To categorize the different phenotypes, the assay was performed on 3 different non-induced MIC mice; the cysts were grown for 8 days, and were then fixed with 4% PFA at days 0, 2, 4, 6, and 8 post doxycycline addition. 3D structures were stained with fluorescent phalloidin to mark the apical membrane and nuclei were labeled with Hoechst 33342. Images of all the cysts (or up to 50 images/well) were taken and categorized manually in the different categories according to two criteria, shape of the lumen (single prominent lumen or collapsed microlumen) and layering of the cells around the lumen (monolayer, multilayer, and full/collapsed cyst).

To prepare cells for live imaging, lentivirus containing cDNA for mCherry-H2B was used to infect cells before embedding, and mark the nuclei. Spindle orientation of dividing cells was then measured using ImageJ.

To knock-down p190-B (*Arhgap5*), shRNA containing lentivirus was used to infect the single cells before embedding. The shRNA used were purchased from ThermoFisher Scientific (sh1p190B TRCN0000012703, sh2p190B TRCN0000012704, sh3p190B TRCN0000012705, sh4p190B TRCN0000012706, and sh5p190B TRCN0000012707) and a non-targeting scrambled shRNA was used as a control. Cells were mixed with the lentivirus in media at a multiplicity of infection (MOI) of 10, and were left spinning at 300rpm for 3 hours at room temperature. The infected cells were then plated with the virus-containing media on top of a layer of 100% GelTrex in media supplemented with 2% GelTrex. Media was changed after 3 days of plating, then every 2 days until the cells were fixed. PyMT was induced using 2µg/ml doxycycline 6-8 days after plating, for an additional 5-7 days

2.4.7 Live Imaging

Organotypic cultures were treated with 2µg/ml doxycycline or water and imaging was immediately started using an automated Zeiss LSM700 confocal microscope with a 20X 0.8NA objective lens. Z-stacked image series of were collected every 15 min for 72-96 hrs. Non-induced samples were used as negative controls and were collected in parallel with induced samples using an automated stage.

2.4.8 Orthotopic Transplants

Following primary mammary epithelial cell extraction and infection, cells were added to mammosphere media plated in suspension for 3 days. The mammosphere medium consists of Epicult-B mouse medium (StemCell Technologies #05610), Knockout Serum Replacement (Gibco # 10828010), Pen/Strep, 20 ng/ml EGF, 20 ng/ml FGF, and 4 µg/ml Heperan Sulfate. Then, the cells were spun down and resuspended in PBS with 10% Geltrex and 10% Trypan Blue, and injected into the cleared mammary fat pad of a 3-week-old FVB female mouse. Each injection was 10-12 µl in volume.

2.4.9 Statistical Analysis

Comparisons of multiple means was performed by ANOVA, using Tukey's *post-hoc* test in general, or Sidak's *post-hoc* test specifically for the shp190-B knock-down cysts. An alpha of 0.05 was used for determining statistical significance. Comparison of two unpaired independent means was performed using a student's t-test. Statistics were determined using excel, SPSS, and GraphPad Prism. All images are representative of more than 5 fields from at least three mice or 5 human tumour samples.

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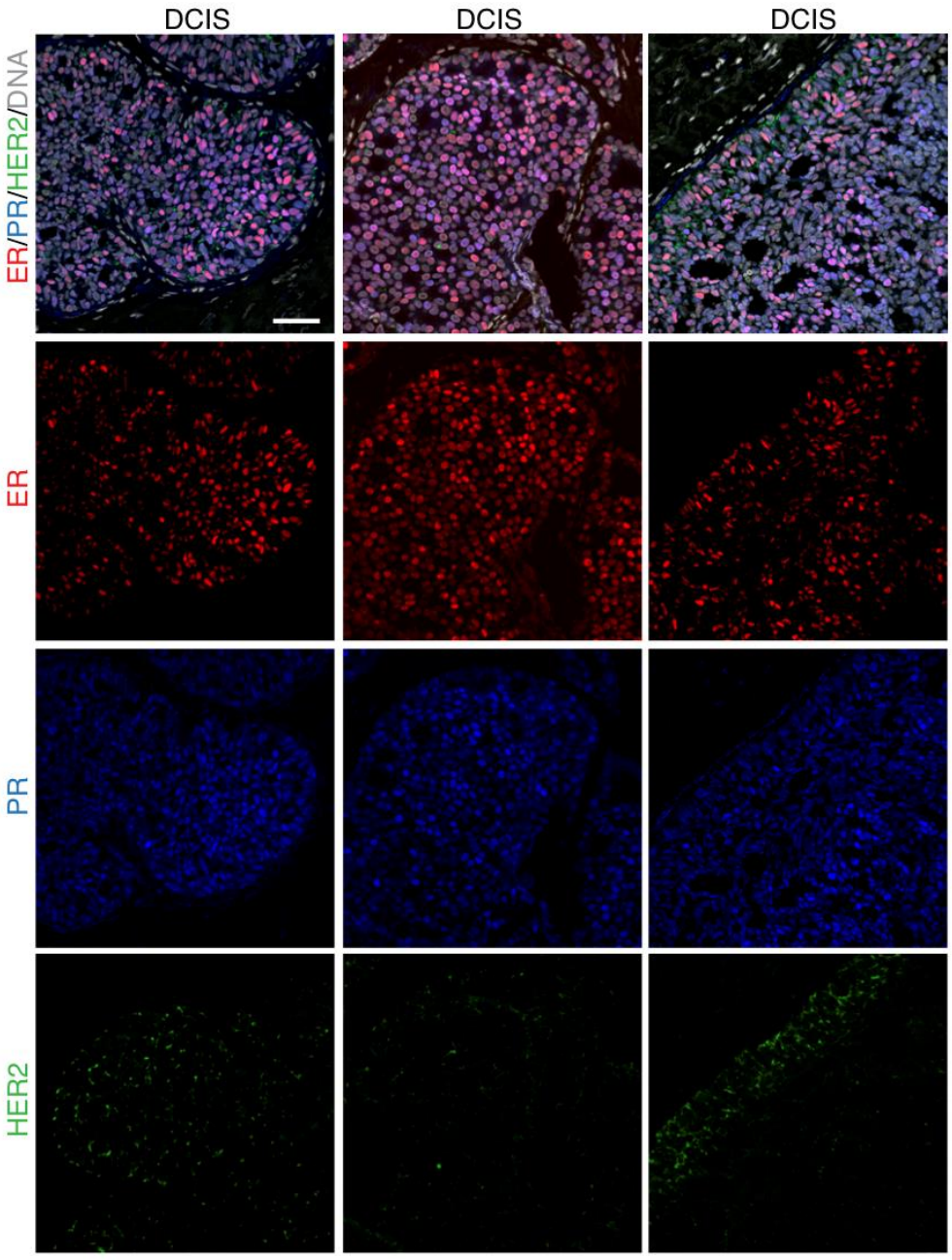
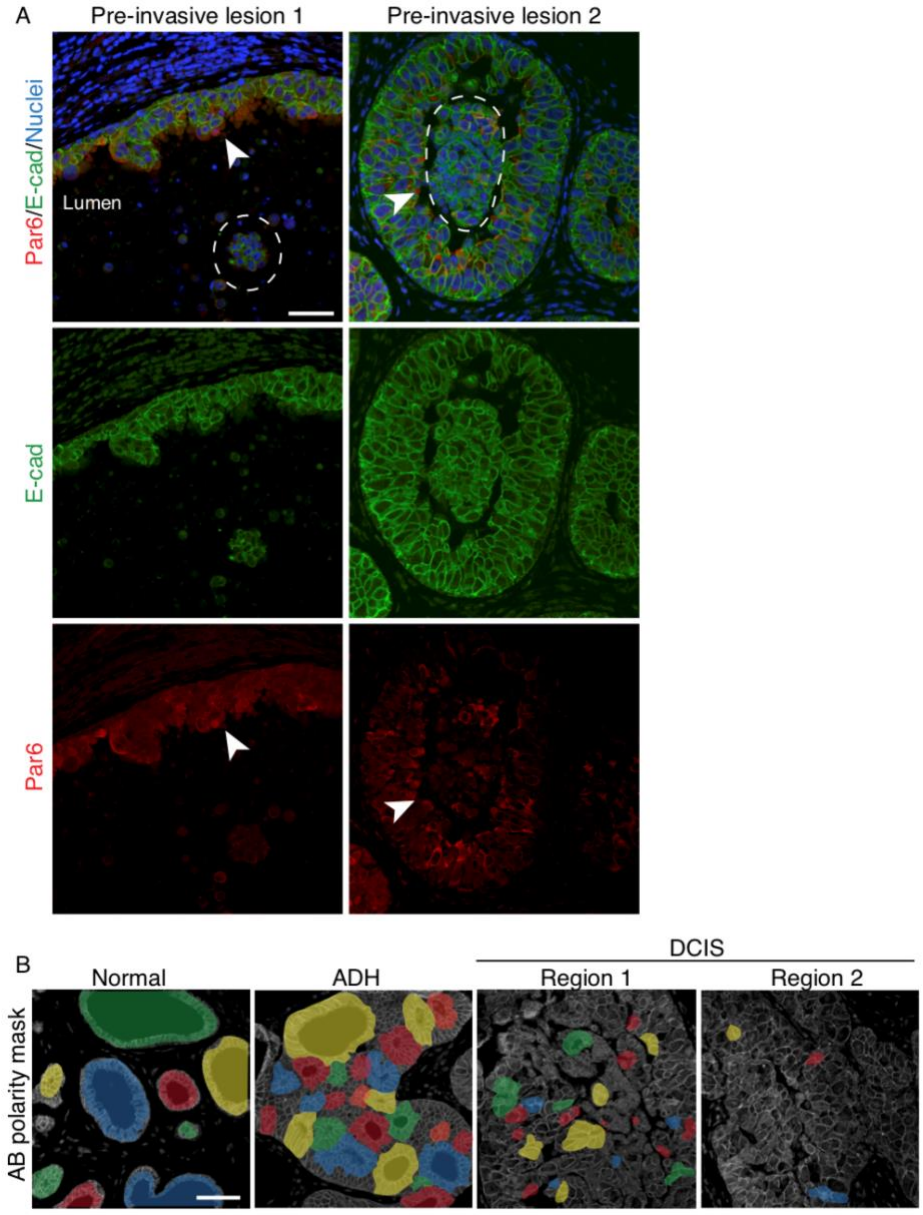
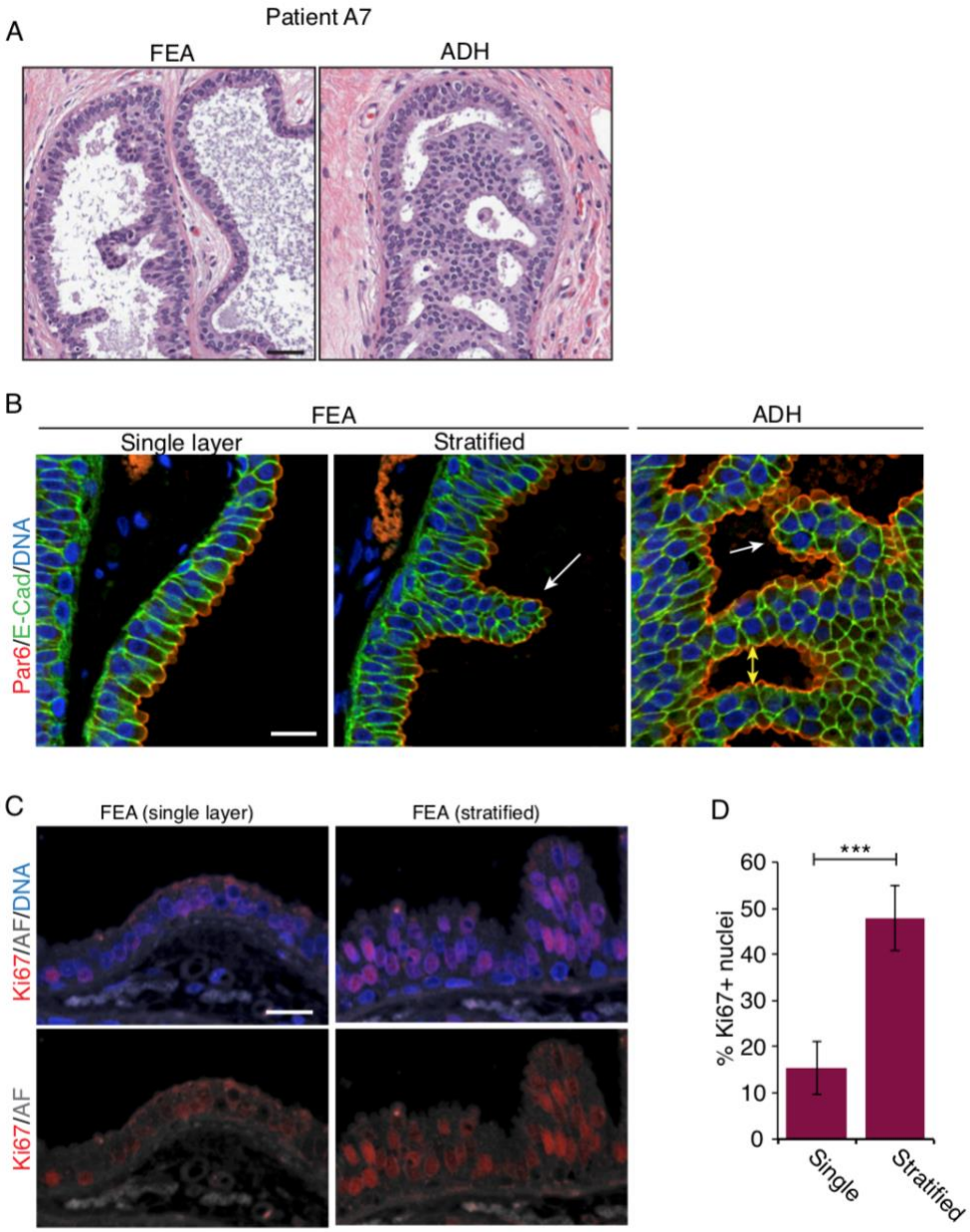
2.7 Supplemental Figures



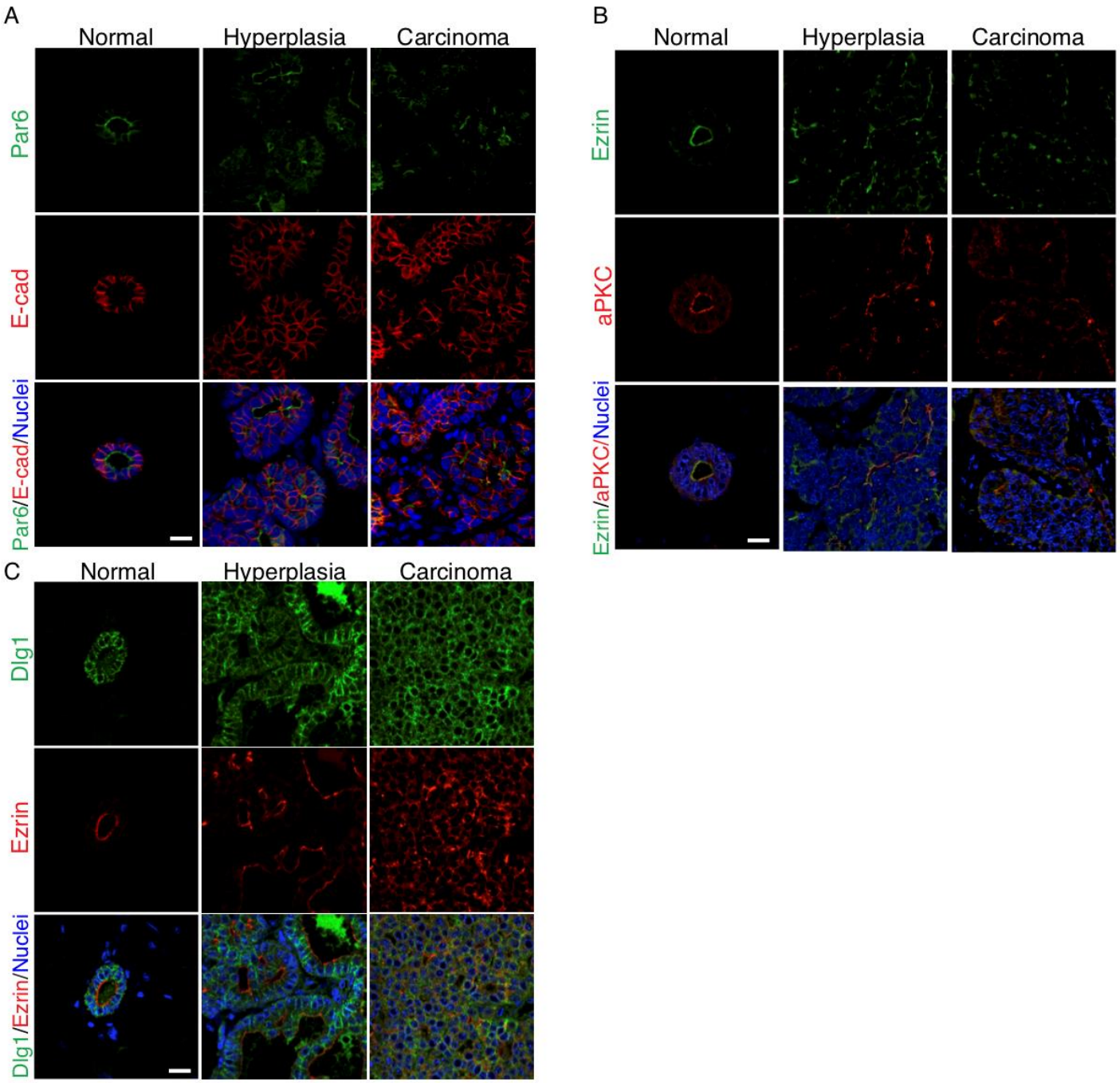
Supplementary Figure S2.1: Expression of ER, PR, and HER2 in luminal DCIS. Related to Figure 2.1. (A) Representative images of DCIS immunostained for estrogen receptor alpha (ERα, red), progesterone receptor (PR, blue), and HER2 (green). Bar: 50μm.


A
Pre-invasive lesion 1
Pre-invasive lesion 2
Par6/E-cad/Nuclei
Lumen
E-cad
Par6
B
Normal
ADH
DCIS
Region 1
Region 2
AB polarity mask

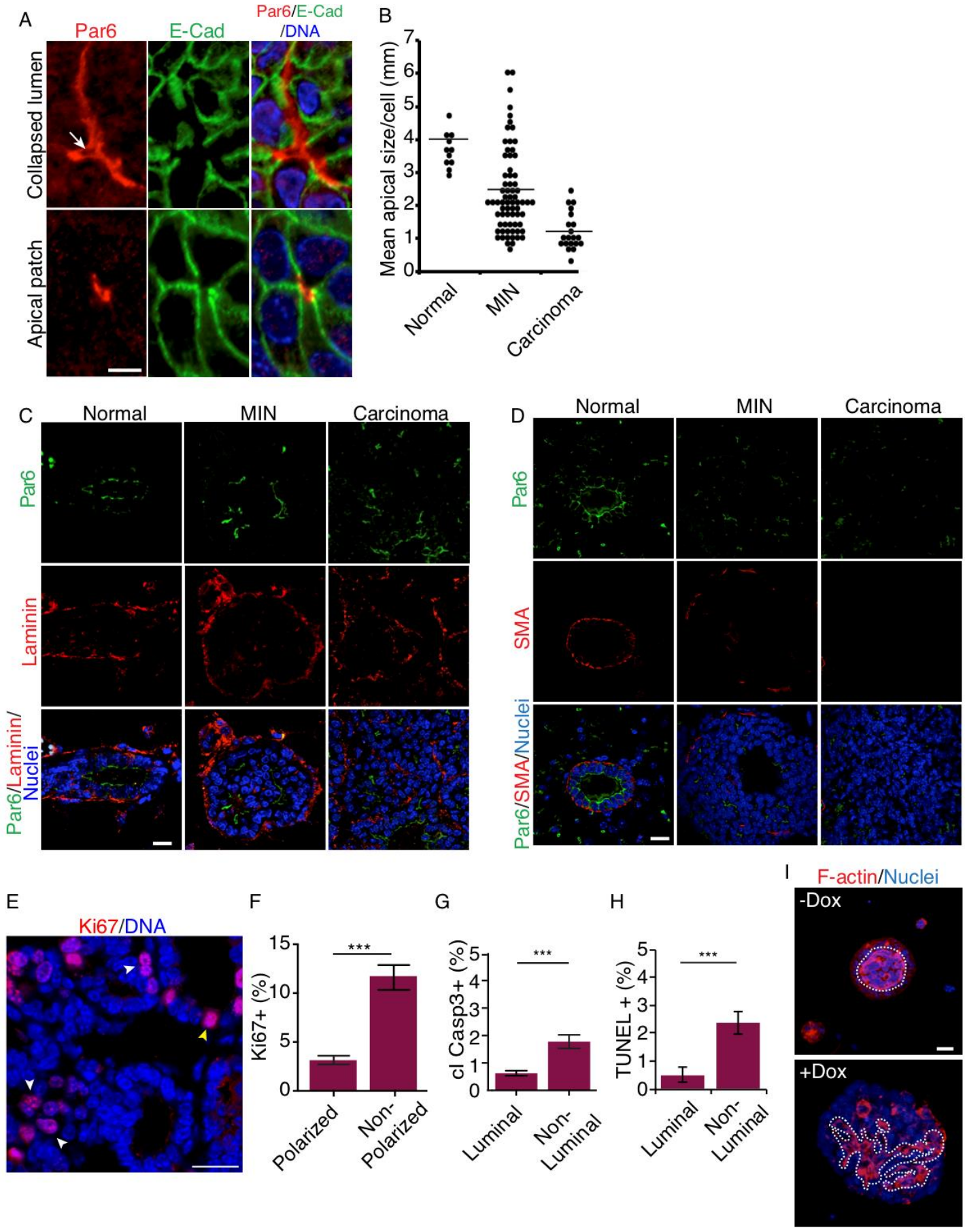
Supplementary Figure S2.2: Loss of apical-basal polarity in pre-invasive breast lesions. Related to Figure 2.2. (A) Images of pre-invasive lesions immunostained for Par6 (red) and E-cadherin (green). Arrowheads show apical domain with disrupted polarity. Dotted lines outline cells shed into the lumen. (B) Images of normal, ADH, and DCIS progression from a single biopsy sample showing colored overlay masks of cells with apical polarity. Each colored shape covers all polarized cells contacting a single lumen. Colored regions are polarized and non-colored regions are not polarized. Bars: A, 50μm; B, 100μm.



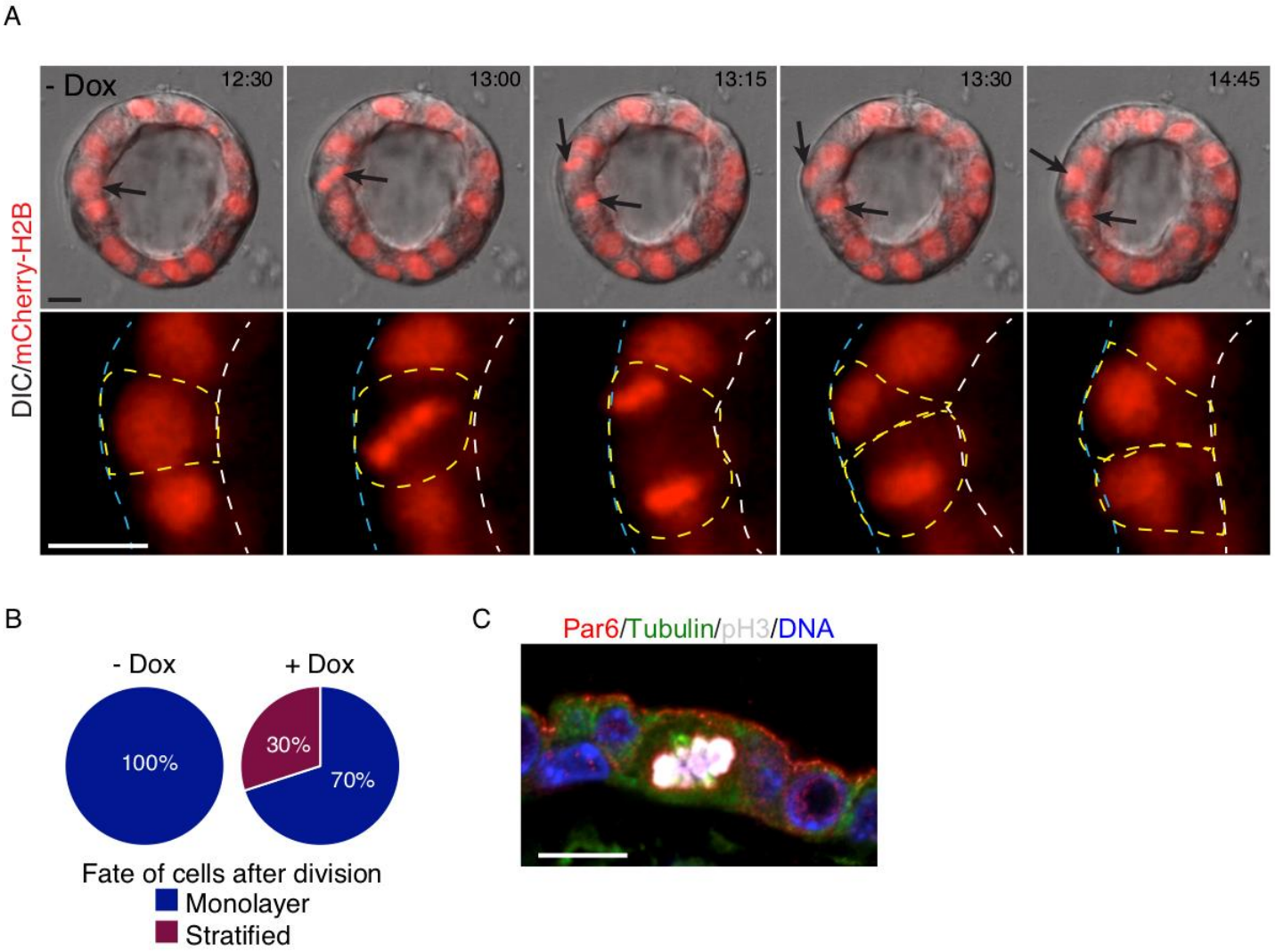
Supplementary Figure S2.3: Collective invasion contributes to lumen splitting in atypical ductal hyperplasia. Related to Figure 2.2. (A) Histological section of FEA and adjacent ADH. (B) representative fluorescence images of tissue samples immunostained for Par6 (red), E-cadherin (green). White arrows show polarized cells invading into the lumen. Yellow arrows show epithelial bridges that split the primary lumen. (C) Immunofluorescence images of tissue samples immunostained for Ki67 (red). Tissue autofluorescence (AF) was used to show tissue outline. (D) Quantification of Ki67 positive cells in single layer and stratified FEA from 4 subjects. ***, $p<0.001$. Bars: A, 100μm; B, 50μm; C, 20μm.



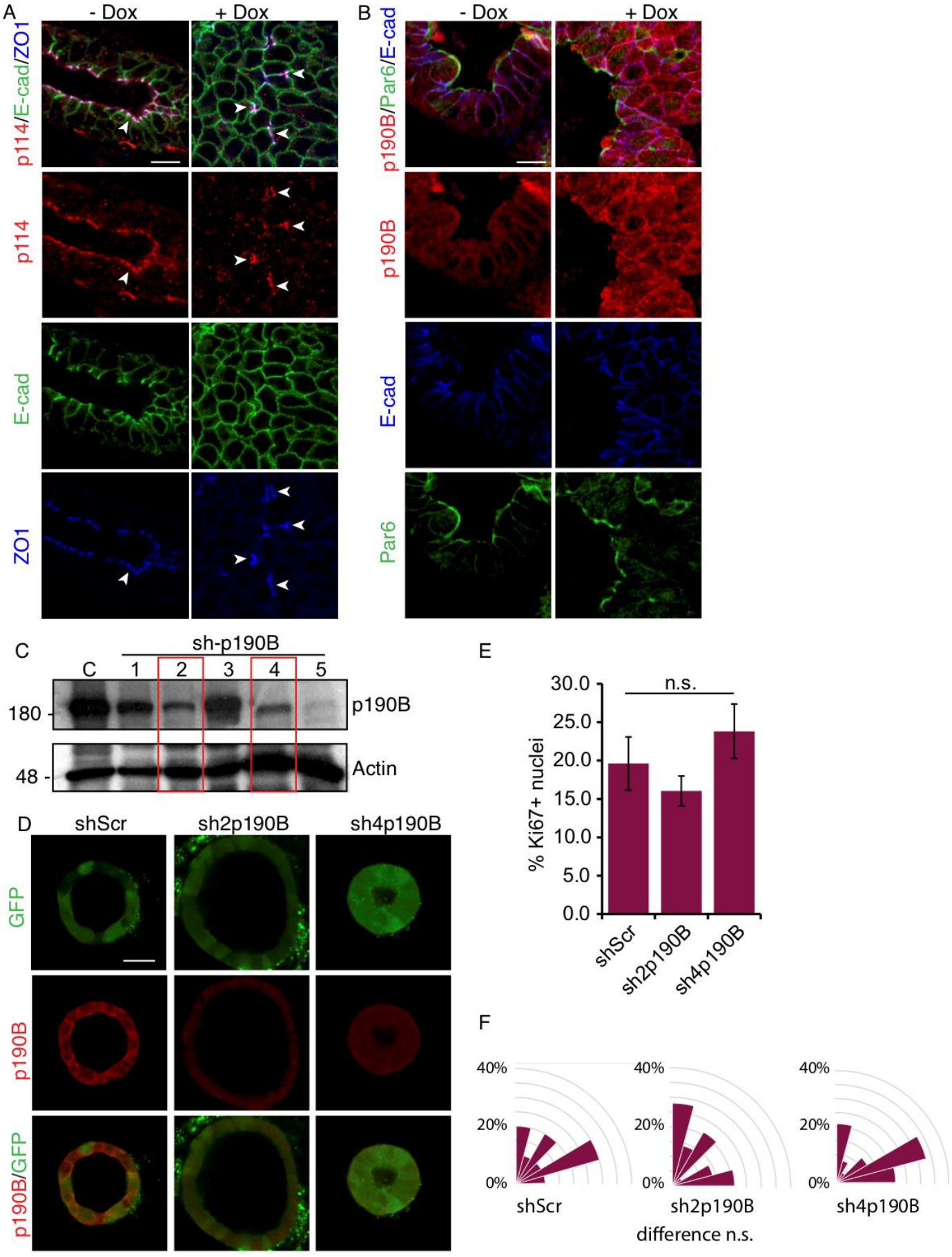
Supplementary Figure S2.4: Additional markers of apical-basal polarity. Related to Figure 2.3. (A-C) Images of normal or PyMT-expressing mammary gland sections, immunostained for polarity markers. (A) Par6 (green)/E-Cadherin (red); B, Ezrin (green)/aPKC (red); C, Ezrin (red)/Dlg1(green). Bars: 20μm.


A
Par6
E-Cad
Par6/E-Cad
/DNA
Collapsed lumen
Apical patch
B
Mean apical size/cell (mm)
Normal
MIN
Carcinoma
C
Normal
MIN
Carcinoma
Par6
Laminin
Par6/Laminin/
Nuclei
D
Normal
MIN
Carcinoma
Par6
SMA
Par6/SMA/Nuclei
E
Ki67/DNA
F
Ki67+ (%)
***
Polarized
Non-
Polarized
G
cl Casp3+ (%)
***
Luminal
Non-
Luminal
H
TUNEL + (%)
***
Luminal
Non-
Luminal
I
F-actin/Nuclei
-Dox
+Dox

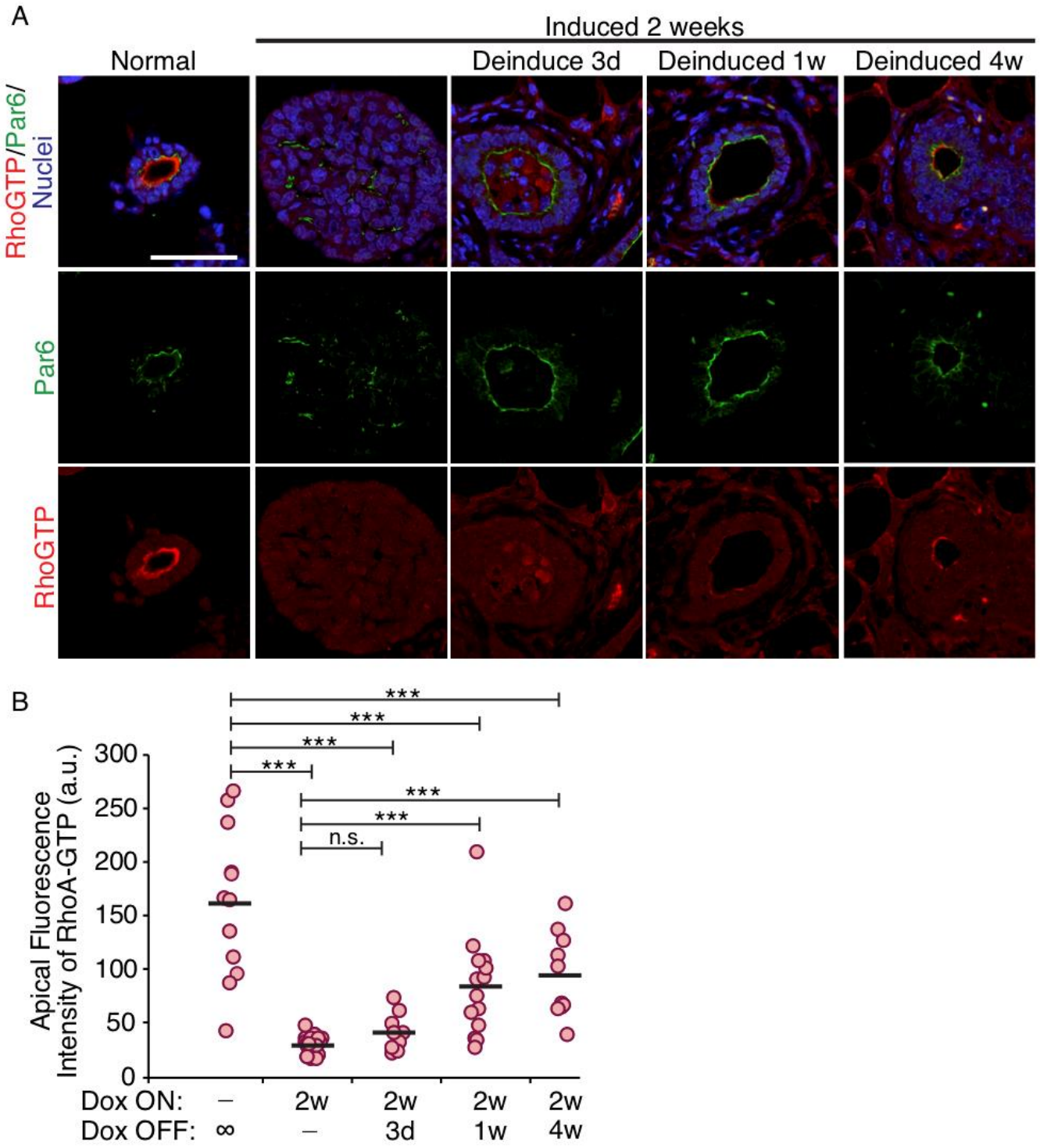
Supplementary Figure S2.5: Analysis of basement membrane, myoepithelium cells and apoptosis during mammary tumour progression. Related to Figure 2.3. (A) Images from PyMT tumours showing collapsed lumen and apical patches. The white arrow shows luminal space. (B) Quantification of the apical membrane length of polarized cells at the indicated stages from PyMT mice. Each dot represents the average apical size per lumen (lumen length/number of cells). (C and D) Images of normal and PyMT tumour sections immunostained for (C) Par6 (green) and laminin (red) or (D) Par6 (green) and smooth muscle actin (SMA, red). (E) Immunofluorescence image showing a PyMT tumour section immunostained for Ki67. Yellow arrowheads indicate polarized proliferating cells, white arrows show non-polarized proliferating cells. (F) Quantification of Ki67-positive cells in polarized and non-polarized populations. (G) Quantification of the percentage of cells expressing cleaved-Caspase-3 that were adjacent to lumen (luminal) or in stratified regions (non-luminal). (H) Quantification of the percentage of TUNEL positive cells that were adjacent to lumen (luminal) or in stratified regions (non-luminal). (I) Image of 3D-projection of organotypic cultures in the absence or presence of Dox-induced PyMT expression stained with phalloidin (F-actin) to mark the lumen. White dotted lines show continuous lumen. Bars: 20μm.



Supplementary Figure S2.6: Asymmetric cell divisions regulate stratification in hyperplastic epithelia. Related to Figure 2.3. (A) Image series from time-lapse microscopy showing cell division in 3D organotypic cultures from uninduced MIC-MTB. Arrows show an oblique cell division that resolves to a monolayer structure. White dotted lines mark the apical surface and blue dotted lines mark the basal surface. Yellow dotted lines outline cells during division. (B) Quantification of cell fates. Dividing cells from organotypic cultures (n=24) were tracked after division and their positional fates (monolayer or stratified) were determined. (C) Image of PyMT-expressing mammary gland section immunostained for Par6 and mitotic markers phospho-Histone H3 and tubulin. Bars: 10μm.


A
- Dox
+ Dox
p114/E-cad/ZO1
p114
E-cad
ZO1
B
- Dox
+ Dox
p190B/Par6/E-cad
p190B
E-cad
Par6
C
sh-p190B
C
1
2
3
4
5
180
p190B
48
Actin
D
shScr
sh2p190B
sh4p190B
GFP
p190B
p190B/GFP
E
% Ki67+ nuclei
30.0
25.0
20.0
15.0
10.0
5.0
0.0
n.s.
shScr
sh2p190B
sh4p190B
F
40%
20%
0%
shScr
sh2p190B
difference n.s.
sh4p190B

(A and B) Images of mammary gland sections before or after doxycycline-induced expression of PyMT. (A) Immunostaining for p114RhoGEF (red), E-cadherin (green) and ZO1 (blue). Arrows show p114 colocalizing with apical ZO1 in normal cells and at collapsed lumen in tumours. Bars: 10μm. (B) Immunostaining for p190-B (red) E-cadherin (blue), and Par6 (green). Bars: 10μm. (C) Immunoblot of p190-B and tubulin (loading control) to show knockdown efficiency of different shRNA. (D) Images showing sh2- and sh4-p190B knock-down in non-induced 3D organotypic cultures from MIC-MTB. (E) Quantification of the number of cleaved Caspase-3 positive cells in mouse mammary glands with induction of PyMT and expression of p190B-RhoGAP shRNA. (F) Quantification of the cell division angle in these glands relative to the plane of the lumen; n=10 for shScr, n=20 for sh2p190B, and n=24 for sh4p190B. Bars: 20μm.



Supplementary Figure S2.8: RhoA-GTP expression following oncogene deinduction. Related to Figure 2.6. (A) Images of mouse mammary gland following Dox induction/deinduction cycles and immunostained for Par6 (green) and active RhoA (RhoGTP; red). (B) Quantification of active RhoA (RhoGTP) at the apical membrane from indicated induction/deinduction cycles. Bars: 50μm.

Conceptual link between Chapters 2 and 3

While establishing the cellular mechanism for breast cancer progression, we observed tissue stratification induced by mitotic spindle orientation perpendicular to the plane of the epithelial duct. While other groups have observed altered cell division orientation in cancer mouse models, it occurs subsequent to loss of cell polarity. Our data demonstrate that disrupted cell division orientation leads to loss of cell polarity in luminal type breast cancers, the most prevalent type. Although altered cell division orientation correlates with early steps of cancer development, an outstanding question is whether or not disrupted cell division orientation is necessary to initiate breast cancers. This question drove our next study, described in the coming chapter. A major challenge to address this is that many, if not all, breast oncogenes disrupt cell division orientation along with numerous other oncogenic properties, and therefore coupling them with models that disrupt the cell division orientation machinery are unlikely to affect tumour progression. To overcome this, we evaluated PyMT models in which gene-depletion studies resulted in loss of tumour formation and identified PyMT-Src-deficient lesions that failed to stratify and do not form mammary tumours (i.e. they rescue tissue from tumour formation). We characterized cell division orientation in this model, and disrupted cell division orientation specifically in the mammary epithelium to initiate mammary tumours (i.e. unrescued tumour formation).

Chapter 3: Disrupted cell division orientation is essential for breast cancer initiation

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Running title: Spindle misorientation in cancer development

Keywords: epithelial, cancer progression, spindle orientation, breast cancer, duct

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Abstract

Loss of cell and tissue organization is a hallmark of epithelial cancers, as the tissue transforms from its normal ductal morphology progressing into a solid mass. Disrupted tissue organization is an early event that presents as epithelial stratification in many cases. We and others previously reported that epithelial stratification is associated with cells dividing perpendicular to the epithelial plane. However, whether disruption of cell division orientation is essential to tumour formation, has not been definitively reported. Using the inducible PyMT mouse model (MIC), which we previously showed resembles early breast cancer progression in humans, we found that depletion of Src tyrosine kinase fails to produce mammary tumours. The MIC/Src$^{-/-}$ mammary glands form enlarged ducts that fail to generate stratified epithelia, with no direct effect on proliferation. We report that Src-deficient mammary ducts re-establish proper mitotic spindle orientation. To examine if disrupted spindle orientation is necessary for tumour progression, we depleted the spindle orientation regulator LGN from the mammary epithelium. In normal tissue, LGN$^{-/-}$ glands resulted in randomized mitotic spindles, but the knock0out was not sufficient to induce tumours or even disrupt tissue organization. However, LGN-deficient mammary glands unblocked tumour formation in MIC/Src$^{-/-}$ mice. This supports that spindle mis-orientation is a necessary and early step in breast cancer initiation.

3.1 Introduction

Loss of cell and tissue organization is a hallmark of epithelial cancers, as the tissue transforms from its normal organized morphology into a solid mass (Bergstralh and St Johnston 2012; Huebner et al. 2014; Halaoui and McCaffrey 2015). In breast cancer, this mass often presents as solid ducts at the ductal carcinoma in situ (DCIS) stage, which can subsequently progress to invasive disease (IDC). Tissue polarity is initially broken and the normal morphology disrupted, which is associated with early epithelial stratification (Halaoui and McCaffrey 2015). The latter is observed both in normal tissue remodeling and during the progression of various cancers, and often occurs through regulation of cell division orientation (Martin-Belmonte and Perez-Moreno 2011; Huebner et al. 2014).

Ductal Breast cancer develops in a step-wise manner, going through defined but non-obligatory pre-cancerous stages, beginning with Flat Epithelial Atypia (FEA), where the ducts exhibit a dilated appearance (Sgroi 2010). Next, the ducts are stratified at the Atypical Ductal Hyperplasia (ADH) stage, become solid at DCIS, and once the basement membrane is breached, the lesion becomes an invasive IDC (Sgroi 2010). Previously, we described cellular and molecular mechanisms for this stepwise progression (Halaoui et al. 2017). Interestingly, we reported that asymmetric divisions occur early in cancer progression and are responsible for generating stratified mammary ducts and a population of depolarized cells that proliferate to occupy solid ducts (Halaoui et al. 2017). We and others have speculated that altered cell division orientation to generate stratified ducts is a necessary precursor to cancer progression (Huebner et al. 2014; Seldin and Macara 2017; Zhou et al. 2019).

In fact, spindle orientation changes have been observed in many cancer types (Thoma et al. 2009; Quyn et al. 2010; Hehnly et al. 2015). Mutated colorectal cancer oncogene APC has been shown to cause an increase in perpendicular divisions (Fleming et al. 2009; Matsumura et al. 2012), as well as ABL1 (Matsumura et al. 2012) and Src (Sun et al. 2018). Many breast oncogenes like ErbB2, aPKCi,

disrupt epithelial architecture as well (Huebner et al. 2014; Linch et al. 2014). Some oncogenes even lead to the loss of polarity proteins in cancers, which is linked to altered oriented divisions, such as Par3 (Williams et al. 2014), and Scribble (Godde et al. 2014). This offers much circumstantial evidence, but a direct causative link has yet to be established between oncogenic activation or tumour suppressor loss, spindle misorientation, and tumourigenesis.

A spindle orientation complex is responsible for orienting the mitotic spindle through coupling the polar astral microtubules to the cell cortex. The core of this complex is made up of three proteins: LGN, an adaptor protein binds to the spindle microtubules on one side, while binding a NuMA/dynein complex from the other side, which in turn anchor to the cortex through myristoylated Gαi.

Several properties may control cell division orientation, from mechanical cell shape and tension to biochemical manipulation of the cell orientation machinery. Although many studies have demonstrated the importance of orientated cell divisions in proper tissue morphogenesis and function of mammalian epithelial cells (Byrd et al. 2016; Seldin et al. 2016; Shafer et al. 2017), genetic ablation of the process's regulatory proteins seems to contradict this paradigm because even though altered cell division orientation is observed in these tissues, these are rarely associated with long-term developmental defects (Postiglione et al. 2011; Williams et al. 2014; Chiu et al. 2016; Lacomme et al. 2016). Moreover, we are not aware of any reports stating that the disruption of cell division orientation is sufficient to induce cancer. Therefore, the importance of cell division orientation in cancer progression is unclear.

One challenge with understanding the role for cell division orientation in cancer initiation is that many driver oncogenes themselves alter cell division orientation, as mentioned above, and therefore studies combining an oncogene with disrupted oriented cell division machinery are difficult to interpret. However, we do know that spindle mis-orientation alone does not cause tumour growth in flies unless

apoptosis is blocked (Nakajima et al. 2013), which is a property of all oncogenes, indicating that there exists many layers of complexity during tumour development. Whether all oncogenes need to manipulate mitotic spindle orientation, however, remains to be elucidated.

Here, we report that disrupting the oncogene c-Src in the PyMT breast cancer model blocks cancer progression by preventing the generation of stratified epithelia, with no direct effect on proliferation, resulting in enlarged single layered ducts that retain apical-basal polarity and rarely progress to palpable tumours. Interestingly, re-instating spindle mis-orientation by depleting regulators of the spindle orientation complex enables progress into solid cancers. Therefore, this provides compelling evidence to supports an essential role for cell division orientation in the initiation of breast cancers.

3.2 Results

3.2.1 Src-deficiency halts mammary tumour progression

We previously conducted an extensive characterization of the early progression of an inducible polyoma virus middle-T antigen (PyMT) MIC/MTB mouse model (hereafter referred to as MIC) (Rao et al. 2014; Halaoui et al. 2017). The MIC mouse model forms mammary tumours that resemble human luminal cancers and follow a similar progression through early stages of breast cancer progression (Rao et al. 2014). To further understand events required for early breast cancer progression, we sought a modified PyMT model that failed to produce mammary tumours. It was previously reported that Src-deficient PyMT mice fail to form mammary tumours (Guy et al. 1994). To determine if Src was required for early mammary tumour progression, we induced PyMT expression in MIC/$Src^{+/+}$ and MIC/$Src^{-/-}$ mice for 2 weeks, a time-point when ducts are typically stratified or solid in the MIC model (Halaoui et al. 2017). Whereas 84% of ducts from MIC/$Src^{+/+}$ mice exhibited stratified and solid phenotypes, ducts from MIC/$Src^{-/-}$ mice were dilated, but none were stratified or solid (Figure 3.1 A,B). Therefore, Src is required to generate stratified and solid mammary ducts in this model.

Since mammary tumour progression is halted prior to stratification in MIC/$Src^{-/-}$ mice, we wanted to further investigate cell and tissue architecture by staining for polarity proteins Par6 and E-cadherin, which mark the apical and basolateral membranes, respectively (Figure 3.1C). Consistent with the idea that epithelial polarity is lost during stratifying asymmetric cell divisions, polarized Par6 and E-cadherin were retained in MIC/$Src^{-/-}$ mammary glands that fail to stratify (Figure 3.1D).

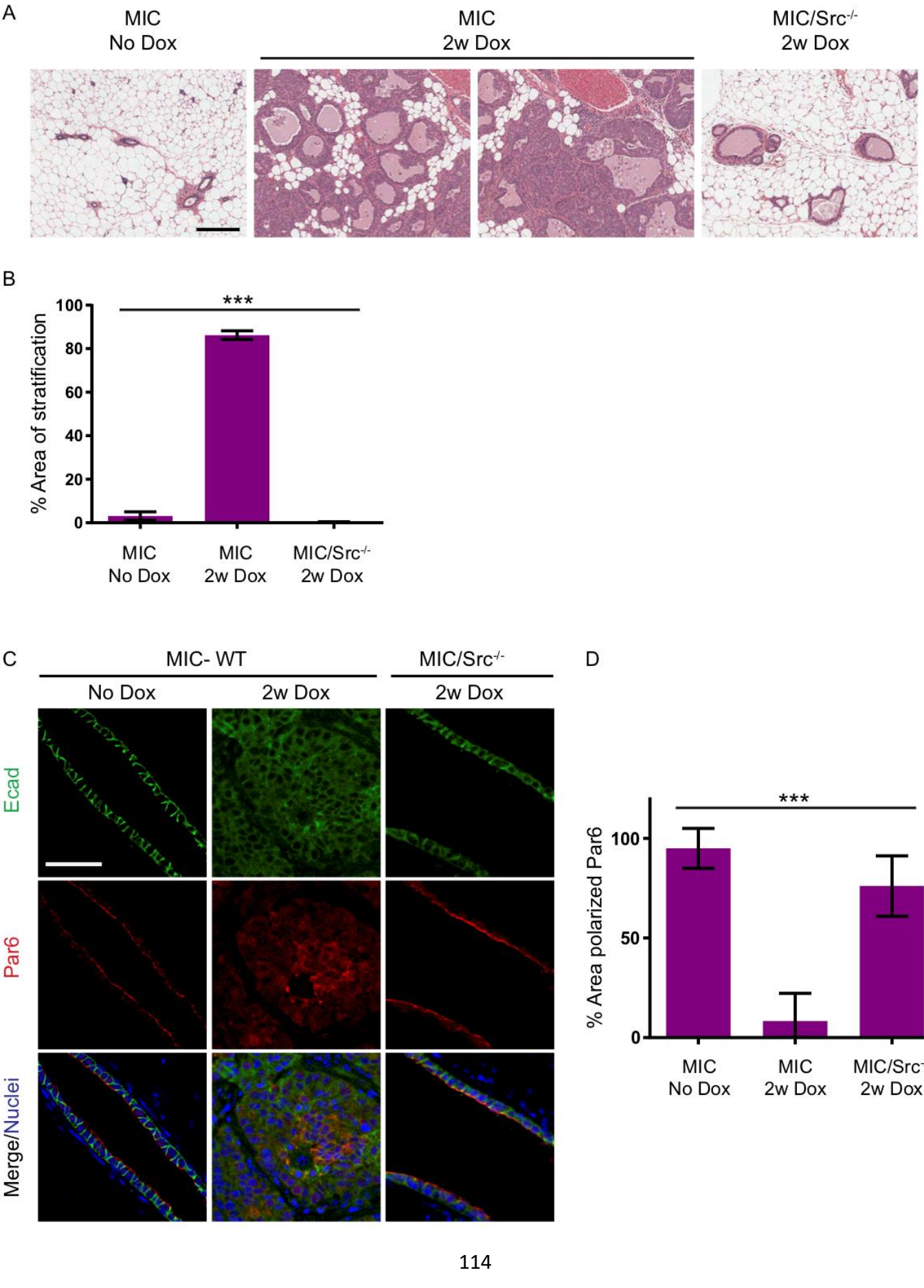

A
MIC
No Dox
MIC
2w Dox
MIC/Src-/-
2w Dox
B
***
% Area of stratification
100
80
60
40
20
0
MIC
No Dox
MIC
2w Dox
MIC/Src-/-
2w Dox
C
MIC- WT
MIC/Src-/-
No Dox
2w Dox
2w Dox
Ecad
Par6
Merge/Nuclei
D
***
% Area polarized Par6
100
50
0
MIC
No Dox
MIC
2w Dox
MIC/Src-/-
2w Dox

Figure 3.1: Characterizing the mammary glands of the MIC/Src$^{-/-}$ mouse model

(A) Histological sections of mouse mammary glands stained with H&E of control non-induced and 2-week induced MIC-WT, and 2-week induced MIC-Src$^{-/-}$. (B) Quantification of the area of stratification of the ducts shown in A. Respectively, the number of ducts counted per condition is 26, 52, and 45. (C) Histological sections of mouse mammary glands of control non-induced and 2-week induced MIC-WT, and 2-week induced MIC-Src$^{-/-}$, stained with E-cadherin and Par6. (D) Quantification of the percentage of polarized ducts exampled in C. At least 5 images from 4 mice were counted per condition. Scale bars: A, 200μm; C, 50μm . Error bars represent SEM, $p < 0.0001$.

3.2.2 Src does not directly affect proliferation or apoptosis in PyMT mammary glands

Src has been implicated in proliferation (Wheeler et al. 2009), and the reduced mammary tumour progression observed in the Src-deficient MIC mammary glands could result from reduced proliferation. We therefore immunostained tissue sections from non-induced controls and 2-week-induced MIC/$Src^{+/+}$ and MIC/$Src^{-/-}$ for the proliferation marker Ki67 (Figure 3.2A). As expected for normal mammary tissue, few cells expressed Ki67, whereas ~37% of the cells were Ki67 positive in MIC glands. Src-deficient MIC mammary ducts were intermediate, with 13% of cells positive for Ki67 (Figure 3.2B). We previously reported that epithelial cell proliferation in early stages of mammary tumour formation in the MIC model and in human pre-cancerous breasts lesions were not equally distributed in all cells (Halaoui et al. 2017). Instead, cells in stratified regions had a significantly higher proportion of Ki67-positive cells, indicating that polarized cells were growth restricted compared to adjacent stratified regions (Halaoui et al. 2017). Therefore, we more closely examined proliferation in induced Src-proficient MIC glands that had not yet stratified and were still a single epithelial layer. Importantly, we observed that only 15% of MIC cells in non-stratified regions glands were Ki67-positive, which is similar to Src-deficient mammary ducts. This supports that the tissue structure influences proliferation rates, and that the basal proliferation rates of Src-proficient and Src-deficient MIC mammary glands are not different (Figure 3.2B).

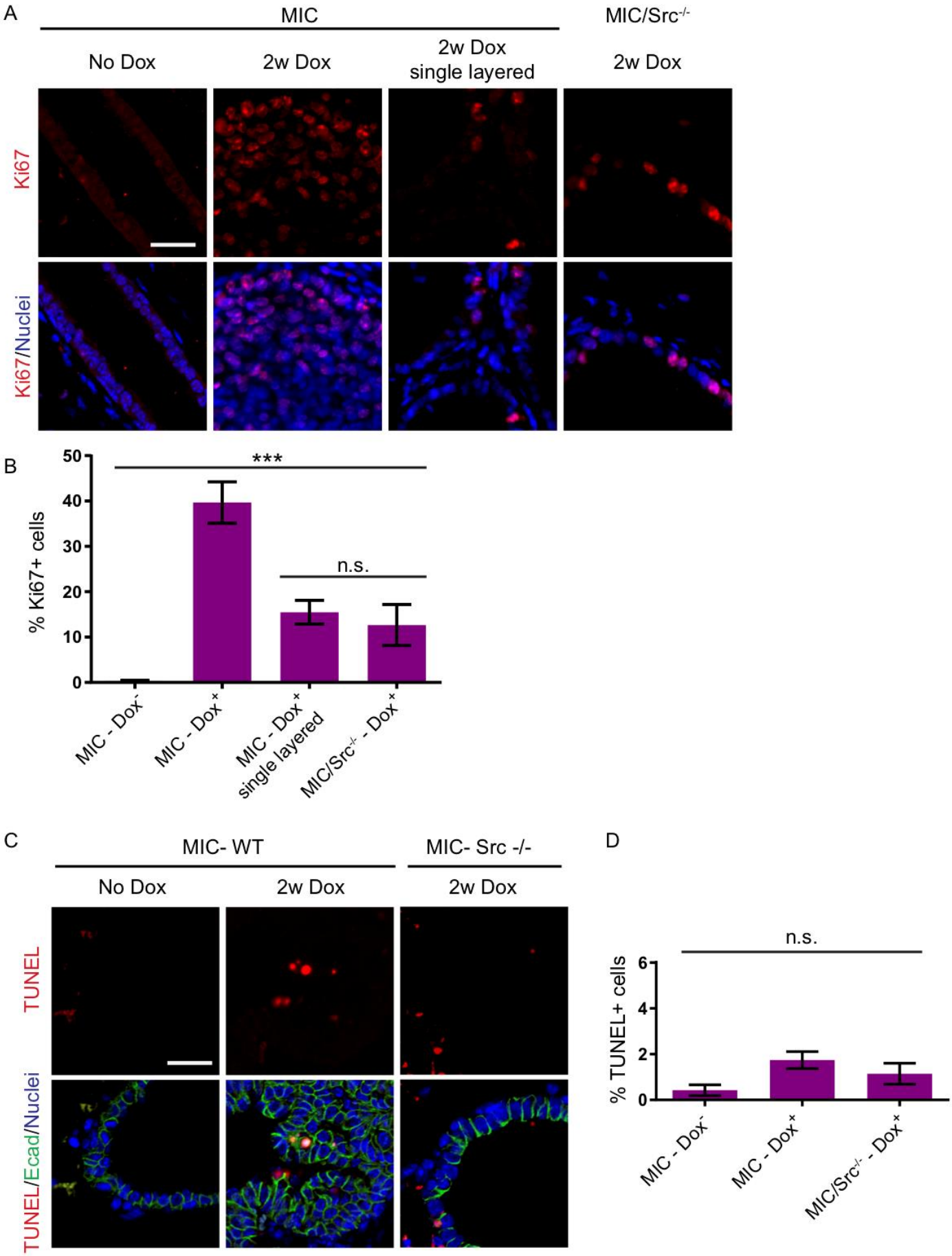

A
MIC
MIC/Src-/-
No Dox
2w Dox
2w Dox single layered
2w Dox
Ki67
Ki67/Nuclei
B
***
n.s.
% Ki67+ cells
50
40
30
20
10
0
MIC - Dox-
MIC - Dox+
MIC - Dox+ single layered
MIC/Src-/- - Dox+
C
MIC- WT
MIC- Src -/-
No Dox
2w Dox
2w Dox
TUNEL
TUNEL/Ecad/Nuclei
D
n.s.
% TUNEL+ cells
6
4
2
0
MIC - Dox-
MIC - Dox+
MIC/Src-/- - Dox+

Figure 3.2: Src does not directly impact proliferation or apoptosis in early mammary tumours

(A) Histological sections of mouse mammary glands stained with Ki67 and Hoescht (Nuclei) of control ducts from non-induced MIC-WT, stratified ducts from 2-week induced MIC-WT, single layered ducts from 2-week induced MIC-WT, and ducts from 2-week induced MIC-$Src^{-/-}$. (B) Quantification of the percentage of proliferating cells exampled in A. At least 5 images from 3 mice were counted per condition, $p < 0.0001$ for ***, and $p > 0.05$ for n.s. (C) Histological sections of mouse mammary glands stained with E-cadherin and TUNEL of control non-induced and 2-week induced MIC-WT, and 2-week induced MIC-$Src^{-/-}$. (D) Quantification of the percentage of apoptotic cells in the glands shown in C, $p = 0.64$. At least 6 images from 3 mice were counted per condition. Scale bars: A, C, 25μm. Error bars represent SEM.

Apoptosis has been implicated in cavitation and generating hollow lumen (Martin-Belmonte et al. 2008). Therefore, we examined whether Src deficiency affected apoptosis and if this could explain the hollow phenotype of Src-deficient MIC mammary ducts. For this, we performed TUNEL staining on mammary gland tissue sections from non-induced, and 2-week induced MIC/$Src^{+/+}$ and MIC/$Src^{-/-}$ (Figure 3.2C). Although MIC expression showed a trend towards increased apoptosis compared to non-induced tissue, we did not observe a significant difference between Src-proficient and Src-deficient MIC glands (Figure 3.2D). We conclude that delayed progression in Src-deficient MIC glands is unlikely caused by increased apoptosis.

3.2.3 Spindle orientation control is re-established in MIC-$Src^{-/-}$ mammary glands

We previously reported that stratification of MIC mammary ducts correlated with an increase in non-planar dividing cells (Halaoui et al. 2017). We therefore examined whether Src-deficiency re-established planar divisions, as a potential mechanism to block epithelial stratification and mammary tumour progression. For this, we measured mitotic spindle orientation of single-layered luminal cells in the glands of induced Src-proficient and Src-deficient MIC mammary ducts. We previously reported that luminal cells in the mammary normally divide at an angle parallel to the lumen (0^0-30^0 (Halaoui et al. 2017)). Consistently, induced MIC/$Src^{+/+}$ glands increase the proportion of oblique (30^o-60^o) and perpendicular (60^0-90^0) divisions (Figure 3.3A). Conversely, luminal cells in MIC/$Src^{-/-}$ glands divided at angles similar to normal, mostly at 0^0-30^0 (Figure 3.3B). This suggests that Src is required to disrupt spindle orientation that promotes epithelial stratification in early mammary cancer progression in the MIC model.

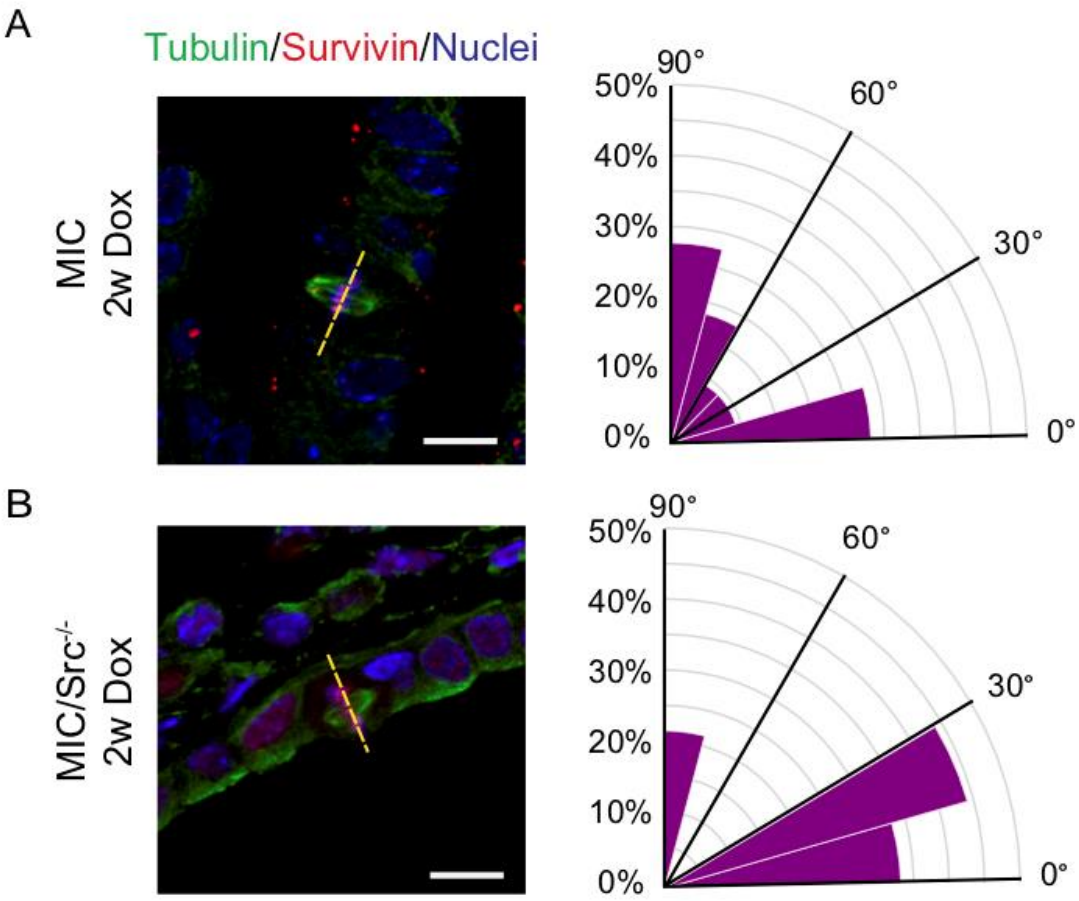


Figure 3.3: Src is required to restrict spindle orientation in MIC mammary epithelium

Histological sections of mouse mammary glands of (A) 2-week induced MIC-WT and (B) 2-week induced MIC-Src$^{-/-}$, stained with Tubulin and Survivin adjacent to the quantification of the cell division angle relative to the plane of the lumen; n=11 for MIC-WT and n=9 for MIC-Src$^{-/-}$. Scale bars: 10μm, $p < 0.0001$.

3.2.4 LGN regulates spindle orientation in the mammary gland

Our data support that altered mitotic spindle orientation is crucial for epithelial stratification and progression of mammary tumours. To determine if disrupting the spindle orientation machinery is sufficient to induce stratification of the mammary epithelium, we examined LGN-deficient ($LGN^{-/-}$) mice, previously described in (Lacomme et al. 2016), in which LGN is depleted from all cells. LGN is part of the spindle orientation complex, being the adaptor protein responsible for anchoring the astral microtubules to the cell cortex. Overall, the mice have no gross morphological phenotype, but do have spindle orientation defects in the retina (Lacomme et al. 2016). To study if the disruption this complex alters the orientation of cell divisions in the mammary gland, we measured the cell division angles in adult virgin mice and compared them to heterozygous mice as controls. Whereas luminal cells in heterozygous controls divided predominantly in the planar orientation (~55% were 0-30^{O}), cells from $LGN^{-/-}$ mice divided more often perpendicular to the lumen (~58% were 60-90^{O}) (Figure 3.4A). Therefore, LGN is required for orienting the mitotic spindle in the mammary epithelium. Next, we wanted to further investigate whether altered spindle orientation had an effect on tissue architecture in the mammary gland. For this, we examined carmine alum stained mammary wholemounts (Figure 3.4B). Interestingly, the ductal branching appeared normal and ductal trees filled the mammary fat pad by 12 weeks. In pubertal glands at 6-weeks of age, $LGN^{-/-}$ mammary ducts had elongated slightly less than $LGN^{+/-}$ glands (Figure 3.4C).

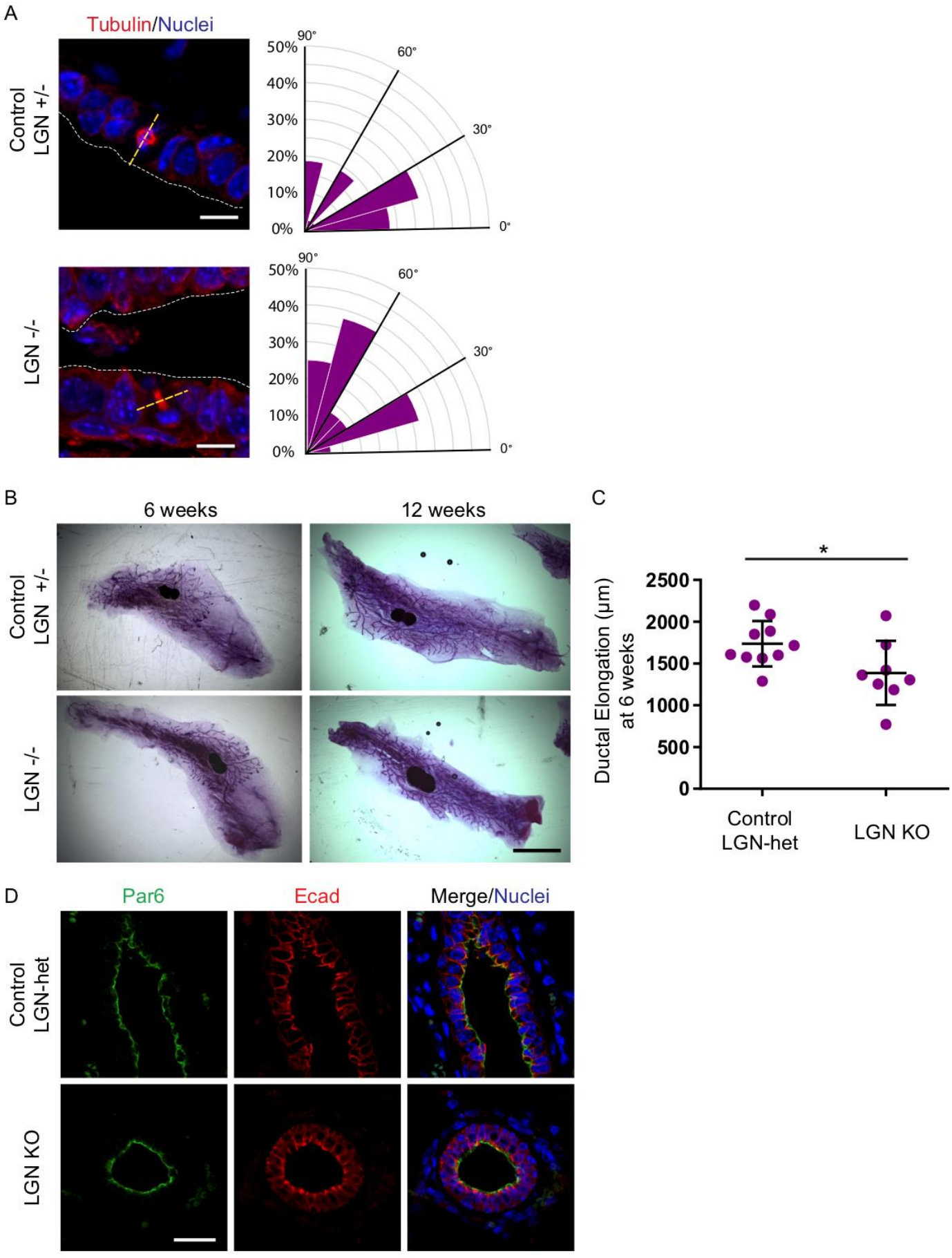

A
Tubulin/Nuclei
Control
LGN +/-
LGN -/-
90°
60°
30°
0°
50%
40%
30%
20%
10%
0%
B
6 weeks
12 weeks
Control
LGN +/-
LGN -/-
C
Ductal Elongation (µm)
at 6 weeks
2500
2000
1500
1000
500
0
*
Control
LGN-het
LGN KO
D
Par6
Ecad
Merge/Nuclei
Control
LGN-het
LGN KO

Figure 3.4: LGN-deficiency inducing spindle misorientation, but does not affect tissue organization

(A) Histological sections of mouse mammary glands of control $LGN^{+/-}$ and experimental $LGN^{-/-}$, stained with Tubulin, adjacent to the quantification of the cell division angle relative to the plane of the lumen; n=31 for $LGN^{+/-}$ and n=20 for $LGN^{-/-}$. (B) Whole mounts of mammary glands of $LGN^{+/-}$ and $LGN^{-/-}$, stained with Carmine Alum. (C) Quantification of the ductal elongation of the glands illustrated in B. (D) Histological sections of $LGN^{+/-}$ and $LGN^{-/-}$, stained with Par6 and E-cadherin. Scale bars: A, 10μm; B, 500μm; D, 25μm.

We further examined the morphology of mammary ducts. Surprisingly, despite increased perpendicular cell divisions, we did not observe large regions of epithelial stratification in LGN$^{-/-}$ mammary glands. Loss of apical-basal cell polarity is a feature in stratified epithelia (Huebner et al. 2014; Halaoui et al. 2017). We therefore examined if cell polarity was altered in the LGN-deficient mammary gland. Consistent with a lack of overt stratification, luminal epithelial cells retained the apical-basal polarity marker Par6. (Figure 3.4D). Therefore, LGN regulates spindle orientation in the mammary epithelium, but does not result in epithelial stratification of ducts during normal development. Furthermore, mice were maintained for 18 months, during which time no mammary tumours formed, indicating that loss of LGN is not sufficient to initiate mammary tumours.

3.2.5 Intraductal delivery of AAV specifically targets luminal mammary cells

Our data above indicate that Src does not directly block proliferation or promote apoptosis, but does affect spindle orientation. However, Src may have additional functions. To further determine the contribution of spindle orientation to mammary cancer progression, we reasoned that disrupting the spindle orientation machinery specifically may enable Src-deficient MIC mammary glands to progress to cancer. LGN is a core element of the spindle orientation complex, with no other known functions. We therefore decided to deplete LGN to determine if it could un-rescue Src-deficient MIC glands to promote progression. The breeding strategy using LGN-null mice crossed to Src-deficient MIC mice genetically modified mice proved challenging to generate sufficient mice for experimentation, so we instead developed a mammary-specific CRISPR/Cas9 strategy using intraductal injection of adeno-associated virus (AAV) carrying Cas9 and gRNA. AAV has been successfully used in gene therapy techniques (Naso et al. 2017), since its infection is transient and its viral DNA does not integrate into its host's genome.

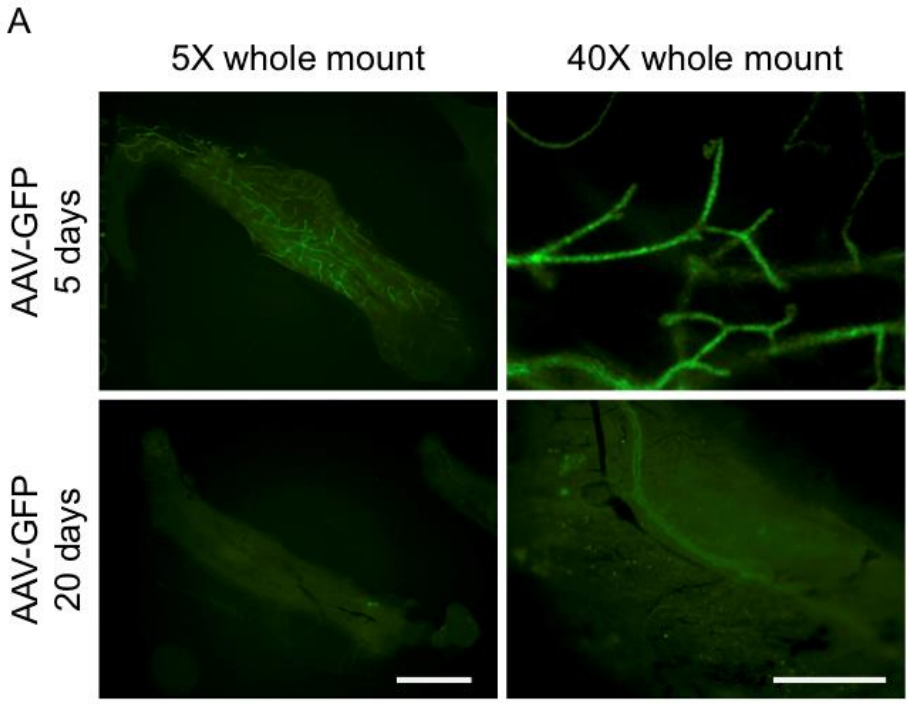
To evaluate the efficiency of AAV in infecting the mammary epithelium and the duration of expression we first injected an AAV-GFP virus intraductally and examined the mammary glands after 5 and 20 days (Figure 3.5A). The virus is expressed by day 5 and the expression subsides by day 20, consistent with the transient expression of AAV. To determine how deep the infection penetrates, we sectioned the glands expressing GFP as well as control glands injected with PBS, stained them with a GFP antibody, and co-stained with a Keratin 8 antibody to mark the luminal cells (Figure 3.5B). Only K8+ luminal cells are GFP positive, indicating that intraductal injection of AAV is specific to luminal cells. This is advantageous experimentally, since the majority of breast cancers arise in the luminal compartment.

3.2.6 Altered spindle orientation enables mammary tumour progression in MIC/Src$^{-/-}$ mice

Although spindle misorientation alone does not initiate tumour growth, we hypothesize that it is a crucial step for tumour initiation. This prompted us to investigate the role spindle orientation plays in tumour progression *in vivo*. We used sgRNA for LGN to deplete it from mammary luminal cells in MIC/Src$^{-/-}$ mice. As further validation, we established a second model in which we depleted a different spindle orientation factor (NuMA) using a similar strategy. For these experiments we cloned sgRNA for each gene into AAV-Cas9 plasmids, generated virus and injected it intraductally into mature virgin MIC/Src$^{-/-}$ and MIC/Src$^{flox/flox}$. The latter control mice do not contain the MTB gene; as they do not express rtTA, they lack the ability to induce PyMT and Cre expressing upon Doxycycline administration. We induced PyMT expression and Src-deletion by adding Doxycycline the animal's drinking water for 4 weeks, and then initially examined histological sections of the glands (Figure 3.6A). In Src-proficient control tissue not expressing PyMT, LGN^{CRISPR} glands did not display a stratified phenotype, similar to our observations in LGN$^{-/-}$ transgenic mice described above. In contrast, NuMA^{CRISPR} mammary glands exhibited duct stratification in all glands observed. In Src-deficient MIC ducts, stratified and solid

nodules were observed in all LGN^{CRISPR} (4/4) and NuMA^{CRISPR} (5/5) mice but not in the control MIC/Src$^{-/-}$ glands (0/3).

The myoepithelial layer is marked by smooth muscle actin (SMA) and is a marker for in situ disease, whereas loss of the myoepithelial layer indicates invasive cancer. Above, we indicated that whereas MIC mice lose the myoepithelium early, non-stratified Src-deficient MIC mice retain the myoepithelium and are therefore not invasive. In both LGN^{CRISPR} and NuMA^{CRISPR} non-tumour tissue, the myoepithelial layer was intact, even in stratified regions of the NuMA^{CRISPR} glands (Figure 2.6C). In contrast disruption of the myoepithelial cell layer was observed in the MIC/Src$^{-/-}$/NuMA^{CRISPR} and MIC/Src$^{-/-}$/LGN^{CRISPR} mice, indicating that the tumours have progressed to invasive cancer, further supporting that disrupting spindle orientation unblocked tumour formation in Src-deficient MIC mice. This indicates that disruption of oriented cell division has an essential role in tumour progression.


A
5X whole mount
40X whole mount
AAV-GFP
5 days
AAV-GFP
20 days

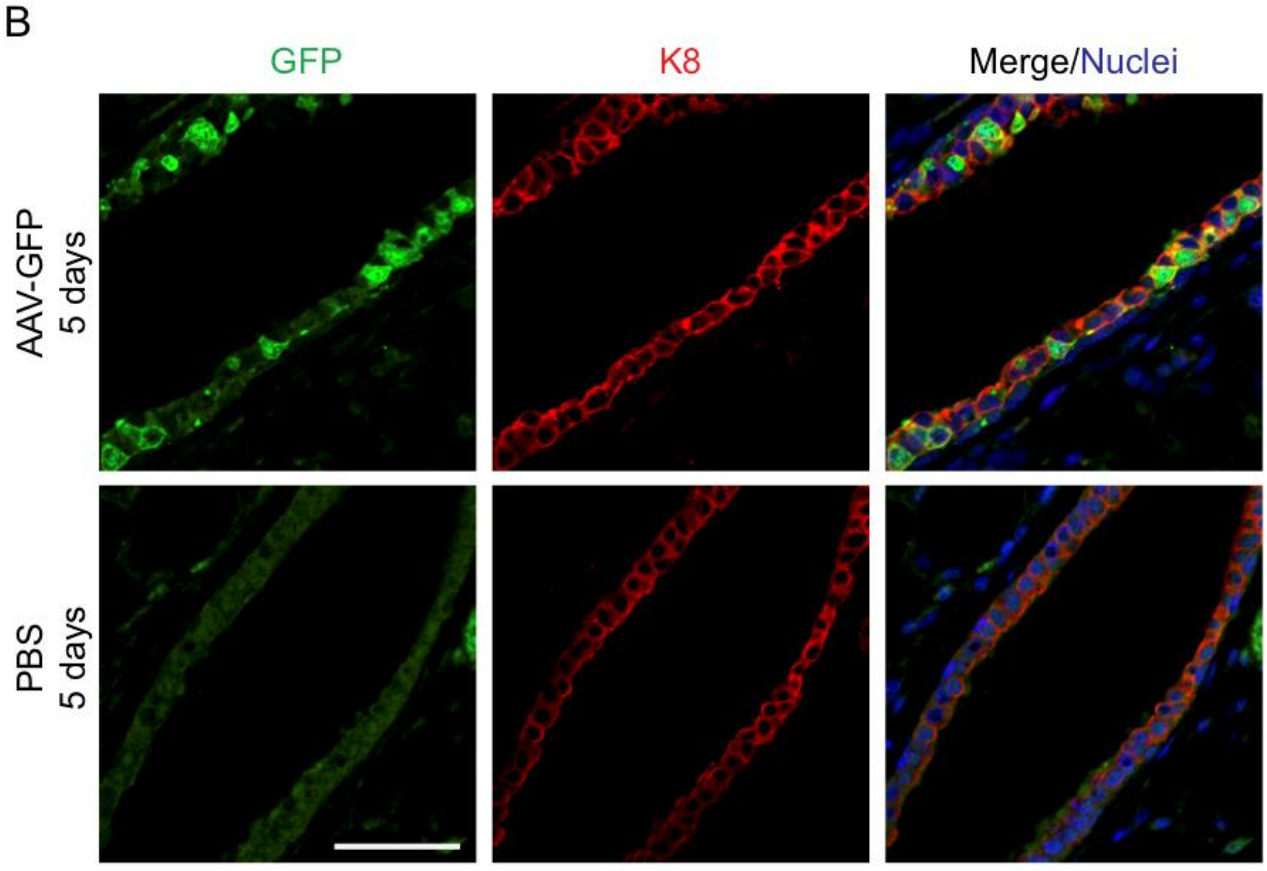

B
GFP
K8
Merge/Nuclei
AAV-GFP
5 days
PBS
5 days

Figure 3.5: Establishing intraductal injections of virus into the mammary gland

(A) Whole mounts of mouse mammary glands from FVB mice intra-ductally injected with AAV-GFP 5 days and 20 days post-injection. (B) Histological sections mouse mammary glands intraductally injected with AAV-GFP 5 days post injection, stained with GFP and Keratin-8. Scale bars: A, 5X 5mm, 40X 1mm; B, 50μm.

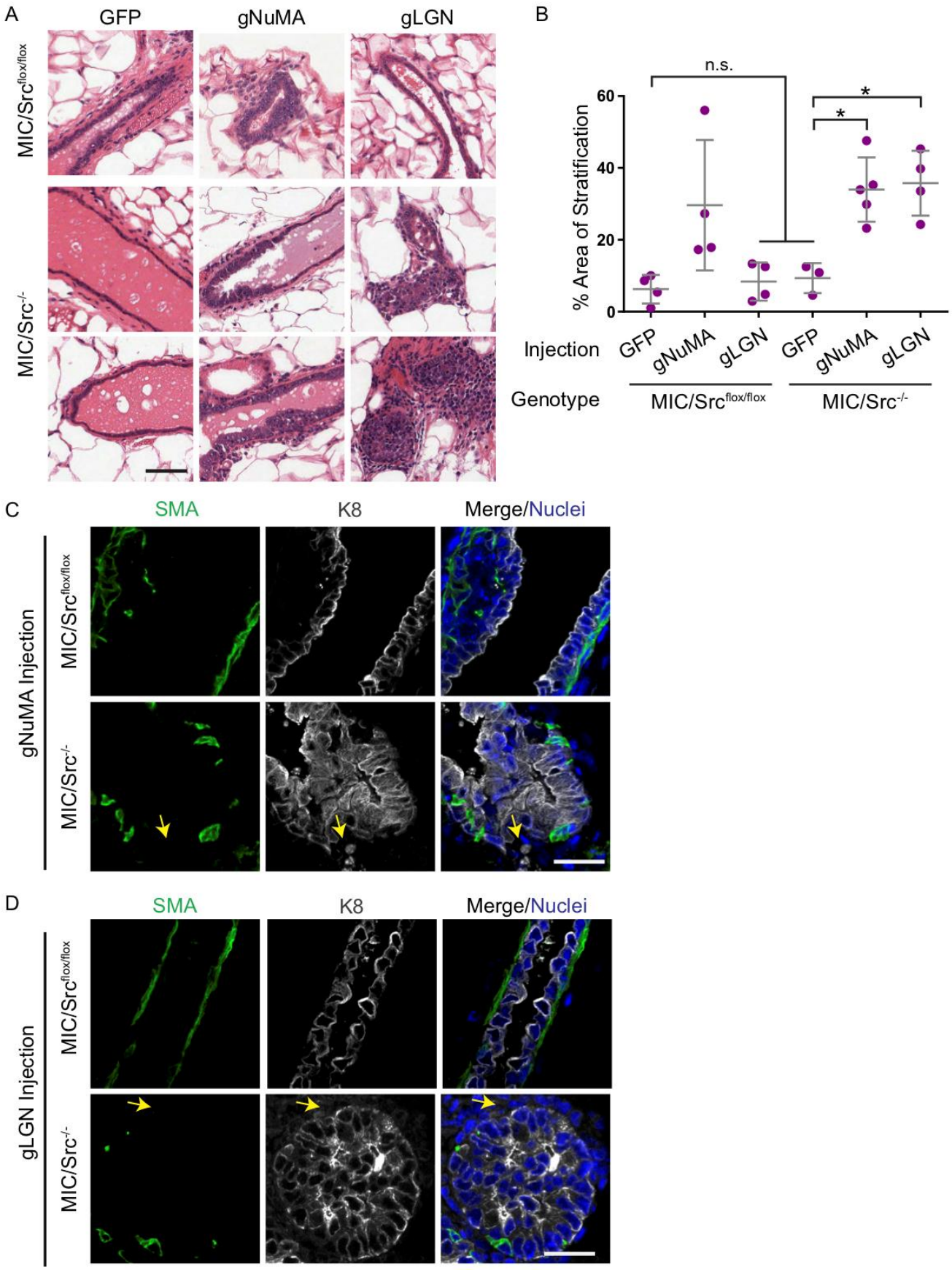

A
GFP
gNuMA
gLGN
MIC/Src^flox/flox
MIC/Src^-/-
B
n.s.
*
*
% Area of Stratification
60
40
20
0
Injection
GFP
gNuMA
gLGN
GFP
gNuMA
gLGN
Genotype
MIC/Src^flox/flox
MIC/Src^-/-
C
SMA
K8
Merge/Nuclei
gNuMA Injection
MIC/Src^flox/flox
MIC/Src^-/-
D
SMA
K8
Merge/Nuclei
gLGN Injection
MIC/Src^flox/flox
MIC/Src^-/-

Figure 3.6: Inducing spindle misorientation in vivo in a susceptible field causes cancer progression

(A) Histological sections of mouse mammary glands stained with H&E of MIC-WT and MIC-Src$^{-/-}$ intraductally injected with AAV-GFP, AAV-gLGN-Cas9, and AAV-gNuMA-Cas9. (B) Histological sections of mouse mammary glands from C injected with AAV-gNuMA-Cas9 stained with K8 and SMA. (C) Histological sections of mouse mammary glands from C injected with AAV-gLGN-Cas9 stained with K8 and SMA. The yellow arrows in B & C point to the absence of SMA from the basal side of the lesion. Scale bars: A, 50μm; B&C, 25μm.

3.3 Discussion

In this study, we report major findings describing the importance of spindle orientation during early tumourigenesis. We have found that Src-deficient PyMT cells fail to form tumours, which is associated with planar cell divisions and halts tumour growth at the early stages before stratification in the MIC/MTB model. Although inducing spindle mis-orientation alone does not cause tumour development or even ductal stratification, disrupting proper cell division orientation in the MIC/Src$^{-/-}$ context, which we can consider to be an activated epithelium context, is able to re-establish stratification, leading to tumour formation in these mice.

We and others have previously reported (Godde et al. 2014; Huebner et al. 2014; Halaoui et al. 2017) a correlation between spindle orientation and cancer development by inducing a stratified epithelium. Here, we establish the importance of this mechanism during tumour initiation, whereby oncogenes need to manipulate mitotic spindle orientation to cause stratification of the tissue, consequently leading to tumourigenesis. Interestingly, some oncogenes have been previously linked to deregulated spindle orientation. Indeed, Src itself has been implicated in decoupling cell division orientation from cell shape in cultured mammalian cells, whereby when the kinase is over-expressed, it is able to cause misoriented divisions leading to stratification (Sun et al. 2018). However, the mechanistic role of Src kinase in this disruption remains to be elucidated. Furthermore, mutated APC is an oncogene that may initiate intestinal cancer via multiple mechanisms, and mice heterozygous for this mutation exhibit abnormal mitotic spindle orientation (Fleming et al. 2009). Breast cancer oncogene ErbB2 has also been linked to spindle orientation defects by binding the Par complex and disrupting apical-basal cell polarity, causing the spindle orientation complex to no longer be selective to which membrane it binds the cortex (Huebner et al. 2014). This suggests that manipulating spindle

orientation could be a widespread property of oncogenes. Src acts downstream of many of these oncogenes, but whether spindle regulation by all oncogenes is dependent on Src is unknown.

A hallmark of cancer is loss of growth control and over-proliferation. Here, we report a global increase in proliferation in the MIC tumours, when compared to the MIC/Src$^{-/-}$. However, when comparing proliferation occurring in the similar context of a single layered epithelium, rates are comparable. Therefore, this indicates that the apparent difference in proliferation is at least partly due to differences in tissue organization and stratification. This suggests that increased proliferation occurring in a stratified epithelium enables tumour growth, whereas in a single-layered epithelium, the presence of hyperproliferation alone is not sufficient for cancer development. Therefore, this study further argues that the context in which increased proliferation occurs is important and that the development of epithelial cancers is initiated by coupling loss of growth control and disrupted tissue organization. In support of this, expression of cyclin D1 promotes constitutive proliferation, however only about half of MMTV-Cyclin D1 mice form mammary tumours after a very long latency (~1.5 years) in the mammary gland (Wang et al. 1994). Most of the tissue is enlarged hyperplastic ducts that are not stratified and resemble MIC/Src$^{-/-}$ glands we observed.

We propose that epithelial stratification is a key step in the process of tumour initiation, since stratification leads to solid ducts and proliferation is elevated in stratified epithelia. This supports a model in which altered cell division orientation produces non-polarized cells that have a growth advantage that eventually populate the emerging tumour. We show here that when stratification does not take place, the tissue is not able to fully transform, and progression is halted. In fact, Src- family inhibitor Dasatinib has been proven successful in delaying tumour onset in mice, with no effect on proliferation (Karim et al. 2013), directly supporting our data, and probing itself useful as a preventative therapy. Furthermore, re-instating spindle misorientation to a cancerous field restores tissue

stratification and formation of *in situ* cancer lesions, providing direct support that altered regulation of spindle orientation promotes cancer in certain contexts. Ongoing studies are aimed at evaluating whether these cancers progress to invasive and metastatic disease.

Interestingly, although $LGN^{-/-}$ mice disrupt cell division orientation, there are no major effects on tissue structure. This differs from mammary tissue in which NuMA is targeted, which form stratified epithelia. This may indicate that the tissue can compensate for the perpendicular divisions and re-organize into a single-layered epithelium. This is supported by our previous live imaging data where we observed cells reorganizing following perpendicular divisions (Halaoui et al. 2017). It could be that $NuMA^{CRISPR}$ glands are less efficient at reorganizing, or that NuMA has additional functions that contribute to the stratified phenotype. Future experiments will help address these possibilities.

We propose that it is essential for oncogenes to be able to manipulate tissue organization, thus adding a new characteristic to the definition of an oncogene, a new possible avenue for preventative therapeutic targeting. We previously demonstrated that early stage cancers are reversible and that the tissue is able to re-organize into a normal state when oncogene expression is eliminated, indicating that epithelial tissues have remarkable plasticity, and that oncogenic signals are necessary to maintain disrupted tissue organization (Halaoui et al. 2017). Patients with high risk of disease recurrence could benefit from preventative therapies, which currently are limited and need to be improved since they have many undesirable side effects. One avenue that these high-risk patients might benefit from treatment that could restore tissue organization, potentially by manipulating pathways that control spindle orientation. What these pathways are, however, remains unknown and should be prompted for further investigation because of their potential as therapeutic targets.

3.4 Materials & Methods

3.4.1 Mouse Models

The MIC-MTB mice used were in an FVB background, the LGN-KO mice were in a C57BL/6 background. For mice with inducible expression of PyMT, mT-IRES-Cre (MIC) mice were crossed with MMTV-rtTA (MTB) mice (Rao et al. 2014), and pups heterozygous for both genes were selected. The *Src* gene was flanked with loxp sites in some of these mice, and Src is knocked-out when Cre is activated, upon induction of the PyMT gene. Mice 8-weeks of age and older were given 2mg/ml of doxycycline in their water and were sacrificed after 2-weeks induction for hyperplasia/MIN. All procedures involving animals were approved by the McGill University Animal Care Committee.

3.4.2 Immunostaining and imaging

Mouse mammary tissue was fixed in either 4% PFA, as described previously (McCaffrey et al. 2012) and cut at 8μm thickness. Deparafinization and immunostaining was performed as previously described (McCaffrey et al. 2012). The primary antibodies were used as follows: cleaved-Caspase-3 1/300 (Cell Signaling #9661), Cytokeratin-8 1/250 (Developmental Studies Hybridoma Bank #TROMA-I-c), E-Cadherin 1/500 (BD Transduction #610181), Ki67 1/300 (Abcam #ab15580), Par6B 1/300 (Santa Cruz # sc-67393), Survivin 1/400 (Cell Signaling #2808), and α-Tubulin 1/250 (Millipore #MAB1864). Secondary antibodies (Alexa Fluor 488, 555, and 647) were used at a 1/750 dilution at room temperature for one hour. For TUNEL staining, tissue sections were prepared and stained using the TumourTACs kit according to the manufacturer's instructions (Trevigen), except 3,3'-Diaminobenzidine (DAB) chromogen was replaced with AlexaFluor-555 Tyramide Signal Amplification (ThermoFisher) for fluorescence detection. Confocal imaging was performed for fluorescence imaging using LSM700 from Zeiss with 20X/0.8NA or 40X/1.4 NA lenses. H&E sections were imaged using the Slice Scanner courtesy

of the Goodman Cancer Research Centre Histology Core. Whole mount mammary glands were stained with Carmine Alum and imaged using the ZEISS AxioZoom V16 microscope.

Image processing of brightness and contrast was performed in ImageJ and was applied uniformly to the whole image.

3.4.3 Tissue section measurements

To measure the number of polarized ducts in the mouse tissue, sections were stained with apical marker Par6 tight and basolateral marker E-Cadherin. For each mammary duct or lesion, we traced the plasma membrane encircling the nucleus and scored it as polarized or not-polarized. To be considered polarized in our study, ducts met all of the following criteria: 1) Both apical and basolateral membrane markers were detected in all cells of the duct; 2) the apical marker was enriched on a part of the membrane and excluded from another part of the membrane of the cells; 3) the basolateral marker was enriched on a part of the membrane of the cells and was excluded from another part of the membrane; and 4) enrichment of apical and basolateral proteins on regions of the plasma membrane were mutually exclusive. A protein was considered enriched if the intensity was higher on the membrane than in the cytoplasm of that cell. A protein was considered excluded from the membrane if intensity at the membrane was equal to or less than the cytoplasmic intensity in that cell. Measurements were taken from at least 2 sections from 3 mice per condition.

To measure the number of proliferating cells, tissue sections were stained with the Ki67 proliferation marker and counter-stained with Hoescht. In the MIC-SrcWT sections at hyperplasia/MIN, proliferation was measured in stratified and in single layered epithelia separately. Using ImageJ, counts were performed on at least 3 mice per condition, with 3 different fields of view per section at 2 weeks of induction.

To measure the area of stratification of the ducts, sections from non-induced control and MIC-MTB and MIC-MTB-Src$^{-/-}$ mice were stained with H&E and imaged using a slide scanner. Using ImageJ, we utilized the whole tissue section for quantification to trace around the areas of interest and obtain an area measurement. We measured the area of the duct with multi-layered cells and divided that by the total area of the duct. We measured at least 26 ducts from sections from at least 3 mice per genotype.

To measure the spindle orientation of dividing cells, sections from non-induced control and MIC-MTB and MIC-MTB-Src$^{-/-}$ mice were stained for survivin to mark the centrosome and α-tubulin to visualize the spindle pole. The lumen was used as the planar reference, and only dividing cells in regions that were not stratified were measured. Using ImageJ, a line was drawn tangent to the lumen, and another through the spindle poles; the resulting angle was measured. We measured the angles of at least 10 dividing cells from 6 glands from 3 mice per genotype.

3.4.4 Adeno-Associated Virus (AAV) Production

HEK293LT cells below the passage number of 15 were plated at a 50% confluency in a 15 cm^2 dish (Nunc). After 24 hours of plating, the cells were transfected with 3 AAV vectors using calcium phosphate: the vector of interest was suspended with the packaging plasmids pHelper and pRep/Cap and added dropwise to the cells. The media was removed and fresh serum-free media was added 6-8 hours later. After 3-day incubation, the virus was concentrated using 40% polyethylene glycol (PEG8000, BioShop Canada) and incubated at 4°C overnight. The concentrated virus was spun down and the pellet was re-suspended PBS and stored at -80°C.

3.4.5 Intraductal Injections

A volume of 10 μl of the virus was loaded into a Hamilton syringe, with a 1-inch 33-gauge needle attached. Under a dissecting miscroscope, the tip of the mouse's nipple is cut, the syringe is inserted

into the nipple, and the volume is slowly injected into the mammary ducts. This was done on the 3rd, 4th, and 5th mammary glands.

3.4.6 Statistical Analysis

Comparisons of multiple means was performed by ANOVA, using Tukey's *post-hoc* test in general, and a Fisher's LSD test for the mammary intra-ductal injection analysis. An alpha of 0.05 was used for determining statistical significance. Comparison of two unpaired independent means was performed using a student's t-test. Statistics were determined using excel, SPSS, and GraphPad Prism. All images are representative of more than 5 fields from at least three mice.

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Chapter 4: Discussion and Future Directions

In this body of work, I describe and characterize the mechanisms for the progression of a normal tissue into a malignant lesion, from a cellular biology perspective. First, we provided models for two types of progression, the less common early loss of polarity and the predominant progressive loss of polarity. We went on to further describe the cellular mechanisms of the latter. Our findings indicate that asymmetric cell divisions generating non-polarized cells are followed by the collapse of luminal architecture and generation of solid ducts with microlumen, frequently observed in DCIS. Further progression occurs with the gradual loss of apical-basal polarity as lesions advance. Asymmetric divisions are the result of cell divisions oriented perpendicular to the plane of the duct, which we demonstrate are necessary for the formation of stratified epithelium during early tumourigenesis. Luminal collapse and condensation in the transition from ADH to DCIS is due in part to reduced apical tension mediated by RhoA/myosin signaling. Remarkably, we found that disruption of tissue and cell polarity was reversible, which raises the possibility that early breast lesions may be manipulated pharmacologically to restore or maintain cell and tissue polarity to prevent progression of pre-malignant lesions or recurrence of breast cancer in the future.

Many concepts were discussed above in the individual Chapters 2 and 3. Here, I will expand on these concepts while integrating them together, discuss additional ideas that cover the general scope of the project, and highlight additional questions raised and future studies that would be helpful to answer them.

4.1 Progressive Loss of Polarity Occurs Through Tissue-level Rearrangements

Luminal filling has previously been proposed as a mechanism by which ducts become solid (Taraseviciute et al. 2010; Leung and Brugge 2012b; Venugopalan et al. 2014).; however, it was

characterized predominantly in cell lines that lack physiologic properties of ducts (e.g. apical-basal polarity) and lack *in vivo* confirmation. Our integrative analysis of human and mouse pre-invasive luminal breast lesions indicate that it is not the primary mechanism that generates solid lesions in HR+ DCIS. Instead, we found that perpendicularly oriented divisions lead to stratified ducts and progressive loss of apical-basal polarity; the ducts then collapse to generate microlumen within solid ductal structures. The differences in these cellular mechanisms show that the cellular context and environment are essential in defining the contribution of different mechanisms leading to carcinogenesis.

Based on our observations, as well as genetic and histological data from human tissues, we propose a model by which stepwise progression through FEA, ADH, and DCIS is a non-obligate precursor of invasive carcinoma (Sgroi 2010; Sinn et al. 2010). Starting from a normal duct, increased proliferation initially expands the number and size of ducts (dilation), while maintaining relatively normal tissue organization. This is consistent with flat epithelial atypia (FEA) one of the earliest stages of pre-cancerous stages observed in patients, which is characterized by dilated ducts in the absence architectural structures like cribiform patterns (Sgroi 2010; Sinn et al. 2010). Perpendicular divisions are then needed for further progression, as it involves breaking tissue polarity and creating a stratified epithelium enabling the lesion to progress to ADH. Altered spindle orientation alone, however, is not sufficient to cause tumour growth. At this stage, concomitant luminal splitting and reduction of ductal size are followed by luminal collapse creating solid lesions, termed DCIS. Afterwards, a breach of the basement membrane of the ducts marks the transition into IDC. However, not all ADH lesions progress to DCIS and subsequently invasive disease; although several studies have shown that an ADH diagnosis increases the overall risk of breast cancer by 30% (Kader et al. 2018). Several transcriptional and molecular events have been described illustrating the changes that transpire during the transitions

from ADH to DCIS and DCIS to IDC, although the former is very much underrepresented in the literature. Transcriptional elevation of ERBB2 and ER regulators FOXO1 and GATA3 have been observed during the transitions from early breast neoplasia to carcinoma, regardless of clinical subtypes (Brunner et al. 2014). Furthermore, many of the changes observed in ADH are maintained through DCIS, which gradually acquires and increasing number of subtype specific alterations before progression to IDC (Kader et al. 2018). Microenvironmental changes have also been described in the DCIS to IDC progression, such as rearrangement of myoepithelial cells, stromal cells, and the surrounding extra-cellular matrix (Gorringe and Fox 2017).

Whether this mechanism of progression we describe is common to many cancer types is not known, though we could predict that some other epithelial cancers, such as prostate, lung, or kidney, could possibly follow a similar progression route. In a subset of human lesions, we observed a different mechanism that involved early loss of cell polarity and shedding of cells into the lumen. We do not currently know the mechanisms underlying the progression of these lesions; however, we did not observe intermediates such as FEA or ADH in these DCIS. This could result from lesions that progress much faster, and therefore earlier intermediate stages are not readily observed, since polarity loss seems to be permissive to cancer development. Indeed, processes such as epithelial stratification become easier and occur faster in the absence of an apical membrane, which defines proper spindle orientation anchoring during cellular division (Nakajima 2018). Alternatively, some intermediate steps may be omitted in the progression of some breast lesions. For example, completely solid ducts are not necessary, as direct FEA to DCIS transitions have been observed (Sgroi 2010). This could possibly be explained by the luminal filling hypothesis. In fact, other studies have shown cells shedding in the lumen when apical polarity is lost, *in vitro* using MCF10A cells by Leung et al. (Leung and Brugge 2012b) and *in vivo* by McCaffrey et al. in very early stages of hyperplasia (McCaffrey and Macara 2009a). What

remains unknown is whether cells shedding into the lumen is effectively occurring throughout carcinogenesis, whether it is a true contributor to the formation of solid ducts, as well as in what context it occurs, and if the modes of progression are tumour subtype-dependent. In addition, there could be other alternative mechanisms of progression of which we are not aware. These questions could be answered through a more extensive evaluation of human samples exhibiting these phenotypes of progression to see how they may differ with prognosis, subtype (particularly HER2+ and triple negative), and tumour grade. This could be supplemented with studies in corresponding mouse models to identify and compare the cellular and molecular mechanisms driving them.

4.2 Early Tumour Heterogeneity and the Implications of Apical Membrane Loss

The development of breast carcinoma is generally associated with loss of growth control and disrupted tissue organization in the form of solid ducts that lack a central lumen (Chatterjee and McCaffrey 2014). This has been previously associated with a loss of polarity in the cells but had not yet been properly examined by localizing the polarity proteins inside the cell (Chatterjee and McCaffrey 2014). After examining apical-basal polarity markers in hormone receptor positive DCIS, we made the unexpected discovery that although tissue organization is largely disrupted in DCIS, individual cells can retain elements of cell polarity. However, these cells are unlikely normal, and they may have other aspects of polarity disrupted.

Invasive breast cancers are heterogeneous, which has a significant impact on therapeutic response and relapse (Zhang et al. 2017), but heterogeneity in pre-invasive lesions is less well characterized. We report that within pre-invasive lesions, cellular heterogeneity exists with respect to apical-basal polarity, an important regulator of cell behavior (Halaoui and McCaffrey 2015), therefore, disrupted polarity cues may contribute to heterogeneous cell behaviors in the progression of pre-

invasive breast lesions that may promote development of invasive carcinoma. Moreover, although pre-invasive lesions in which some cells retain apical-basal polarity was the most common phenotype in our dataset, we also observed other phenotypes, including basolateral or cytoplasmic Par6, and rare ducts in which apical-basal polarity was lost and cells shed into the lumen. Whether these different phenotypes are associated with different potential to progress to invasive carcinoma is not known at present and will need to be evaluated in the future.

Interestingly in these tissues, the subapical marker Ezrin was undetectable in most cells by immunostaining, whereas Par6 was mis-localized, indicating that the cells had lost their apical membrane. The apical membrane of epithelial cells has a central role in maintaining cellular homeostasis through its many functions. Facing the external environment, it is constantly exposed to hostile settings from stomach acid to viruses and bacteria; it is also a hub for the absorptive and secretory actions of the cell (Schuck and Simons 2004). In the mammary gland specifically, the apical membrane is responsible for transporting nutrients and secreting milk during lactation (Chatterjee and McCaffrey 2014). These properties make it vital for epithelial cells to establish and maintain its apical membrane when needed. In fact, this membrane is a hub for protein sorting; a basal level of endocytosis therefore exists where any protein that does not belong on the apical membrane will get internalized and correctly re-sorted. Interestingly, the whole membrane can be internalized and replaced within four hours, highlighting how essential it really is for the cell to resist any disturbance in its integrity (Schuck and Simons 2004). Many studies have attempted to clarify how epithelial cells sort their plasma membrane into apical and basolateral domains. The apical membrane is established shortly after the concentration of the Par6-aPKC complex in one area on the cellular membrane (Hutterer et al. 2004), and that suppressing the Par complex from the apical membrane leads to its disassembly (Yamanaka et al. 2006). In addition, the disruption specifically of the Par complex has been previously linked to cancer

progression. In fact, the oncogene ErbB2 associates with the Par6-aPKC polarity complex in order to modulate the disruption of the acinar organization of breast epithelia (Aranda et al. 2006a), and when uncoupled from aPKC, Par6 is able to associate with TGFβ to activate an Epithelial-to-Mesenchymal Transition (EMT) program.

This data joined with my observations of Par6 mis-localization in a subset of cells at pre-cancerous stages indicate that Par6 could not only be a prognostic marker for these early stages but could also play a role in their progression to IDC. The mere presence of Par6 mis-localization and number of cells exhibiting this phenotype have the potential to be indicators of the severity of the disease that will arise as well as the timing of progression to IDC. This is essential information that could be drawn from biopsies displaying these early stages in order to determine if the patients would benefit from immediate therapeutic intervention or if watchful waiting could be an option. Further studies are necessary evaluate the therapeutic and prognostic strength of Par6 localization, or other polarity markers, as a biomarker for breast or other cancers.

4.3 Cancer Field Theory: Creating a Permissive Environment through an Activated Epithelial State

The accumulation of mutations in cells, their de-polarization, mis-orientated divisions, and tissue rearrangement come together to illustrate the generation of a cancerous field in a tissue, bringing up the question of the origin of cancers. There exist two main opposing theories attempting to explain cancer initiation. The somatic mutation theory of cancer is the prevalent one, which the scientific community has focused on in the past century. It proposes that cancer is a clonal, cell-based disease, whereby the cells acquire increasing mutations in their DNA that control proliferation and inhibit apoptosis (Soto and Sonnenschein 2011). However, our data do not fully support this. In the MIC/MTB

mouse model, the PyMT oncogene activates many oncogenic pathways including the Src, Shc-MAPK, phospholipase C, and PI3K-pAKT pathways that all control proliferation and survival; when simply Src is knocked-out, no tumours arise.

Furthermore, many emerging studies speak of the importance of factors external to the mutated epithelial cell, such as the tumour microenvironment (Lochhead et al. 2015; Belli et al. 2018). The latter contains various cell types including fibroblasts and immune cells that can condition the epithelial tumour cells to promote tumourigenesis, invasion, and metastasis. Interestingly, suppression of polarity protein expression in tumour cells enables dendritic cell or macrophage conditioned medium to induce invasion, potentially by upregulating epidermal growth factor receptor (EGFR) and Akt signaling (Chatterjee et al. 2012). This is not observed in normal cells, indicating that apical–basal polarity proteins act as a barrier to invasion induced by cytokines secreted by the tumour stroma. In addition, the tumour microenvironment and extracellular matrix are critical regulators of cancer progression, and breaching the laminin-rich basement membrane is a feature of invasive carcinomas. Basement membrane protein gels, like Matrigel, act as a barrier to tumour cell invasion; however, cells readily invade in collagen I, which is highly expressed in many tumours (Nguyen-Ngoc et al. 2012a). Remarkably, even normal epithelial cells can become invasive in collagen, indicating that the composition of the ECM is a critical modulator of invasive behavior (Nguyen-Ngoc et al. 2012a). This demonstrates the importance of the environment and its impact on cell behavior; therefore, there needs to be extra layers of complexity to the process of tumour initiation rather than just the genetic factor that is a mutation.

On the other hand, the tissue organization field theory states that carcinogenesis is based at tissue-level, rather than at the level of cell proliferation, similar to normal tissue morphogenesis (Soto and Sonnenschein 2011). It also declares that the default state of cells is proliferative, and when normal

tissues come in contact with neoplastic ones, this state may be un-inhibited in the normal cells, or vice-versa thus creating an activated epithelial state or a cancer field (Soto and Sonnenschein 2011). In our studies, the PyMT oncogene in our mouse models causes an increase in basal proliferation rates that seem to drive hyper-proliferation. In the initial steps of mammary tumourigenesis, the gland accommodates the increased number of cells by increasing ductal diameter and increasing branching morphogenesis, mimicking the response to increased proliferation induced by puberty, leading to FEA (Ewald et al. 2008). Therefore, as mentioned above, simply increasing proliferation is not sufficient to initiate tumour formation, especially in the mammary gland. This could be a phenomenon specific to tissues that are less dense such as the fatty mammary gland, where the open ductal tree allows for expansion of the tissue. A question that arises is whether tissue level effects alone, with no change in mutation status and proliferation, are enough to initiate tumourigenesis. However, this seems unlikely, since many major oncogenic pathways affect proliferation. The mammary gland provides and interesting view in attempting to resolve this question. It undergoes cyclic expansion and regression throughout the lifetime of the animal, easily acclimating to the changes in proliferation rates and tissue rearrangements. Additionally, our *in vitro* organoid imaging data shows normal cells dividing in the incorrect orientation are able to re-integrate into the tissue without causing stratification, whereas this is not true in an oncogenic context. Increased proliferation can be deterrent to the corrective mechanisms the tissue has in place, as not enough time is allowed between divisions for the correction to occur. Thus, I believe our data supports a hybrid theory between the Somatic Mutation and the Tissue Organization theories for cancer initiation.

4.4 An Active Epithelial State May Explain the Transition from Pre-cancerous to Invasive Lesions.

One of the best characterized hallmarks of cancer is loss of growth control from over-proliferation. The work I present in this thesis argues that the context in which proliferation occurs is important, and that the development of epithelial cancers is initiated by coupling loss of growth control and disrupted tissue organization through oncogene activation and misoriented divisions, for example, that break tissue symmetry (Wang et al. 1994). In support of this, expression of cyclin D1, a putative oncogene that is frequently upregulated in breast cancer, promotes constitutive proliferation. However, only about half of MMTV-Cyclin D1 mice form mammary tumours after a very long latency (~1.5 years) in the mammary gland (Wang et al. 1994). Most of the tissue is enlarged hyperplastic ducts that are not stratified and resemble the FEA-like structures in the PyMT-Src$^{-/-}$ glands we observed. Therefore, excessive proliferation alone does not appear sufficient for tumour formation. This supports our model in which altered cell division orientation produces non-polarized cells with a growth advantage that eventually populate the emerging tumour. Our data show that when stratification does not take place, the tissue is not able to fully transform, and progression is halted. Furthermore, I report here that re-instating spindle misorientation to this cancerous field restores tissue stratification and formation of *in situ* cancer lesions, providing direct support that altered regulation of spindle orientation promotes cancer in certain contexts. Ongoing studies are aimed at evaluating whether these cancers progress to metastatic disease.

The formation of a stratified epithelium seems to un-block cancer progression in our model, allowing for tissue-level rearrangements to take place, and likely driving tumour formation. Therefore, tissue stratification may initiate an activated epithelial state that permits dynamic tissue remodeling during progression to later stages. This may reflect changes that resemble developmental programs.

In a normal context, studies have shown that the generation of a stratified epithelium is coincident with dynamic cell rearrangements that promote branching morphogenesis and ductal growth during mammary development (Huebner and Ewald 2014). Stem/progenitor cells in the adult mammary gland divide asymmetrically and perpendicular to the lumen at every estrous cycle and during pregnancy in order to get the gland ready for lactation (Huebner and Ewald 2014). This could explain the baseline percentage of perpendicular divisions we observe in normal glands, as it may be a product of the continuous cyclical expansion and involution that the mammary gland goes through. These perpendicular divisions could also be a random event considered a "mistake" that is able to fix itself by inciting the daughter cell positioned towards the lumen to re-integrate into the duct, as we have seen in our *in vitro* live imaging of organoids. This developmental process, in a cancerous tissue context, may initiate an activated epithelial state that permits dynamic tissue remodeling during progression from early to late stages in cancer. Indeed, collective migration from stratified epithelial structures has been observed in advanced carcinoma (Nguyen-Ngoc et al. 2012b; Cheung et al. 2013). Consequently, cancer cells residing in the primary pre-cancerous lesion gaining epithelial plasticity early on can profit from a competitive advantage and cause the neighbouring "normal" cells exhibiting no mutations to participate in the tissue re-arrangements seen (Soto and Sonnenschein 2011; Lochhead et al. 2015). Therefore, there exists endogenous tissue-level mechanisms that are at play during normal tissue development and morphogenesis that can be repurposed in early pre-cancerous lesions and may be tissue level drivers of a cancerous field.

Furthermore, cancer migration and metastasis are often associated with EMT, where the cancer cells are able to enter a less differentiated state and gain plasticity; they thus profit from the advantage of reinstating this program utilized mainly during embryonic development (Nieto 2013). EMT is associated with a down-regulation of tight junctions and a loss of apical basal polarity. In our model of

cancer progression, the latter occurs through concomitant mis-oriented divisions, eventually leading to fully asymmetric divisions where only one daughter cell inherits the apical membrane while the other cell becomes depolarized and more proliferative, heading one step closer to a mesenchymal transition. EMT inducers such as ZEB and Snail are known to regulate apical-basal polarity through transcriptional and post-transcriptional mechanisms (Moreno-Bueno et al. 2008), and loss of polarity in epithelial cells is able to activate EMT pathways, notably through mis-localized Par6 associating with TGFβR and inducing the Par6- TGFβ polarity pathway which mediates TGFβ-induced EMT (Viloria-Petit and Wrana 2010). Moreover, apical-basal polarity suppresses EMT in order to inhibit metastasis by promoting the degradation of EMT-associated transcription factor SNAIL (Jung et al. 2019). We have not directly examined EMT markers in our studies, but epithelial markers such as E-cadherin were always expressed by the cells in both human and mouse tissue samples. However, epithelial and mesenchymal states are not binary, but rather a spectrum of reversible cell states in transition (Yeung and Yang 2017). Therefore, retention of polarity in early lesions, as our data shows, could be seen as a positive prognosis while detection of depolarized cells at this stage could be an indication of progression to malignant stages.

Blocking EMT, on the other hand, would logically be seen as a worthy therapeutic strategy, as it inhibits metastasis from taking place; however, studies have found that this does not always translate *in vivo* once the primary cancer has already been established (Husemann et al. 2008; Rhim et al. 2012). In some cancer types, metastasis could be a very early event during tumourigenesis which transpires before the cancer is even diagnosed, and blocking EMT in these cases could favour the formation of secondary tumours from early disseminated cells.

4.5 Cancer Progression as a Result of Cell Competition

We know from some cancer types such as breast and prostate, that not all pre-invasive lesions progress to invasive (Bell et al. 2015; Gorringe and Fox 2017). The heterogeneity in cell polarity status that we see at these early stages is quite interesting, as it brings up the concept of cell competition. Cell competition is present in both development and cancer. It is a well-conserved cell fitness-sensing mechanism that eliminates cells that are less fit than their neighbours. It is both an intrinsic property of the cell and an extrinsic one depending on the fitness of the neighbouring cell (Di Gregorio et al. 2016). Factors that competition depends on are growth differences, signaling differences, and the loss of polarity and epithelial integrity. In my studies, I have observed a growth advantage of non-polarized cells versus their polarized counterparts, whereby they are able to proliferate at higher rates. This growth advantage is important, as cells undergo mitosis with mis-oriented spindles, leading to an increasing amount of un-polarized daughter cells. These daughter cells are able to divide more often and populate the emerging tumour, outgrowing the remaining polarized cells which ultimately become extinct in invasive cancer.

Moreover, at the tissue level, cell competition may occur during tumourigenesis. Normal cells are able to suppress cancer cells into quiescence until some of them acquire additional abilities and are then dubbed “super-competitors” able to prompt adjacent normal cell clearance (Di Gregorio et al. 2016). Therefore, normal tissue surrounding mutated cells exhibits a tumour suppressor role in pre-cancerous lesions until sufficient events take place for the cancer cells to take control. We currently have little understanding of how these cells acquire increased fitness, but the first step to studying them is through their detection, and perhaps depolarization could be used as a marker for this. Cell competition as a whole arises through important mechanisms about which we do not have definitive knowledge. Studies in Drosophila have attempted to tackle the topic of cell competition during

development and have established three categories of mutations leading to differences in cell fitness depending on their effect on the cell: 1) mutations causing growth disparities, 2) mutations causing significant signaling differences, and 3) mutations causing apical-basal polarity disruption (Di Gregorio et al. 2016). Cell fitness sensing mechanisms come into play, also divided into three theories: 1) cells competing for growth factors could end up with the losers being eliminated via apoptosis, 2) cells could sense and communicate fitness amongst themselves and the less-fit cells are eliminated through an innate immune-like response or through differential expression of a code at the cell surface activating downstream elimination pathways, or 3) mechanical sensing leading to the extrusion and cell death of the less-fit cells. Therefore, the comprehension of cell competition mechanisms in mammalian cells is an essential key to uncovering the reasons behind the transitions from each pre-cancerous stage to the next, and more importantly the transition from pre-cancerous to invasiveness in hopes of no longer over-treating patients with early lesions such as DCIS. Modern techniques such as single cell sequencing could be used on pre-cancerous lesions in order to better understand the different types of cells co-existing in these early heterogeneous lesions, as well as their transition to more advanced stages.

4.6 Loss of Apical-Basal Polarity and Tissue Organization is Reversed upon Oncogene Removal.

The ability for a tissue akin to the mammary gland to undergo systematic and repetitive growth and regression is an interesting concept, as the tissue has in place mechanisms that ensure these tissue level changes properly occur, not only controlling the transition from one state to another, but knowing the limits and baseline of each tissue state. We therefore sought to examine whether this concept could be applied to tumour regression.

De-induction of oncogene expression in PyMT and other transgenic mouse models results in tumour reversion *in vivo*, but it is not known if cell polarity and ductal organization is restored (Liu et al. 2011; Rao et al. 2014). Remarkably, we found that disruption of tissue and cell polarity was reversible, and that de-inducing PyMT resulted in polarized ducts, even after advanced carcinoma that mostly lacked cell polarity were established. This suggests that polarity was not irreversibly suppressed in these lesions, but that cells were transiently impaired in their ability to maintain cell polarity. Re-establishing ductal organization with a lumen was rapid and involved apoptosis of internal cells to clear the luminal space. The literature has described some mechanisms of lumen formation from a solid structure. Clearing of a lumen through apoptosis was observed for other oncogenes in 3D culture of primary cells, where oncogene cessation caused internal cells to lose their mitochondrial polarity and undergo caspase-3-induced apoptosis (Jechlinger et al. 2009). Another study using the MCF10A cell line describes lumen formation from a solid cyst by cavitation, an active morphogenic process that happens during embryonic development (Debnath et al. 2002). In fact, apoptosis protein BIM is found to regulate apoptosis-driven lumen formation *in vivo* during mammary gland morphogenesis (Mailleux et al. 2007; Mailleux et al. 2008). Since we have found that apoptosis is also involved in re-establishing ductal structures during tumour regression, there may be a common underlying mechanism between regression and morphogenesis.

Our finding that pre-invasive stages are reversible and that the tissue is able to re-organize into a normal state when oncogene expression is eliminated, indicates that epithelial tissues have remarkable plasticity, and that oncogenic signals are necessary to maintain disrupted tissue organization. Indeed, spontaneous regression of cancers have been observed and described for most types of solid cancers, even at the advanced metastatic stage (Kitai et al. 2015; Parks et al. 2015; Ito et al. 2016). Suspected mechanisms for such remarkable cancer remissions include immune system

mediation, tumour inhibition by growth factors/cytokines, induction of differentiation, elimination of a carcinogen, tumour necrosis/angiogenesis inhibition, psychological factors, apoptosis, and epigenetic mechanisms (Papac 1998; Bodey 2002; Ito et al. 2016). We could then infer that these mechanisms have interesting implications for cancer therapies, as their pathways would make specific targets for anti-cancer treatments. Of recent interest, spontaneous regressions, although very rare, could be used to understand the context in which immune therapy would be most beneficial (Parks et al. 2015). The role of immune cells in the progression of cancers cannot be ignored, but their role in regressions and response to therapies has not been sufficiently explored. We are beginning to understand how the immune system and growing tumours are intertwined, acting through mechanisms such as Immunoediting to promote cancer growth (Candeias and Gaipl 2016). Immune cells are also able to manipulate the protective myoepithelial layer in breast cancer in order to promote basement membrane breaching and metastasis. Indeed, myoepithelial cells are thought to be natural tumour suppressors (Pandey et al. 2010), and their loss is an early event in tumour growth as carcinomas are predominantly composed of luminal cells (Hilton et al. 2013). However, what deserves a closer look is if and how immune cells can be utilized to promote pre-cancerous lesion and early tumour regression, how the myoepithelial cell layer is involved, and how to manipulate them to clear out the lesion or prevent it from developing further. The aim is to find a way through pharmacological manipulation to eliminate mutated cells with driver oncogenes, restore or maintain organized ducts thus preventing progression of pre-malignant lesions, promoting their regression, or halting recurrence of breast cancer in the future.

4.7 How to be an Oncogene

An oncogene is currently defined as a protein which has the ability to form a cancerous tumour, giving cells the ability to sustain proliferation, evade growth suppressors, resist death, replicate immortally, induce angiogenesis, invasion, and metastasis, reprogram metabolism, and evade the immune system (Hanahan D, cell 2011). We propose that it is essential for oncogenes to be able to manipulate tissue organization, thus adding a new characteristic to the definition of an oncogene, a new possible avenue for therapeutic targeting.

In addition to regulating cell division orientation, other mechanisms are likely to be used by oncogenes to disrupt tissue architecture, such as cell shedding, and cellular rearrangements. It is essential to catalogue these processes and understand the contribution of each one to cancer development, and the context in which these contributions change, thus expanding our understanding of "how to be an oncogene". With this information, we are able to gain a better understanding of the cellular processes guiding early carcinogenesis. Whether these detected pre-cancerous masses will progress to malignancy is unknown, which often instigates over-treatment of patients. Patients with high risk of disease recurrence could benefit from preventative therapies, which currently are limited and need to be improved since they have many undesirable side effects. One avenue that these high-risk patients might benefit from treatment that could restore tissue organization, potentially by manipulating pathways that control early cellular processes such as spindle orientation. They need to be prompted for further investigation because of their potential as therapeutic targets for pre-cancerous lesions.

Chapter 5: Final Conclusion

We initiated these studies with a goal in mind: to better understand early stages of cancer in order to instigate the development of good therapies for women with pre-cancerous breast lesions, including high-risk women. We hypothesized that polarity disruption is a necessary step in tumour development. To answer this, we have taken a fundamental approach in our work, where we have described the cellular mechanisms behind the changes in tissue morphology of pre-cancerous mammary gland lesions.

Our first aim was to evaluate polarity loss and characterize the cellular mechanisms behind early tumourigenesis. Notably, we have described the novel mechanism of luminal collapse for the generation of solid ducts, whereby the epithelium stratifies then undergoes a luminal splitting phenomenon, and these micro-lumen lose apical tension and collapse on themselves. With this, we have identified a key feature of tissue remodeling in carcinogenesis and expanded on some of the cellular mechanisms at play. Understanding the formation of pre-cancerous lesions brings us one step closer to differentiating between the ones that will advance from the ones that will not, and prevent over-treatment of women.

Next, we aimed to examine the contribution of spindle orientation to cancer initiation. We went on to investigate the role of epithelial stratification in tumour development. We found that the change in tissue morphology is a necessary step in the process of carcinogenesis of epithelial cancers. This tissue remodeling seems to create the necessary cancer field allowing for the progression of lesions and could be an avenue of treatment to prevent local recurrences. More importantly, this novel finding sheds light on one of the cellular processes and tissue rearrangements required for an oncogene to perform in order to grow into a tumour.

Finally, we assessed whether tissue polarity is lost in the growing lesions. We showed that the cellular rearrangements and tissue remodeling ensued from carcinogenesis is reversible, and that upon inactivation of the driver oncogene, the tumour is able to regress and the tissue revert back to a normal morphological state. This novel finding demonstrates that the cells and the tissue do not completely lose their polarity, but it is merely transiently suppressed by the oncogene or encompassing cancer field, and they are intrinsically able to reestablish normal morphological features. Importantly, the development of drugs targeting these early lesions may be a better treatment option compared to tumour resection.

Altogether, this collection of findings is fundamental as it brings us one step closer to understanding tumour initiation, from pre-cancerous lesions to their transitioning into invasive cancers, and expands our knowledge of early stages of tumour cell biology. (Halaoui et al. 2017)

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